CARCINOGÉNESE DO CARCINOMA DA MAMA MASCULINA

MARIA DA SAUDADE PEREIRA ANDRÉ

Tese para obtenção do grau de Doutor em Medicina

na especialidade de Genética, Oncologia e Toxicologia Humana (Anatomia Patológica)

na Faculdade de Ciências Médicas | NOVA Medical School da Universidade NOVA de Lisboa

Setembro 2019

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This research was approved by:

The Ethical Committees

Instituto Português de Oncologia de Francisco Gentil (IPOFG), Lisboa - ref. UIC/821

Faculdade de Ciências Médicas da Universidade Nova de Lisboa (CEFCM) – ref. 24/2014/CEFCM

Financial support

Prémio NOVARTIS | NMS-FCM 2015

(Bolsa Excellence in Fundamental Medical Research 2015)

UDHC- Unidade de Diagnóstico Histológico e Citológico, Lda

Ao meu pai

Manuel André

a pessoa que, nesta vida, mais orgulho teve em mim

Prefácio

Figura 1 - Primavera no IPOLFG em frente ao Laboratório de Citopatologia (2019).

Considero-me uma pessoa solitária. Cresci a contar apenas com a minha força, a conseguir tudo por mim. No entanto, agora que já vivi bastante tempo, avalio-me feliz por saber que pude e posso contar com muitas pessoas que ao longo destes anos se constituíram como famílias, representando lugares onde posso sempre voltar e alguns mesmo onde volto com entusiasmo todos os dias. Embora haja aqueles que já partiram, o seu lugar permanece enquanto eu permanecer, enquanto tiver memória. Agradeço muito tudo o que recebi e recebo.

Refiro, em especial:

- o meu pai Manuel, a minha mãe Isaura, os meus filhos João e Leonor, o Fernando (em relação a quem todos os excelentes adjetivos que eu possa utilizar serão sempre insuficientes), a minha irmã Dina e a minha sobrinha Luna.

- a minha tão importante família da UDHC.

- a minha família da Anatomia Patológica do IPO de Lisboa.

É da vivência no IPO que nasce e se forma este trabalho. O IPO de Lisboa é, há mais de 30 anos, a minha família mais numerosa e também a mais dinâmica, com membros já não fisicamente presentes, outros que vão chegando. O IPO é muito a minha casa. Às vezes esforço-me para chegar muito cedo, antes de toda a gente, para sentir o fresco da manhã. Muitas vezes fico feliz com o sol do entardecer quando parto. Alegro-me com as ninhadas de patos na Primavera, no lago mesmo ao pé da Citopatologia. Gosto

muito da luz nas árvores, do cheiro do jardim, de todos os animais que vão fazendo parte dele, dando-lhe toda uma vida efervescente de recomeços que apaziguam a tristeza e o cansaço que vão passando nos rostos. Ainda gosto de "ser" do IPO, gosto das pessoas com quem me cruzo há anos, que vão envelhecendo nesta comunidade que a vivência oncológica torna tão especial, da qual desde sempre senti fazer parte e isso é algo singular que muito agradeço.

Ao Serviço de Anatomia Patológica do IPO de Lisboa (médicos, técnicos, administrativos e auxiliares) devo um dia-a-dia de amizade calorosa, de companheirismo e de gratificante apoio. Os Patologistas do IPO, muito particularmente os que me acompanham desde o início da especialidade, são amigos verdadeiros de uma vida, em quem confio e de quem muito me orgulho. Em relação aos mais recentes, considero um privilégio que façam parte da equipa.

Saliento, com muito orgulho e carinho, a minha muito valiosa equipa do Laboratório de Citopatologia.

Ao Professor Jorge Soares devo o fundamental apoio e estímulo à minha dedicação à Patologia Mamária e a aprendizagem do rigor científico.

No contexto deste estudo, quero manifestar um agradecimento muitíssimo especial à minha grande amiga e orientadora, a Professora Ana Félix. O equilíbrio entre o hábito de tudo fazer de forma independente e apresentar resultados na cadência certa nem sempre foi fácil para mim. Agradeço todo o apoio, disponibilidade e boa vontade da co-orientadora Professora Jacinta Serpa, sempre presente quando precisei. Ao Dr. António Pinto devo a serena, mas entusiástica, meticulosa e imprescindível colaboração em grande parte das publicações feitas ao longo de muitos anos, com ênfase especial em dois dos artigos desta tese. À Doutora Fernanda Silva, agradeço muito toda a simpatia, disponibilidade e esforço. Um agradecimento também ao Giovani Silva por toda a paciência e gentileza na realização da análise estatística.

Um grande agradecimento à Professora Carmen Jerónimo e ao Professor Rui Henrique, mentores do segundo estudo desta tese. Nunca esquecerei a amizade e simpatia com que me receberam. Em relação à sua colaboradora Sandra Nunes, devo a base de apoio à realização do segundo artigo.

Não posso deixar de referir a agradável colaboração com a Catarina Brito, a Marta Estrada, a Ana Cartaxo e o Giacomo Domenici do Instituto de Biologia Experimental e Tecnológica de Oeiras (IBET), a continuar com promessa de excelentes resultados.

Agradeço também aos médicos da Radiologia, Cirurgia, Radioterapia e Oncologia Médica da Clínica da Mama do IPO, que me inspiram a fazer sempre o melhor. Não posso esquecer todos os médicos exteriores ao IPO com quem tenho colaborado ao longo da minha vida profissional, quer os que partilharam e partilham o meu gosto pela Patologia Mamária, quer todos os que me ajudaram e ajudam a preservar a minha atividade privada, a minha UDHC, que tanto prezo como sinónimo da minha independência e cujo apoio económico tornou possível este trabalho.

Com este trabalho saúdo também, com amizade, os patologistas portugueses, em particular os da minha geração. Conheço os nossos caminhos. Sei que ser patologista é parte integrante das pessoas que somos.

Fechando o círculo e voltando a mim, ao meu trabalho e à minha carreira profissional até agora, acredito que os valores da minha vida são ser construtiva e honrar todos os que me amaram e todos os que acreditaram e confiaram em mim. Agradeço à vida toda a força e toda a fragilidade, toda a capacidade de trabalho e todas as circunstâncias que me permitiram ser quem sou. Este trabalho de tese é mais um pequeno contributo a inserir neste agradecimento.

Publications

This thesis is based on three principal research papers and, additionally, in other previous publications and communications within the same scope, made over several years.

The three principal scientific papers:

Saudade André, Teresa Pereira, Fernanda Silva, Patrícia Machado, Fátima Vaz, Mariana Aparício, Giovani L. Silva, António E. Pinto.
Male breast cancer: Specific biological characteristics and survival in a Portuguese cohort.
Mol Clin Oncol. 2019 Jun;10(6):644-654. doi: 10.3892/mco.2019.1841. Epub 2019 Apr 8. Pubmed PMID: 31031981. PubMed Central PMCID: PMC6482395

Saudade André, Sandra P. Nunes, Rui Henrique, Ana Félix, Carmen Jerónimo.
Epigenetic alterations in homologous recombination DNA repair genes in male breast cancer
(submitted to International Journal of Molecular Sciences)

Saudade André, António E. Pinto, Giovani L. Silva, Fernanda Silva, Jacinta Serpa, Ana Félix.
Male Breast Cancer – Relevance of FASN, FASN and Collagen IV
(submitted to Translational Recearch)

Abbreviations

AJCC/UICC - American Joint Committee on Cancer/ Union for International Cancer Control

AJCC/TNM staging system - Tumor size and extent (T), involvement of regional lymph nodes (N), presence of absence of distant metastasis (M)

AR - Androgen Receptors

AS - Anatomic Stage

ASCO - American Society of Clinical Oncology

ATF3 - Activating Transcription Factor 3

ATM - Ataxia Telangiectasia Mutated

BC - Breast Cancer/ Breast Carcinoma

BRCA1 - Breast Cancer, early onset 1

BRCA2 - Breast Cancer, early onset 2

CAP - College of American Pathologists

CpG - C -Cytosine; p - phosphodiester, G - Guanine

CS - Clinical defined Subtypes

DFS - Disease Free Survival

ERα - Estrogen Receptor alpha

ERBB2 - Epidermal Growth Factor Receptor 2

EGFR - Epidermal Growth Factor Receptor

FASN - Fatty Acid Synthase

FATP1 - Fatty Acid Transporter Protein 1

FFPE - Formalin-Fixed, Paraffin-Embedded

FH - Family History

G1 - Well differentiated, grade 1

G2 - Moderately differentiated, grade 2

G3 - Poorly differentiated, grade 3

hCG - Human chorionic gonadotropin

HRR - Homologous Recombination Repair

IARC/ Globocan - International Agency for Research on Cancer/ Global Cancer Observatory

IHC - Immunohistochemistry

IPOLFG - Instituto Português de Oncologia de Lisboa Francisco Gentil

M - Distant metastasis

NBPN - Non-Breast Primary Neoplasm

NGS - Next-generation sequencing

OS - Overall survival

PALB2 - Partner and Localizer of BRCA2

PARP - Poly (ADP-ribose) polymerase

PCR - Polymerase chain reaction

PD-L1 - Programmed death-ligand 1

PI3K/AKT - Phosphatidylinositol 3-kinase/ serine/threonine kinase

pN - Pathological evaluation of axillary nodal status

PR - Progesterone Receptors

pT - Pathological evaluation of tumor size

p16 - $p16^{INK4a}$, Cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

RAD51B - Recombinase 51 homolog B

SEER - Surveillance, Epidemiology and End Results

TMAs - Tissue Microarrays

TN - triple negative carcinoma

TP - triple positive carcinoma

WHO - World Health Organization

XRCC3 - X-Ray Repair Cross Complementing 3

β integrins - beta integrins

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Abstract

Introduction: Male breast carcinoma (male BC) is an uncommon neoplasm with rising incidence, emerging in the last years as a multifactorial and distinct subtype of BC. It is frequently diagnosed in anatomic advanced stage disease and has no individualized strategies for diagnosis, therapeutics and follow-up. The existent ones are based in female BC. Our main objective was to identify biological factors with potential to innovate the clinical management of male BC. To achieve this aim, the thesis integrated three principal studies. In a first study, we collected a series of 196 male BC cases, evaluated clinicopathological parameters currently used as prognostic and predictive factors, as well as germline *BRCA* mutation (g*BRCA*) and DNA ploidy status, and analyzed their association with disease-free survival (DFS) and overall survival (OS). The central purpose was to obtain and to characterize a well-defined series of cases with detailed clinicopathological data for additional research. In the second study, we considered the relevance of altered homologous recombination repair (HRR) and explored the potential of aberrant promoter methylation of five HRR genes in male BC detection and/or monitorization, also because there is an increasing interest in understanding the molecular basis of sensitivity to PARP inhibitors (PARPi) in patients with deficient HRR and no g*BRCA1/2* mutations. Finally, in the 3rd study, using immunohistochemistry (IHC), we aimed to identify IHC epithelial and stromal phenotypes with prognostic and/or predictive value and potential eligibility for application in the clinical management of male BC.

Material and Methods: We profiled 196 patients (1970-2018; mean follow-up: 171.3 months), using clinicopathological review and molecular assessment (g*BRCA* status, immunohistochemistry, *in situ* hybridization, DNA flow cytometry). Additionally, we used formalin-fixed and paraffin-embedded tissue samples from 128 male BC carcinomas, paired adjacent normal tissue and 19 gynecomastia cases, for the evaluation of aberrant promoter methylation of *ATM*, *BRCA1*, *PALB2*, *RAD51B* and *XRCC3* by quantitative methylation-specific PCR. In addition, we selected 40 of the invasive male BC series with no neoadjuvant therapy, to evaluate IHC in Tissue Microarrays (TMAs) with Androgen Receptor (AR), Activating Transcription Factor 3 (ATF3), p16 and Cyclin D1, Fatty Acid Synthase (FASN), Fatty Acid Transport Protein 1 (FATP1), β1, β3, β4 and β6 integrins, Collagen I and Collagen IV. In the first and third evaluations, Pearson's

χ^2 and Fisher's exact tests of independence and a Cox proportional hazards regression model were employed for statistical analysis. Non-parametric tests were used in the second study to compare methylation levels between tumor and non-tumor samples and to seek for associations with clinicopathological variables. As gynecomastia is the most frequent benign condition in male breast, we used gynecomastia as comparative model in these two studies.

Results: The median age of the patients was 66.5 years. The majority of cases were invasive carcinoma of no special type, histological grade 2 (G2), estrogen receptor α (ERα) and progesterone receptor (PR) positive, ERBB2 negative, high Ki67, Luminal B-like and aneuploid. Thirteen of the 44 (29.5%) g*BRCA* evaluated patients had g*BRCA2* mutations, significantly linked with family history, bilaterality, high Ki67, absence of PR and Luminal B-like. Bilaterality was associated with the occurrence of non-breast primary neoplasms (NBPT). The 5/10-year disease-free survival (DFS) rates of 145 patients (excluding M1 patients at diagnosis, patients with non-breast primary neoplasms and with *in situ* carcinomas) were 65.9% and 58.2% and overall survival (OS) rates were 77.5% and 59.2%, respectively. In univariate analysis, age at diagnosis ≥70 years and HER2-like carcinomas were significantly associated with low OS and G3 carcinomas were associated with short DFS. Also, in univariate analysis, pT 2+3 and pT4, pN1, anatomic stage II and III, high Ki67, Luminal B-*like* carcinomas and g*BRCA2* mutations were significantly related with short DFS and low OS. In multivariate analysis, bilaterality and G3 carcinomas were significantly associated with short DFS as well as family history and Luminal B subtype with low OS. In addition, AS II and III were significantly associated with short DFS and low OS. In the second study, *RAD51B* and *XRCC3* disclosed significant differences between tumor and gynecomastia (p<0.0001 and p=0.020, respectively). Assembled in a panel, the promoter methylation levels of these genes discriminated male BC from gynecomastia with 91.5% sensitivity, 89.5% specificity and 91.2% accuracy. No associations were found between epigenetic alterations and clinicopathological features. The IHC study showed that homogeneous and intense epithelial staining of p16, ATF3, β6 integrin, FASN and FATP1 was significantly intercorrelated, and significantly associated with high Ki67. All these biomarkers, with the exception of FASN, stained fibroblasts in a different proportion, and p16 also stained fibroblasts in malignant epithelial negative cases. Kaplan-Meier/log-rank

tests showed significant associations of homogeneous phenotype of FASN with DFS and OS, of ATF3 with OS, as well as diffuse and intense stromal Collagen IV staining with DFS. No homogeneous epithelial pattern or intense Collagen IV stromal staining were identified in gynecomastia tissue.

Conclusions: Male BC was commonly diagnosed in high anatomic stages and has low OS rates. The significant relation of g*BRCA2* mutations with family history, bilaterality, Luminal B subtype, negative progesterone receptors, high Ki67 and worst prognosis, identifies a group of male BC deserving a particular clinical approach. In multivariate analysis, bilaterality and G3 carcinomas were significantly associated with short DFS and family history as well as Luminal B subtype with low OS. Moreover, AS II and III were significantly associated with short DFS and low OS. The quantitative promoter methylation of *RAD51B* and *XRCC3*, evaluated as a panel in tumoral tissue, constitutes a promising biomarker for detection and monitorization of male BC, and should be considered in clinical trials involving PARPi in men belonging to hereditary cancer families and with no detect g*BRCA1/2* mutations. We highlight the value of the intratumoral homogeneity of malignant epithelial cells in biomarkers with distinct biological functions, of their interactions and their significant association with high proliferation. The prognostic relevance of homogeneous phenotype FASN and ATF3, as well as the diffuse and intense Collagen IV stromal staining may contribute to identify carcinomas of Luminal subtypes with worse outcome. These biomarkers are eligible for investigation as with potential to innovate the clinical management of male BC and, eventually, of Luminal subtypes female BC.

Keywords: Male breast carcinoma; Clinicopathologic characteristics; Molecular markers; Immunohistochemistry; Epigenetics; Homologous recombination; DNA repair; Detection; Survival; Prognosis; Gynecomastia

Resumo

Introdução: O carcinoma da mama masculina é uma neoplasia pouco comum, mas com incidência crescente, que se tem destacado nos últimos anos como um subtipo de carcinoma da mama, multifatorial e com características próprias. É frequentemente diagnosticado em estádio avançado e não existem estratégias individualizadas para diagnóstico, terapêutica ou seguimento clínico, que se baseiam no carcinoma da mama feminina. O nosso objetivo *major* é contribuir para a caracterização desta neoplasia, pela identificação de fatores biológicos com potencial para inovar a abordagem clínica. Para conseguir este objetivo integrámos nesta tese, três artigos principais. No primeiro, selecionámos uma série de 196 casos de carcinoma da MM, avaliámos parâmetros clínico-patológicos utilizados na prática clínica como fatores prognósticos e preditivos, pesquisámos o *status* da mutação germinal *BRCA* (g*BRCA*) e a ploidia do ADN, identificámos a associação destes parâmetros com o intervalo livre de doença (ILD) e com a sobrevivência global (SG). O objetivo foi obter uma série de casos bem estruturados para estudo subsequente. No segundo estudo, considerámos a importância da recombinação homóloga na reparação do ADN e explorámos o potencial da metilação aberrante do promotor de 5 genes na deteção e/ ou monotorização do carcinoma da mama masculina, tanto mais porque há um interesse crescente na compreensão da base molecular da sensibilidade aos inibidores da PARP (PARPi) em doentes com deficiente reparação por recombinação homóloga sem mutações *BRCA1/2*. No terceiro estudo, o nosso propósito foi utilizar a imunohistoquímica (IHQ) em TMAs para avaliar padrões de biomarcadores epiteliais e do estroma com potencial valor prognóstico e/ou preditivo e passíveis de aplicação na prática clínica do carcinoma da mama masculina.

Material e Métodos: No primeiro estudo, obtivemos o perfil de uma série de 196 pacientes (1970-2018; média de seguimento clínico: 171,3 meses), fizemos a revisão clínico-patológica e a avaliação de características moleculares (status da mutação g*BRCA*, IHQ, hibridização *in situ*, citometria de fluxo) e a análise estatística. No segundo estudo, avaliámos os níveis de metilação aberrante do promotor dos genes *ATM*, *BRCA1*, *PALB2*, *RAD51B* e *XRCC3* por PCR quantitativo, em amostras de tecido fixado em formol e incluído em parafina de 128n carcinomas desta série, respetivo tecido normal adjacente e 19 casos de ginecomastia. No terceiro estudo, construímos vários TMAs com 40 destes

carcinomas invasivos sem terapêutica neoadjuvante, para avaliar o padrão da IHC com Receptores de Androgénios, Ativador do Fator de Transcrição 3 (ATF3), p16, Ciclina D1, Fator de Síntese de Ácidos Gordos (FASN), Proteína de Transporte de Ácidos Gordos 1 (FATP1), Integrinas β1, β3, β4 e β 6, Colagénio I e Colagénio IV. Foram ainda utilizados 8 casos de ginecomastia como modelo comparativo. No primeiro e terceiro estudo, utilizámos os testes de Pearson χ^2 e Fisher e o modelo de Cox para análise estatística. No segundo estudo utilizámos testes não paramétricos para comparar os níveis de metilação entre amostras tumorais e não tumorais e para procurar associações com variáveis clínico-patológicas. Como a ginecomastia é a condição benigna mais frequente na mama masculina, utilizámos a ginecomastia como modelo comparativo nos dois últimos estudos.

Resultados: A média de idade dos doentes foi de 66,5 anos e observámos um predomínio de carcinomas invasivos sem tipo especial, grau histológico 2 (G2), com receptores de estrogénios α (REα) e receptores de progesterona (RP) positivos, com ERBB2 negativo, com Ki67 alto, de subtipo Luminal B-*like* e aneuplóides. Treze dos 44 (29,5%) pacientes avaliados para risco genético apresentaram mutações germinais no *BRCA2*, que estavam significativamente relacionadas com história familiar (HF), bilateralidade, valores elevados de Ki67, ausência de RP e subtipo Luminal B-*like*. A bilateralidade associou-se à ocorrência de neoplasias primárias não mamárias. As taxas de ILD em 5/10 anos de 145 doentes (excluindo os doentes M1, os doentes com neoplasias primárias não mamárias e os doentes com carcinomas *in situ*) foram de 65,9% e 58,2% e as taxas de SG foram de 77,5% e 59,2%, respetivamente. Em análise univariada, a idade ao diagnóstico ≥70 anos e os carcinomas ERBB2 positivos associaram-se significativamente com menor SG e os carcinomas G3 associaram-se a menor ILD. Ainda, em análise univariada, os carcinomas pT 2+3 e pT4, pN1, estádios anatómicos II e III, Ki67 alto, carcinomas Luminal B-*like* e as mutações g*BRCA2* associaram-se significativamente a menor ILD e menor SG. Em análise multivariada, a bilateralidade e os carcinomas G3 associaram-se significativamente a menor ILD e a história familiar assim como o subtipo Luminal B-*like* a menor SG. Ainda, os estádios anatómicos II e III associaram-se a menor ILD e menor SG. No segundo estudo, apenas a metilação dos promotores dos genes *RAD51B* e o *XRCC3* revelou diferenças significativas entre tumor e ginecomastia ($p<0,0001$ e $p=0,020$, respetivamente). A

metilação simultânea dos promotores dos genes *RAD51B* e *XRCC3* discriminou o carcinoma da mama masculina da ginecomastia, com 91,5% de sensibilidade, 89,5% de especificidade e 91,2% de acuidade. Além disso, os níveis de metilação do promotor destes genes foram menores nas amostras dos tecidos não tumorais, em comparação com as amostras dos respetivos tumores. Não foram encontradas associações entre alterações epigenéticas e características clínico-patológicas. O estudo com IHC mostrou que a positividade homogénea das células epiteliais malignas em relação com os biomarcadores p16, ATF3, integrina β6, FASN e FATP1 estava significativamente inter-relacionada e significativamente associada com Ki67 alto. Todos estes biomarcadores, com exceção do FASN, mostraram positividade nos fibroblastos do estroma tumoral em diferentes percentagens e o p16 também marcou fibroblastos nos casos p16 negativos nas células epiteliais. Os testes de Kaplan-Meier/ log-rank mostraram associações significativas do fenótipo homogéneo do FASN com o ILD e com a SG, do ATF3 com a SG, assim como a marcação difusa e intensa do Colagénio IV com o ILD. Não identificámos nenhum padrão homogéneo das células epiteliais nem marcação intensa com Colagénio IV no tecido da ginecomastia.

Conclusões: O carcinoma da mama masculina é comumente diagnosticado em estádios avançados e apresenta baixa sobrevivência global. A relação da mutação g*BRCA* com a história familiar, bilateralidade, subtipo Luminal B, carcinomas progesterona negativos, Ki67 alto e com pior prognóstico, identifica um subgrupo de carcinoma da mama masculina a merecer avaliação clínica específica. Em análise multivariada, a bilateralidade e os carcinomas G3 associaram-se de forma significativa a menor ILD, assim como a história familiar e o subtipo Luminal B a menor SG. Os estádios anatómicos II e III associaram-se significativamente a menor ILD e menor SG. A quantificação dos níveis de metilação dos promotores dos genes *RAD51B* e *XRCC3*, avaliada em conjunto no tecido tumoral, constituiu um biomarcador de carcinoma da mama masculina, a considerar particularmente em ensaios clínicos que envolvam terapêutica com inibidores da PARP em homens pertencentes a famílias com cancro hereditário e sem mutações germinais *BRCA1/2*. Evidenciámos o valor do padrão homogéneo das células epiteliais malignas em biomarcadores com funções biológicas distintas, a sua inter-relação e a associação com o aumento da proliferação. O significado prognóstico do padrão homogéneo do FASN e ATF3, assim com da marcação intensa do estroma tumoral com

Colagénio IV podem contribuir para identificar carcinomas Luminal-*like* com pior prognóstico e estes biomarcadores são elegíveis para investigação pelo potencial de poder inovar a terapêutica do carcinoma da mama masculina e, eventualmente, do carcinoma Luminal-*like* da mama feminina.

Palavras-chave: Carcinoma da mama masculina; Características clínico-patológicas; Marcadores moleculares; Imunohistoquímica; Epigenética; Recombinação homóloga; Reparação do ADN; Deteção; Sobrevida; Prognóstico; Ginecomastia

Contents

Chapter I

Figure 2 - Marble bust with the head of an unknown man (*Le gallerie degli Uffizi* - Greece 54-68 d.C.) (Verão 2019 - Florença)

Introduction

1.1 - The mammary gland in men, until puberty, is anatomically and histological similar to the gland mammary in women, with small ductal structures, surrounded by specific stroma and fatty tissue. In men, the sex hormone changes in puberty do not induce the development of lobules nor the increase the mammary tissue. Therefore, breast tissue in men is located in the subareolar region and histologically comprises a fibrofatty stroma and ductal structures without terminal duct lobular units or lobules (1). Male breast is not exposed to cyclic variations common in women through the menstrual cycle, nor undergoes modifications during pregnancy, lactation and menopause. A male breast tissue contains receptors for estrogens and androgens and, while estrogens stimulate the proliferation of the mammary ducts, the androgens inhibit this process (2). The testes are responsible for the secretion of 95% of the testosterone, 15% of the estradiol and 5% of the estrone that are produced daily (2). In healthy normal men, the serum concentration of estrogens is very low, and mostly (80%) are due to the peripheral conversion of androstenedione and testosterone in estrone and estradiol, respectively, by the enzyme aromatase (2). Peripheral conversion occurs in the mammary and subcutaneous fat, but

also in the liver, skin, muscles and kidneys (2). The aromatase activity increases with age and with the elevation of the body mass index (2). Also, this condition may occur in a familial setting (3). Several families have been described as showing estrogen excess due to activating mutations of the aromatase gene and the development of pubertal gynecomastia (2).

Gynecomastia and breast carcinoma are the most frequent disorders in male breast and the study of gynecomastia biology may be important to the knowledge of male breast carcinoma (male BC), as both conditions share the risk factors related to high estrogen levels (1,2).

1.2 - Gynecomastia is a common, non-neoplastic, often reversible, growth of the mammary tissue, due to proliferation of ductal and mesenchymal components (World Health Organization/WHO) (4). This condition is the most common benign proliferation in male breast.

1.2.1 - The pathogenesis of gynecomastia is attributed to the imbalance between estrogens and androgens. The estrogen/ androgen balance may be disturbed by many different factors, namely:

- Increased levels of free estrogens secreted by the testes or adrenal glands.
- Extraglandular aromatization of estrogen precursors.
- Decreased estrogen degradation.
- Exposure to estrogen-like chemicals or exogenous estrogens.
- Use of drugs that cause displacement of more estrogen than androgen from sex hormone-binding globulin.
- Decreased androgen production in the testes.
- Increased binding of androgens relative to estrogens by sex hormone-binding globulin.
- Altered androgen metabolism.
- Drug-induced displacement of androgens from their receptors and androgen receptor defects.

1.2.2 - Clinical findings - Gynecomastia may be focal or diffuse, unilateral or bilateral, is often asymmetric and can occur at all ages (5). Lipomastia (pseudogynecomastia) is a separate entity that consists in the enlargement of breasts due to the increase of adipose tissue without fibro-glandular proliferation. It is common in obese men and should not be considered true gynecomastia. However, true gynecomastia

may also be found in obese men (6). On the basis of the mammographic appearance, there are three subtypes of gynecomastia: nodular, dendritic and diffuse (5).

1.2.3 - Histological features of gynecomastia include, in an initial phase, ductal epithelial hyperplasia and stromal fibroblastic proliferation accompanied by the increase in stromal connective tissue and in a late phase, a slight increase in the number of ducts and marked stromal fibrosis (7). An intermediate phase with heterogeneous pattern is commonly found on the morphological analysis of specimens (Figure 3). The identification of lobular structures on histological analysis of gynecomastia tissue is very rare. The development of lobular structures appears to be linked with progesterone exposure. Progesterone acts in synergy with insulin growth factor-1 to form true glandular acini (7).

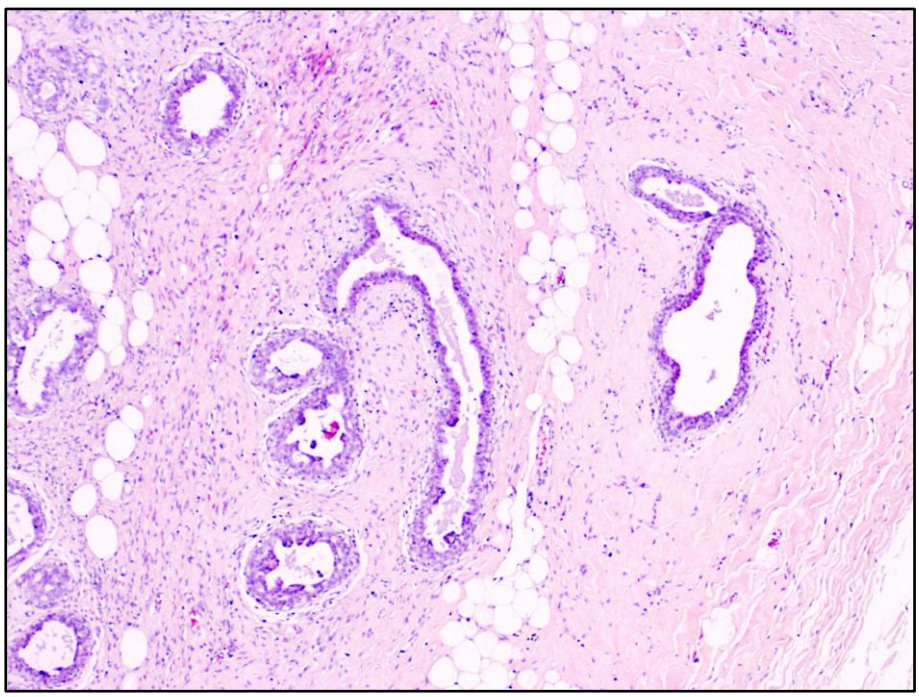

Figure 3 - Gynecomastia: histological section of male breast with proliferation of epithelial cells inside some tubules and an irregular fibroblastic proliferation. Scarce adipose tissue is present (H&E x 100).

1.2.4 - Three clinical subtypes of gynecomastia are described, with no reported specific histological findings:

a. Physiologic gynecomastia is detected in neonatal, pubertal or aging periods of life (4, 5, 6). Neonatal gynecomastia is attributable to the exposure to maternal hormones and usually regress in few weeks (4). Pubertal gynecomastia has been estimated to occur in 30% to 60% of boys during adolescence. Mild degrees of pubertal gynecomastia generally appear at 13 or 14 years of age, last for 6-12 months and spontaneously regress in 95% of the cases. The highest prevalence of physiologic gynecomastia is found in old age and occurs in up to 65% of men over 65 years of age (6). Aging gynecomastia often co-exists with relative hypogonadism, a decline in plasma testosterone levels, elevation of sex hormone-binding globulin, decrease in free testosterone, and progressive adiposity favoring peripheral aromatase activity (6).

b. Pathologic gynecomastia has been related to numerous clinical conditions and medications regimes (3, 6, 7, 8). The most common are further indicated:

- Testicular Sertoli or Leydig cell tumors that may secrete estradiol.

- Choriocarcinoma, other germ cell tumors producing hCG and paraneoplasic syndromes associated with tumors such as carcinomas of the lungs, liver, stomach and kidneys, stimulate testicular Leydig cells to secrete estradiol.

- Pituitary adenomas producing prolactin and adrenocortical tumors with direct secretion of estrogens and steroid precursors.

- Severe hyperthyroidism increases serum sex hormone-binding globulin. Since estradiol binds less avidly to sex hormone-binding globulin than testosterone, this condition results in gynecomastia in 10 to 40% of the patients.

- Primary gonadal failure as a result of testicular trauma, chemotherapy, mumps and orchitis.

- Klinefelter syndrome, a chromosomal disorder (47 XXY karyotype) is associated with hypogonadism, infertility and gynecomastia in almost 70% of the cases.

- Liver cirrhosis is associated with decreased serum testosterone and increased estradiol levels leading to gynecomastia.

- Chronic renal failure is often associated with hypogonadism and may also have associated gynecomastia.

- In men with HIV, gynecomastia occurs in 2-3%, which can be activated by lipodystrophy or highly active antiretroviral therapy.

- Drug-induced gynecomastia may account for 25% of all cases of new-onset gynecomastia in adults. Even though the mechanisms through which many drugs can cause gynecomastia are not fully clarified, they are commonly associated with estrogen-like function, stimulation of testicular production of estrogens, inhibition of testosterone synthesis or blockade of androgen action.

- Androgen deprivation therapy for prostate cancer frequently presents gynecomastia as a side effect in 40-70% of the cases, depending on the type and duration of the hormone therapy.

- Cocaine, heroin and amphetamines are commonly associated with gynecomastia and marijuana is believed to interfere with estrogen receptors and acts as a phytoestrogen.

- The use of anabolic androgenic steroids by male athletes commonly induces gynecomastia.

c. Idiopathic gynecomastia (with no evident cause) is found in 25% to up to 50% of the cases depending on the series, which suggests that multiple environmental endocrine factors may be involved in excessive breast development in men (3, 9).

As previously mentioned, estrogen excess due to activating mutations of the aromatase enzyme have been described and the aromatase excess syndrome is a familial disorder inherited in an autosomal dominant manner (9, 10).

1.2.5 - In gynecomastia treatment intervention, the main purposes are to identify underlying conditions that could be solved and to relieve symptoms (6, 7). In most cases, gynecomastia regress within 2 years without therapy (7). Evaluation of gynecomastia must include a detailed medical history, clinical examination, specific blood tests and imaging tests. Biopsy or aspiration cytology may be an option in the differential diagnosis with male BC or other entities. For patients with non-physiologic gynecomastia, treatment is directed toward the underlying cause or to discontinuing the use of the etiologic agent (7). If gynecomastia is associated with pain, discomfort and psychological distress, medical and surgical options are available (6, 7). While surgery can be performed at any time with similar results, medical treatment is more effective if used as early as possible after signs or symptoms are noted (7). The major medical options are androgens, anti-estrogens, in particular tamoxifen and aromatase inhibitors (6, 7).

1.3 - Male breast carcinoma (Male BC) is a malignant epithelial neoplasm in the male breast. It is a rare entity although, in last years, a slight increase in incidence has been reported in some countries (5, 11, 12). The unviability of screening due to its rarity is one of the factors that contribute to the persistent high percentage of diagnosis of male BC in advanced anatomic stage (5). Also due to its very low frequency, male BC is not as well characterized as female BC and the therapeutic options are based on the recommendations for BC in women (11, 12).

However, in recent years, the available data on male BC push away the hypothesis that male patients only exhibit endocrine-associated BC identical to postmenopausal women, and male BC has emerged as a distinctive subtype of BC requiring its own guidelines (11-18).

1.3.1 - Incidence is one of the most important differences between BC in men and in women.

BC is the most frequent cancer in women worldwide, with an estimated incidence of 1.7 million cases and 521,900 deaths in 2012, accounting for 25% of all cancers and 15% of female cancer deaths (19).

In Portugal, BC is the most frequent cancer and the leading cause of cancer mortality among women, with an estimated 6088 new cases and 1570 deaths in 2012, accounting for 30% of all cancer cases and 16% of all cancer deaths (20). According to Globocan data (21), in 2018, the number of new cases of BC in both genders, in Portugal, was 6984 (Figure 4).

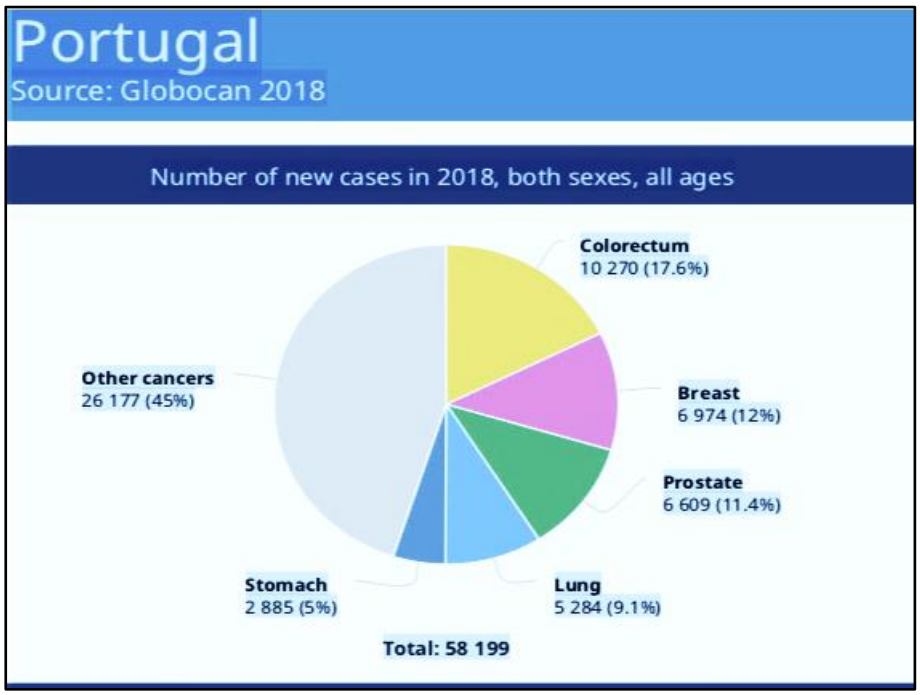

Figure 4 - Incidence of breast cancer new cases in Portugal, in 2018 – Globocan. (21. Portugal – Global Cancer Observatory. Available at https://gco.iarc.fr › factsheets › 620-portugal-fact-sheets)

In opposition, male BC is a rare entity (about 1% of female BC and 1% of all malignancies in men in Western countries). The lifetime risk of BC for men is about 1 in 833 as compared with 1 in 8 for women (21) As in female BC, geographic and ethnic variations have been reported, with the lowest rates of incidence seen in Asian men and the higher rates in men living in West Africa and African Americans. The high number of cases in Africa is thought to be due to endemic infection diseases such as schistosomiasis and hepatitis causing chronic liver damage that leads to high estrogen levels. The highest incidence in the world has been reported in Israel and the lowest in Taiwan (1, 11, 22).

The incidence has been rising in the past few decades and continues to rise (1, 11, 12). Data from the Surveillance, Epidemiology and End Results (SEER) program in USA indicate that the age-adjusted incidence rate has increased from 0.85 cases per 100.000 men in the general population in 1975 to 1.43 cases per 100,000 in 2011 (23). In addition, data from UK, Canada and Australia are consistent with an increase in the incidence of

male BC over the last decades (24). The American Cancer Society projected the occurrence of 2550 cases of male BC in 2018 and 2670 new cases in 2019, compared to 900 cases diagnosed in 1991 (23).

In Portugal, the annual male BC gross incidence rate in 2010 and 2011 was 1.23 and 1.77, respectively, and the gross mortality rate was 0.34 and 0.51 (25).

1.3.2 - Multiple risk factors have been described in male BC and their interaction is likely to occur. Old age, obesity, hormonal imbalances, genetic risk factors including a positive family history (FH) of BC and mutations in predisposing genes, may act together with environmental and occupational exposures (11, 26, 27)**.**

a. Aging and obesity trends - The male BC rising incidence has been attributed to the aging of the population and also to the obesity trends (1, 27, 28). The relationship between aging and epithelial malignancies is frequent, complex and multifactorial, but the impact of age in male BC was described to be even greater than in female BC (27). The usual co-existence of old age and relative hypogonadism, progressive adiposity and consequent high estrogen levels may be important influences. As aging gynecomastia, the incidence of male BC reaches the highest point in the seventh decade of life and, in most countries, men diagnosed with BC tend to be 5-10 years older than women detected with BC (23). In Portugal, in elderly men, obesity is usual (22% and 14.9% in men between 50-59 and 60-64 years old, respectively) (29) confirming the common co-existence of obesity and old age.

b. The heredo-familiar susceptibility is another relevant risk factor for male BC according to population-based studies and several types of susceptibility have been identified (11, 30). Up to 20% of male BC cases arise within families with a background of familial breast and ovarian cancers (5). The risk is particularly increased in cases of an affected sister (relative risk 2.25) or both mother and sister (relative risk, 9.73) (4). Almost half of familial BC have a known genetic cause, whereas the other half remains unexplained. As described in BC in women, it is likely that a large proportion of familial male BC with unknown cause is due to the combinations of moderate and low penetrance mutations, independent of or in combination with environment risk factors (31, 32).

BRCA1 and *BRCA2* are high-penetrance tumor suppressor genes that carry a genetic susceptibility for BC. Men carrying a germline *BRCA* (g*BRCA*) mutation have an estimated increased lifetime risk of developing breast cancer at the age of 70 of 6.8% for

BRCA2 and 1.2% for *BRCA1*, which should be compared with the lifetime risk in the general male population of 1 in 833 (33).

BRCA2 was identified in 1995. It is located on chromosome 13q, consists of 26 coding exons, and plays an important role in the homologous repair process in response to double strand breaks (34). An increased frequency of truncated *BRCA2* mutations in familial male BC compared with *BRCA2* female BC has been described and *BRCA2* is the strongest risk factor for male BC, suggesting a specific etiopathogenesis of BC in male gender (30, 31). The reported rates of g*BRCA2* mutations in male BC vary significantly from 3.7 to 40%, depending on the population studied, on sample sizes, different type of mutations and differences in the sensitivities of mutation screening methods (35). In populations with founder mutations, g*BRCA* mutations account for the higher percentage of cases. The *BRCA2* c.156_157 insAlu mutation is a Portuguese founder mutation, unique to hereditary breast/ovarian cancer families of Portuguese ancestry, accounts for the majority of the *BRCA2* mutations and for about one-third of all deleterious germline mutations in Portuguese hereditary breast/ovarian cancer families (36, 37, 38).

The genophenotypic profile of g*BRCA1* is also different between genders. The risk of BC in a male g*BRCA1* carrier is significantly lower when compared with females. In addition, carcinomas arising through loss of g*BRCA1* have specific clinicopathologic characteristics in female (early-onset carcinomas and association with basal type) comparing with male BC (almost all cancers arise in middle aged to old men and have a Luminal phenotype) (31).

Despite the increase in the use of multigene panel testing in female BC, a limited number of studies have investigated male BC susceptibility genes. In one of the most recent studies, a germline investigation was performed by next-generation sequencing (NGS) focusing on coding and intron-exon regions of 24 cancer predisposition genes in a well characterized series of 81 male BC (33). This study identified germline mutations in 22 patients (23%) in 4 genes*: BRCA2, BRITP1, MUTYH* and *PMS2*. They also found that a positive family history was a strong predictor of g*BRCA2* mutations in male BC and g*BRCA2* accounted for the highest percentage of pathogenic variants identified (22.2%) (33). Other mutations associated with a moderate risk of BC in women, such as those in *PALB2* (partner and localizer of *BRCA2*, which encodes a *BRCA2*-interacting

protein) and *CHEK2* (encodes a cell-cycle checkpoint kinase involved in DNA-repair pathways), have conflicting results regarding the relevance for the risk of male BC (33). Patients with Klinefelter's syndrome also had an increased risk of developing BC (4).

c. Hormonal risk factors are similar to those described in gynecomastia. Hyperestrogenism derived from liver diseases, alcoholic habits, Klinefelter's syndrome, exogenous estrogen use for the treatment of prostate cancer and androgen deficiency due to testicular disease such as hypogonadism, cryptorchidism, testicular trauma and orchitis, seems to magnify the risk of the disease (4). Obesity is one of the most common causes of high estrogen levels in males, because of peripheral aromatization of testosterone in adipose tissue (27). In the MBC Pooling Project, a consortium of 11 case-control and 10 cohort investigations involving 2405 case patients and 52 013 control subjects, the risk of male BC was significantly associated with circulating estradiol levels, weight and body mass index, with evidence that recent rather than remote body mass index was the strongest predictor for male BC (28). However, adolescent overweight has been also associated with an increased risk of male BC (39).

d. Radiation was also found to be a risk factor for male BC (23). The relative risk after radiotherapy was calculated as 7.2. The risk increases in 20-35 years after initial exposure and declines after 35 years (35). Risk factors for male BC are shown in Table 1.

Table I - Risk Factors for Breast Cancer in Men.

Genetic	**Hormonal**
BRCA2	Increased serum estradiol
BRCA1	Liver disease
CHEK2	Obesity
PALB2	Testicular abnormalities
PTEN	Exogenous estrogen exposure
ATM	Treatment related (prostate carcinoma)
Family history of breast cancer	
Epidemiologic	**Environmental**
Increasing age	Radiation exposure
Geographic and ethnic variations	High-temperature
Obesity	Electromagnetic fields

1.3.3 - Clinical presentation of most men with BC is a painless, retro-areolar nodule. Other clinical signs may include nipple retraction, bleeding from the nipple or skin ulceration (4, 23, 30). Unlike gynecomastia, male BC tends to be located eccentrically in relation to the nipple (5). It is usually unilateral but may be bilateral in 0-1.9% of the cases (4). Paget disease of the nipple has been described and palpable axillary lymph nodes are detected in about 50% of cases (4).

The pattern in age distribution of BC at diagnosis differs between sexes and has different prognostic value. The distribution in female BC is bimodal, with one peak corresponding to early-onset of the disease and the other peak to later age at onset, while in male BC the curve has only one peak at 71 years of age (4, 35). Furthermore, in female BC, young age is associated with aggressive tumors and to an unfavorable prognosis (40). On the contrary, in male BC, old age is associated with low 5 and 10-year overall survival (OS) in multiple studies while young age is a favorable prognostic factor (12, 14, 41). A recent study comparing male BC patients aged < 40 years and ≥ 40 years identified in SEER database between 1988 and 2012 confirmed that patients <40 years had a significant overall survival benefit (42).

In current clinical practice, after a clinical examination suspicious of BC, diagnostic confirmation is done by mammography or ultrasonography with histologic examination of core biopsy. Both core biopsy or fine-needle aspiration cytology may be used to evaluate axillary lymph nodes. Although BC in men has become more frequently diagnosed at early anatomic stages, a persistent high percentage of diagnosis in advanced anatomic stages is still reported (12, 15, 18, 43-46).

Beyond the lack of mammographic screening due to male BC rarity and the referred prevalence in old age groups, other factors contribute for a diagnosis at advanced stage, such as the high incidence of lipomastia in obesity and nodular true gynecomastia that may overlap clinical presentation; the particular anatomic characteristics of the male breast; the absence of publicly-available information about the disease; and the fact that males are less likely to report symptoms that would guide to an early diagnosis (5). In addition, for anatomical reasons, men with BC have a higher susceptibility for lymphatic invasion and also for skin and nipple involvement (5).

TNM staging system (AJCC/ UICC) (47) is a powerful prognostic tool. Although the independent prognostic significance of the individual factors has some controversial results in male BC, increased tumor size and nodal involvement have been shown to be associated with worse prognosis in univariate and multivariate analysis (13, 43).

The proportion of *in situ* (pTis) carcinomas in men, is variable among studies, but is significantly low compared with the proportion of *in situ* carcinomas described in women (30, 47, 48). A study from SEER database study revealed that the rate of *in situ* BC rose by 12.3% in men between 1973-2001 (despite the absence of screening mammography in men) compared to 55,5% in women in the same period (49).

Pathologic M category (distant metastasis) is used if distant metastases are biopsy proven (47). It is universally accepted that M1 patients have incurable disease and low survival rates.

Pathological anatomic stage includes all data used for anatomic clinical staging, plus data from surgical exploration and resection, as well as pathological gross and microscopic examination of the primary carcinoma and regional lymph nodes (47). Anatomic stage remains an indispensable tool of the staging process, as a link for comparison of studies and patient populations and a universal terminology for doctors regardless of resources or country. However, the addition of biologic parameters is indispensable for prognosis and therapeutic management (47).

1.3.4 - Clinicopathologic characteristics - Beyond the age at diagnosis and anatomic stage, predictive and prognostic clinicopathologic characteristics, include, as in female BC, the histological type (WHO), histological grade (Elston and Ellis criteria), ER, PR, ERBB2 and Ki67 status. These last 4 factors, evaluated by immunohistochemistry, allow the classification of male BC in clinically defined subtypes. In appropriate settings, a multigene genomic panel testing is additionally proposed for assigning therapy and prognosis (47).

a. Using histological classification as per WHO 2012 criteria (4), invasive carcinoma of no special type (Figure 5) is the most common histological type in both genders and have identical heterogeneous morphology (4). In this group, as well as in other histological invasive subtypes, prognosis and predictive factors are heterogeneous and clinical management depends from other variables (4).

However, the frequency of invasive histological special subtypes show some different distribution male and female BC. Papillary invasive carcinomas, although rare, are more common in males than females (2-4% and 1%, respectively) (4). In addition, the papillary morphology of carcinoma *in situ* in men was described in 74% of the cases in the study of Hittmair, being significantly higher than in women (50). The frequency of papillary morphology either in situ or invasive carcinomas, may be associated with the common subareolar localization in male BC.

In contrast, lobular carcinomas are rarer in males (1-2% and 5-12%, respectively) and have been associated with exposure to estrogens and progesterone (4). Mucinous carcinoma accounts for 2% of all BC and for 1-4% of male BC. Despite the unknown pathogenesis, this histological type has a favorable prognosis in the pure form in female BC, and it seems to present a more homogeneous genetic profile than invasive carcinomas of no special type (51, 52).

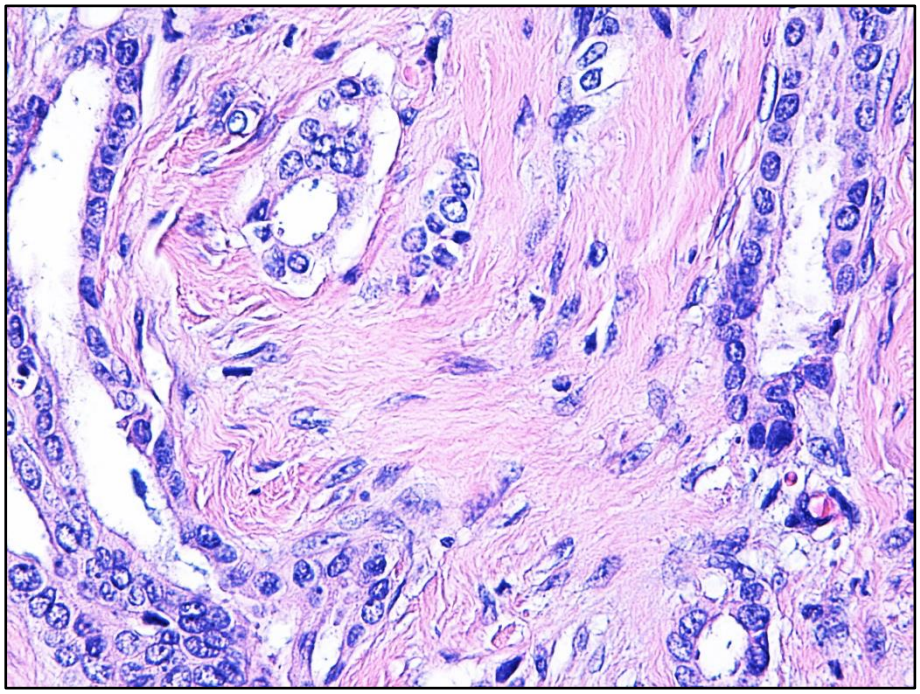

Figure 5 - Invasive male breast carcinoma of no special type, well differentiated (G1) (H&E x 400).

b. Histological grade is considered an important prognostic factor, and according with AJCC recommendations, is included in the pathology reports of BC (4, 47). All invasive breast carcinomas, including male BC, are routinely graded based on tubule formation, nuclear pleomorphism and mitotic count, with a value from 1 (favorable) to 3 (unfavorable) for each feature and totalizing the scores for the three categories as G1 (3 to 5 points - low combined histological grade - favorable), G2 (6 to 7 points - intermediate combined histological grade - moderately favorable) and G3 (8 to 9 points – high combined histological grade – unfavorable) (4). Some studies have found high histological grade to be an independent factor for poor prognosis in male BC while others have not (13, 53, 54). An analysis of data from the SEER program of the National Cancer Institute has shown that histological grade is a valuable prognostic factor, independent of tumor size or number of positive lymph nodes (47). In male BC, the prognostic value of tumor grade was confirmed in multivariate analyses, in a study of Masci *et al*. (53), as the only factor with a statistically significant effect on 5-year OS (p=0.015).

c. Estrogen receptor-alpha (ERα) and progesterone receptor (PR) are predictive and prognostic biomarkers that play a major role in determining the responsiveness to hormonal target BC therapies. ERα corresponds to a steroid receptor protein that is activated by the three main estrogens: estrone, estradiol and estriol. It functions as an intracellular transcription factor and is involved in cell growth and survival. ERα also regulates the expression of PR and the presence of PR usually indicates that ERα pathway is functionally intact (8). The higher the level of expression of ERα and PR, the greater the benefit of hormonal therapies (47). Male BC cases are more frequently hormone-receptor-positive than female BC. In a comparison of existing literature on receptor status in male and female BC, Korde (55) found an ERα positivity of 82% *versus* 69% and a PR positivity of 75% *versus* 56%, in male BC and female BC, respectively (4, 5, 14, 16, 23, 53, 55, 56).

d. The proto-oncogene *ERBB2* codes a tyrosine kinase receptor that belongs to the human epidermal growth factor receptor family. The amplification of ERBB2 leads to proliferation, growth signals independence, increased invasive capacity, increased angiogenesis, and is associated with poor prognosis in both N0 and N1 patients (47). The available data on ERBB2 positive cases and triple negative cases (ER/PR/ERBB2 negative) in male BC are controversial, but the number of ERBB2 positive cases and triple negative cases is usually lower than the reported in female BC (53). Before using ERBB2 target therapy, the outcomes of patients with triple negative and ERBB2 positive carcinomas were similar. The development of ERBB2 targeting agents for the treatment of positive cases combined with chemotherapy, is associated with higher rate of complete responses rates to treatment, and changed completely the outcomes for female patients with the disease (12, 47). In men, prospective clinical trials are unlikely and the limited number of ERBB2 positive cases do not allow retrospective definitive conclusions (53). A case of a male with an HER2-positive locally advanced disease was reported, with incomplete response to a combination of the use of platinum-based chemotherapy and dual HER2 blockade (57).

The importance of predictive and prognostic value of ERα, PR and ERBB2 in BC lead to the jointly published guidelines for their evaluation by American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) (58, 59).

e. Ki67 is a cell proliferation marker expressed by all proliferating cells in the body, in all phases of the cell cycle, except the G0 - quiescent phase. However, its function is still relatively unknown. It is the most commonly used marker to evaluate proliferation, although it lacks standardized methodology or generally accepted cut-offs. Ki67 is included in pathology reports of BC as an additional piece of information to use in clinical decisions (47). With a cut-off of ≥20%, previous studies identified a predominance of low Ki-67 values in male BC (12, 53), and Masci (53) found a poor OS of cases with Ki67 >20% in univariate analysis.

f. DNA ploidy has been reported to be a prognostic marker for BC in women in numerous studies, but conflicting data have limited the prognostic value of DNA flow cytometry and the extent of its clinical use. Discrepant data appears to rely on technical differences, diverse study design, analytical and interpretative methods and quality control programs to ensure the intra- and inter-laboratory reproducibility of results. In addition, intratumoral heterogeneity may also be implicated in the variability of the results. Despite these limitations, the prognostic significance of DNA ploidy in female BC is relatively consensual, at least in univariate models (60). Various studies of the prognostic relevance of DNA cytometry in BC at the IPOLFG (25-year experience) have consistently identified a strong association between histological grade and DNA ploidy in female and male BC, as most G1 carcinomas are diploid and the vast majority of G3 carcinomas are aneuploid (60). Furthermore, our result that aneuploidy can identify subgroups of patients with G1 and G2 tumors with poor prognosis, highlights the significance of the clinical application of DNA ploidy analysis as a complementary or alternative tool to conventional histological grading, providing additional and less subjective prognostic information (60). Bezić *et al* (61) observed aneuploidy in 78% of 31 male patients with BC. In a previous comparative study between sexes (50 cases each), our group also demonstrated a significantly higher frequency of DNA aneuploid tumors in males compared with females (80% *versus* 46%) (62).

1.3.5 - Clinically Defined Subtypes of BC - A molecular classification of female BC was proposed by Perou *et al.* in 2000, using microarray-based gene expression profiling (63). Although gene expression profiling has become a commonly used laboratory technique, whose cost has decreased significantly, it is still not generally validated (47). Therefore, instead of gene expression–based molecular subtypes of BC, clinically defined subtypes (Table 2) are currently used to guide therapeutic decisions. A

relatively high concordance (75-90%) between the intrinsic gene expression subtypes and IHC subgroups is present, although definitions for immunohistochemical cut-off have changed over time. As the criteria for the IHC definition of clinically defined subtypes has been in continuous progress, with consequent difficulties to the comparison of results, some controversy exists about the prognostic role of tumor subtypes in male BC (12, 17, 54). As PR, ERBB2 and Ki-67 are important prognostic and predictive factors, the inclusion of carcinomas with positive or negative PR and/or ERBB2 expression patterns and different Ki-67 cut-offs in the same subtype have been key factors contributing to discordant results.

Table II - Clinically Defined–Treatment Oriented Subtypes of Breast Cancer (Adapted from AJCC Cancer Staging Manual (Eighth Edition, 2017)

Subtypes	**Characterization**
LUMINAL-LIKE	Hormone receptor-positive and HER2-negative luminal disease as a spectrum
(**Luminal A-like**) High receptors, low proliferation	Multiparameter molecular marker of "favorable prognosis", if available high ER/PR and clearly low proliferation rate (low Ki67, low mitotic count); commonly histological grade 1 or 2
(**Luminal B-like**) Low receptor, high proliferation	Multiparameter molecular marker of "unfavorable prognosis", if available; lower ER/PR with high proliferation rate (high Ki67, high mitotic count); histological grade 2 or 3
HER2-LIKE HER2 positive	HER2 positive and hormone receptor negative or HER2 positive and hormone receptor positive; commonly histological grade 3
BASAL-LIKE Triple negative	ER, PR and HER2 negative; commonly histological grade 3

1.3.6 - Commercial multigene panels tests were designed to evaluate levels of expression of a variable number of genes in the BC tissue, most often by measuring the levels of messenger RNA present in the neoplasm (RNA expression profile). Several multigene panels testing have been approved for clinical use such as Oncotype®, Mammaprint and PAM50 (Prosigna). AJCC proposed Oncotype® to be used as a genomic test for assigning prognosis to patients with T1-2, N0, M0, ER positive and ERBB2 negative carcinomas with level of evidence I (47).

There is some debate on the use of multigene panels, once they predominantly represent a substitute for measuring proliferation (47). The AJCC recommendation on the eighth edition is that they should be incorporated into the staging system only on certain subsets of BC (47).

Massarweh *et al.* found that both men and women with lower 21-gene Breast Recurrence Score (Oncotype®) have low mortality from ER-positive BC, and many can be spared to the risks associated with chemotherapy overtreatment (64). Most treatment trials are limited to female BC but recent efforts have shown that clinical trials in male BC are feasible and essential to improve the standard care in these patients (65).

1.3.7 - Therapeutic procedures for male BC are based on the recommendations for BC in women (65). However, mastectomy rather than breast-conserving surgery is performed in the vast majority of cases. Breast-conservative surgery is considered less appropriate in male BC patients mainly due to the scarcity of breast tissue, the frequent central location of the neoplasm, and the common advanced stage at diagnosis (5, 38, 65). Few studies have been carried out on male BC to compare the outcome of patients undergoing mastectomy to those undergoing breast-conservative surgery and equivalent survival rates were found (66). The detection of sentinel node biopsy is also a reliable tool for surgical treatment in male BC (66, 67).

Adjuvant radiotherapy in female and male patients is administrated mainly to eliminate possible residual microscopic disease after surgery and is recommended in male BC patients. No randomized trials have evaluated the role of radiotherapy after mastectomy in men, but population-based observational studies suggested a beneficial effect in a postoperative setting (68).

Systemic treatment includes hormonal therapy, chemotherapy and other target drugs. Most male BC are “dependent” on estrogen for their “survival” and target ER

pathway blockage can be done by inhibiting the ER with tamoxifen or by removing the ligand estrogen with aromatase inhibitors such as anastrozole, letrozole and exemestane. However, the use of tamoxifen on male BC may be limited by the incidence of adverse effects, including weight gain, hot flashes, sexual dysfunction, neurocognitive deficits and thromboembolic events, and in one study, 20-25% of the male BC patients discontinued tamoxifen therapy due to side effects (65, 69). Eggemann *et al*. considered that the risk of thrombotic event markedly increased in the first 18 months of tamoxifen therapy, and is significantly higher in patients older than 71 years (69).

Chemotherapy planning depends on the distinct settings. In a neoadjuvant setting, the objective of chemotherapy is to shrink the tumor to make it operable, in an adjuvant setting is to target possible micrometastases, and in a palliative setting the intention is to shrink the tumor and metastases in order to prolong life with improved quality. Targeted therapy uses drugs that target specific molecules with the purpose of blocking the growth and spread of the tumor and, in the same way as chemotherapy should be offered to men with risk for recurrence or death (65). Examples of these are drugs directed against ERBB2, Poly ADP-ribose polymerase or PARP inhibitors, PI3K/ AKT pathway inhibitors and blocking antibodies to PDL1. PARP inhibitors have been shown to selectively induce cells death enhancement of defective double-stranded DNA break repair mechanisms (synthetic lethality). These inhibitors are interesting for the treatment of metastatic BC with a g*BRCA1* or g*BRCA2* mutation (65). However, few male BC, if any, were included in these studies and the results in men are largely unknown and may not be identical to female BC. For example, although the majority of male BC corresponds to Luminal subtypes, PIK3CA somatic genetic alterations are found less frequently in male than in female BC (5).

1.3.8 - Survival has been a debated issue in male BC. The majority of studies have demonstrated a poorer outcome in male compared with female patients, but others revealed that there is no difference in the prognosis of the two sexes, when paired according to specific groups. A similar disease-free survival (DFS) and OS to pre/peri-menopausal females, but poorer as compared with post-menopausal female BC, was described in male patients with BC, and even a lower risk of mortality with comparable females, despite the frequent presentation in elderly and more advanced disease in male

BC (17, 56, 70-78). In a recent paper, Liu *et al.* included a total of 289.673 BC cases (2054 in male patients) diagnosed from 2005 to 2010, using an analysis of SEER data, and concluded that in recent years, male BC patients have had worse survival outcomes compared to women with BC (Figure 6) (75). The explanation for these high death rates in men with BC may be related to the diagnosis in advanced stage and older age (with consequent comorbidities).

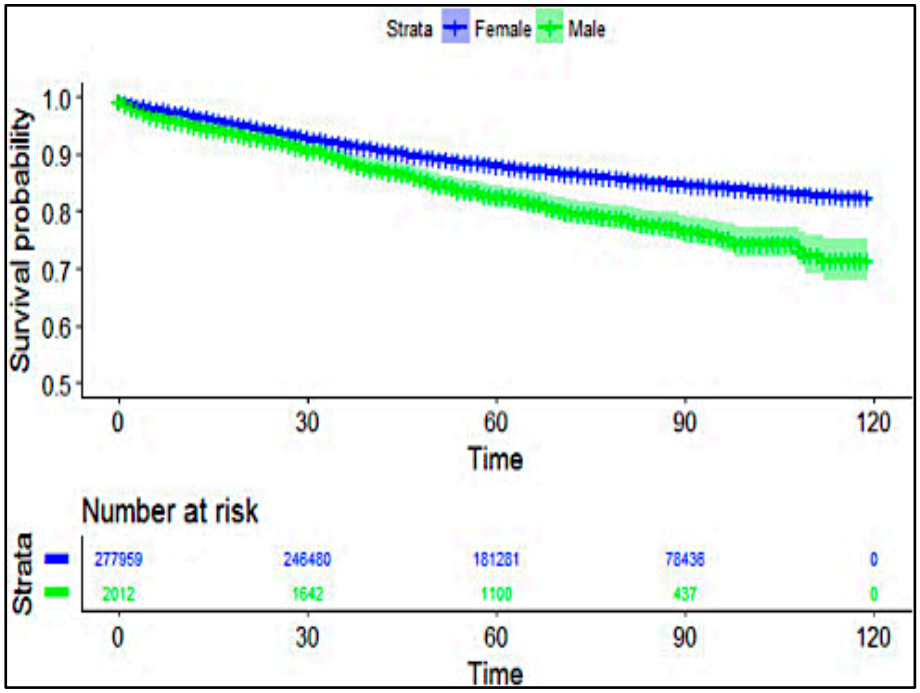

Figure 6 - Kaplan-Meier survival curves for BC patients between genders, diagnosed from 2005 to 2010 (75. Liu N, Kimberly JJ, CynMa CX. Male Breast Cancer: An Updated Surveillance, Epidemiology, and End Results Data Analysis. Clin Breast Cancer. 27, 2018. doi:10.1016/j.clbc.2018.06.013).

1.4 - Epigenetics is described as genome alterations, heritable during cell division, that do not implicate a change in the DNA sequence (79). The most common epigenetic modification is methylation of CpG islands. The cytosine (C) and guanine (G) residues are linked by a phosphodiester (p) bond, and they frequently occur in clusters, so called CpG rich islands, mainly in genomic regions that are relevant for gene expression regulation (80). Through methylation of the CpG islands, the binding of histones and transcriptional protein complexes is disturbed or the recruitment of inhibitor

complexes of methylation binding proteins occurs, which causes the promoter to be less accessible or completely inaccessible for transcription (epigenetic silencing) (81). Methylation regulates several genes, sometimes with such a degree that the gene is silenced, which is called hypermethylation. Hypermethylation occurs in hereditary BC families as well as in sporadic neoplasms (82).

Male BC epigenetic alterations, in particular aberrant DNA methylation**,** although widely acknowledged as an early and relevant event in tumorigenesis, have been seldom reported and with different aims in male BC (82-86).

Kornegoor *et al*. examined promoter methylation of 25 genes in 108 male BCs using methylation specific multiplex ligation dependent probe amplification and concluded that promoter methylation is common in male BC and that a high methylation status correlates with an aggressive phenotype and poor outcome (82). In this study, *BRCA1* and *BRCA2* promoter hypermetilation was found in 2% and 18% of male BC, respectively, comparing with 18% and 64% of female BC, pointing to differences in carcinogenesis between genders (82). Subsequently, Pinto *et al*. found different expression patterns in male and female familial BC in a set of 27 familial BC cases, using quantitative methylation-specific PCR (83). Johanssen et al. performed a genome-wide methylation profiling of 47 male BC, underscoring the heterogeneity of this entity and suggesting that male BC should not be defined using conventional criteria applied to female BC (84). Using methylation-sensitive high resolution, Deb *et al*. assessed a panel of 10 genes in 60 male BCs, concluding that *BRCA2*-associated male BC is characterized by high gene methylation and that the average methylation index might be a useful prognostic marker (85). Finally, Rizzolo *et al*. assessed promoter methylation in 69 male BC patients and concluded that alterations in methylation patterns were common in male BC and might identify specific subgroups related to *BRCA1/2* mutation status and some clinicopathologic parameters (86).

1.5 - Homologous recombination DNA repair (HRR) system is a major surveillance mechanism in the preservation of genome integrity, acting on repair of DNA double-strand breaks, which occur during replication (Figure 7) (87). *BRCA1* and *BRCA2* genes are the most important genes identified in familial BC and are involved in DNA repair (87). *BRCA2* plays an important role in regulating RAD51, thereby being part of the homologous repair process in response to double strand breaks (87). *ATM, PALB2*,

and *RAD51* paralogs also play important roles in HRR pathway (87). *RAD51* paralogs encode for proteins that structurally resemble *RAD51* and congregate *in vivo* into three subcomplexes, comprising *BCDX2 (RAD51B, RAD51C, RAD51D, XRCC2), CX3 (RAD51C, XRCC3),* and the Shu complex (*SWSAP1, SWS1*) and the balance between *BRCA2*, *RAD51* and *RAD51* paralogs seems to be essential in HRR (88, 89). Mutations in HRR genes, either somatic and/ or in the germline occur in multiple conditions, including hereditary breast and ovarian cancer susceptibility syndromes, in which there is also an increased male BC risk (90, 91).

HRR deficiency may be mediated by DNA repair genes aberrant promoter methylation. Owing to the eventual relevance of HRR deficiency in male BC and the lack of systematic studies on altered methylation patterns of HRR genes in this specific context, the epigenetic signature of HRR genes may identify novel detection, diagnostic and/or prognostic biomarkers that might improve the clinical management.

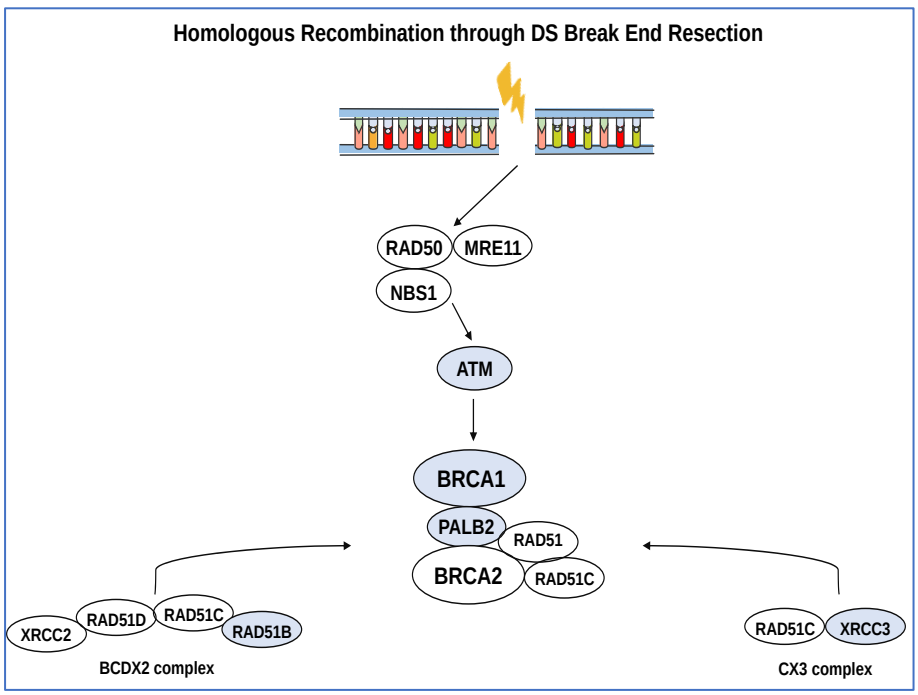

Figure 7 - Homologous recombination through double-strand break end resection (created by Sandra Nunes for the second study / Figure 1. Graphical abstract).

1.6 - Intertumoral and intratumor heterogeneity versus homogeneity seems to be critical in the management and prognosis of BC and an important factor to consider in the efforts to develop combination therapies with curative purposes (92, 93).

The intertumoral heterogeneity was first achieved by the clinicians, who stated the common diverse prognosis of BC, leading to the identification of hormone dependent carcinomas and to the grouping in clinical anatomic stages. Pathologists recognized intertumoral and intratumoral heterogeneity and constructed the histological type classification and immunohistochemistry classification with ER, PR and ERBB2 carcinomas. Finally, genetic and epigenetic data revealed the complexity in the genomic abnormalities of individual malignant cells and also a significant intratumoral molecular spatial and temporal heterogeneity (92, 93, 94).

The intratumor heterogeneity, routinely observed in morphological and IHC patterns, may be responsible for pathological diagnostic inconsistencies and may have clinical relevance on different outcomes and for BC therapy response (92, 93). Breast tissue shows an exuberant high degree of phenotypical heterogeneity, present in benign and malignant conditions. Intratumor heterogeneity designates the coexistence of different clones of malignant cells that vary in their genetic, phenotypic or behavioral characteristics within a given primary tumor (spacial heterogeneity), and between a given tumor and its metastases or recurrences (temporal heterogeneity) (92).

The intratumor phenotypic diversity may result from distinct genetic aberrations in distinct cell clones or from other mechanisms such as epigenetic events or pathway alterations in genetically similar areas (93).

In a recent study, Reiter *et al.* evaluated the extent of genetic heterogeneity and its clinical relevance in different types of untreated epithelial cancers, and found that the homogeneity of predicted functional mutations in driver genes, in primary tumors and respective metastasis, is the rule rather than the exception (95).

Tumor microenvironment (TME) has also emerged as having a crucial role in modulating the evolution of carcinomas and as a factor of tumor heterogeneity. Cumulative evidence has showed that disease outcome is dependent on the intrinsic features of malignant epithelial cells, but TME also provides an active contribution (96). Biologic functions of stromal constituents, specifically of fibroblasts, although complex and not totally understood, seems to be important in neoplastic progression. Stromal

specific component of carcinomas includes not only fibroblasts ("cancer associated fibroblasts"), but also extracellular matrix, immune cells, endothelial cells and adipocytes. The fibroblasts have been proposed to have several roles such as altering the organization of the basement membrane, making it permissive for malignant epithelial cells invasion, inducing the degradation and remodeling of the extracellular matrix, activating epithelial mesenchymal transition and modifying angiogenesis (96), as well as accounting for carcinoma metabolic remodeling (97). At the morphological level, there are different phenotypes of stroma associated with male BC. Some carcinomas are associated to an important desmoplastic reaction while others do not initiate a stromal response and other have a prominent lymphoid infiltrates or tumor associated macrophages. Importantly, fibroblasts seem to be a relatively stable component of TME, representing a potential therapeutic target with low chances of developing drug-resistance (96). However, the translation of therapeutic management that disrupts the dynamics of TME, requires a better understanding of their different components (96).

1.7 - Gynecomastia and male BC are both multifactorial conditions and share risk factors related to high estrogen levels.

In mammography, nodular gynecomastia appears as a subareolar mass and may mimic BC (5). Atypical ductal hyperplasia, carcinoma *in situ* and invasive carcinoma, can be found in resection specimens of gynecomastia, but with a very low prevalence (each <1% of the cases) (98). Inversely, the reported incidence of patients with male BC and coexisting gynecomastia is difficult to assess and has a large variation in literature (99) At present, gynecomastia is not considered a risk factor for male BC, but a possible relationship between gynecomastia and male BC remains to be established (100). Gynecomastia has been used in male BC research as a benign male breast tissue control (27, 100). Accordingly, we analyzed epithelial and stromal characteristics of gynecomastia as a comparative model, since it is the most common benign condition in male breast, related to high estrogen levels, also occurring in old men, and also because its discrimination from BC may be clinically and radiologically challenging (5).

1.8 - The relevance of studying male BC is due to the asymmetry in the knowledge of this condition in relation to female BC. Beyond the factors used in current clinical practice for BC in women, biological specifiers for disease control in men are undetermined. Furthermore, the low incidence of this condition has not encouraged efforts in improving research and prevention. Later detection, neoplastic dissemination

and drug resistance are still obstacles to male BC treatment efficacy and cure, and worse survival outcomes compared to women with BC have been reported. This reality reinforces the need to ongoing in the study of BC in men.

In this thesis, we seek to demonstrate that male BC is a clinical entity with specifiers that distinguishes it from female BC, and therefore it ought to be analyzed in a specific way. To contribute to this task, we investigated a retrospective series of male BC. In a first step we assessed the clinicopathologic and molecular characteristics that are currently the basis of therapeutic strategies, as well as g*BRCA* mutations and DNA flow cytometry status, and we estimated their association and relationship with prognosis. In order to deepen research and achieve innovate results, we performed an epigenetic evaluation of promoter methylation levels in samples from male BC, the corresponding adjacent normal tissues and some cases of gynecomastia cases with the purpose of identifying novel detection, diagnostic and/ or prognostic biomarkers.

Furthermore, using immunohistochemistry, we appraised some phenotypical malignant epithelial homogeneity or heterogeneity burden in tumor progression as well as the concomitant characteristics of TME fibroblasts, by analyzing the pattern of different markers previously implicated in gene expression, tumor proliferation, differentiation, invasion, migration, metastasis, and survival and, in general, considered potential tools for targeted therapy. We estimated their interrelation and association with prognosis.

Chapter II

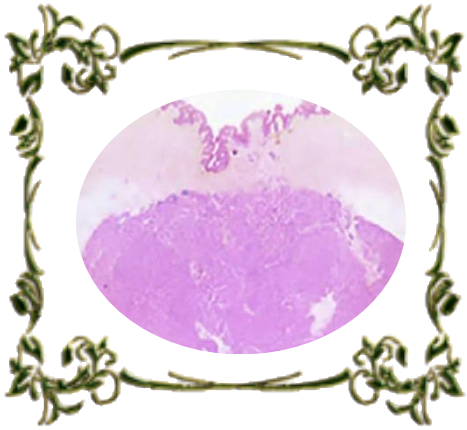

Figure 8 - Male breast carcinoma (H&E x2)

Aim

As a consequence of scientific progress, the vast and complex knowledge achieved in the oncology field in recent years stresses the important task of undertaking a personalized approach to disease management.

Male breast carcinoma (male BC), a multifactorial and distinctive neoplastic disease with low but rising incidence, usually detected in advanced stages and with therapeutic strategies following the recommendations for BC in females, is a practical example of how personalized medicine setting is not yet assured.

Our thesis hypothesis was that male BC has a specific carcinogenesis that confers clinical and phenotypical particular features that can be used to improve diagnosis and allow better prognostication and therapy selection.

The aim of this study was to contribute to a better characterization of clinical, pathological, molecular and epigenetic parameters, gather more data for improving the scientific knowledge of male BC, enabling its early detection, an accurate assessment of prognosis and a personalized definition of therapeutic strategy.

The specific objectives of this study are:

To identify and profile the clinicopathological parameters of a series of male BC cases and obtain a solid cohort for additional research.

To access the impact of germline *BRCA* status.

To evaluate the epigenetic regulation of homologous recombination repair (HRR), a major surveillance mechanism in the preservation of genome integrity in male BC.

To assay potential biomarkers candidates for application in the clinical practice for better prognostication and therapy targets.

To establish a male BC cell line to be used as *in vitro* research.

By accomplishing these specific objectives, we hope to contribute to identify biological factors eligible for further investigation and with potential to innovate the clinical management of male BC.

Chapter III

Figure 9 - Blocks of male BC used in this thesis

Materials and Methods

This thesis is based upon three scientific communications presented in the Chapter IV entitled Results, and each one has its own Material and Methods described in detail. Briefly, we highlight that the material used in this thesis is from the archives of the Pathology Department of IPOLFG and the main procedures were as follows:

Identification and selection of cases in the files

Consultation of clinical records to obtain clinical data

Confirmation of all histological diagnosis and selection of tumor and non-tumor areas to further studies.

Identification of germline *BRCA* status data

Performance of the immunohistochemistry study

Evaluation of *ERBB2* gene amplification status by dual silver *in situ* hybridization (SISH)

Quantification of tumor DNA ploidy by flow cytometry

Construction of Tissue Microarrays (TMAs) (Figure 10)

Evaluation of immunohistochemical scoring

Extraction of tumoral and normal DNA from paraffin blocks

Quantitative methylation-specific PCR (qMSP) of tumor and normal samples

Performance of the statistical analysis

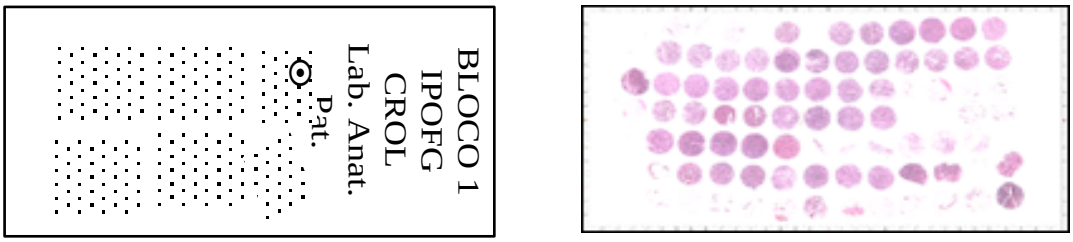

Figure 10 - Tissue Microarray used in this thesis.

Chapter IV

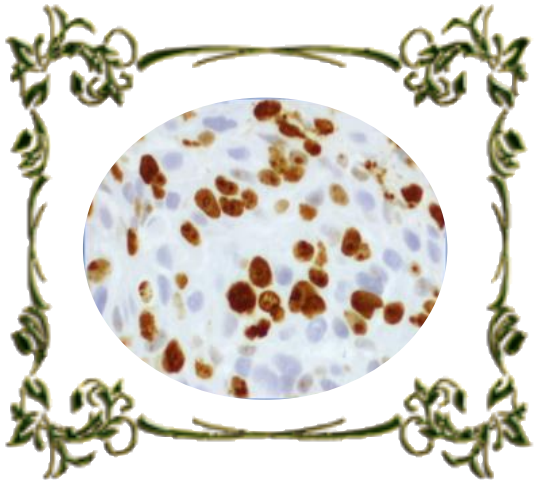

Figure 11 – Ki67 immunostaining in male BC (x400)

Results

Each of the first three scientific papers presented in this Chapter has the results described in detail (4.1 - Male breast cancer: Specific biological characteristics and survival in a Portuguese cohort. 4.2 - Epigenetic alterations in homologous recombination DNA repair genes in male breast cancer. 4.3 - Male Breast Cancer – Relevance of FASN, ATF3 and Collagen IV).

However, the characterization of g*BRCA2* mutations variants present in men with BC was not fully described in these articles.

The importance of g*BRCA2* mutations in male BC leads us to present a table with g*BRCA2* mutation variants and also to highlight one scientific article and three previously published abstracts within this subject (4.4 - *BRCA2* germline mutations).

Chapter IV

4.1 - Saudade André, Teresa Pereira, Fernanda Silva, Patrícia Machado, Fátima Vaz, Mariana Aparício, Giovani L. Silva, António E. Pinto.

Male breast cancer: Specific biological characteristics and survival in a Portuguese cohort.

Mol Clin Oncol. 2019 Jun;10(6):644-654. doi: 10.3892/mco.2019.1841. Epub 2019 Apr 8. Pubmed PMID: 31031981. PubMed Central PMCID: PMC6482395.

4.1 - 

Male breast cancer: Specific biological characteristics and survival in a Portuguese cohort

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Received September 19, 2018; Accepted March 29, 2019 DOI: 10.3892/mco.2019.1841

Abstract

Male breast cancer (BC) represents an individual subtype of BC, with therapeutic procedures based on female BC therapy results. The present study evaluated the parameters currently used for the characterization and therapy of male BC, and their association with disease-free (DFS) and overall survival (OS), aiming to obtain a comprehensive basis to improve the personalized care of male BC. A total of 196 patients from March 1970 to March 2018 (mean follow-up, 84.9 months) were profiled, using clinicopathological review, molecular assessment [BRCA1/2, DNA repair associated (BRCA1/2) status, immunohistochemistry, fluorescence *in situ* hybridization and DNA flow cytometry] and Cox regression statistical analysis. The median age of patients was 66.5 years. At presentation, 39.2% of patients with invasive carcinomas were in anatomic stage (AS) I. Patients exhibited primarily invasive carcinomas of no special type, histological grade 2, estrogen receptor α-(ERα) and progesterone receptor (PR)-positive, receptor tyrosine kinase erbB-2-negative, high Ki-67, Luminal B-like and aneuploid

tumors. A total of 13 of the 44 (29.5%) BRCA-evaluated patients exhibited BRCA2 mutations, significantly associated with family history (FH), bilaterality, high Ki-67 expression, absence of PR and Luminal B-like tumors. Bilaterality was associated with the occurrence of non-breast primary neoplasms (NBPN). The 5 and 10-year DFS rates, excluding patients with distant metastasis, NBPN and *in situ* carcinomas (n=145) were 65.9 and 58.2%, respectively, and the 5 and 10-year OS rates were 77.5 and 59.2%, respectively. In the univariate analysis, Luminal B-like subtype, BRCA2 mutations, high Ki-67 expression, and AS II and III were significantly associated with shorter DFS and OS. In addition, age >70 years was associated with low OS. In the multivariate analysis, FH, AS II and III, and Luminal B-like subtypes were associated with poorer OS. In conclusion, the data from the present study emphasize the high incidence of BRCA2 mutation in male BC, and its association with FH, bilaterality, high Ki-67 expression, negative PR expression and Luminal B-like subtypes, and with shorter DFS and OS in univariate analysis.

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Keywords: male breast cancer, clinicopathological characteristics, molecular features, survival

Introduction

Breast cancer (BC) is a heterogeneous and complex disease, with a great variation in clinical outcomes. BC is the most frequently diagnosed cancer in females and second in causes of cancer mortality in both sexes, as metastatic BC remains an almost incurable disease. Incidence of male BC is rare (~1% of numbers of female BC and 1% of all malignancies in males in Western countries); however, in previous years, a slight increase in incidence has been observed in certain countries (1-5). BC in males has become most frequently diagnosed at an early anatomic stage (AS), and an improvement in overall survival (OS) has been observed (6-9). However, the lack of information regarding male BC and the unviability of screening have contributed to a persistently high percentage of diagnoses at advanced AS. In Portugal, the annual male BC gross incidence rates in 2010

and 2011 were 1.23 and 1.77 per 100,000 people, respectively, and the gross mortality rates were 0.34 and 0.51, respectively (10).

Despite increasing interest, the biology and optimal management of male BC remain poorly understood, and contradictory data are often identified. Certain common epidemiological risk factors, which remain to be identified in either sex, may be relevant in the understanding and prevention of the disease (11). Genetic predisposition appears to be an important contributor to risk and may have clinical implications (8, 12, 13). Family history (FH) is also relevant, and a positive FH in a masculine family member is strong indication for genetics consultation (13). In addition, genetic mutations may be identified in patients without FH and should be routinely screened in male BC (13). In contrast to those identified in females, mutations in the BRCA2, DNA repair associated (BRCA2) gene are predominant in male BC, while BRCA1, DNA repair associated (BRCA1) gene mutations are infrequent (8, 12, 14, 15).

Obesity is one of the most common causes of hyperestrogenism in males, and adolescent overweight has been associated with an increased risk of male BC (2, 12, 16). In addition, liver diseases, alcoholism, Klinefelter's syndrome, exogenous estrogen use (namely for the treatment of prostate cancer) and androgen deficiency due to testicular disease including hypogonadism and orchitis, appear to increase the risk of disease (2, 4, 7). Occupational and environmental exposures to radiation, and heat and electromagnetic fields have also been implicated as potential risk factors (3, 8, 12).

Male BC is diagnosed by mammography or ultrasonography and confirmed by core biopsy, which are always performed following a suspicious clinical examination. Therapeutic procedures are based on the recommendations for female BC, but mastectomy rather than breast-conserving surgery is performed in the vast majority of cases. In addition, hormone therapy is less tolerated in males compared with in females, and side effects including weight gain, depression, deep venous thrombosis, decreases in libido and impotence, with high rates of discontinuation, were described (4,5,17,18).

Molecular testing in BC, through the use of sophisticated techniques including deep sequencing analysis, has led to an improved understanding of this disease. Concomitantly, the identification of targeted therapies has reinforced the requirement for improved stratification in BC subtypes (13). The present study investigated a

retrospective series of male BC cases, assessed the clinicopathological and molecular characteristics that are currently the basis for therapeutic strategies, and estimated their association and significance in disease-free survival (DFS) and OS of male BC.

Patients and methods

Patient selection and clinicopathological evaluation

The present retrospective study involved 196 male patients with BC diagnosed and treated according to therapeutic protocols from March 1970 to March 2018 (mean follow-up time, 84.9 months), at the Portuguese Institute of Oncology of Lisbon (Lisbon, Portugal). The institutional Ethical Committee of the Portuguese Institute of Oncology of Lisbon approved the study. Patient data, including age, obesity, FH, bilaterality, presence of non-breast primary neoplasms (NBPN), presence of distant metastasis and therapeutic modalities were obtained by review of the clinical records. All slides were reviewed. AS classification included pathological tumor size (pT), pathological axillary nodal status (pN) and distant metastasis (M), and was registered according to the TNM classification system recommended by the 8th edition of the American Joint Committee on Cancer (AJCC) staging system (19). The histological type was re-evaluated as per the World Health Organization 2012 classification (20). Histological grade of differentiation (G) was assessed using the Elston-Ellis grading system criteria (21).

BRCA status

Nucleic acids were obtained from peripheral blood nucleated cells. DNA was extracted with the EZ1 Bio Robot and the EZ1 DNA blood kit (Qiagen GmbH, Hilden, Germany) and RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 44 patients were pre-screened for the c.156_157insAlu *BRCA2* Portuguese founder mutation, analyzed for *BRCA1/2* point mutations by next generation sequencing (NGS) with a CE-IVD MASTR BRCA molecular diagnostic assay (Multiplicom; Agilent Technologies, Inc., Santa Clara, CA, USA) in a MiSeq Instrument (Illumina, Inc., San Diego, CA, USA) and for large rearrangements with a Multiplex Ligation-dependent Probe Amplification (MLPA) assay using P002 BRCA1 and P045 BRCA2/CHEK2 kits (MRC-Holland, Amsterdam, The Netherlands). Variant Studio v.2.2 (Illumina, Inc, San Diego, CA, USA) and DNAnexus, Inc, Mountain View, CA, USA were used to analyze the NGS data, and Coffalyzer (MRC-Holland, Amsterdam,

The Netherlands) software was used for the MLPA data. In addition to the information provided in the Variant Studio and DNAnexus programs, the Breast Cancer Information Core database and the Universal Mutation Database were used to clarify certain variants. Prior to NGS screening, patient samples were analyzed by conformation sensitive capillary electrophoresis or conformation sensitive gel electrophoresis, and samples with different patterns (fragment pattern comparison between the 44 patient samples analyzed in the same batch and also comparison with a negative control) were sequenced by Sanger sequencing using an ABI 3130 instrument as described previously (22,23).

Estrogen receptor α (ERα), progesterone receptor (PR), receptor tyrosine kinase erbB-2 [(ERBB2), antigen Ki-67 (Ki-67) immunohistochemistry (IHC) and ERBB2 in situ hybridization (ISH)]

IHC was performed using a peroxidase-indirect-polymer technique performed on a Ventana BenchmarkTM ULTRA instrument (Ventana Medical Systems, Inc.; Roche Diagnostics, Basel, Switzerland) on formalin-fixed paraffin embedded tumor tissues. All cases were re-analyzed under the same conditions for all samples within this study and all kits were used according to the protocol of the manufacturer. The levels of ERα clone SP1 (Ventana Medical Systems, Inc.; Roche Diagnostics; cat. no. 790-4324) and PR clone IE2 (Ventana Medical Systems, Inc.; Roche Diagnostics cat. no. 790-2223) were recorded as the percentage of positively-stained neoplastic cell nuclei, using a ≥1% cut-off value as the criterion for positivity. The staining intensity was not evaluated (24). The ERBB2 clone 4B5 (Ventana Medical Systems, Inc.; Roche Diagnostics cat. no. 790-2991) was used for ERBB2 evaluation. The quantification of ERα, PR and ERBB2 oncoprotein overexpression (0, 1+, 2+ and 3+) was based on the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guide- lines (25). The Ki-67 index was recorded as the percentage of positively-stained cells, using the Ki-67 clone 30-9 (Ventana Medical Systems, Inc.; Roche Diagnostics; cat. no. 790-4286), and 'hot spots' were classified as those areas containing 100 malignant cells. The threshold for high proliferation was 20%, based on the adaptation of the 2013 St. Gallen consensus guidelines (26). ERBB2 gene amplification was determined by FISH using a BenchMarkTM Ventana® system (Ventana Medical Systems, Inc.; Roche Diagnostics) or, in samples collected from 2009, by silver *in situ* hybridization (SISH) with evaluation

of chromosome 17 (Dual-Color SISH red ISH/BenchmarkTM ULTRA Ventana®; Ventana Medical Systems, Inc.; Roche Diagnostics) in 77 cases, which included 38 IHC-negative (0/1+), 35 equivocal (2+) and 4 positive (3+) cases, according to the latest ASCO/CAP guidelines. The IHC pattern, complemented with ERBB2 ISH, allowed the identification of clinically-defined, treatment-oriented subtypes (CS), according to AJCC, 8th Edition (19), and were as follows: Luminal A-like (high hormone receptors and low proliferation level), Luminal B-like (low hormone receptors and high proliferation level), HER2-like (ERBB2-positive and negative or positive hormone receptor expression) and Triple negative (TN; ER-, PR- and ERBB2-negative).

DNA flow cytometry

DNA flow cytometric analysis was performed on representative paraffin-embedded tissue according to the method described by Hedley *et al* (27), with slight modifications (50 μm sections and propidium iodide DNA staining were used). DNA content of the neoplastic cells was determined in 79 invasive carcinomas with no neoadjuvant therapy. The cell cycle analysis of DNA histograms was assessed using the Multicycle software program (32-bit version; Phoenix Flow Systems, San Diego, CA, USA). The male BC cases were also classified as diploid vs. aneuploid according to their nuclear DNA ploidy status.

Statistical analysis

A survival study with an initial descriptive analysis and subsequent nonparametric, semiparametric and parametric statistical techniques was elaborated. The statistical analysis was performed using the software R Core Team 2018 (28). Pearson's χ^2 and Fisher's exact tests of independence were used to evaluate the association between categorical variables. Fisher's exact test was used when the number of observations was small ($n<20$). Identical conclusions were obtained following the use of each test. Survival curves were estimated using Kaplan-Meier analysis, and the differences between curves were assessed by the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference. The DFS represented the remission time until a relapse event. The OS was defined as the interval between pathological diagnosis and the occurrence of mortality due to BC. Patients without disease recurrence during the study period and those who succumbed to other causes, or those who were lost to follow-up, were considered as

censored observations. The OS was evaluated in the subgroup of patients with M1 disease, and the DFS and OS in the whole series, excluding patients with M1 disease, NBPN and *in situ* carcinomas. OS and DFS were also evaluated and compared in the following groups: Patients with vs. patients without NBPN, and patients diagnosed in the years 1970-1998 (group A; n=84) vs. those diagnosed in the years 1999-2018 (group B; n=132) (prior and subsequent to the introduction of taxane chemotherapy). The variables describing the type of treatment were excluded due of the variability of protocols used. A Cox proportional hazards regression model was employed to assess the independent prognostic value of the variables. Initially, the model was calculated for each variable to evaluate their effects on OS time and remission time of disease, as a simple regression analysis. Following the determination of significant variables, a Cox regression model was performed with all variables simultaneously, as a multiple regression analysis. A backward stepwise procedure based on Akaike's Information Criterion (29) was used to select auxiliary variables. The statistical significance was obtained by the Wald test, and complementary inference was calculated as relative risk and 95% confidence intervals for each category. The quality of the models was assessed using a residual analysis, and a test of proportionality of risk functions was conducted for Cox regression models associated with OS and DFS.

Results

Descriptive analysis and associations.

The clinicopathological and therapeutic characteristics of the series are summarized in Table I. *BRCA* mutation, hormone receptors and Ki-67 protein expression, ERBB2 overexpression and amplification, CS and DNA ploidy data are presented in Table II. Significant associations between variables are indicated in Table III.

The mean and median age of patients at diagnosis was 65.2 and 66.5 years (range, 31-89 years), respectively. The majority of the patients (n=108; 55.1%) were between 40 and 69 years, and older patients (≥70 years) comprised 41.3% of the sample. Old age (≥70 years) exhibited a significant association with pT4 (P=0.021). Body mass index was not evaluated in the majority of the cases; however, from review of the clinical records, obesity was observed in ~20% of the patients.

A confirmed FH of BC was obtained in 30 patients (15.3%). FH is significantly associated with G2/G3 carcinomas, high Ki-67, Luminal B-like subtype, high anatomical stage, presence of *BRCA2* mutations and bilaterality. A total of 7 patients (3.6%) exhibited bilateral carcinomas, and 1 patient exhibited synchronous tumors. FH and bilaterality were significantly associated with *BRCA2* mutations. Bilaterality was also associated with the presence of NBPN. The occurrence of NBPN was identified in 28 patients (14.3%). One patient exhibited 3 other carcinomas, in the prostate, colon and kidney, and 2 patients exhibited 2 carcinomas, in the prostate and bladder and in the prostate and kidney. From the remaining patients, 12 had prostate carcinomas, 3 presented with colon-rectal carcinomas, 3 exhibited head and neck squamous cell carcinomas, 3 had gastric carcinomas, 1 had papillary thyroid carcinoma, 1 exhibited chronic lymphocytic leukemia, 1 had Hodgkin disease and 1 exhibited soft tissue histiocytic sarcoma. In the majority of cases, BC was the first neoplasm recorded. In 5 patients, it was the second neoplasm observed; 2 of these patients had exhibited lymphoma previously. A total of 2 patients with prostate carcinoma also had bilateral BC.

A total of 79 patients (40.3%) presented with pT4 carcinomas and 110 (56.4%) with axillary lymph node metastasis. The majority of the patients (n=178; 90.8%) had no distant metastasis at presentation (M0). At diagnosis, 61 patients (31.1%) were diagnosed with AS III disease. Nodal status and AS were significantly associated with Ki-67. Distant metastases at presentation (M1) were associated with ERBB2-positive carcinomas.

Regarding the histological type, 177 (90.3%) invasive carcinoma of no special type (NST) were identified, ~25% of which exhibited a range of proportions of *in situ* components, and the other most frequent invasive subtypes were mucinous carcinoma (1 pure and 4 mixed) and papillary carcinoma (4 cases). The case of pure mucinous carcinoma belonged to a 37 years old patient with no FH, diagnosed with a pT2/pN0/M0 tumor, G2, ER/PR-positive, ERBB2-negative and BRCA indeterminate, who survived with no recurrence during a follow-up of 96 months. A total of 2 patients (1%), at 51 and 64 years old, were diagnosed with lobular invasive carcinoma, one with a FH and the other with a pathogenic BRCA2 mutation.

The majority of the male BC cases were graded as G2. A total of 45 cases (23.7%) were classified as G1, and only a minority of the cases (18.4%) was integrated in the G3 group. High grades were associated with high Ki-67 levels.

During the present study, therapeutic strategies for male BC followed the patterns of the recommendations for BC in females. The majority of patients (90.3%) underwent surgery, but only 9 patients (4.6%) performed breast-conserving surgery. A total of 33 patients (16.8%) received neoadjuvant therapy. Adjuvant radiotherapy was used in 63.3% of the patients, adjuvant hormonotherapy in 60.2% and chemotherapy in 37.2% of the patients. ERBB2-targeting agents were used in 2 patients in the cohort.

BRCA2 mutations were identified in 13 (29.5%) of the 44 patients examined and, in addition to the associations with FH and bilaterality, were also significantly associated with high Ki-67 and negative PR expression levels. A total of 10 (76.9%) confirmed BRCA2 mutated carcinomas belonged to the surrogate Luminal B-like subtype and 2 cases were HER2-like, according to the AJCC 8th edition classification system (19). No BRCA1 mutations were identified in the series.

The majority of male breast carcinomas were ER-positive/ PR-positive/ERBB2-negative, and 14 (7.4%) were TN. All PR-positive cases were ER-positive, and 34 cases (75.6%) of PR-negative carcinomas were ER-positive ($P<0.0001$). Using IHC, 35 ERBB2-equivocal (2+) cases and 6 positive (3+) cases were identified. From the equivocal cases, 5 (14.2%) became positive. In total, 11 ERBB2-positive cases were identified, 10 cases of which were triple-positive and 1 case was ER-positive and PR-negative. Positive ERBB2 expression was significantly associated with M1 carcinomas at presentation, high AS and high Ki-67 expression.

High Ki-67 (n=113; 59.5%) was significantly associated with positive FH, high grades, pN1, high AS, ER-negative, PR-negative and ERBB2-positive expression, and the presence of BRCA2 mutations.

The incidence rates of the 4 clinically-defined CS using IHC, according to AJCC 8th Edition (19), were as follows: Luminal A-like (40.5%), Luminal B-like (45.3%), HER2-like (6.8%) and TN (7.4%).

DNA ploidy pattern was analyzed in 79 cases, revealing a high percentage of aneuploid carcinomas (88.6%). As shown in Table III, aneuploid carcinomas were significantly associated with bilaterality.

Table I - Clinicopathological and therapeutic characteristics of the patients cohort.

Characteristics	**N**	**N (%)**
Age, years	196	
31-39		7 (3.6)
40-69		108 (55.1)
0-89		81 (41.3)
Family history (FH)	196	
No/not evaluated		166 (84.7)
Yes		30 (15.3)
Bilaterality	196	
No		189 (96.4)
Yes		7 (3.6)
Non-breast primary neoplasms (NBPN)	196	
No		168 (85.7)
Yes		28 (14.3)
Tumor size (pT)	196	
pT*is*		7 (3.6)
pT1		52 (26.5)
pT2		58 (29.6)
pT4		79 (40.3)
Axillary nodal status (pN)	195	
pN0		85 (43.6)
pN1-3		110 (56.4)
Distant metastasis (M)	196	
M0		178 (90.8)
M1		18 (9.2)
Anatomic stage (AS)	196	
0		7 (3.6)
I		52 (26.5)
II		58 (29.6)
III		61 (31.1)
IV		18 (9.2))
Histological type (HT)	196	
In situ		6 (3.1)
Invasive no special type (NST)		177 (90.3)
Other invasive subtypes		13 (6.6)
Histological grade (G)	190	
G1		45 (23.7)
G2		110 (57.9)
G3		35 (18.4)
Therapy	196	
Surgery		177 (90.3)
Radiotherapy		124 (63.3)
Hormonotherapy		118 (60.2)
Chemotherapy		73 (37.2)

N, number of patients; (%), percentage.

Table II - Molecular characteristics of the series.

Characteristics	**N**	**N (%)**
***BRCA2* mutations**	44	
Indeterminate		31 (70.5)
Positive		13 (29.5)
Estrogen receptors (ER)	190	
Positive		176 (92.6)
Negative		14 (7.4)
Progesterone receptors (PR)	190	
Positive		143 (75.3)
Negative		47 (24.7)
***ERBB2* (IHC + ISH)**	190	
Negative		179 (94.2)
Positive		11 (5.8)
Ki-67	190	
Low		77 (40.5)
High		113 (59.5)
Clinically defined subtypes (CS)	190	
Luminal A-like		77 (40.5)
Luminal B-like		86 (45.3)
HER2-like		13 (6.8)
TN		14 (7.4)
DNA ploidy	79	
Diploid		9 (11.4)
Aneuploid		70 (88.6)

N, number of patients; (%), percentage; BRCA2, BRCA2 DNA repair associated; IHC, immunohistochemistry; ISH, fluorescence *in situ* hybridization; ERBB2/HER2, receptor tyrosine kinase erbB-2; Ki-67, antigen Ki-67.

Table III - Significant associations between clinical and molecular characteristics of the patient cohort (Pearson's chi-square test).

Characteristics	**P-value**
Age (< 40, 40-69, ≥ 70yrs)	
pT	0.021
Family history (FH) (no vs. yes)	
G	0.009
AS	0.011
Ki67	0.002
CS	0.001
BRCA mutations	0.002
Bilaterality (no vs. yes)	
FH	0.009
Non-breast primary neoplasms (NBPN)	<0.001
DNA ploidy	0.004
BRCA2 mutations	0.008
Tumor size (pT) (pT1 vs. pT2-3 vs. pT4)	
pN	<0.001
M	<0.001
AS	<0.001
PR	0.029
Nodal status (pN) (pN0 vs. pN1)	
M	0.002
AS	<0.001
Ki67	0.003
CS	0.030
Distant metastasis (M) (M0 vs. M1)	
AS	<0.001
CS	0.030
ERBB2	0.009
Anatomic stage (AS) (I vs. II/III vs. IV)	
Ki67	0.004
CS	0.009
ERBB2	0.015
Histological type (HT) (NST vs. others)	
pT	<0.001
pN	0.012
AS	<0.001
Histological grade (G) (G1 vs. G2 vs. G3)	
Ki67	<0.001
CS	0.002
BRCA mutations	
FH	0.002
Bilaterality	0.008
PR	<0.001
Ki67	0.047
CS	<0.001
Estrogen receptors (ER) (positive vs. negative)	
PR	<0.001

Progesterone receptors (PR) (positive vs. negative)	
BRCA2 mutations	<0.001
Ki-67 (low vs. high)	
CS	<0.001
ER	0.004
PR	<0.001
ERBB2	0.011
BRCA2 mutations	0.047
Clinically defined subtypes (CS	
FH	0.001
pN	0.030
M	0.030
AS	0.009
G	0.002
BRCA2 mutations	<0.001
Ki67	<0.001

FH, Family history; G, Grade; AS, anatomic stage; KI-67, antigen Ki-67; CS, subtype; BRCA2, BRCA2 DNA repair associated; M, Distant metastasis; ER, Estrogen receptors; PR, Progesterone receptors; ERBB2, receptor tyrosine kinase erbB-2.

Survival analysis

The 5 and 10-year DFS rates of patients, excluding patients with M1 carcinomas, patients with non-primary breast neoplasms and *in situ* carcinomas (n=145) were 65.9 and 58.2%, respectively, and the 5 and 10 year OS rates were 77.5 and 59.2%, respectively. Mean and median remission times were 75.6 and 50 months (range, 0-312), respectively, and mean and median survival times were 87.8 and 72 months (range, 3-396), respectively. Of the 18 patients with distant metastasis at presentation, only 1 was alive with bone metastasis after 34 months of follow-up. All other 17 patients succumbed to the disease, with the mean and median survival times for all patients with distant metastases (M1) being 18.7 and 15.5 months (range, 1-38 months). The occurrence of NBPN did not decrease OS, as patients with NBPN exhibited 5 and 10-year OS rates of 92.3 and 92.3% compared with 75.5 and 59.2% of patients without NBPN.

Kaplan-Meier estimates indicated that a longer DFS and an improved OS were significantly associated with pT1/pN0/stage I (all $p<0.001$), low Ki-67 carcinomas ($P=0.030$ and $P=0.010$, respectively; Fig. 1 and Tables IV and V), while a shorter DFS and poorer OS were associated with Luminal B-like subtype ($p=0.002$) and the presence of BRCA2 mutations ($P=0.003$ and $p<0.001$, respectively) (Fig. 2). Patients with G3

carcinomas also exhibited a shorter DFS (p=0.020). Additionally, a longer OS was associated with young age (<40 years; p=0.010 and Fig. 3). The patients diagnosed prior to the introduction of taxane chemotherapy exhibited significantly decreased 5 and 10-year DFS (p=0.030) and OS (p=0.050) compared with those diagnosed in the years following the introduction of taxane chemotherapy.

The results of the univariate Cox model analysis (Table IV) were consistent with the Kaplan-Meier analysis. The categories pT2-3 and 4, pN1, AS II and III, high Ki-67, Luminal B-like and BRCA2 mutations were significantly associated with shorter DFS and OS. In addition, G3 and ages >70 years were significantly associated with lower DFS and poorer OS, respectively.

In the multivariate Cox regression analysis (Table V), bilaterality, G3 and AS II and III carcinomas were the significant factors associated with a higher risk of disease recurrence. The presence of FH, AS II and III and Luminal B-like subtype were the significant characteristics associated with low OS.

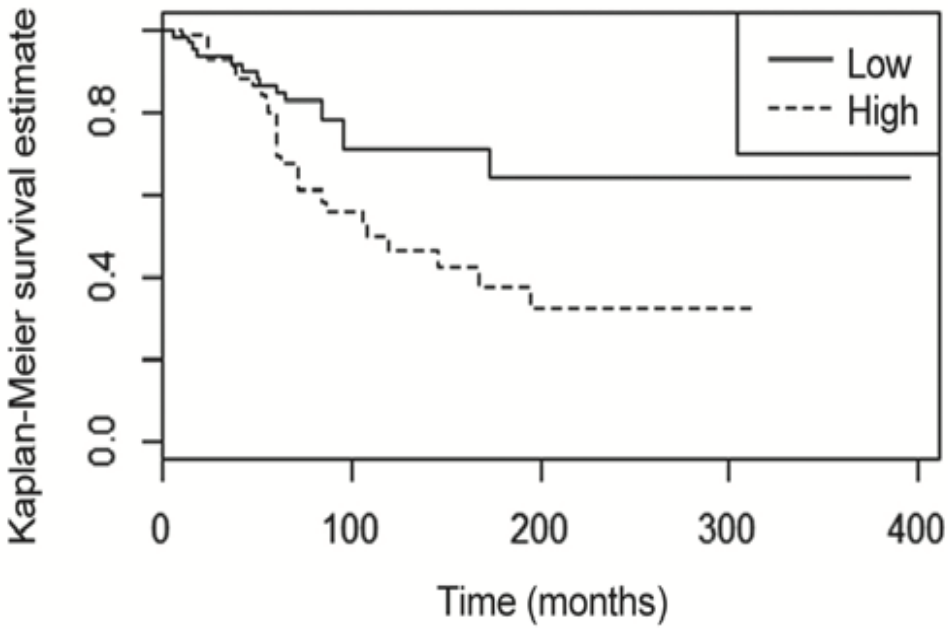

Figure 1 - Kaplan-Meier overall survival curve for Ki-67 index. Log rank tests were used to analyze the curves (p=0.010).

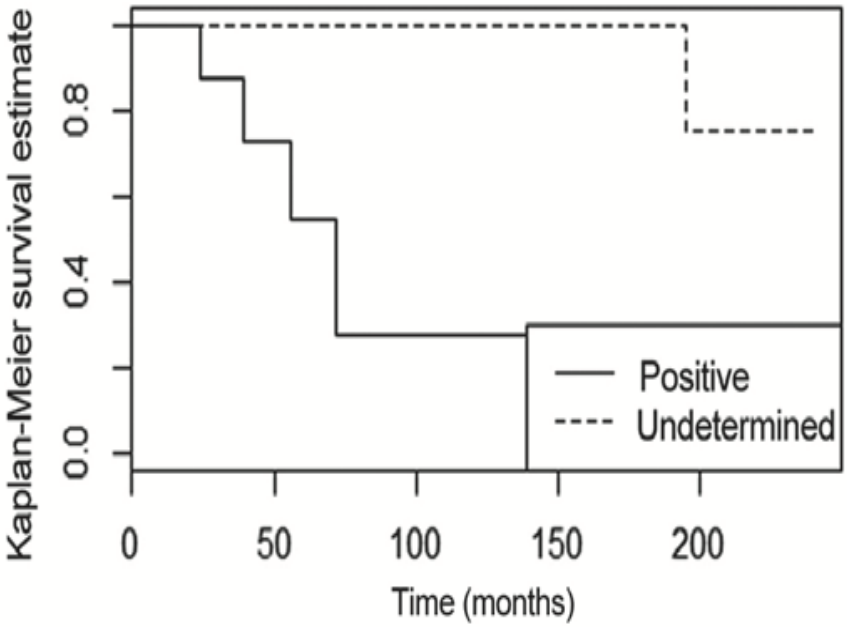

Figure 2 - Kaplan-Meier overall survival curve for BRCA2 mutations. Log rank tests were used to analyze the curves ($p<0.001$).

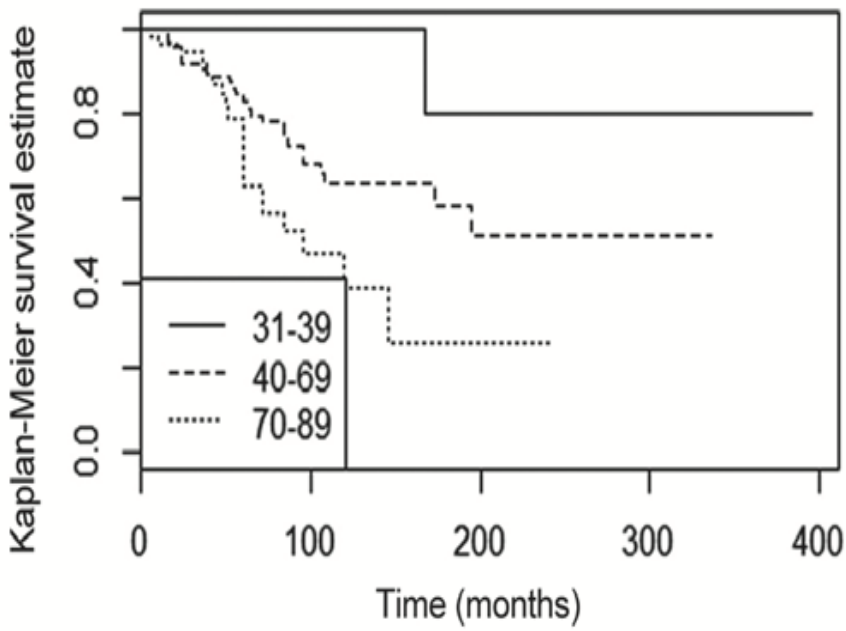

Figure 3 - Kaplan-Meier overall survival curve for age (years) of the patients. Log rank tests were used to analyze the curves ($p=0.010$).

Table IV - Univariate Cox simple regression analysis in relation to DFS and OS.

Variables	Disease-free survival			Overall survival		
	RR	95% CI	P value	RR	95% CI	P value
Age (years)						
31-49	1	-	-	1	-	-
40-69	4.37	0.59-32.3	0.148	4.05	0.54-30.1	0.172
70 -89	6.35	0.84-48.3	0.074	8.02	1.06-60.8	0.044
Grade (G)						
G1- G2	1	-	-	1	-	-
G3	2.14	1.12-4.05	0.020			
Tumor size (pT)						
pT1	1	-	-	1	-	-
pT2-3	3.19	1.23-8-27	0.017	3.55	1.14-11.0	0.029
pT4	6.10	2.50-14.9	<0.001	9.10	3.19-26.0	<0.001
Nodal status (pN)						
pN0	1	-	-	1	-	-
pN1-3	6.92	4.03-19.7	<0.001	5.92	3.21-10.9	<0.001
Anatomic stage (AS)						
I	1	-	-	1	-	-
II	6.71	3.11-14.5	<0.001	7.81	3.28-18.6	<0.001
III	4.80	1.01-22.7	0.048	11.1	2.75-44.9	<0.001
Ki-67						
Low	1	-	-	1	-	-
High	1.89	1.05-3.39	0.033	2.14	1.16-3.96	0.015
Clinically subtypes (CS)						
Luminal A-like	1	-	-	1	-	-
Luminal B-like	2.67	1.45-4.90	0.002	2.97	1.58-5.60	<0.001
HER2-like	0.81	0.11-6.08	0.837	1.25	0.16-9.15	0.010
Triple negative	0.52	0.12-2.23	0.379	0.64	0.15-2.80	0.556
BRCA2 mutation						
Indeterminate	1	-	-	1	-	-
Positive	0.11	0.02-0.63	0.013	0.06	0.01-0.52	0.011

RR, relative risk; CI, confidence interval.

Table V - Multivariate Cox regression analysis in relation to DFS and OS.

Variables	**Disease-free survival**			**Overall survival**		
	RR	95% CI	P value	RR	95% CI	P value
Bilaterality						
No	1	-	-	-	-	-
Yes	6.16	1.30-29.3	0.022	-	-	-
Family history (FH)						
No		-	-		-	-
Yes				0.33	0.13-0.90	0.030
Histological grade (G)						
G1- G2	1	-		1	-	-
G3	2.20	1.10-4.42	0.026	2.06	1.0-4.24	0.051
Anatomic stage (AS)						
I	1			1		
II	4.08	1.53-10.9	0.005	8.95	3.65-21.9	<0.001
III	6.79	1.15-40.2	0.035	45.7	9.92-2.11	<0.001
Clinically defined subtypes (CS)						
Luminal A	1	-	-	1	-	-
Luminal B	1.72	0.92-3.23	0.091	2.05	1.07-3.93	0.030

RR, relative risk; CI, confidence interval.

Discussion

BC is a complex disease that affects women and men, and the primary known difference between sexes is incidence. According to recognized biological heterogeneity of BC, studies comparing female and male BC have demonstrated similarities and differences (1, 3, 7, 30, 31). The understanding of the effects of the clinicopathological, molecular and genomic features in therapy and prognosis is continuously progressing in male BC and, as more data become available, the theory that men only exhibit endocrine-associated BC identical to that in postmenopausal women becomes less credible, and male BC emerges as a distinctive subtype of BC lacking its own guidelines (15, 32-36).

Survival has been a controversial issue in male BC. The majority of studies have demonstrated a poorer outcome in men compared with female patients, but others revealed that there was no difference in the prognosis of the two sexes when paired according to specific groups (15, 30, 32-38). A similar DFS and OS to pre/peri-menopausal women, but poorer compared with post-menopausal female BC, was also described in male BC (32), and even a lower risk of mortality compared with similar female BC was communicated, despite the frequent presentation in elderly and more advanced disease in male BC (1). M1 patients have incurable disease and, in our cohort, all but 1 men succumbed to the disease, with a median survival time of 15.5 months. The present study encompassed a long time period, with slightly longer 10-year OS rates (stages I-III; 59.2%) compared with those demonstrated by Leone *et al* (53.7%) (6), Chen *et al* (40.1%) (38) and Tural *et al* (52.5%) (39).

The risk of developing BC increases with age, similar to the majority of carcinomas at all sites. In the present study, the percentage of the patients aged ≥70 years (41.3%) confirms the high incidence of BC in older males, and also that the average age at diagnosis is ~5-10 years older compared with in females (1,12,36). The high frequency in the elderly population is important, as the therapeutic approach in older male patients is based on studies performed in females of different ages, and comorbidities in the elderly population may result in inadequate treatment. In the present study, elderly patients exhibited larger carcinomas and higher Ki-67 expression levels compared with younger patients, and old age was a prognostic factor significantly associated with low 5 and 10-year OS in Kaplan-Meier estimates, which were concordant with data from previous studies (6, 7, 33, 38, 39). Poorer prognoses in older males may be associated

with tumor biology, late diagnosis, comorbidities and/or inadequate therapeutic management, and constitutes a persistent clinical problem (33, 39). Similar to older patients, obese patients have unknown risk factors affecting the accurate prediction of toxicity of treatments and prognosis (35). Obesity is an important risk factor and the proportion of obese male patients with BC observed in the present study was similar to that identified by Gargiulo *et al* (8, 40).

FH appears to be particularly relevant in male BC. Bouchardy *et al* (41) identified a positive FH in ~20% of male patients with BC, but no significant differences in OS in patients with FH compared with sporadic cases were observed. As the present study included patients diagnosed from 1970 onwards, the majority of patients had no information regarding FH in their clinical records. However, a confirmed family history of BC in a first-degree relative was significantly associated with BRCA mutations, and also to high AS, high grade, high Ki-67 and Luminal B-like subtype. Additionally, in the multivariate analysis, a positive FH was associated with OS. In concordance with previous data (41), positive FH was also associated with bilateral male BC. Bilaterality occurred in 3.6% of the patients in the cohort within the present study, and was significantly associated with BRCA2 mutations and with the presence of NBPN. Male patients with BC also have an increased risk of NPBN, and the long survival times currently observed should be observed cautiously (42-44). A total of ~14% of the patients in the present study exhibited NBPN, and 2 with bilaterality and prostate carcinoma. As described previously (45), prostate carcinoma is the most frequently observed non-breast primary tumor. The risk of head and neck, colon and thyroid carcinomas were demonstrated to be high in male BC (43, 44), and their occurrence was also observed in the present study. A total of 2 patients had previous lymphoma, supporting the observation that males who survive lymphoma may have an increased risk of developing BC (44, 46). Among the factors identified to be responsible for causing a second neoplasm, genetic factors appear to represent an important contribution. These data suggest the requirement of a genetic consultation in male BC. BRCA2 is one of the most frequently mutated genes in male BC, ranging between 4 and 40% depending on the population studied (15); 29.5% of the 44 patients included in the present study exhibited this mutation, while BRCA1 mutations are infrequent; none were observed in the present

study, suggesting a dissimilar genetic etiology between sexes (13). As described previously (8,16), the majority of BRCA2-mutated carcinomas in the present study belonged to the Luminal B-like subtype, and a significant association between BRCA2 mutations and poorer prognosis was observed.

The AS classification systems represents one of the most important established prognostic tools for male BC, as demonstrated in the present study and in previous studies (6,8,34). Male BC is increasingly diagnosed earlier (6, 7), and a predominance of early stages was observed in the present study. However, high rates of advanced stages are frequently observed (1, 6, 8, 12, 33, 36, 37). The unviability of screening due to low incidence rates, the high occurrence of gynecomastia that may exhibit identical presentation symptoms, the fact that males are less likely to report symptoms that would lead to early diagnosis, the absence of publicly-available information about the disease, the incidence in old age with an associated suboptimal access to healthcare, and anatomic and biological differences, may explain the number of diagnoses at high stages observed (4, 36, 39).

The proportion of pure *in situ* carcinomas, one-half with papillary morphology, varies in previous studies, but is significantly decreased compared with the proportion described in females (30, 33, 47). The relative frequency of papillary morphology, either *in situ* or invasive carcinomas, may be associated with the common subareolar localization in male BC. The heterogeneous histological type of invasive carcinoma NST, with an occurrence between 85 and 95% described in previous studies (5, 37), was diagnosed in 90.3% of cases in the present study. The percentage of associated *in situ* components was similar to the proportion demonstrated in females (48). Mucinous carcinoma accounts for 1-4% of male BC, has a favorable prognosis in the pure form, but the pathogenesis is not understood (30, 39, 49, 50). In the present study, 5 cases (2.5% of all cases) were observed, one being the patient with a pure form, unusually young for the described in mucinous carcinomas. Invasive lobular carcinoma, the second most frequent histological type in females (10% of the cases), is exceptionally rare in males (1%) and its etiology remains unexplained considering the lack of development of terminal lobules in males (6, 51). A total of 2 invasive lobular carcinomas (1%) were identified in the present study, both with negative epithelial-cadherin staining.

G2 carcinomas were predominant in the present study, similar to other previous studies (5, 7, 30, 33, 52). High histological grade (G3) is commonly associated with poor prognosis, but this is not always statistically significant (6, 7). In the present study, G3 carcinomas occurred in 18.4% of the cases, and were demonstrated to be significantly associated with FH, high Ki-67 expression, Luminal B clinical subtype and poorer prognosis in univariate and multivariate Cox regression analyses.

The lack of randomized trials in male BC explains why therapy is based on the guidelines for BC in females. However, due to primarily anatomical and hormonal reasons, the management is not exactly the same, highlighting the requirement to improve the personalized care of male BC (17, 38). Breast-conserving surgery vs. mastectomy may be performed in early stages, but is rarely used due to the paucity of breast tissue and the frequent subareolar location of carcinomas associated with the distribution of epithelial breast tissue (6-8, 49). Tamoxifen is the most frequently employed systemic treatment (38, 49), but low tolerance, side effects and high rates of discontinuation have been described (4, 17, 38, 49). The relatively low rate of hormonotherapy compared with the high percentage of ER-positive carcinomas identified in the present study, and demonstrated in previous studies (7,31), may be associated with the fact that the use of tamoxifen in males was only recently standardized (7). Different chemotherapy agents and regimens have been used and the introduction of taxanes marked a significant advance in the treatment of metastatic disease in females, but there are no specific evidence-based guidelines for male BC (33, 35, 49). In addition to the therapeutic effects of the treatment, the improvement in DFS and OS observed in the present study when comparing the groups of patients diagnosed prior and subsequent to taxane chemotherapy may be associated with early diagnosis, standardized clinicopathological evaluation and improved follow-up observed in recent years (1).

Biomarker evaluation by IHC has resulted in differing data among male BC studies, primarily due to different methodologies, the development of scoring systems and the range of cut-off values used, but the high frequency of ER-positive/PR-positive expression and the low frequency of TN carcinomas are concordant (7,8,38,45,52). ER-positive expression is associated with improved prognoses at 5-year OS (6), but certain

clinically aggressive male BC cases do not appear to have an active ER pathway (16). In the present study, negative PR expression status was associated with BRCA mutations.

Despite the different estimates described, ERBB2 positivity has a low frequency in males (7, 51). Using IHC and ISH, 6.8% of the cases in the present study were identified as HER2-like clinical subtypes, according to AJCC 8th Edition (19), and significantly associated with a high Ki-67 expression level and high AS. ERBB2 positivity is generally associated with aggressive phenotypes, but survival of HER2-like carcinomas has improved in previous years, due to specific treatment with associated ERBB2-targeting agents (19, 33).

Cell proliferation is also an important biological factor, usually associated with poor outcome (53). Ki-67, a nuclear protein present during all phases of the cell cycle, is the most commonly used marker to evaluate proliferation, although it lacks standardized methodology or generally accepted cut-offs. With a cut-off of ≥20%, previous studies identified a predominance of low Ki-67 values (7, 8). By contrast, the present study identified a slight predominance of high Ki-67 values, significantly associated with old age, positive FH, high grade, pN1, high AS, CS and poor prognosis in the univariate Cox regression analysis.

The criteria for the definition of BC molecular subtypes used clinically for decisions regarding therapy are continuously progressing, resulting in difficulties when comparing data (5, 7, 15, 30, 38, 52). Using IHC surrogates, Luminal A-like and Luminal B-like subtypes were identified in the majority of BC in males and females, usually with a poorer outcome for Luminal B-like (7,15,26). As PR, ERBB2 and Ki-67 are important prognostic and predictive factors, the inclusion of carcinomas with positive or negative PR and/or ERBB2 expression statuses and different Ki-67 cut-offs in the same subtype are key factors contributing to discordant results.

In the present study, according to the AJCC 8th edition (19), Luminal B-like subtype exhibited the poorest OS.

Bezić *et al* (54) observed aneuploidy in 78% of 31 male patients with BC. In a previous comparative study between sexes (50 cases each), our group demonstrated a significantly higher frequency of DNA aneuploid tumors in males compared with females (80 vs. 46%) (55). The high percentage of aneuploidy, which was observed to be

increased in the present study, suggested a distinctive genomic instability in the carcinogenesis of male BC.

The present study has the limitations of a retrospective study from a single institution conducted over a long time period. However, the results are consistent with those of large and/or multi-institutional studies, confirming that studies involving smaller, but well-characterized clinicopathological and molecular subgroups, diagnosed and followed in multidisciplinary departments within single institutions, are important in improving the understanding of this disease.

In conclusion, the present study demonstrated that male BC was more likely to be diagnosed in older patients (with consequent associated comorbidities and suboptimal therapy), and exhibited poorer prognosis in elderly and in high anatomical stages. BRCA2 mutations were frequent, associated with FH, bilaterality, high Ki-67, PR negativity and Luminal B-like subtype, and with shorter DFS and OS in univariate analysis. In addition, male patients with BC were at high risk for NBPN. In the multivariate analysis, FH and Luminal B carcinomas were associated with poorer OS. These data underline the importance of early diagnosis and genetic screening in male BC. As sex may be a crucial feature to improve personalized care, additional studies investigating male BC are warranted and may lead to the development of relevant management approaches for BC in males and females.

Acknowledgements

Not applicable.

Funding

Professor Giovani Silva was partially funded by FCT-Portugal project (grant no. UID/MAT/00006/2019).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SA and AEP discussed experimental design, interpreted and discussed the data and wrote the manuscript. TP and FS performed IHC experiments. PM and FV performed BRCA analysis. MA and GLS analyzed and interpreted statistical data.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of the Portuguese Institute of Oncology Lisbon Center.

Competing interests

The authors declare that they have no competing interests.

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Chapter IV

4.2 - Saudade André, Sandra P. Nunes, Rui Henrique, Ana Félix, Carmen Jerónimo.

Epigenetic alterations in homologous recombination DNA repair genes in male breast cancer.

(submitted to International Journal of Molecular Sciences)

4.2 - Epigenetic alterations in homologous recombination DNA repair genes in male breast cancer

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Received: date; Accepted: date; Published: date

Abstract

Background: Male breast cancer (BC) is a distinct neoplasm with low but rising incidence, frequently diagnosed as advanced stage disease. Considering the relevance of altered homologous recombination repair (HRR) in male BC, we aimed to explore the biomarker potential of aberrant promoter methylation of *ATM*, *BRCA1*, *PALB2*, *RAD51B* and *XRCC3*. *Methods:* Formalin-fixed paraffin-embedded (FFPE) tissue samples from 128 male BC patients, paired adjacent normal tissue and 19 gynecomastia cases were collected and assessed by quantitative methylation-specific PCR (qMSP). Non-parametric tests were used to compare methylation levels between tumor and non-tumor

samples and to seek for associations with clinicopathological variables. *Results*: Only *RAD51B* and *XRCC3* disclosed significant differences between tumor and gynecomastia (p<0.0001 and p=0.020, respectively). Assembled in a panel, *RAD51B* and *XRCC3* promoter methylation discriminated male BC from gynecomastia with 91.5% sensitivity, 89.5% specificity and 91.2% accuracy. Moreover, promoter methylation levels were lower in paired non-tumor tissues, comparing to tumor samples. No associations between epigenetic alterations and clinicopathological features were found. *Conclusion*: Quantitative promoter methylation of *RAD51B* and *XRCC3* constitutes a promising and reliable biomarker for male BC. Validation in larger series, using immunohistochemistry and/or liquid biopsies is warranted to confirm its usefulness in monitoring settings.

Keywords: Male breast cancer; Epigenetics; Homologous recombination DNA repair; Detection

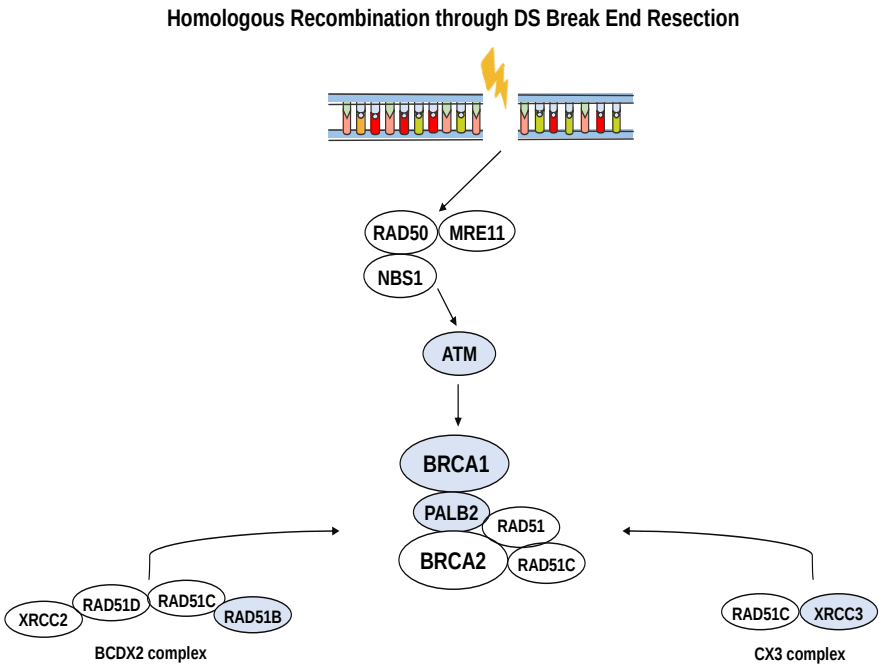

Figure 1 - Graphical abstract.

Introduction

Male breast carcinoma (male BC) is a multifactorial neoplasm lacking specific guidelines for detection, therapy and surveillance. Although it constitutes a rare entity, comparatively to its female counterpart, incidence has been rising over the last decades [1, 2]. Furthermore, advanced stage disease is rather common at diagnosis [1]. Although several genetic, hormonal and environmental risk factors have been acknowledged, an in-depth understanding of the biologic peculiarities of male breast carcinogenesis is clearly lacking [3]. Consequently, treatment strategies rely on the recommendations for BC in women [1].

The process of carcinogenesis is complex, resulting from the accumulation of multiple genetic and epigenetic alterations [4]. The best characterized epigenetic change in cancer consists on altered methylation of CpG dinucleotides, impacting on genome stability and regulation of gene expression [5]. Aberrant methylation, occurring mostly at gene promoter regions, is associated with gene transcription repression [5]. This alteration is among the most common and earliest events involved in cancer initiation and promotion, being easily measured [6].

Homologous recombination repair (HRR) is a major surveillance mechanism in the preservation of genome integrity, acting in repair of DNA double-strand breaks, which occur during replication [7]. *BRCA2*, the most common high penetrance susceptibility gene for male BC, but also *ATM, BRCA1*, *PALB2*, *RAD51* and *RAD51* paralogs play important roles in HRR pathway [8]. *RAD51* paralogs encode for proteins that structurally resemble RAD51 and congregate *in vivo* into three subcomplexes, comprising *BCDX2 (RAD51B, RAD51C, RAD51D, XRCC2), CX3 (RAD51C, XRCC3*), and the Shu complex (*SWSAP1, SWS1*) [7-12]. Indeed, the balance between BRCA2, RAD51 and RAD51 paralogs seems to be essential in HRR [8, 12, 13]. Mutations in HRR genes, either somatic and/or germline occur in multiple conditions, including hereditary breast and ovarian cancer susceptibility syndromes, in which there is also increased male BC risk [2,8,14,15,16,17]. Nonetheless, HRR deficiency may also be mediated by DNA repair gene aberrant promoter methylation. Although altered DNA methylation has been seldom reported in male BC, it might constitute a novel biomarker for disease monitoring, allowing for more personalized clinical care [4, 18, 19, 20, 21].

Poly (ADP-ribose) polymerases (PARPs) proteins are enzymes whose functions include to help repair damaged DNA single strand breaks. Because malignant cells with

germline *BRCA* (g*BRCA*) gene mutations already have troubling repairing damage DNA, blocking the PARP proteins leads to the dead of these cells. PARP inhibitors (PARPi) are effective in carcinomas harboring dysfunctional HRR, included g*BRCA1/BRCA2* related BC, and were approved by the Food and Drug Administration [22]. The use of PARPi could be extended to a wider group of BC patients with defective HRR, beyond detected g*BRCA* mutations [23]. In addition, carcinomas from g*BRCA* mutation carriers may restore HRR and acquire PARPi therapy resistance, which may be a serious problem [25]. Thus, there is an increasing interest in understanding the molecular basis of sensitivity to PARPi in patients with deficient HRR and no g*BRCA2* gene mutation or of resistance mechanisms to PARPi therapeutic approach [24, 25].

Owing to the relevance of HRR deficiency in male BC and the lack of systematic studies on altered methylation patterns of HRR genes in this specific context, we aimed to explore the epigenetic signature of the HRR genes *ATM, BRCA1, PALB2, RAD51B* and *XRCC3* in a large, well characterized (clinically and pathologically) series of male BC patients, to identify novel detection, diagnostic and/or prognostic biomarkers that might perfect clinical management.

Additionally, 19 cases of gynecomastia were added as benign comparative model. Gynecomastia is the most common benign disease in male breast and shares risk factors with male BC, including high estrogen levels [18, 26]. However, gynecomastia is not considered by itself a risk factor for male BC [26].

Results

Clinical and pathological data

This study included 128 male BC, matched normal tissues (66 normal breast tissue and 62 axillary lymph nodes) and 19 patients with gynecomastia. Detailed clinicopathologic characterization of the male BC cases is provided in Table 1. The mean age of patients with breast cancer at diagnosis was 66.7 years (range: 37-87 years). About 20% of the male BC patients had a familial history (FH) of breast cancer. Germline *BRCA1* mutations were not found in this series. Germline *BRCA2* mutations were found in 12 of the 37 patients (32.4%) evaluated. Ten (83.3%) of these 12 patients had a FH of breast cancer. Six patients (4.7%) had bilateral carcinoma and 20 patients (15.6%) had non-breast primary neoplasm (NBPN), most of them (eight patients – 40%) corresponding to prostate cancer. Germline *BRCA* mutations were evaluated in 12

patients with NBPN and g*BRCA2* was identified in four (33.3%) of these patients, all with a FH of BC.

Table I - Clinicopathological characteristics of male breast cancer patients.

Characteristics	**Total of cases**	**Number (%)**
Age (years)	128	
37-69		67 (52.3%)
≥70		61 (47.7%)
Familial history (FH) of breast cancer	128	
No		102 (79.7%)
Yes		26 (20.3%)
Germline *BRCA2* mutations	37	
Indeterminate		25 (67.6%)
Positive		12 (32.4%)
Bilateral breast cancer	128	
No		122 (95.3%)
Yes		6 (4.7%)
Non-breast primary neoplasms	128	
No		108 (84.4%)
Yes		20 (15.6%)
Tumor size (pT)	128	
pTis		8 (6.2%)
pT1		31 (24.2%)
pT2		43 (33.6%)
pT3		2 (1.6%)
pT4		44 (34.4%)
Axillary nodal status (pN)	128	
pN0		60 (46.9%)
pN1		68 (53.1%)
Distant metastasis (M)	128	
M0		122 (95.3%)
M1		6 (4.7%)
Anatomic stage (AS)	128	
0		8 (6.2%)
I		23 (20%)
II		47 (36.7%)
III		43 (33.6%)
IV		7 (5.5%)
Histological type (HT)	120*	
Invasive no special type (NST)		112 (93.3%)
Other invasive subtypes		8 (6.7%)

Histological grade (G)	120*	
G1		20 (16.7%)
G2		74 (61.7%)
G3		26 (21.6%)
Estrogen receptor (ERα) status	128	
Positive		125 (97.7%)
Negative		3 (2.3%)
Progesterone receptor (PR) status	120*	
Positive		97 (81%)
Negative		23 (19%)
ERBB2 (IHC & ISH) status	120*	
Negative		111 (92.5%)
Positive		9 (7.5%)
Ki67 immunoreactivity	120*	
Low		77 (64.2%)
High		43 (35.8%)
Clinically defined subtypes	120*	
Luminal A-like		44 (36.7%)
Luminal B-like		64 (53.3%)
HER2-like		9 (7.5%)
Triple negative		3 (2.5%)
Follow-up	128	
Died of disease		40 (31.3%)

*Excluding eight *in situ* carcinomas

Patients with gynecomastia were younger, with a mean age of 34.3 years (range: 16-69 years). None of the patients with gynecomastia had FH of breast cancer. Twelve cases were bilateral. Five patients had non-breast primary neoplasms, two of which were prostate carcinomas. One patient with gynecomastia and prostate carcinoma harbored a g*BRCA2* mutation.

Gene promoter methylation levels

ATM, BRCA1, *PALB2*, *RAD51B* and *XRCC3* promoter methylation levels were evaluated in male BC and paired normal breast tissue or adjacent lymph nodes, and in gynecomastia tissue samples.

Only *RAD51B* and *XRCC3* disclosed statistically significant differences between tumor and gynecomastia tissues, with higher methylation levels observed in gynecomastia tissue samples (Figure 2, Table II).

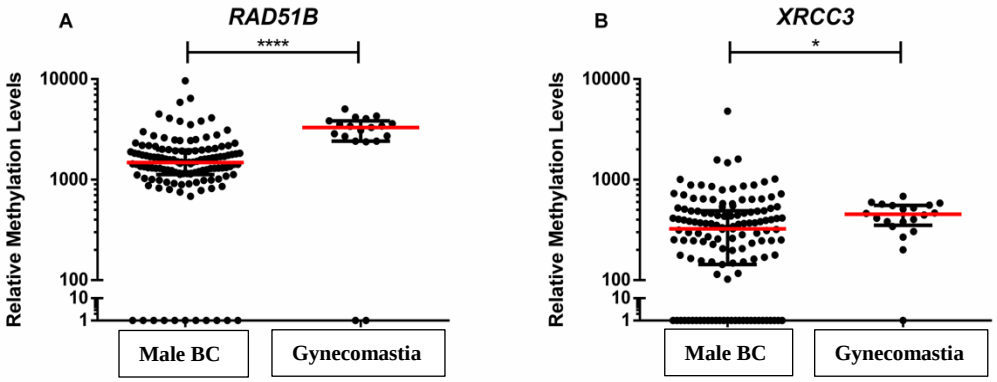

Figure 2 - Scatter plot of the distribution of (**A**) *RAD51B* and (**B**) *XRCC3* promoter methylation levels [(gene/β-Actin) × 1000] in male BC (n=128) and gynecomastia tissue (n=19) samples. Red horizontal line represents the median levels and the black lines the interquartile range. *p* value derived from Mann-Whitney U test, * $p < 0.05$ and **** $p < 0.0001$.

Table II - Statistical significance of differences in gene promoter methylation levels between male BC and gynecomastia tissue samples.

Gene	***p* value**
ATM	0.749
BRCA1	0.289
PALB2	0.436
RAD51B	**<0.0001**
XRCC3	**0.020**

Furthermore, *XRCC3* promoter methylation levels were lower in normal adjacent tissue comparing to male BC tissue (p=0.002), whereas *RAD51B* promoter methylation levels were higher in male BC samples, although not reaching statistical significance (p=0.968) (Figure 3). No differences were depicted for the remainder genes.

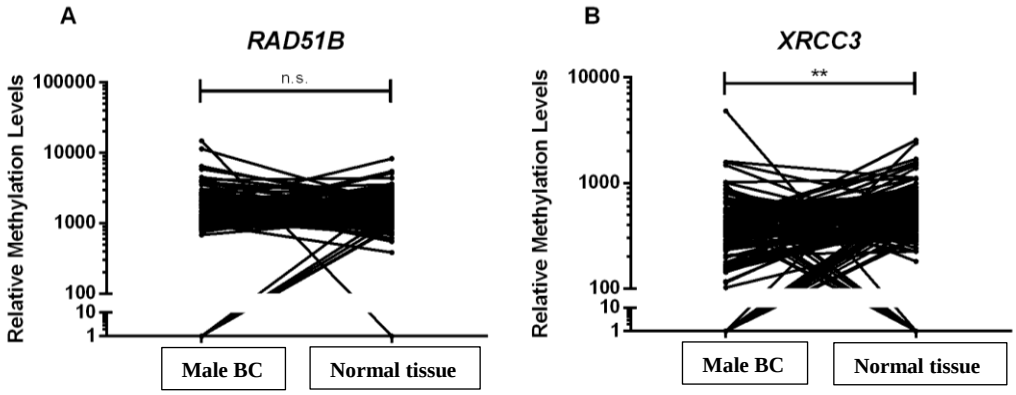

Figure 3 - Relative methylation levels distribution of (**A**) *RAD51B* and (**B**) *XRCC3* of male BC (n=128) and normal (n=128) adjacent tissue samples. *p* value derived from Wilcoxon signed-rank test, n.s. $p > 0.05$ and ** $p < 0.01$.

Biomarker performance

The gene promoters that showed statistically significant differences between tumor and gynecomastia samples (*RAD51B* and *XRCC3*) were evaluated as potential biomarkers for male BC. Individually, *RAD51B* displayed over 80% sensitivity and specificity, whereas *XRCC3* correctly identified 43.4% of the tumor samples with 94.7% specificity.

Table III - Biomarker performance of *RAD51B* and *XRCC3* promoter methylation levels in tissue samples.

Validity estimates	***RAD51B***	***XRCC3***
Sensitivity (%)	82.9	43.4
Specificity (%)	94.7	94.7
Positive predictive value (%)	98.9	97.9
Negative predictive value (%)	50.0	23.1
Accuracy (%)	84.7	51.2

When the two genes were assembled in a panel, sensitivity increased to 91.5%, with 89.5% specificity and 91.2% accuracy for identification of male BC vs. gynecomastia (Figure 4, Table IV).

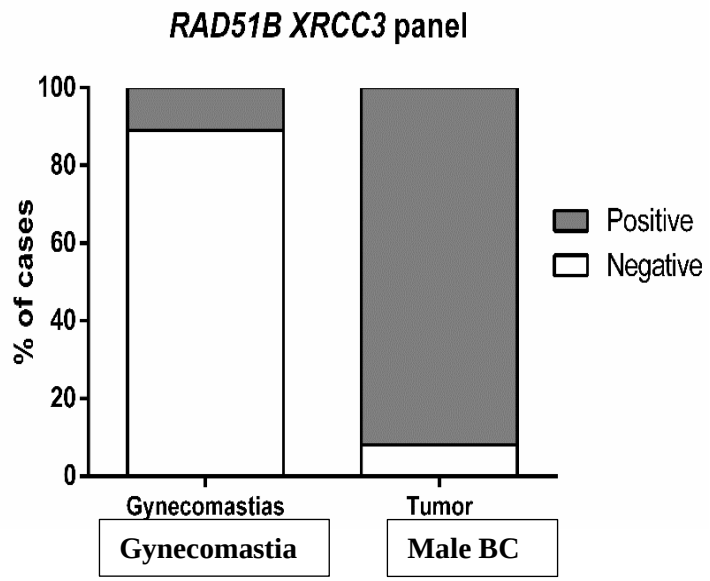

Figure 4 - Proportion of cases disclosing positivity for *RAD51B* & *XRCC3* methylation panel in male BC (Positive 92%, Negative 8%) and in gynecomastia (Positive 11%, Negative 89%) tissue samples. RAD51B cut-off = 2345,7 and XRCC3 cut-off = 262,0.

Table IV - Biomarker performance of the panel *RAD51B* & *XRCC3* promoter methylation levels in tissue samples.

Validity estimates	***RAD51B & XRCC3***
Sensitivity (%)	91.5
Specificity (%)	89.5
Positive predictive value (%)	98.0
Negative predictive value (%)	65.4
Accuracy (%)	91.2

No statistically significant associations between epigenetic alterations in the tested HRR genes and clinicopathological parameters were depicted, either in male BC or gynecomastia cases.

Discussion

Male BC is a multifactorial and distinctive neoplasia with low, but rising, incidence, requiring a personalized approach and warranting optimal care [1, 27]. To achieve this aim**,** detailed knowledge of genetic and epigenetic alterations, as well as of other specific characteristics of BC in the male gender are mandatory [27]. Population-based mammographic screening in males has no role considering the rarity of male BC, although it may be useful in selected high risk groups [1, 28]. In current clinical practice, male BC is diagnosed by mammography or ultrasonography and confirmed by core biopsy, which is always performed following a suspicious clinical examination and frequently at advanced disease stage [1]. Furthermore, specific biomarkers that might assist in early disease detection, diagnosis, therapy and prognostication are clearly lacking and constitute an unmet need. We, thus, assessed the methylation status of five genes (*ATM, BRCA1, PALB2, RAD51B* and *XRCC3*) involved in HRR (which is deficient in a large proportion of male BC cases) looking for biomarkers that might be useful for clinical management.

Notwithstanding the biological peculiarities of male BC, the uselessness of mammographic screening due to low incidence rates, the high incidence of gynecomastia that may have overlapping clinical presentation, the particular anatomic characteristics of the male breast, the absence of publicly-available information about the disease, the prevalence in old age groups and the fact that males are less likely to report symptoms that would guide to an early diagnosis, contribute to the significant number of advanced stage disease at diagnosis and, consequently, the high mortality rates of male BC [29]. Indeed, in this cohort, 39.1% of the patients presented at stages III and IV and 31.3% died of disease, underlining the importance of early diagnosis. In addition, evidence of significant hereditary predisposition was found in this cohort (FH of breast cancer, g*BRCA2* mutations and non-breast primary neoplasms, in 20.3%, 32.4% and 15.5% of cases, respectively) which is in line with published literature [30, 31]. The fact that Portuguese population has a founder mutation may justify the incidence achieved in this

series [32]. Furthermore, the clinical and pathological characteristics of the patient population in this study are also similar to those previously published [3, 21, 33], which further validates our dataset.

Although the acquisition of epigenetic alterations, in general, and aberrant DNA methylation, in particular, is widely acknowledged as an early and relevant event in cancerigenesis [34], they have been seldom reported and with different purposes, in male BC [4,18,19,20,21]. Kornegoor *et al*. examined promoter methylation of 25 genes in 108 male BCs using methylation specific multiplex ligation dependent probe amplification and concluded that promoter methylation was common in male BC and high methylation status correlated with aggressive phenotype and poor outcome [18]. Subsequently, Pinto *et al.* found different expression patterns in male and female familial BC in a set of 27 familial BC cases, using quantitative methylation-specific PCR [19]. Johanssen *et al.* performed a genome-wide methylation profiling of 47 male BC, underscoring the heterogeneity of this entity and suggesting that male BC should not be defined using conventional criteria applied to female BC [20]. Using methylation-sensitive high resolution, Deb et al. assessed a panel of 10 genes in 60 male BCs, concluding that *BRCA2*-associated male BC was characterized by high gene methylation and that the average methylation index might be a useful prognostic marker [4]. Finally, Rizzolo *et al.* assessed promoter methylation in 69 male BC patients and concluded that alterations in methylation patterns were common in male BC and might identify specific subgroups related to *BRCA1/2* mutation status and some clinicopathologic parameters [21].

Among the five gene promoters tested, only two - *RAD51B* and *XRCC3* – disclosed statistically significant differences between tumor and gynecomastia tissue samples, whereas *ATM*, *BRCA1* and *PALB2* did not. Moreover, *RAD51B* and *XRCC3* promoter levels were higher in tumor tissues compared to normal breast or lymph node, although with statistical significance for *RAD51B* only. Globally, these results are in line with those of Kornegoor *et al*, which found that *ATM* and *BRCA1* promoter methylation did not seem to play a key role in male BC genesis [18].

RAD51B and *XRCC3* promoter methylation has been reported in association with the inflamed phenotype of squamous cell carcinomas of the head and neck, lung and cervix, warranting further investigation as predictive biomarkers of response to immunotherapy [35]. Defects in DNA HRR result in a higher number of mutational

events in malignant cells with a consequent production of neoantigens that might explain the improved response to immunotherapy in HRR defective tumors. Therefore, carcinomas with defects in HRR may be responsive to both PARPi and immune-checkpoint inhibitors. Preclinical studies demonstrated that PARPi upregulates PD-L1 and combinations of both PARPi and immune-checkpoint inhibitors are presently tested for different tumors [36]. Also, detection of RAD51 nuclear foci, used as a surrogate marker of HRR functionality, was reported to correlate with PARPi resistance in patients with gBRCA tumors [37] and the immunoexpression of XRCC3 was described as a possible radiosensitivity predictor [38].

Gynecomastia is a common benign proliferation of the breast that shares with male BC the risk factors related to high estrogen levels and its discrimination from BC is clinically challenging, although is not considered a risk factor for male BC [26]. Thus, this entity was selected as control for determining the biomarker performance of gene promoter methylation as this constitutes a clinical scenario in which specific biomarkers might aid in differential diagnosis and monitoring. Remarkably, the methylation panel combining *RAD51B* and *XRCC3* accurately discriminated male BC from gynecomastia, in tissue samples. This might prove useful in the diagnostic context of biopsies with limited tissue representativeness. The translation of this evaluation into monitoring scenarios requires the validation of this performance in liquid biopsies. Nevertheless, it should be emphasized that this gene methylation panel constitutes the first discriminative biomarker in this setting.

Normal breast tissues disclosed *RAD51B* and *XRCC3* promoter methylation, although with lower median promoter methylation levels compared to male BC, suggesting the existence of a cancerization field effect. This phenomenon reflects the susceptibility of normal tissue to undergo early genetic and epigenetic alterations leading to tumor development [39]. Field cancerization was hypothesized to explain the development of multifocal areas of premalignant change, multiple primary tumors and local recurrence [40] and more recent studies demonstrate that aberrant DNA methylation patterns, either hyper- or hypomethylation, are potential biomarkers of field cancerization and may be useful for risk stratification [41]. Surprisingly, however, higher *RAD51B* and *XRCC3* promoter methylation levels were disclosed in gynecomastia comparing to male BC. This finding might be related with proliferation, high estrogen levels or other yet unknown risk factors. To fully understand the biological consequences of these

observations, detailed expression analysis of those genes is required. Indeed, promoter methylation acts in concert with other epigenetic mechanisms (*e.g.*, histone post-translational modifications and chromatin remodeling) to achieve effective gene silencing. Thus, although *RAD51B* and *XRCC3* promoter methylation levels might be higher in gynecomastia, histone-related factors might preclude effective gene silencing, contrarily to BC. Notwithstanding the elusive biological significance of this finding, *RAD51B* and *XRCC3* promoter methylation stand as candidate biomarkers for male BC, requiring further investigation, namely in liquid biopsies (with special interest in men with no detect g*BRCA1/2* mutations and with familial history of BC) and in immunohistochemical expression as a surrogate marker of HRR functionality. The availability of a larger amount of biological data and the investigation of *RAD51B* and *XRCC3* as predictive biomarkers to the response to PARPi and/or immune-checkpoint inhibitors may provide a better therapeutic approach for male BC, based in homologous recombination defects beyond gBRCA2 mutations or in the context of therapy resistance.

Materials and Methods

Patients and samples collection

A cohort of 128 male BC patients, diagnosed and treated at the Portuguese Oncology Institute of Lisbon (Lisbon, Portugal), between 1978 and 2018 were enrolled, after informed consent. Routine sampling for standard pathological examination by H&E and immunostaining was performed, allowing for tumor classification, grading and staging [42]. A representative formalin-fixed, paraffin-embedded (FFPE) tumor tissue sample was made available for molecular analyses. The corresponding adjacent normal breast tissue and lymph nodes were also included in the study, as controls. For comparison purposes, 19 cases of gynecomastia were used. Patient data, including age, family history, tumor bilaterality, presence of non-breast primary neoplasms, information about distant metastasis and follow-up were obtained from clinical records. Germline mutational *BRCA2* status was previously evaluated in 37 cases of male BC and in one case of gynecomastia, as previously described [29]. This study was approved by the Ethics Committee of Portuguese Oncology Institute of Lisbon.

DNA extraction and sodium-bisulfite modification

Areas of interest (breast cancer, normal breast and gynecomastia) were delimited in H&E slides by a dedicated Pathologist (S.A.), macrodissected from 10μm tissue sections, deparaffinized with xylene (VWR, Radnor, PA, USA) and rehydrated using 100% ethanol (Merck Millipore, Burlington, MA, USA). DNA was extracted using the FFPE RNA/DNA Purification Plus Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's recommendations. DNA samples were eluted in 20 μL of sterile distilled water and stored at -20ºC until further use. DNA was quantified using the Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA, USA), using the manufacturer's recommendations.

Sodium-bisulfite modification was performed in all samples using the EZ DNA Methylation-Gold™ (Zymo Research, Orange, CA, USA) following the manufacturer's recommendations. 150 ng of extracted DNA were used and eluted in 60 μL of sterile distilled water. Additionally, 1 ng of CpGenome™ Universal Methylated DNA (Millipore, Temecula, CA, USA) was sodium-bisulfite converted for control purposes and eluted in 40 μL of sterile distilled water. All sodium-bisulfite converted DNA was stored at -80ºC until further use.

Quantitative methylation-specific PCR (qMSP)

ATM, *BRCA1*, *PALB2*, *RAD51B* and *XRCC3* promoter methylation levels were assessed by qMSP, using *β-Actin* as a reference gene. The reactions were carried out in 384-well plates using the LightCycler 480 Instrument (Roche Diagnostics, Manhaeim, Germany) and the sodium-bisulfite modified DNA was used as a template. The primers' volumes and conditions used for each gene are listed in Supplementary Table 1. Per well, 2 μL of sodium-modified DNA and 5 μL of Xpert Fast SYBR (GRiSP, Porto, Portugal) were added. All samples were run in triplicate. In order to generate a standard curve for DNA relative quantification and plate efficiency calculation, the sodium-bisulfite modified CpGenome™ Universal Methylated DNA was subjected to serial dilutions (5x dilution factor). Efficiency values above 90% were considered for each plate. Relative methylation levels were obtained by calculating the ratio between the methylation levels of each gene and the respective value of *β-Actin*, multiplying by 1,000 for easier tabulation.

Statistical analysis

Non-parametric tests were used to compare the methylation levels between tumor and non-tumoral samples and to assess associations with clinicopathological variables (Kruskall-Wallis test for three or more groups, followed by pairwise comparisons using Mann-Whitney U test with Bonferroni's correction, when applicable, and Wilcoxon signed-rank test for paired samples). Correlations between age and genes' methylation levels were evaluated by Spearman's nonparametric test. Receiver operating characteristic (ROC) curve analysis was performed and the validity estimates [sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy] were calculated to assess biomarker performance. Carcinoma samples of patients subjected to neoadjuvant therapy were not considered for this analysis. Samples were categorized as methylated (positive) or unmethylated (negative) based on the cut-off determined by ROC curve analysis corresponding to the highest sensitivity and specificity (Youden's J index) [43].

Conclusions

In this study we demonstrated that promoter methylation levels of *RAD51B* and *XRCC3* differ between male BC and gynecomastia tissues, suggesting their usefulness, in a panel, as male BC biomarkers. Further analyses in liquid biopsies are mandatory to assess the potential of this panel for early detection, in at-risk populations, and disease monitoring.

Supplementary Materials: The following materials are available online at www.mdpi.com/xxx/s1, Table S1:

Author Contributions: S.A. designed the study, reviewed all clinical and pathological data, and drafted the manuscript. S.P.N. performed DNA extraction, qMSP and analyzed the data. R.H.; A.F. and C.J. supervised the study and revised the manuscript. All authors read and approved the final manuscript.

Funding: This research was funded by Prémio NOVARTIS | EXCELLENCE in Fundamental Medical Research da NOVA(Prémio Novartis | NOVA) 2015. S.P.N. was supported by a fellowship from Liga Portuguesa contra o Cancro/ Pfizer.

Acknowledgments: The authors gratefully acknowledge the patients who accepted to participate in this study.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter IV

4.3 - Saudade André, António E. Pinto, Giovani L. Silva, Fernanda Silva, Jacinta Serpa, Ana Félix.

Male Breast Cancer – Relevance of FASN, ATF3 and Collagen IV.

(submitted to Translational Research)

4.3 - Male Breast Cancer – Relevance of FASN, ATF3 and Collagen IV

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Abstract

Context: Male breast carcinoma (male BC) is an uncommon neoplasia without individualized strategies for diagnosis and therapeutics. Low overall survival rates are commonly reported, mostly associated to the detection in advanced stage and older age. Intratumoral heterogeneity versus homogeneity of malignant epithelial cells seems to be an important factor to consider for the development of combination therapies with curative intention.

Objective: We aim to identify immunophenotypical patterns that could contribute to improve the clinical management of male BC.

Design: In a series of 40 male BC patients, we evaluated Androgen Receptor, Activating Transcription Factor 3 (ATF3), p16, Cyclin D1, β1, β3, β4 and β6 integrins, Fatty Acid Synthase (FASN), Fatty Acid Transport Protein 1 (FATP1), Collagen I and Collagen IV, and their interactions. We used gynecomastia tissue as a comparative model.

Results: Homogeneous epithelial staining of ATF3, p16, β6 integrin, FASN, and FATP1 are significantly intercorrelated and associated with high Ki67. These markers

also stained tumor stromal fibroblasts. Statistical prognostic analysis shows significant associations of FASN with disease-free (DFS) and overall survival (OS), as well as ATF3 with OS and Collagen IV with DFS.

Conclusions: Despite preliminary, this study highlights FASN, ATF3 and Collagen IV as particular phenotypes eligible for further investigation, with potential to improve the management of male BC.

Keywords: male breast carcinoma; gynecomastia; immunohistochemistry; molecular markers; prognosis.

Introduction

Male breast carcinoma (male BC) is a complex group of malignant epithelial proliferations with specific stromal microenvironment. Although rare, the incidence has been rising by 20%-25% in the past few decades and continues to rise (1). This has been partially attributed to the aging of the population and the obesity trend (2-4). In addition, lower overall survival rates in male BC as compared to women BC have been reported, associated with detection in advanced stage and older age (2). Despite recent improvements, current clinical and pathologic parameters are still insufficient to a personalized and accurate treatment efficacy, and male BC requires comprehensive studies to identify additional markers able to assure an optimal clinical care.

A main challenge in cancer biology is the intratumoral heterogeneity of neoplastic tissues, which contain diverse and dynamic interacting epithelial and stromal subpopulations, including fibroblasts. The intratumor heterogeneity versus homogeneity of malignant epithelial cells seems to be critical in the management and prognosis of BC and is an important factor to consider in the efforts to develop combination therapies with curative purposes (5).

In this series of previously evaluated estrogen receptor alpha (ERα) and progesterone receptor (PR) positive male BC, we sought to complete the hormonal characterization with androgen receptors (AR) and make a comprehensive study of the eventual interactions between fatty acids metabolism, a mediator of cellular stress response, cell cycle regulation proteins, cell-surface proteins, and components of extracellular matrix.

Immunohistochemistry (IHC) has increasing relevance in breast pathology for solving diagnostic difficulties and is used as a surrogate tool for mutational evaluation in determining response to therapy and prognosis (6). The IHC analysis of ERα, PR, HER2 (human epidermal growth factor receptor-2 / ERBB2) and Ki67 is now standard care to evaluate BC. The tumoral phenotypical expression by IHC can also contribute to understand molecular characteristics of malignant epithelial cells and stroma, and to assess intratumor heterogeneity versus homogeneity (5, 6). Diverse biomarkers appraised by this technique have been implicated in BC tumoral gene expression, proliferation, differentiation, invasion, migration, metastasis and survival, being considered potential therapy targets. The matching pursue of genetic and epigenetic parameters with staining data to optimize IHC surrogates is indispensable to improve the IHC accuracy in clinical practice (7).

In this study, we analyzed the IHC pattern of the following markers: AR, a nuclear transcription factor member of the steroid hormone nuclear receptor family; activating transcription factor 3 (ATF3), member of the activator protein 1 family of transcription factors; cell cycle regulation proteins: p16 and Cyclin D1; cell-surface proteins: β1, β3, β4 and β6 Integrins; Fatty Acid Synthase (FASN), the enzyme for endogenous synthesis of fatty acids; Fatty Acid Transport Protein 1 (FATP1), the first described element of the six members of FATPs family; and the structural components of the extracellular matrix Collagen I and Collagen IV. Our intention was to evaluate positivity as a marker of widely common expression “all or not all”. We assessed different cores from different morphologic areas and our results underlined the presence of homogeneous positivity. All these markers have been previously evaluated in female BC.

Gynecomastia is a benign proliferation of ducts and mesenchymal components in male breast. As male BC, gynecomastia is a multifactorial condition and both entities share risk factors related to high estrogen levels and old age . Gynecomastia is not considered a risk factor for male BC, and a possible relationship between gynecomastia and male BC remains to be established (3, 8 -10). In addition, the reported incidence of patients with male BC and coexistent gynecomastia is difficult to assess and has a great variation in literature (8). Gynecomastia has been used as a male BC control (4, 10), and accordingly, in this study, we included gynecomastia tissue as a comparative model.

We aim to identify IHC epithelial and stromal patterns that may contribute to better characterize the biology of male BC, and consequently, may represent promising tools for improving the clinical management in this subtype of BC.

Material and Methods

Study cohort

This study comprises 40 invasive male BC selected from a series of 198 male BC patients diagnosed and treated at the Portuguese Institute of Oncology (IPO) Lisbon Center. The patients included in this series were retrieved from the larger series using the following criteria: no neoadjuvant therapy and diagnosed in the last 10 years to homogenize the fixation conditions. All the cases were reviewed to ensure standardized characterization.

The Institutional Ethical Committee of IPO Lisbon Center approved the study (UIC/821). The clinical data (age, obesity, presence of concomitant gynecomastia, family history, bilaterality, presence of non-breast primary neoplasia, and follow-up) were obtained by review of the clinical records. All male BC were previously characterized by histologic type, grading, and staged according to the TNM/AJCC classification system (8^{th} edition) (11, 12). Immunohistochemistry (IHC) analysis of ERα, PR, ERBB2 and Ki-67 allowed the identification of clinically defined, treatment-oriented subtypes (clinical subtypes), previously assessed for pathologic diagnosis and basis of therapy. Germinal mutational *BRCA* (g*BRCA*) status has been assessed in formerly evaluated 22 cases of male BC, as described in our previous study (13).

Tissue Microarrays (TMAs)

Representative formalin-fixed, paraffin-embedded (FFPE) tissue cores were inserted in 4 TMA paraffin blocks. Three or four 1.5 mm diameter cores for each case were included to account for the heterogeneity of the lesions and used to perform an additional immunohistochemical study.

Antibody reagents and conditions

IHC used a peroxidase-indirect-polymer technique performed on a Ventana Benchmark^{TM} ULTRA instrument (Ventana Medical Systems, Inc.; Roche Diagnostics, Basel, Switzerland). Paraffin sections (3 µm) were stained with hematoxylin and eosin staining (H&E) (Hematoxylin, Cat. Number CS700, Dako; and Eosin, Cat. Number

CS701, Dako). We performed IHC with AR, ATF3, $p16^{INK4a}$, Cyclin D1, β1, β3, β4 and β6 Integrins, FASN, FATP1, Collagen I and Collagen IV in an automatic staining platform (Ventana Medical Systems), using OptiView DAB IHC Detection Kit (Ventana Medical Systems) with diaminobenzidine as the chromogen to detect antigen expression (Table I).

Tissue sections were counterstained with Mayer's hematoxylin before mounting. All antibody dilutions were made in Antibody Diluent Reagent Solution (Cat. Number 003218, Life Technologies). Image acquisition was performed in Digital Microimaging Device Leica DMD108 (version 1.15 Build 704, Leica Microsystems).

Table I - Antibody reagents and conditions

Antibody	Manufacturer	Clone	Dilution	Pre-treatment
ERα	Ventana Medical Systems, Inc.; Roche Diagnostics	SP1	Pre-diluted 28 min	ULTRA CC1-64 min
PR	Ventana Medical Systems, Inc.; Roche Diagnostics	IE2	Pre-diluted 36 min	ULTRA CC1-64 min
ERBB2	Ventana Medical Systems, Inc.; Roche Diagnostics	4B5	Pre-diluted 60 min	ULTRA CC1-76 min
Ki67	Ventana Medical Systems, Inc.; Roche Diagnostics	30-9	Pre-diluted 20 min	ULTRA CC1-40 min
AR	Ventana Medical Systems, Inc.; Roche Diagnostics	SP107	Pre-diluted 32 min	ULTRA CC1-64 min
ATF3	Santa Cruz Biotechnology	C19	1:150 - 28 min	CC1-56 min
p16	CINtec Histology	E6H4	Pre-diluted 32 min	ULTRA CC1-64 min
Cyclin D1	Thermo Scientific	SP4	1:30 - 20 min	CC1-20 min
β1 Integrin	Cell Signaling	D2E5	1:100 - 28 min	ULTRA CC1-92 min
β3 Integrin	Cell Signaling	D7X3P	1:100 - 28 min	ULTRA CC1-56 min
β4 Integrin	Atlas antibodies	ITGB4	1:200 - 28 min	ULTRA CC1- 56 min
β6 Integrin	Atlas antibodies	ITGB6	1:350 - 28 min	ULTRA CC1- 48 min
FASN	Sigma	Not indicated	1:800 - 28 min	CC1-56 min
FATP1	R&D system	308420	1:200 - 16 min	CC1-24 min
Collagen I	Abcam	EPR7785	1:300 - 20 min	ULTRA CC1-16 min
Collagen IV	DAKO	CIV22	1:10 - 20 min	ULTRA CC1-16 min

ER, estrogen receptor; PR, progesterone receptor; ERBB2, human epidermal growth factor receptor-2; FASN, fatty acid synthase; ATF3, activating transcription factor 3; AR, androgen receptor; FATP1, fatty acid transport protein 1; min, minutes.

Scoring criteria and patterns

The staining pattern was recorded in the benign and malignant epithelial cells, stromal fibroblasts and interstitial stroma. Some of the antibodies also marked vessels and adipose cells, but these structures were not evaluated. The immunoexpression in the malignant epithelial cells was scored in three subgroups: 1 ("homogeneous phenotype") - positive staining in ≥95% of epithelial cells and strong or moderate intensity; 2 ("heterogeneous phenotype") - positive staining in ≥1% and <95% of epithelial cells and weak or moderate, or focally strong intensity; 3 (negative) - no staining or staining in <1% of cells with weak intensity.

The immunoexpression of fibroblasts was also scored in three subgroups: 1 - positive staining >10% of fibroblasts with strong or moderate intensity; 2 - positive staining ≥1% and ≤10% of fibroblasts/strong and moderate intensity; 3 - negative <1% stained fibroblasts.

Interstitial stroma was classified in two subgroups as: 1- positive diffuse staining with strong, moderate or weak intensity; and 2 – negative staining.

The patterns of the antibodies expression was the following: FASN showed an epithelial cytoplasmatic staining with cell membrane reinforcement and also fibroblasts expression. Androgen Receptors, ATF3 and Cyclin D1 were present as a nuclear staining in epithelial cells and also in fibroblasts. p16, when present in epithelial cells, showed a nuclear and a cytoplasmic staining. β1, β3, and β4 integrins were expressed in the cell membranes of epithelial malignant cells, or around epithelial malignant cell clusters and in myoepithelial cells. β6 integrin was expressed in epithelial cytoplasmatic staining with cell membrane reinforcement or, in some male BC, as a granular cytoplasmic staining. FATP1 was expressed as nuclear staining in epithelial cells and fibroblasts, and occasionally, as light cytoplasmic staining in the epithelial cells. Collagen I and Collagen IV may have a stromal diffuse staining and had no epithelial cell expression. Collagen IV had a positive basement membrane staining of benign ducts and vessels.

Data and Statistical Analysis

For male BC, we made a descriptive analysis, and subsequently, used nonparametric, semiparametric and parametric statistical techniques, employing the software R Core Team 2018 (14). Pearson's χ^2 test of independence and Fisher's exact test were used to evaluate the association between categorical variables, with similar

conclusions in both tests. We show the Fisher's exact test results, because they are more applicable for the small sample size of the series. Survival curves were based on the Kaplan-Meier non-parametric estimator and the differences among the category curves were evaluated by the log-rank test. Tests with p value <0.05 were considered significant. Disease-free survival (DFS) corresponded to the remission time up to recurrence, and overall survival (OS) to the interval since pathologic diagnosis until the occurrence of death due to male BC. Patients without disease relapse during the study period and those who died from other causes or were lost for follow-up, were considered as censored observations. A Cox simple regression model was fitted for each clinicopathologic and IHC variable to evaluate their prognostic influence on both DFS and OS. Following the determination of significant variables, a Cox regression model was performed with all variables simultaneously, as a multiple regression analysis. As the aim of this study is to determine the potential value of IHC markers in male BC, we omitted the statistical results of clinicopathologic characteristics related to prognosis. Because the evaluation of fibroblasts was difficult in some cases due to the above mentioned reasons, we did not consider the immunophenotypically subgroups of fibroblasts for statistical analysis.

Results

Descriptive clinicopathologic analysis

Male BC clinicopathologic characteristics are summarized in Table II. The mean age at diagnosis was 66.7 years (range, 37-84 years). Patients ≥ 70 years categorized 47.5% of the sample. Obesity was recorded in clinical files in 9 patients (22.5%). A confirmed family history of BC was obtained in 11 patients (27.5%). Three patients (7.5%) have metachronous bilateral carcinomas and 8 patients (20%) had non-breast primary neoplasia, most frequently prostate carcinoma (3 cases). One of the patients with prostate carcinoma had also bilateral BC. g*BRCA2* mutations were presented in 27.3% (6 out of 22 tested patients). No g*BRCA1* mutations were found in the 22 tested patients. Fifteen patients (37.5%) presented with pT2 carcinomas and 57.5% with axillary lymph node metastasis. At diagnosis, no patient had distant metastasis and 45% of the patients were in anatomic stage II. Thirty-four carcinomas (85%) were classified as invasive carcinoma of no special type. More than half of the carcinomas (65%) were graded as G2. All patients underwent mastectomy. Adjuvant radiotherapy was used in 26 patients (65%), adjuvant hormonotherapy in 36 (90%), adjuvant chemotherapy in 22 patients

(55%), and ERBB2-target agents in 2 patients (5%). The majority of male BC (90%) were ERα+/ PR+/ ERBB2- and 57.5% had high Ki67 (≥20% of marked cells). This IHC characterization allowed the identification of clinical subtypes as Luminal A-like (42.5%), Luminal B-like (50%) and HER2-like (7.5%). No Triple Negative carcinomas were diagnosed. Nine patients (22.5%) with male BC had also gynecomastia.

Table II - Clinicopathologic characteristics of male BC patients (n=40)

Characteristics	**Number (%)**
Age (years)	
<70	21 (52.5%)
≥70	19 (47.5%)
Family history (FH)	
No	29 (72.5%)
Yes	11 (27.5%)
g*BRCA2* mutations	
Not evaluaed	18 (45%)
Indeterminate	16 (40%)
Positive	6 (15%)
Bilaterality	
No	37 (92.5%)
Yes	3 (7.5%)
Non-breast primary neoplasms (NBPN)	
No	32 (80%)
Yes	8 (20%)
Tumor size (pT)	
pT1	11 (27.5%)
pT2	15 (37.5%)
pT3	2 (5%)
pT4	12 (30%)
Axillary nodal status (pN)	
pN0	17 (42.5%)
pN1	23 (57.5%)
Anatomic stage (AS)	
I	8 (20%)
II	18 (45%)
III	14 (35%)
Histologic type (HT)	
Invasive no special type (NST)	34 (85%)
Other invasive subtypes	6 (15%)
Histologic grade (G)	
G1	1 (2.5%)
G2	26 (65%)
G3	13 (32.5%)
Estrogen receptors α (ERα)	
Positive	40 (100%)
Progesterone receptors (PR)	
Positive	36 (90%)
Negative	4 (10%)
ERBB2 (IHC + ISH)	
Negative	37 (92.5%)
Positive	3 (7.5%)
Ki67	
Low	17 (42.5%)
High	23 (57.5%)
Clinically defined subtypes	
Luminal A-like	17 (42.5%)
Luminal B-like	20 (50.0%)
HER-like	3 (7.5%)

Immunohistochemical results

The results of immunohistochemistry markers staining in male BC are summarized in Table III. Due to their further described relevance in statistical analysis, ATF3, FASN and Collagen IV staining are showed in figures 1, 2 and 3.

AR, p16, Cyclin D1, β1, β3, β4, β6 integrins, FATP1 and Collagen I are depicted as figures in Supplemental Digital Content (SDC).

ATF3 (Fig. 1) was negative in malignant epithelial cells in almost half of male BC cases (46.2%). Positive nuclear malignant epithelial homogeneous phenotype was observed in 20.5% of the cases (Fig. 1a) and heterogeneous phenotype in 33.3% of the cases (Fig. 1b). Fibroblasts positive cases were included in subgroup 1 in epithelial positive cases and were negative in malignant epithelial negative cases (Fig. 1c).

FASN (Fig. 2) stained the cytoplasm (with a cell membrane reinforcement) of epithelial cells in almost all cases, with a homogeneous phenotype in 40% of the cases (Fig. 2a) and heterogeneous phenotype in 57.5% (Fig. 2b). Rare scattered fibroblasts were positive (subgroup 2) in all cases with positive epithelial expression.

Collagen IV (Fig. 3) was present with a diffuse intense stromal staining in 29.7% (Fig. 3a, 3b) of the cases and negative in the remaining (Fig. 3c). Only 5 cases shared an intense staining of stroma with both collagen types. No Collagen I and Collagen IV was present in the epithelial cells.

AR had a malignant epithelial nuclear positivity in 87.5% of the cases: with homogeneous phenotype in 35% (SDC Fig. 1a) and heterogeneous phenotype in 52.5% of the cases (SDC Fig. 1b). Stromal fibroblasts were included in subgroup 1 (>10% staining) in malignant epithelial positive cases and were negative in malignant epithelial negative cases (SDC Fig. 1c).

p16 (see Supplemental Digital Content Fig. 2) was positive in a similar percentage as Cyclin D1 (87.5%), but with a reverse pattern: homogeneous phenotype in 17.5% (SDC Fig. 2a) and heterogeneous phenotype in 70% of the cases (SDC Fig. 2b). Stromal fibroblasts were positive in all cases, even in the malignant epithelial cell negative cases (SDC Fig. 2c).

Cyclin D1 (SDC Fig. 3) was positive in almost all the cases, with a malignant epithelial homogeneous phenotype (SDC Fig. 3a) in 75% and malignant heterogeneous phenotype in 22.5%. Fibroblasts positive cases were included in subgroup 1 in positive

malignant epithelial cells cases and were negative in malignant epithelial negative cases (SDC Fig. 3c).

β1 (SDC Fig. 4a), β3 (SDC Fig. 5a) and β4 integrin chains (SDC Fig. 6a) (see Supplemental Digital Content Fig. 4, 5, and 6, respectively) had an identical staining pattern in most of the cases. Malignant epithelial cells were negative in the majority of the cases (80%, 95% and 59%, respectively) and stromal fibroblasts, when positive, were located around malignant epithelial cells clusters in 17.5%, 22.5% and 33.3% of the cases, respectively. β6 integrin (see SDC Fig 7) showed two staining patterns, one cytoplasmatic with cell membrane reinforcement (SDC Fig. 7a), and the other having a granular cytoplasmic staining (SDC Fig. 7b, 7c). Both types were present in one third of cases (31.6%). Positive stromal fibroblasts (subgroup 1) were present in all positive malignant epithelial cells cases and fibroblasts did not stain in negative cases (SDC Fig. 7d).

Regarding FATP1 expression (SDC Fig. 8), we found a nuclear homogeneous phenotype staining the epithelial cells in 22.5% of the cases (SDC Fig. 8a, 8b) and heterogeneous phenotype in 35% of the cases (SDC Fig. 8c). Cytoplasmic staining was predominantly absent, but occasionally present (SDC Fig 8a). Numerous fibroblasts (subgroup 1) were positive in all male BC cases with positive epithelial expression and did not stain in negative cases (SDC Fig. 8d).

Collagen I (SDC Fig. 9) had a stromal diffuse staining, weak/moderate in 38.5% (SDC Fig. 9c) or intense in 61.5% (SDC Fig. 9a, 9b) of the cases.

In gynecomastia tissue, ATF3 (Fig. 1d, 1e) and FASN (Fig. 2d, 2e) are always present in a heterogeneous type. Collagen IV distinctly highlights the basement membrane of ducts and vessels, and some disperse fibroblasts in the stroma (Fig. 3d). No diffuse interstitial matrix staining was present with Collagen IV in gynecomastia tissue.

We found a positive staining in epithelial cells, always of the heterogeneous phenotype in the epithelial cells within ducts with AR (SDC Fig. 1d, 1e, 1f) and p16 (SDC Fig. 2d, 2e, 2f) (SDC Fig. 1 and 2, respectively). A heterogeneous phenotype was also present in the epithelial cells of all ducts with Cyclin D1 (SDC Fig. 3d, 3e), ATF3 (Fig. 1d, 1e, 1f) and FATP1 (SDC Fig. 8e, 8f) (SDC Fig. 8). β1 integrin, (SDC Fig. 4d) and β3 (SDC Fig. 5d) marked myoepithelial cells, β4 (SDC Fig. 6d) marked myoepithelial cells and some epithelial cells and β6 (SDC Fig. 7e, 7f) showed positive cytoplasmic staining with cell membrane reinforcement in myoepithelial and in the majority of epithelial cells

(see Supplemental Digital Content Fig. 4, 5, 6, and 7, respectively). Rare fibroblasts stained in gynecomastia (subgroup 2) with FASN and p16. Variable number of fibroblasts staining (subgroup 1) was seen with ATF3, AR, β6 integrin and FATP1. The β1, β3 and β4 integrin chains and Cyclin D1 were not present in ≥1% fibroblasts. Collagen I stained the stroma in a diffuse and weak (SDC Fig. 9d), occasionally moderate (SDC Fig. 9e), intensity.

Table III - Immunohistochemical markers staining in male BC

Biomarker	**Number of cases, percentage and staining pattern**					
	Malignant epithelial cells			Stromal fibroblasts		
AR SDC – Fig. 1	14 21 5	35% 52.5%) 12.5%)	homogeneous heterogeneous negative	35	87.5%	subgroup 1
ATF3 Fig. 1	8 13 18	20.5% 33.3% 46.2%	homogeneous heterogeneous negative	21	53.8%	subgroup 1
p16 SDC – Fig. 2	7 28 5	17.5% 70.0% 12.5%	homogeneous heterogeneous negative	40	100%	subgroup 1
Cyclin D1 SDC – Fig. 3	30 9 1	75.0% 22.5% 2.5%	homogeneous heterogeneous negative	39	97.5%	subgroup 1
β1 integrin SDC – Fig. 4	1 32	2.5% 80.0%	heterogenous negative	7	17.5%	subgroup 1
β3 integrin SDC – Fig. 5	2 38	5% 95%	heterogenous negative	9	22.5%	subgroup 1
β4 integrin SDC – Fig. 6	3 23	7.7%) 59.0%	heterogeneous negative	13	33.3%	subgroup 1
β6 integrin SDC – Fig. 7	12 12 14	31.6% 31.6% 36.8%	homogeneous* granular ** negative	24	63.2%	subgroup 1
FASN Fig. 2	16 23 1	40.0% 57.5% 2.5%	homogeneous heterogeneous negative	39	97.5%	subgroup 2
FATP1 SDC – Fig. 8	9 14 17	22.5% 35.0% 42.5%	homogeneous heterogeneous negative	23	57.5%	subgroup 1
Collagen I SDC – Fig. 9	39	100%	negative	34 15	61.5% 38.5	positive intense positive weak
Collagen IV Fig. 3	37	100%	negative	11 26	29.7% 70.3%	positive intense negative

*cell membrane; **cytoplasm.

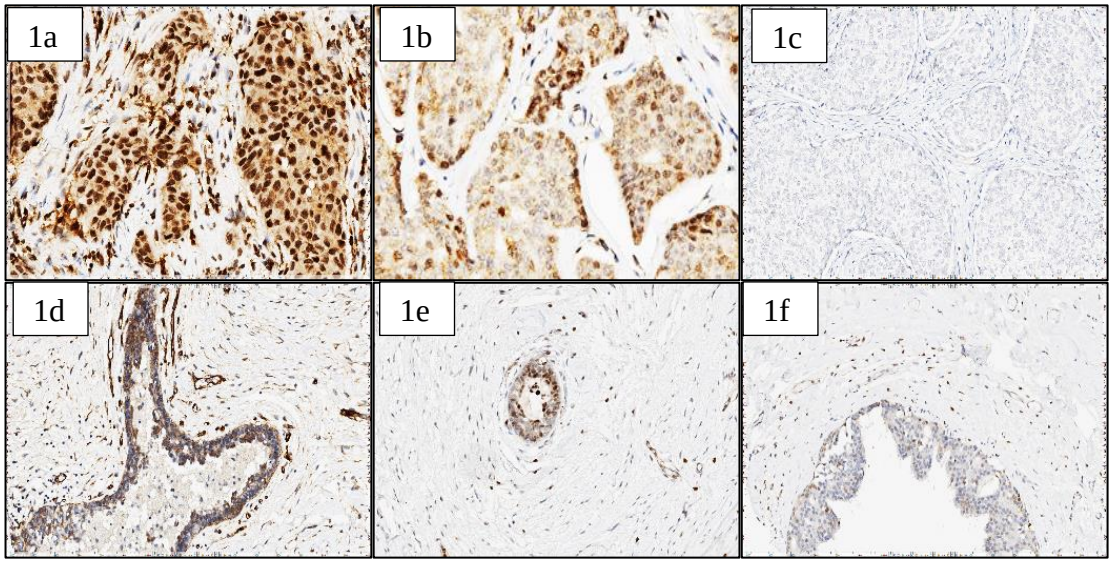

Figure 1 - ATF3 in male BC (1a - positive homogeneous, 1b – positive heterogeneous, 1c - negative) and in gynecomastia (1d, 1e, 1f – heterogeneous cell stain in the same case). Fibroblasts stain irregularly, both in gynecomastia and in male BC epithelial positive cases, and are negative in male BC epithelial negative cases.

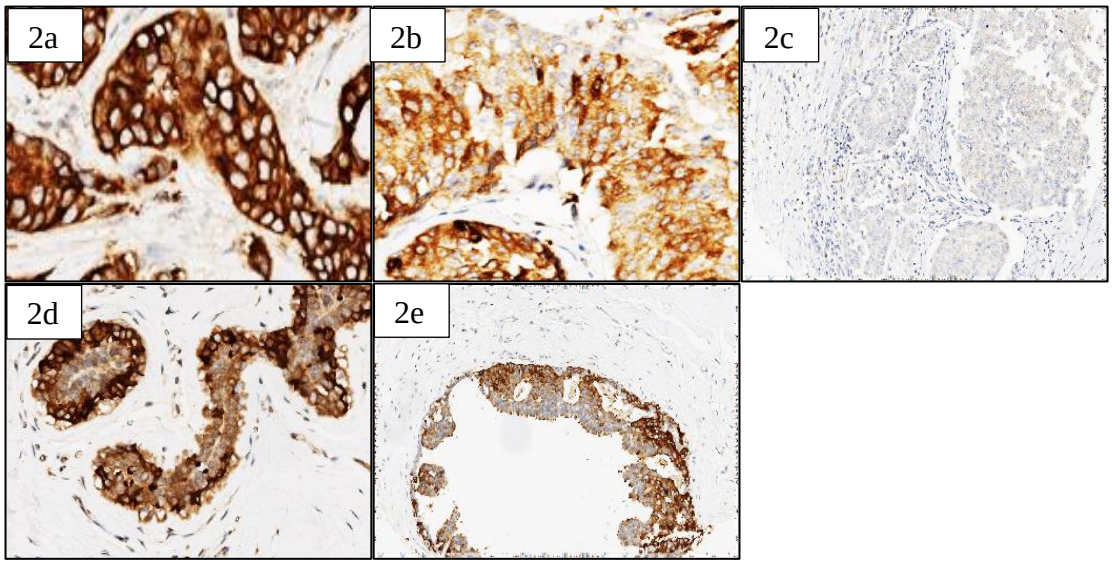

Figure 2 - FASN in male BC (2a – positive homogeneous cytoplasmatic staining, with cell membrane reinforcement, 2b – positive heterogeneous, 2c - negative) and in gynecomastia (2d, 2e – heterogeneous epithelial staining). Fibroblasts stained in gynecomastia tissue and in epithelial male BC positive cases.

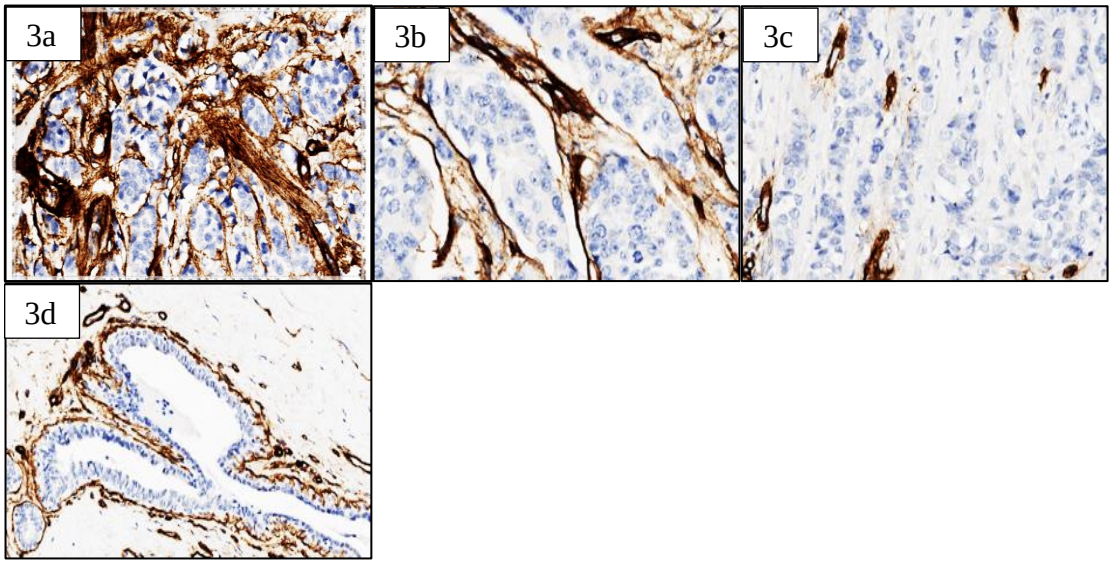

Figure 3 - Collagen IV in male BC (3a, 3b –vascular and diffuse, strong stromal staining around clusters of epithelial cells, 3c – negative/ only vascular basement membrane staining) and in gynecomastia (3d –positive basement membrane staining of ducts and vessels).

Significant associations between malignant epithelial phenotypes

In this series of male BC with epithelial cells previously characterized (13) by ERα positivity (100% of the cases), PR positivity (90%) and high Ki67 (67.5%) (Table II), AR is positive in 87.5% of the cases. We obtained few significant associations between IHC patterns and the clinicopathologic variables and many significant associations between the IHC evaluated biomarkers. All the statistically significant associations found are summarized in Table IV.

All the following associations reach high statistical significance ($p \leq 0.001$): a) between positive homogeneous phenotype of ATF3 and FATP1, b) between p16, β6 integrin, FATP1 and FASN, and c) between FATP1, β6 integrin and FASN. The positive homogeneous phenotype of all these biomarkers was associated with high Ki67 as well. ATF3 positive homogeneous phenotype was significantly associated with positive diffuse and strong Collagen I ($p=0.042$), and FASN homogeneous phenotype with androgen receptor (AR) homogeneous phenotype ($p=0.011$).

ATF3 and FATP1 were the only biomarkers significantly associated with anatomical stage (stage 3) ($p=0.027$ and $p=0.026$, respectively) and intrinsic subtype

(Luminal B) (p=0.006 and p=0.010, respectively). ATF3 was also associated with pN1 status (p=0.025) and the presence of g*BRCA2* mutations (p=0.032).

Table IV - Significant associations between biomarkers in male BC

Biomarker		**p value (Fisher's exact test)**
p16	Ki67	0.004
	β6 Integrin	**<0.001**
	FASN	0.002
	FATP1	**<0.001**
ATF3	pN	0.025
	Stage	0.027
	Ki67	**<0.001**
	Intrinsic subtype	0.006
	β6 Integrin	0.039
	Collagen I	0.042
	FASN	0.011
	FATP1	**<0.001**
	BRCA2	0,032
β6 Integrin	Ki67	0.020
	ATF3	0.039
	p16	**<0.001**
	FATP1	**<0.001**
FASN	Ki67	0.015
	AR	0.011
	ATF3	0.002
	p16	**0.001**
	FATP1	**0.001**
FATP1	Stage	0.026
	Ki67	0.005
	Intrinsic subtype	0.010
	p16	**<0.001**
	ATF3	**<0.001**
	β6 Integrin	**<0.001**
	FASN	**0.001**

Note: In bold, p values ≤0.001

Survival analysis

Seven of 40 patients with BC (17.5%) had disease recurrence and died of disease. Mean and median remission time were 56.9 months and 41 months (range, 6-204), and mean and median survival time were 67.7 months and 50 months (range 7-223), respectively. Beyond the expected and confirmed (not shown) significant prognostic value of "classic" parameters (pT, pN, anatomic stage, grade), Kaplan-Meier estimates (log–rank test) indicate that male BC patients with FASN homogeneous phenotype had shorter DFS (Fig. 4a; p=0.04) and OS (Fig. 4b; p=0.03). Moreover, patients with Collagen IV stromal strong immunoexpression staining had a shorter DFS in univariate analysis (Fig. 5; p=0.05). A shorter OS was observed in patients with tumors with an ATF3 homogeneous phenotype (Fig. 6; p=0.02). The univariate (simple Cox model) analysis (not shown) was consistent with and confirmed the Kaplan-Meier/log-rank tests. In multivariate analysis, Collagen IV was the only of these markers significantly related with disease-free survival (p=0.032).

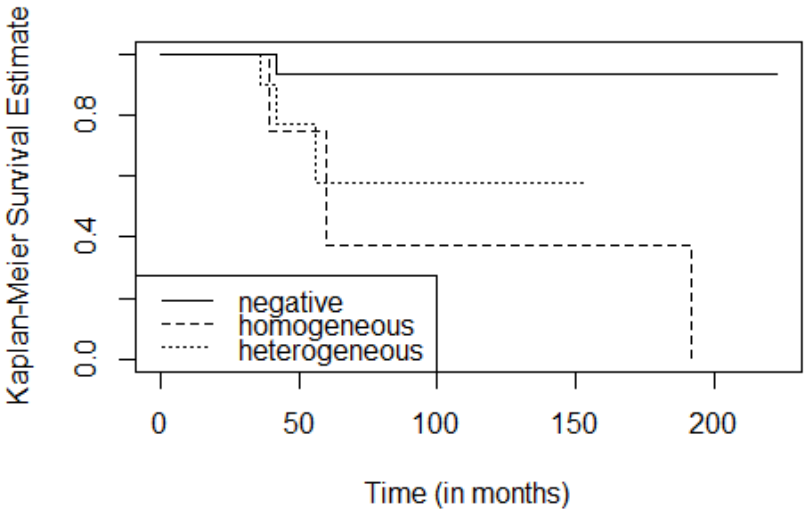

Figure 4 – Kaplan-Meier log-rank test showing that male BC patients with a negative staining for ATF3 have a significant longer OS (p=0.02)

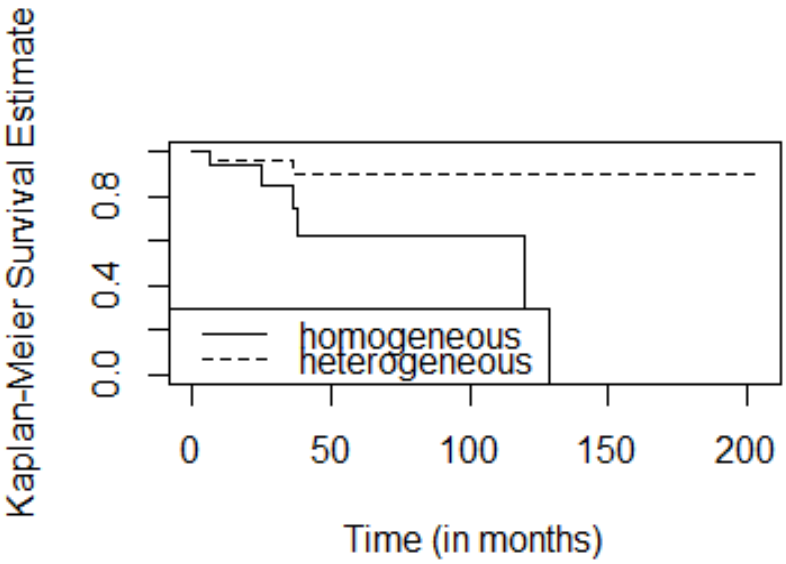

Figure 5 – Kaplan-Meier/log-rank test showing that male BC patients with a strong homogeneous FASN membrane cytoplasmic staining have a significant shorter DFS (p=0.040)

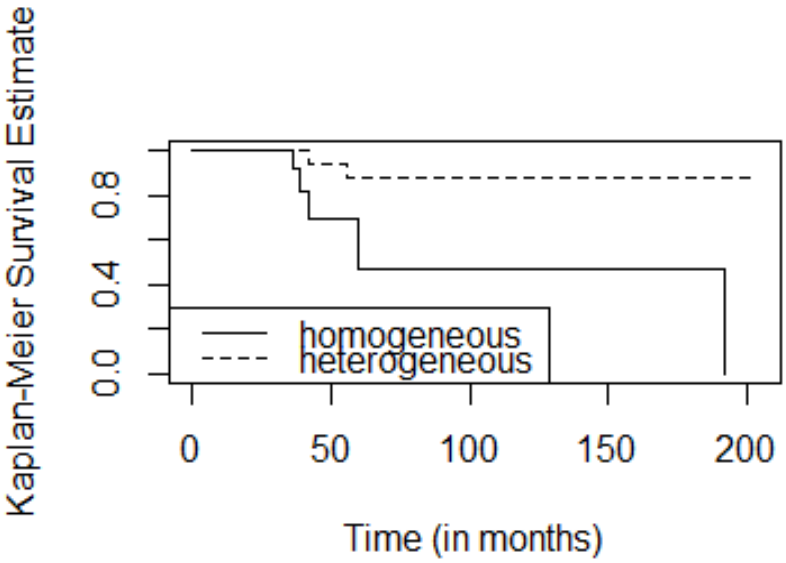

Figure 6 – Kaplan-Meier/log-rank test showing that male BC patients with a strong homogeneous FASN membrane cytoplasmic staining have a significant shorter OS (p=0.030)

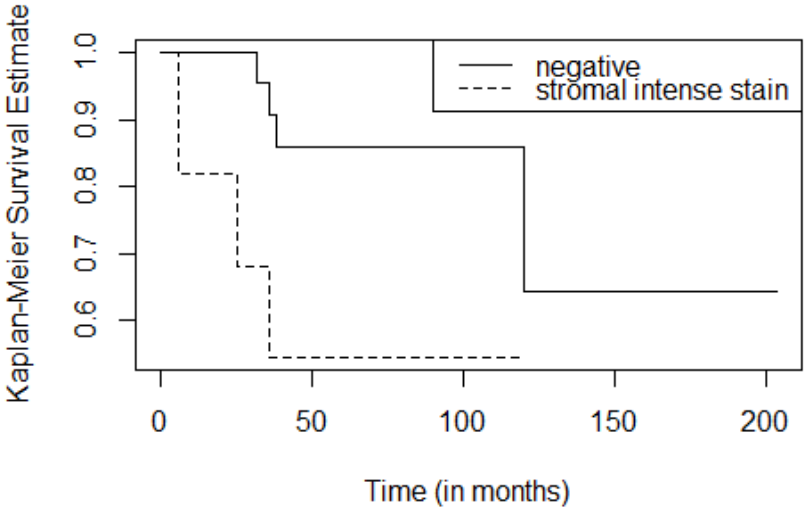

Figure 7 – Kaplan-Meier/log-rank test showing that male BC patients with a negative stromal staining for Collagen IV have a significant longer DFS (p=0.05)

Discussion

This study comprises 40 invasive male BC with no neoadjuvant therapy retrieved from a larger series previously reported (13). Despite the relatively small number of cases, this cohort still appears to be representative of the male BC entity, as shown by the age of diagnosis, presence of risk factors, such as obesity, family history, g*BRCA2* mutations, large predominance of Luminal-like subtype and other clinicopathologic parameters. Our objective was to identify IHC molecular biomarkers that may contribute to better characterize male BC biology, being potentially eligible for further larger and more complex studies in order to improve the clinical management of this entity.

Although the clinicopathologic and treatment factors were taken into consideration in the Cox model and the interaction with these factors has been assessed, our main goal was to highlight the results of the IHC studied biomarkers.

In male BC, biological specifiers for an effective personalized care and cure are undetermined (13). Additionally, the incidence of this rare disease is rising, and persistent poor outcomes have been reported (1, 13, 15). Understanding and simplifying the complexity of epithelial-stromal interaction and the relevance of phenotypical epithelial

homogeneity or heterogeneity burden in tumor progression, may be a clue for a new clinical management (16-19).

IHC is a simple and cost-effective method that demonstrates the presence and location of genes protein expression in epithelial and stromal tissues, allowing information that can help clarifying data obtained by other methods, including gene profile analysis or promoter methylation levels, or indicating the need for additional research. These were primary reasons for performing this immunophenotyping study of invasive male BC.

Although gynecomastia is not considered a pre-malignant lesion, in this cohort of 40 cases, we found that male BC was associated with gynecomastia in 22.5% of the cases. In the literature, this association has been stated as from inexistent to being present in 20-40 % of the cases (8). The complex etiopathogenesis and shared risk factors by both conditions are a cumbersome to clarify this association. Very few studies have been published on the immunoprofile of gynecomastia (4, 9, 20, 21), and a better characterization of gynecomastia, namely of the receptors balance between epithelial and mesenchymal components, may also be important for the clinical management of this entity. Androgen therapy may be an option in males with gynecomastia and hypogonadism (22, 23) and, in fact, AR expression was present in numerous epithelial and stromal fibroblasts of gynecomastia tissue.

AR expression was described to have an anti-proliferative role in normal breast tissue (24). In female BC, AR was reported to be expressed in up to 90% of ERα positive tumors, and its expression was linked to a favorable prognosis (25). Some studies emphasize the role of AR in the regulation of tumorigenesis in female BC via epithelial-mesenchymal signaling, but there are very few data regarding the stromal-epithelial interactions in this condition (24). In male BC literature, AR immunoexpression in the malignant epithelial cells ranges from 34 to 95% and has conflicting data in relation to its prognostic value (26). In this series, malignant epithelial cells and fibroblasts express AR, in the majority of male BC and gynecomastia adjacent tissue, favoring a similar role in both conditions. In accordance with previous studies, the expression of AR in our series, both in the epithelial and stromal cells, was not associated with any prognostic factor. Although the value of AR expression as response predictor to therapy with AR antagonists in male BC patients was not established, Di Lauro *et al.* proposed that

stromal-epithelial interactions may have important effects on their action (26). This question might be answered in a larger series of patients submitted to AR-target therapy, with careful evaluation of the differential AR immunoexpression in malignant epithelial cells and stromal cells and functional studies.

ATF3 transcription factor is considered to be a mediator of cellular stress response and has also been studied in female BC. Several functions were attributed to ATF3, including a role in epithelial cell proliferation (27) and in the promotion of tumor progression as a "breast stroma related gene" (28, 29). No previous studies evaluated ATF3 expression in male BC. In the present series, a 53.8% general positivity was found for ATF3 in the epithelial compartment, together with numerous positive fibroblasts. ATF3 positive homogeneous phenotype, found in 20.5% of the tumors, is significantly associated with *BRCA2* germline mutations (P=0.032), pN1, anatomic stage 3, Luminal B subtype and high Ki67 expression. Accordingly, patients with tumor ATF3 homogeneous score had also a shorter OS. These results seem to be in accordance with the purposed ATF3 role in female BC, particularly in the regulation of cell proliferation and tumor progression. Wang *et al.* reported the existence of an interaction of ATF3 with AR (29), and the possible use of this link to develop a stromal-target therapy in male BC. However, in our series, no correlation between ATF3 and AR immunophenotype was found (p=0.30) to support their results.

p16 and Cyclin D1 have important roles in cell cycle regulation. In female BC, overexpression of Cyclin D1 in epithelial cells has been reported to occur in 35-81% of carcinomas and to be correlated with ERα/PR expression and Luminal subtypes, in contrast to p16, which is usually related to high proliferation activity (30-32). Also, in female BC, Pestell *et al.* demonstrated that stromal Cyclin D1 drives tumor microenvironment signaling and promotes BC growth (33). In male BC, Kanthan *et al.* reported similar positive rates for both markers. In that study, Cyclin D1 positivity was associated with better outcome, and the p16 expression was not of prognostic significance (34). In the present study, we found that these proliferation regulators are commonly expressed in male BC and gynecomastia epithelial cells. However, Cyclin D1 is positive in epithelial and stromal fibroblasts in almost all cases, with a homogeneous phenotype in three quarters of the cases in the epithelial compartment, favoring an important protagonism in male BC. p16 homogeneous phenotype is found in less than a quarter of the cases, is associated with high Ki67 expression (p=0.004) and also with homogeneous

phenotype staining of β6 integrin, FASN and FATP1. In this series, p16 is the only marker with positive stromal fibroblasts in all cases, even in negative malignant epithelial cells cases. Interestingly, in gynecomastia tissue, only rare positive fibroblasts were found to be positive with both Cyclin D1 and p16. These findings may favor an important function of these cell cycle proteins in stromal fibroblasts in male BC, but not in adjacent gynecomastia tissue.

Integrins are glycoproteins composed by 18α and 8β chains that pair and incorporate 24 different heterodimers. They mediate epithelial cell-cell and epithelial cell-extracellular matrix adhesion and organization of the intracellular cytoskeleton. β1, β3 and β4 integrins maintain tissue architecture and contribute to the function of normal breast tissue (35, 36). In female BC, they are linked to tumor progression, immune responses and drug resistance and may be important when considering therapeutic options oriented to tumoral stroma blockage (35-39). In our series, β1, β3 and β4 integrins stained myoepithelial cells in gynecomastia tissue, as reported in female breast hyperplasia and benign tumors (35). In male BC, β1, β3 and β4 integrins have a similar malignant epithelial cell membrane staining, consistent with cell to cell adhesion, in very few cases. All these integrins have an identical peculiar reinforcement in the stroma around aggregates of malignant epithelial cells in some negative epithelial cases, in an identical percentage, although in different cases. The specific immunophenotyping of these β chain integrins deserves further investigation in larger series. β6 integrin staining patterns are different from the other integrins. In gynecomastia tissue, β6 integrin is positive in high number of epithelial and myoepithelial cell membrane as in numerous fibroblasts. In male BC, two different patterns of positivity in malignant epithelial cells are present: homogeneous phenotype of cell membrane and granular cytoplasmic staining. These results may be related with β6 integrin participation in different heterodimers. Moreover, β6 integrin expression was reported to be associated with unfavorable prognosis in different cancer types (40). Although in this series, probably due to the small number of cases, β6 integrin is not directly associated with prognosis, its homogeneous phenotype in male BC cases is associated with high Ki67 and ATF3, p16 and FATP1 homogeneous phenotype, which are related to worse prognosis.

FASN is highly expressed in many conditions, including female BC carcinomas. FASN is a multifunctional protein, involved in the synthesis of long-chain saturated fatty acids. It was reported to be expressed in some benign and pre-invasive female breast in basal/suprabasal cells (41), and we find this pattern in gynecomastia tissue. This marker was also reported to be significantly correlated with grade, stage and worse overall survival in cancers such as ovarian cancer (42). In triple negative female BC, FASN was significantly associated with positive lymph nodes, but not with OS or DFS (43). In proliferating neoplastic cells, fatty acids can be synthesized *de novo* to provide lipids for membrane formation and energy production (44). In female BC, Menendez *et al*. described a complex molecular interaction between endogenous fatty acid metabolism and ERα signaling, occurring at multiple levels, and the capacity of FASN to regulate ERα may represent an effective therapeutic strategy involving FASN inhibitors (45). Interestingly, in our series of ERα positive male BC cases, FASN malignant epithelial cell membrane with homogeneous phenotype is significantly associated with shorter DFS and worse OS, in contrast to the reported in triple negative female BC. Moreover, FASN is also associated with Ki67, AR, ATF3, p16 and FATP1 expression. FASN is positive in rare fibroblasts in both male BC and gynecomastia adjacent tissue, suggesting that its particular relevance is limited to the epithelial cell compartment.

FATP1, encoded by the *SLC27a1* gene, was reported to be expressed in cells and tissues with high-level fatty acid import for metabolism or storage, as the adipose tissue (46). The present series is the only study evaluating FATP1 immunophenotype pattern in BC epithelial cells and fibroblasts. Our group has unraveled, in vitro, a role for FATP1 in the metabolic cross-talk between female BC cells MDA-MB-231 and cancer associated fibroblasts (47). In the present study, according to *in vitro* results, FATP1 stains numerous stromal fibroblasts male BC cases and adjacent gynecomastia tissue, which are also positive in the malignant epithelial cells, favoring the FATP1 modulation between epithelial and stromal components in these conditions.

In normal tissues, lipid droplets are storage organelles for lipids and proteins. These lipids and proteins can traffic between lipid droplets and endoplasmic reticulum, and FATP1 may have a role in facilitating lipid droplet transport at this interface (48-51). The origin and significance of nuclear lipid droplets is uncertain, but the inner nuclear membrane can metabolize lipids and regulate transcription in response to lipid availability (52, 53). Recent understanding of the mechanisms of interaction between chromatin and

lipids suggest that small lipid molecules can regulate main nuclear functions. Lipids that bind to nucleosomes and affect chromatin are likely to be valuable as tools to modify phenotypes at a molecular level (54, 55). Remarkably, in the present study, FATP1 has an unexpected nuclear staining, with a heterogeneous phenotype in gynecomastia, although a concomitant diffuse cytoplasmic staining with variable intensity was observed in some cases. FATP1 homogeneous phenotype in male BC cases is significantly associated to high Ki67 positive cells, with malignant epithelial homogeneous phenotype of FASN, p16, integrin β6 and ATF3, and with high stage and Luminal B carcinomas. The association with high Ki67 sustains the fact that, in highly proliferative lesions, cells have increased metabolic demands undertaken by fatty acid metabolism pathway. This finding should be confirmed by other studies, as clinical inhibitors for different steps in fatty acids pathways already exist (56), and their use could be applied in male BC patients.

Collagens are the major structural component of the stroma and may modulate the genesis and progression of carcinomas. There are 28 collagens organized into subgroups, including the fibrillar-forming collagens and the network-forming collagens. Collagen I is a fibrillar-forming collagen and Collagen IV forms an interlaced network at basement membrane, found at the basal surface of epithelial and endothelial cells, and essential for tissue polarity and molecular filtration function (57-60). Compared to normal tissue, the amount of type I collagen was reported to be augmented in female BC and its dysregulation may affect the behavior of malignant cells (58). In the present series, the intensity of stromal staining in Collagen I and Collagen IV seems to be important in revealing structural differences. Collagen I strong intensity is significantly associated with ATF3 epithelial homogeneous phenotype ($p=0.042$), although collagen I is not associated with prognosis. However, the intense and diffuse homogeneous Collagen IV stromal immunophenotype is significantly associated with a shorter DFS. This finding may be associated with intense stromal remodulation and supports previous studies that showed the potential value of inhibiting collagen IV synthesis or deposition to control female BC progression (61).

As final considerations, we would like to underline that most all the patients included in this preliminary small study had clinicopathologic features classically associated with “good prognosis” such as Luminal-like subtype and anatomic stage I/II.

In consequence, we found a low percentage of disease recurrence and death (17.5%). However, these conditions are commonly found in the current clinical management of male and female BC, emphasizing the importance of the results obtained. The molecular markers p16, ATF3, β6 integrin, FASN and FATP1 are significantly intercorrelated, if homogenous phenotype of epithelial staining is present. They are all also significantly related to high cell proliferation, as assessed by Ki67. With the exception of FASN, all these biomarkers stained >10% of fibroblasts in malignant epithelial positive cases, and p16 also stained fibroblasts in malignant epithelial negative cases. Uniform distribution of ATF3 and FATP1 in malignant epithelial cells was significantly associated with anatomical stage 3 and Luminal B intrinsic subtype. ATF3 was also associated with pN1 status and the presence of g*BRCA2* mutations. Furthermore, the study highlights the significant associations of homogeneous malignant epithelial staining of FASN with DFS and OS, as well as ATF3 with OS and Collagen IV with DFS. No homogeneous epithelial pattern or intense Collagen IV stromal staining were identified in gynecomastia tissue.

Conclusions: This study is preliminary and does not allow definitive assumptions. However, it highlights the potential value of the intratumor epithelial cells homogeneity with biomarkers with distinct biological functions, their interactions and significant association with high Ki67 cell proliferation. The prognostic relevance of the homogeneous phenotype of FASN and ATF3, as well as the diffuse and intense Collagen IV stromal staining may contribute to identify male BC with worse outcome, indicating that these biomarkers may be eligible for further investigation, with potential to innovate the management of male BC.

Funding

Giovani Silva was partially funded by FCT-Portugal project UID/MAT/00006/2019.

Disclosures

The authors have no conflicts of interest or relevant funding to disclose

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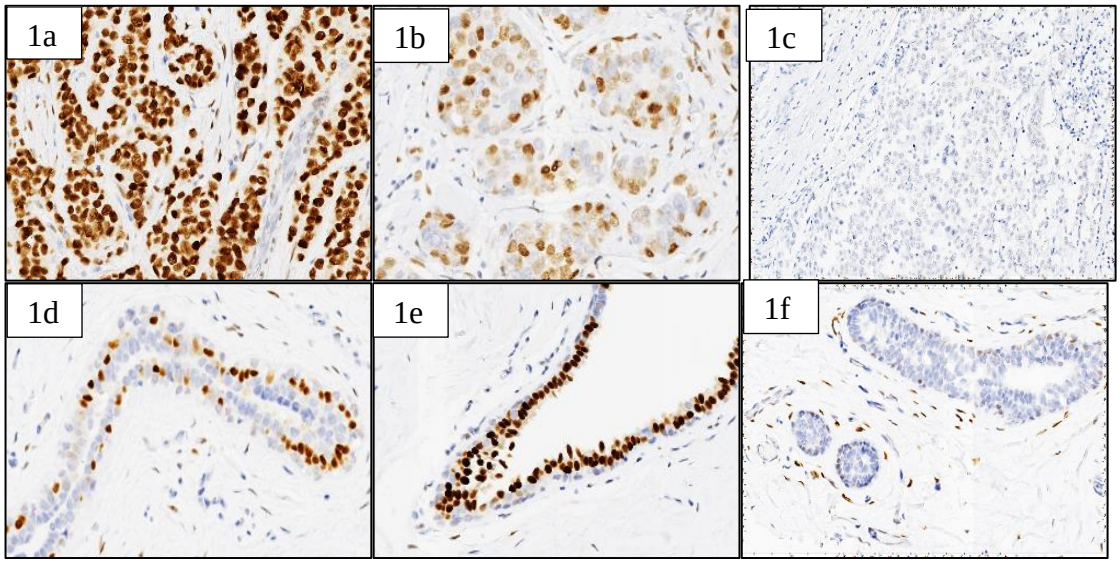
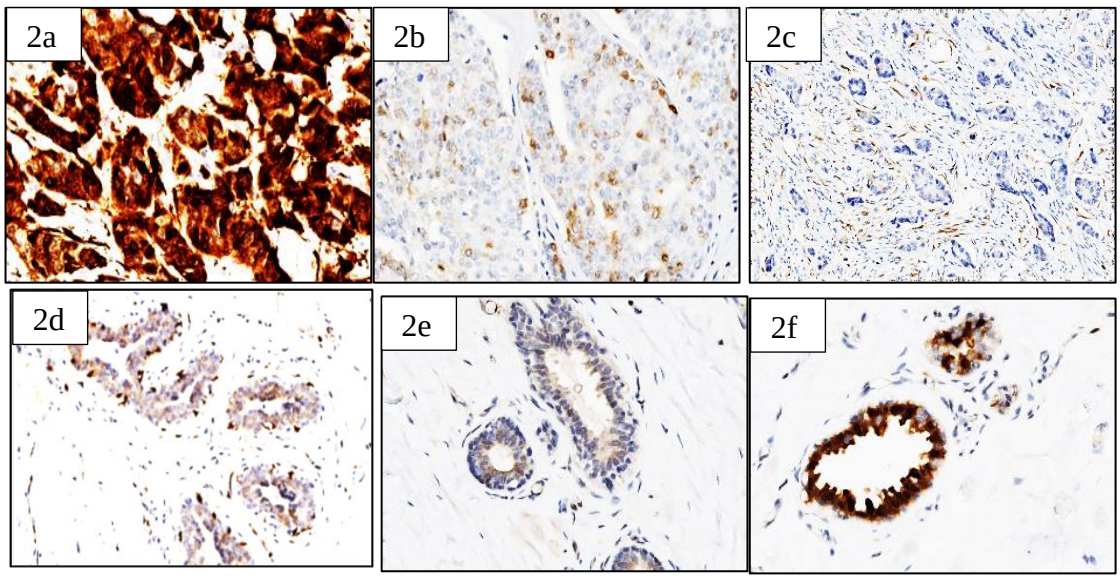
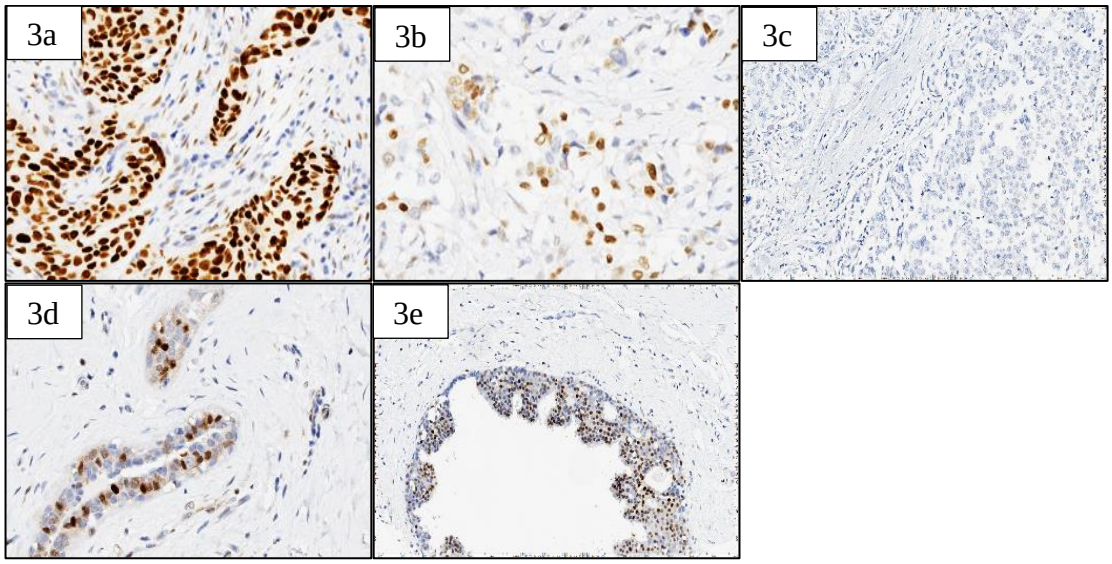
Supplemental Digital Content (SDC)



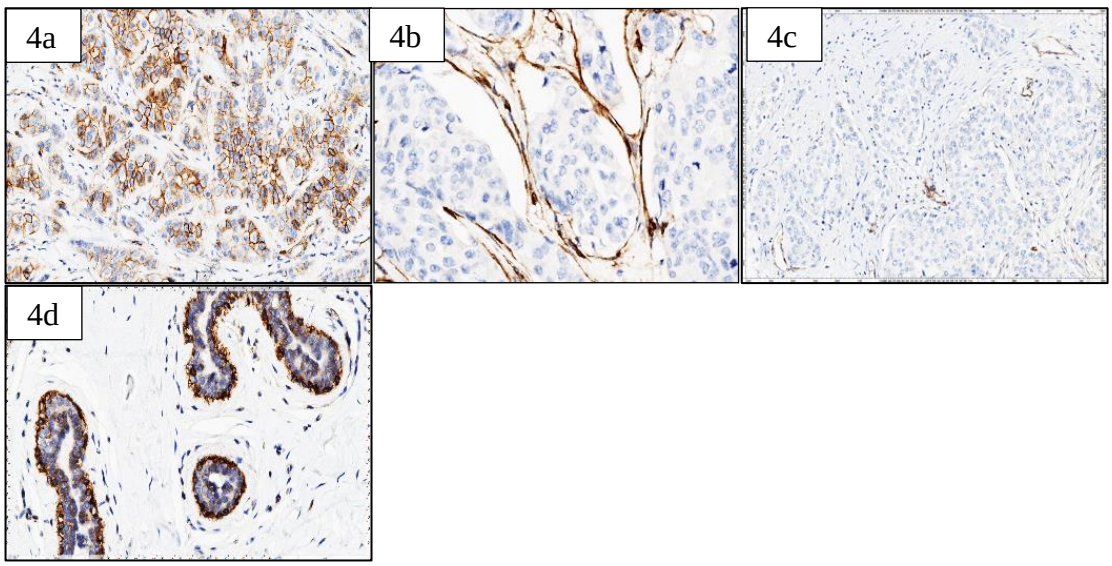
SDC Figure 1 - Androgen receptor (AR) staining in epithelial cells of male BC (1a – positive homogeneous, 1b – positive heterogeneous, 1c - negative) and in epithelial cells of gynecomastia (1d, 1e, 1f – heterogeneous stain in the same case). Fibroblasts stain irregularly in male BC positive epithelial cases and in gynecomastia, and were negative in male BC negative malignant epithelial cases.



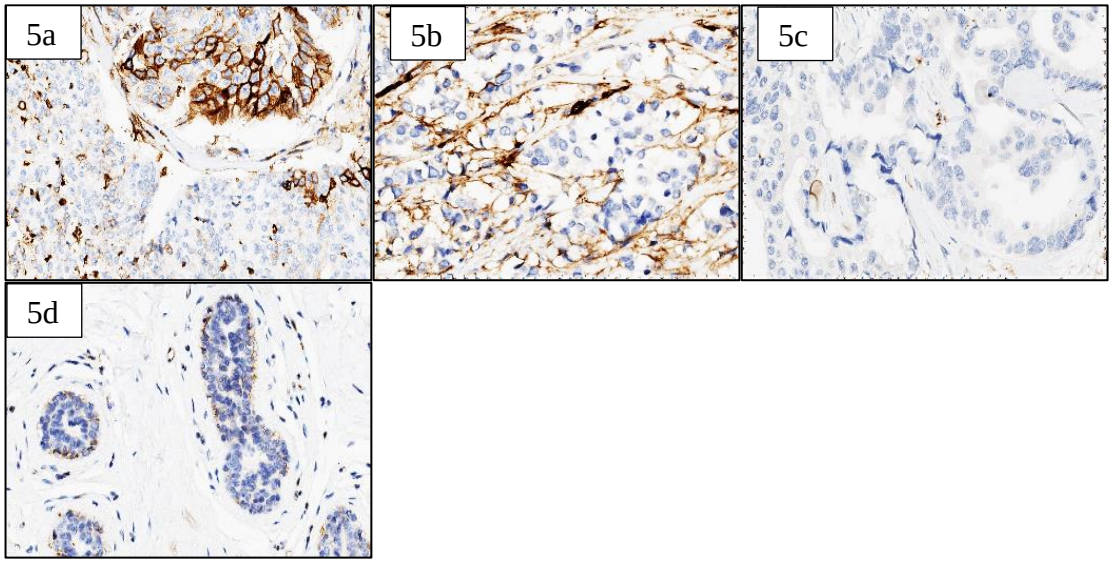
SDC Figure 2 - p16 in male BC (2a - positive homogeneous staining, 2b – positive heterogeneous, 2c - negative) and in gynecomastia (2d, 2e, 2f - heterogeneous staining in the same case). Fibroblasts stained irregularly in gynecomastia and may be numerous in male BC, even in epithelial negative cases (2c).



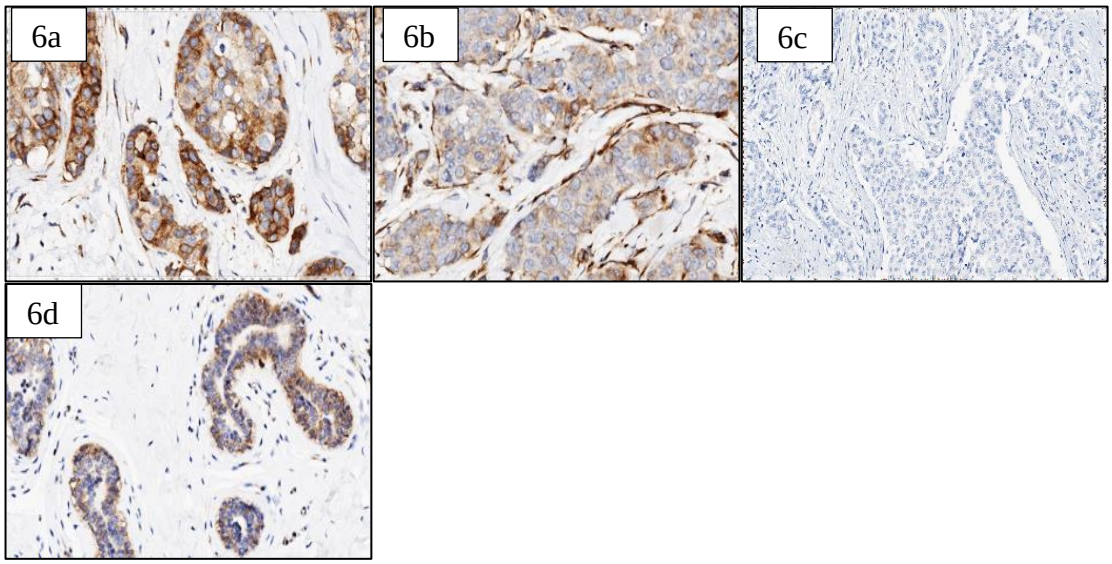
SDC Figure 3 - Cyclin D1 in male BC (3a - positive homogeneous, 3b– positive heterogeneous, 3c - negative) and in gynecomastia (3d, 3e – heterogeneous staining). Fibroblasts stained irregularly in male BC epithelial positive cases and don´t stain in the epithelial negative male BC cases nor in gynecomastia.



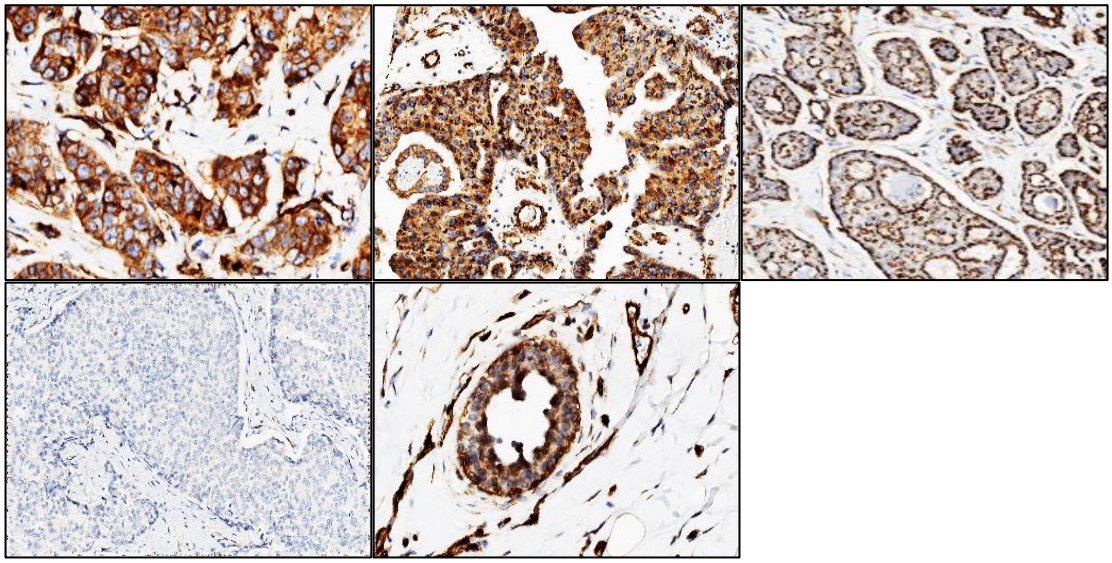
SDC Figure 4 - β1 Integrin in male BC (4a – heterogeneous cell membrane staining, 4b – stromal fibroblast staining around epithelial cell clusters, 4c - negative) and in gynecomastia (4d – myoepithelial cells staining). Fibroblasts staining is negative in gynecomastia.



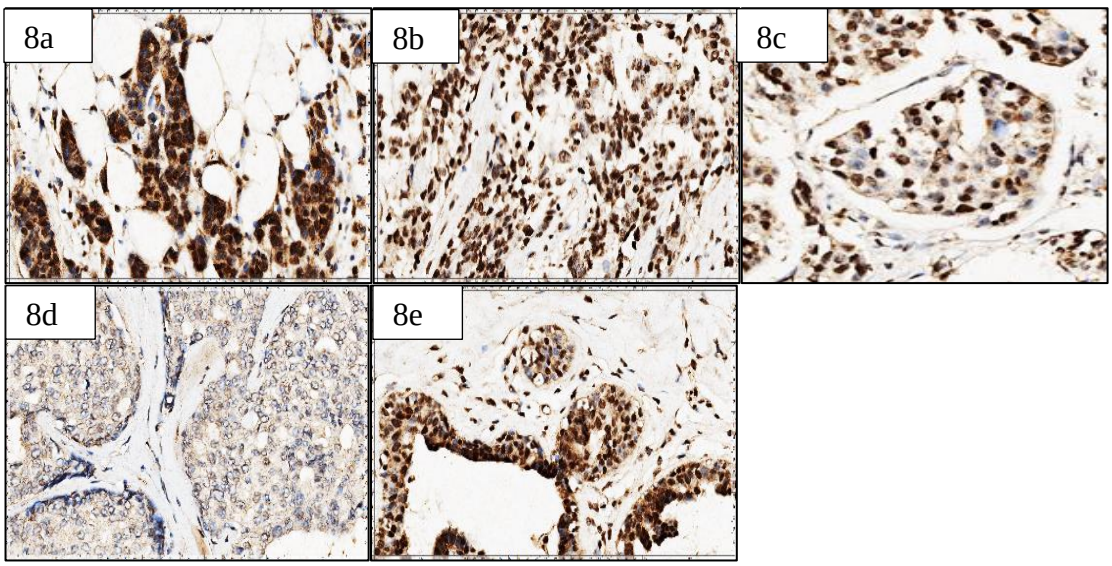
SDC Figure 5 - β3 Integrin in male BC (5a - heterogeneous cell membrane staining, 5b - stromal fibroblast staining around malignant epithelial cell clusters, 5c - negative) and in gynecomastia (5d - myoepithelial cells stain). Fibroblasts staining is negative in gynecomastia.



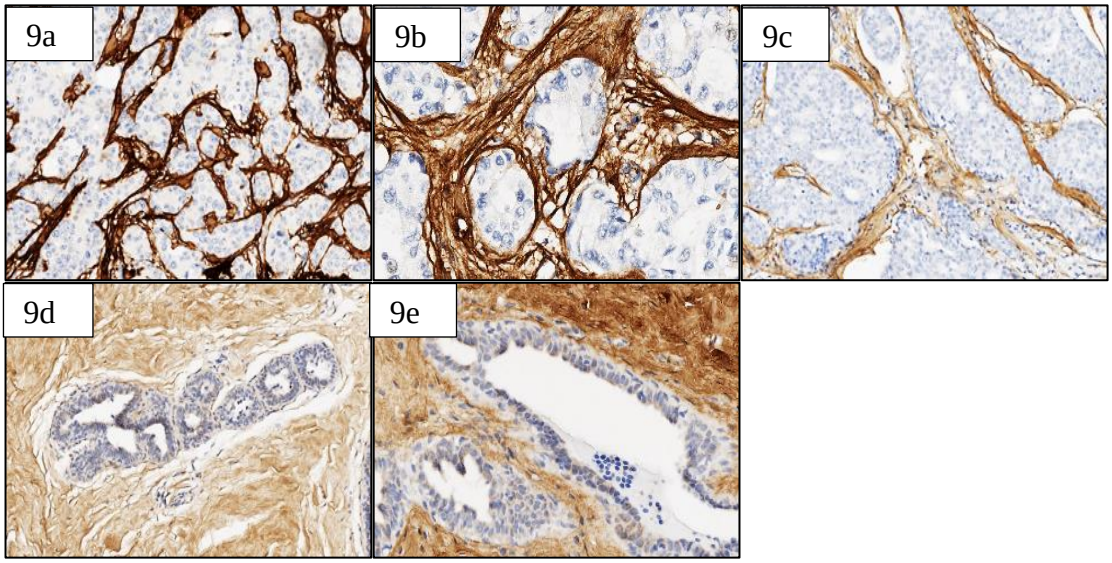
SDC Figure 6 - β4 Integrin in male BC (6a – heterogeneous cytoplasmatic staining with cell membrane reinforcement 6b - stromal fibroblast staining around malignant epithelial cell clusters, 6c - negative) and in gynecomastia (6d – myoepithelial cells and ocasional epithelial cells light staining). Fibroblasts staining is negative in gynecomastia.



SDC Figure 7 - β6 Integrin in male BC (7a – homogeneous epithelial cytoplasmatic staining with cell membrane reinforcement, 7b and 7c – granular cytoplasmatic staining, 7d - negative) and in gynecomastia (7e - heterogeneous epithelial and myoepithelial staining). Positive fibroblasts are numerous in epithelial positive male BC cases and in gynecomastia.



SDC Figure 8 - FATP1 in male BC (8a and 8b – nuclear homogeneous and some cytoplasmatic light staining, 8c - nuclear heterogeneous staining, 8d - negative) and in gynecomastia (8e - nuclear and some cytoplasmatic light heterogeneous staining). Positive fibroblasts are numerous in epithelial positive male BC cases and in gynecomastia.



SDC Figure 9 - Collagen I in male BC (9a and 9b stromal diffuse intense staining, 9c - stromal diffuse weak/ moderate staining) and in gynecomastia (9d and 9e - stromal diffuse weak/ moderate staining).

Chapter IV

4.4 - Germline *BRCA* data

4.4 - Germline *BRCA* data

The importance of genetic data in the study and comprehension of male BC and in the clinical care of patients is undeniable and through all scientific articles previously presented in this chapter (4.1, 4.2 and 4.3), the data from g*BRCA* mutations in the studied populations was included.

In 4.4, we decided to include:

4.4.1 - Table 1 describing the mutation variants and clinicopathologic features found in the g*BRCA2* patients included in all the cohort of this thesis.

4.4.2 - A **scientific article** with an analyses of male patients counselled in IPOLFG Breast Cancer Risk Evaluation Clinic between January 2000 to December 2015. Ana C. Freitas, Ana Opinião, Sofia Fragoso, Hugo Nunes, Madalena Santos, Ana Clara, Sandra Bento, Ana Luís, Jorge Silva, Cecília Moura, Bruno Filipe, Patrícia Machado, Sidónia Santos, Saudade André , Paula Rodrigues, Joana Parreira, Fátima Vaz.

Men seeking counselling in a Breast Cancer Risk Evaluation Clinic.

Ecancermedicalscience. 30;12:804, 2018. doi: 10.3332/ecancer.2018.804. eCollection 2018.

4.4.3 - Three abstracts of previous studies presented in international congress and published in international peer-review journals.

Santos F, Félix A, Carvalho A, Machado P, Vaz F, André S.

***BRCA2* Mutation in Male Breast Cancer.**

Mod Pathol. 28 (s2), 4-573, 2015.

Canas Marques R, Cunha F, Machado P, Vaz F, André S.

Male breast invasive lobular carcinoma: report of two cases

Virchows Arch. 463 (2); 101-352, 2013 (PS-17-066).

Santos F, Cunha F, Machado P, Vaz F, André S.

Bilateral Male Breast Cancer and *BRCA2* Mutation

Virchows Arch. 463 (2); 101-352, 2013 (PS-17-078).

4.4.1 - The mutation variants and clinicopathologic features found in the g*BRCA2* patients included in all the cohort of this thesis (4.1) are described in Table I.

The mean age of the patients is 64.7 years. We would like to emphasize two g*BRCA2* variants, each one found in three patients (23.7% of the cases): the founder mutation c.156_157insAlu, with associated prostate carcinoma in two patients and the mutation c.9098_9099insA, also clinically relevant, associated with prostate and bladder carcinoma in one patient and with neuroendocrine gastric carcinoma in other patient. All these patients, except one with the c.9098_9099insA variant, also had bilateral BC.

We underline that, in the univariate analysis described in 4.1, g*BRCA2* mutations were significantly associated with shorter DFS and OS.

Importantly, in one case evaluated in the Breast Cancer Risk Clinic we identified a patient with a prostate carcinoma and a g*BRCA2* mutation (variant c.1056.1058del) that harbor a gynecomastia, and not a male BC. This case was included as a gynecomastia for comparative group control in the second study (4.2).

Table I - Germline *BRCA2* mutation variants and clinicopathological data.

BRCA2 variant	Age (years)	B	HT	AS	Intrinsic subtype	DFS (months)	Status	OS (months)	NBPN
c.6676delG	72	0	D	1A	HER2+	77	A	77	prostate
c.6468_6469del2	65	0	D	3B	Luminal B	36	D	56	
c.1786G>C/2	64	0	D	3B	Luminal B	72	D	72	
c.9098_9099insA	56	1	D	2A	Luminal B	144	A	252	prostate bladder
c.156_157insAlu	54	1	D	2B	Luminal B	32	A	223	
c.156_157insAlu	58	1	D	3B	Luminal B	120	A	192	prostate
p.E475X	76	0	D	3C	Luminal B	0	D	24	
c.2808_2811del4	66	0	D	2A	Luminal B	168	A	192	prostate colon kidney
c.9098_9099insA	73	1	D	3B	HER2 +	0	DOC	48	gastric
c.4380_4381del2	64	0	L	3B	Luminal B	38	D	39	
c.156_157insAlu	66	0	D	2B	Luminal B	46	A	48	
c.9098_9099insA	67	0	D	3B	Luminal B	25	A	60	
c.793+1G>A	60	0	D	2B	Luminal B	24	A	24	

B, bilaterality; HT, histological type; AS, anatomic stage; DFS, disease-free survival; OS, overall survival; NBPN, non-breast primary neoplasms; A, alive; D, dead of disease; DOC, dead of other causes.

4.4.2 - Patients with rare cancer should be predominantly treated in large referral centers due to the lack of clinical practice guidelines and clinical personal experience. IPOLFG is a referral center for many rare cancers in the south of Portugal and developed a Breast Cancer Risk Evaluation Clinic.

The Breast Cancer Risk Evaluation Clinic presents regularly its clinical and research activity, and has made an important contribution to the original description of the founder mutation of Portuguese origin (c.156_157insAlu) in g*BRCA2* gene (37).

In the following recent scientific article, the analysis of male patients counselled in IPOLFG Breast Cancer Risk Evaluation Clinic found that 102 male patients (84.3%) were *BRCA2* mutation carriers and 51 (50%) of these carriers belong to the c.156_157insAlu families.

4.4.2 - Men seeking counselling in a Breast Cancer Risk Evaluation Clinic

Ana C. Freitas, Ana Opinião, Sofia Fragoso, Hugo Nunes, Madalena Santos, Ana Clara, Sandra Bento, Ana Luís, Jorge Silva, Cecília Moura, Bruno Filipe, Patrícia Machado, Sidónia Santos, Saudade André , Paula Rodrigues, Joana Parreira, Fátima Vaz

Ecancermedicalscience. 30;12:804, 2018. doi: 10.3332/ecancer.2018.804. eCollection 2018.

Abstract

Background - Hereditary breast and ovary cancer syndrome affects both genders but little is known about the uptake of genetic services by men. The objective of this study is to characterize the male population counselled through a multidisciplinary breast/ovarian program.

Methods - Descriptive analysis of male patients counselled from January 2000 to December 2015. Data in this analysis include new cancer diagnoses during prospective follow up.

Results - From 4,320 families registered, 362 male patients were identified: 236 (65.2%) from hereditary cancer families (HCF) and 126 (34.8) from non-HCF. In HCF, 121 patients (51.3%) were mutation carriers (MC): *BRCA2* – 102 (84.3%), *BRCA1* – 16 (13.2%), *CHEK2* – 1 (0.8%) and *TP53* – 2 (1.7%). Non-HCF included 126 patients: 85 (67.5%) belonged to families without pathogenic mutations or with variants of unknown clinical significance; 22 (17.5%) refused testing after counselling and 19 (15.0%) did not meet criteria for testing. Both HCF and non-HCF included patients with previous cancer diagnoses: HCF- Breast Cancer (BC) - 18; prostate cancer (PC) - 13; melanoma - 1; others - 7) and non-HCF (BC - 77; PC - 20; gastric cancer (GC) - 1; melanoma - 8; bladder cancer - 1; others - 22). From the 121 MC identified (including the *TP53* and *CHEK2* carriers), 97 patients (80.2%) adhered to prospective surveillance. With a median follow-up of 36.9 months, 17 cancers were diagnosed in 14 patients, PC being the most frequently diagnosed neoplasia (5 cases). Eleven patients (78.6%) are alive and three patients died of advanced cancer (2 with GC, 1 with disseminated adenocarcinoma).

Conclusion - We observed a high adherence to counselling, genetic testing and active surveillance by men belonging to hereditary BC families. Male carriers of pathogenic DNA variants are at risk for several cancers and should be included in prospective follow-up studies.

Introduction

Family predisposition to cancer has been a clinical, individual and social concern for a long time [1]. With increasing research in cancer genetics, multiple genes and mechanisms have been identified as being involved in inherited cancer syndromes [2]. One of these, the hereditary breast and ovary cancer syndrome (HBOCS), associated with germline mutations in *BRCA1* and *BRCA2* genes, is believed to cause approximately 10–15% of all breast cancers (BCs) [3]. The prevalence of *BRCA1* and *BRCA2* mutations in the general population is estimated to be between 1 in 500 and 1 in 1,000, respectively [4, 5]. Higher prevalence of *BRCA* mutations have been described in certain founder populations, such as the Ashkenazi Jewish and the Icelandic population [6]. Despite these low rates in the general population, pathogenic variants in *BRCA1/2* genes are the most frequent genetic alterations diagnosed in familial BC, being responsible for 3–8% of all BC cases and for 15–20% of all familial BC aggregation [3]. Other high or moderate penetrance genes (that include *CHEK2, PTEN, TP53, ATM, STK11/LKB1, CDH1, NBS1, RAD50, BRIP1* and *PALB2*) have also been described as also contributing to hereditary BC [7–11]. With the increasing use of Next Generation Sequencing and panel multigene testing, this list is increasing [2, 12–14].

Because of the high incidence of BC and the increasing awareness of the possibility of inherited cases (especially since *BRCA* testing is commercially available), the majority of patients counselled and tested in BC Risk Evaluation Clinics are female [15]. However, *BRCA1/2* carriers are also at higher risk for developing gastric and pancreatic cancer and male carriers have higher prostate cancer (PC) risk [16–19]. Skin or uveal melanoma was also associated with *BRCA* mutations, but this association is not conclusive [20]. With this knowledge and since *BRCA1/2* germline mutations have an autosomal dominant transmission, affecting both genders equally, it is expected that men increase their uptake of genetic services [15]. The literature is sparse on this matter, however, and the major focus is more on the distress and psychological needs of male patients getting genetic testing and counselling [21], than on the characteristics of male patients effectively

counselled in genetics clinics. Also, little is known about the surveillance programs performed, and the outcomes and compliance of male patients included in these.

The influence of male *BRCA* carriers in pedigree analysis of potential hereditary BC families has been previously acknowledged [22]. Besides the male-to-female ratio, family size and the individual clinical history of family members (death in young age, surgical procedures for non-oncological reasons that prevent the posterior development of cancer) are factors to consider, when counselling for genetic testing in BC families [23]. Not acknowledging these may prevent the identification of high-risk individuals and their families, women and men, and their participation in specific cancer surveillance programs. The non-systematic inclusion of men from *BRCA1/2* families in the counselling process may also prevent the opportunity to improve health practices and to transmit cancer risk information to offspring [24].

The aim of this study is to characterize the male population counselled during the first 15 years of activity in our BC Evaluation Clinic. Men with a previous diagnosis of BC were included, from the beginning of our program, since we considered male BC as criteria for BRCA1/2 testing, even without other cancer family history. In recent years, we also started inviting healthy men from *BRCA1/2* families for carrier testing, mostly due to high PC risk [16, 18]. Our approach has been to include *BRCA1/2* male carriers in prospective long-term surveillance programs. Men seeking counselling for other hereditary syndromes as well as other male patients seeking counselling due to non- hereditary familial BC, were also reviewed in this study.

Patients and methods

Patients: Review of all consecutive genetic and clinical medical records of male patients counselled through our program, between January 2000 and December 2015. These records include a complete personal and medical history of the patient as well as a pedigree with cancer and genetic information of at least three generations. Most men included in our program were either referred by their physicians because they had breast or PC, or were invited through proband contact when belonging to families found to harbor genetic pathogenic variants.

Genetic counselling: Our criteria for DNA testing are publicly available on the Internet [25]. Briefly, genetic testing was considered in cases of at least 10% combined probability of a *BRCA1/2* mutation or BC diagnosis before 30 years of age or triple

negative BC before 50 or male BC or (since 2014) high-grade serous ovarian cancer. Patients with criteria for genetic testing undergo appropriate counselling. When consenting on molecular diagnosis, they return for a post-test counselling visit, for test result disclosure and post-test management. All male carriers of pathogenic variants were offered the possibility to participate in a prospective surveillance programs. For the purposes of this paper, hereditary cancer families (HCF) are defined as families with a previous identification of a pathogenic germline variant. Non-hereditary cancer families (non-HCF) did not have a germline pathogenic variant identified.

Molecular diagnosis: Until 2014 mutation testing for point mutations in *BRCA1* and BRCA2 genes was performed using methodologies based on heteroduplex analysis: Conformational Sensitive Gel Electrophoresis [26], [27] and Conformational Sensitive Capillary Elec- trophoresis [26]–[28]. After 2014 *BRCA1* and *BRCA2* molecular diagnosis was performed by next generation sequencing (NGS) using the BRCA MASTR Dx (Multiplicom, Niel, Belgium) kit and multigene testing was performed using the Trusight Cancer sequencing panel (Illumina, San Diego, CA, USA) in a MiSeq plattform (Illumina, San Diego, CA, USA) [29]. Large rearrangements in *BRCA1* and *BRCA2* genes were tested by multiplex ligand probe amplification (MLPA, MRC Holland) and the Portuguese founder mutation specifically tested for as previously described [30].

Prospective follow-up: Duration of follow up was defined as the period since the post-test counselling visit until the last registered visit during study period. Data collected included patients' demographics, reason for referral, personal clinical and family history, DNA test results and follow-up data with new cancer diagnoses and survival considered as events of interest. For male *BRCA1/2* mutation carriers (MC) the surveillance program included:

• Annual medical oncology evaluation along with complete physical examination
• Annual urology evaluation with PSA screening
• Annual dermatologic screening for suspicious skin lesions
• Specific surveillance according to the Hereditary Syndrome (e.g., Colon and prostate screening for the *CHEK2* 1100delC carrier and a specific protocol for *TP53* carriers)
• Other clinical investigation according to new symptoms.

Statistics: Descriptive statistics were obtained, using Microsoft Excel, for the distribution of study variables.

Legal and ethical issues: All genetic records are protected with restricted access as per Portuguese law. All men consenting in genetic testing or their legal representatives signed an informed consent form that was approved by the Ethics Committee of our Institute.

Results

Patients characteristics*:* From a total of 4,320 families registered, we identified 362 male patients, with a median age of 53 years (16–86 years). Most of them [236 patients (65.2%), median age 47.5 years] belong to HCF (Figure 1). One hundred twenty-one of these were found to be MC: *BRCA2* (102), *BRCA1* (16), *CHEK2* 1100delC variant (1) and *TP53* (2) (Table 1). Nineteen patients were the first individuals to be tested in their families, all with a previous cancer diagnosis [BC (16), PC (2), colorectal cancer (1)]. All other men in HCF were invited, through proband contact, after the identification of a pathogenic variant in a family relative. Fifty-one (50 %) of all BRCA2 carriers belong to c.156_157insAlu families, a founder mutation of Portuguese origin [30]. The non-MC of HCF was discharged from follow-up after the post-test counselling visit. Six patients in the HCF Group were waiting for test results by the time of the analysis. Their mutational status is included in the sample characterization but they were not included in the follow-up data collection. Three patients refused genetic testing (Figure 1). They were discharged from the clinic with information to their primary care physician. The other group (Non-HCF, Figure 1) includes 126 patients (34.8%), with a median age of 63.5 years (range 1,786 years). More than half (67%) of these men belong to high risk families and were tested as index patients, but were found to have non-pathogenic mutations or variants of unknown clinical significance. Also, in this group, 19 patients asked for counselling due to aggregation of BC in their families, but were found not to have criteria for genetic testing, and 22 refused testing after counselling.

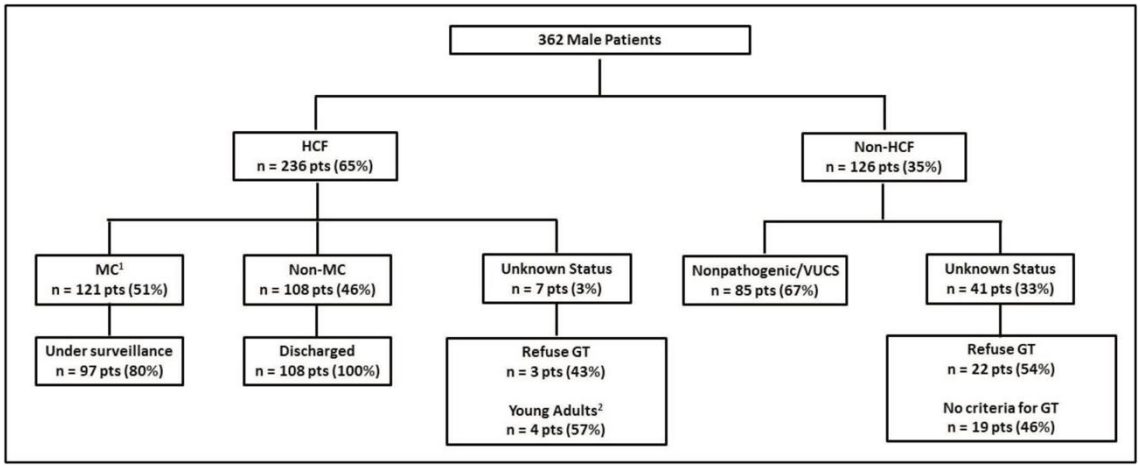


Figure 1 - Study population: HCF – Hereditary cancer families; MC – Mutation carriers; VUCS – Variants of unknown clinical significance; GT - Genetic test [1] Six of these 121 MC were still waiting test results by the time of data analysis. They were not included in the follow-up.[2] Four patients were young adults by the time of first counselling visit, and decided to postpone their genetic testing.

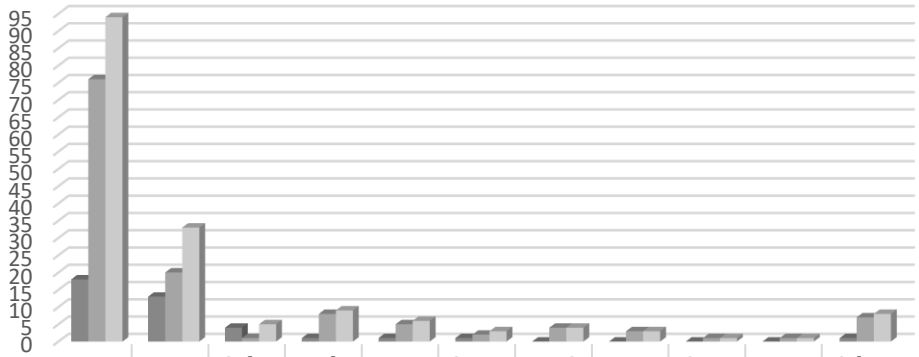
Table 1 – Patients characteristics by mutational status.

	MC	**Non-MC**	**Unknown**	**Total**
Patients (nº)	121	193	48	362
Age at first visit, years				
Mean	44	46	37	47
Range	23-78	16-79	16-64	16-79
Previously affected (nº)	26	83	31	140
Age at first visit, years				
Mean	68	64	58	64
Range	48-77	32-86	16-81	16-86
Previous neoplasia (nº)				
1	12	68	26	106
2	10	14	4	28
3	3	0	1	4
>3	2	1	0	3
Mutations				
BRCA2	102			
BRCA1	16			
P53	2			
CHEK2	1			

MC – Mutation carriers; Non MC - non mutation carriers (*these include true negative carriers for their family mutation and index patients found with non-pathogenic variants or variants of uncertain clinical significance). 1Unknown status: these patients were not tested, either because they did not meet criteria for genetic testing or refused to be tested after counselling.

Regarding cancer diagnoses before genetic testing, 33 patients belonging to HCF (33/236) had been previously diagnosed with a total of 39 cancer cases (0.17 case/patient). BC was the most frequent diagnosis (46%), followed by prostate (33%) and colorectal cancer (10%) (Figure 2). Although these individuals belonged to families with a known hereditary cancer syndrome, only 26 were confirmed carriers of the family mutation. The other seven cancer cases were admitted to be likely sporadic: the other side of the family was not suspicious for cancer heredity, and even when it was (only 1 case) comprehensive *BRCA1/2* analysis did not reveal any pathogenic variant. An example of a family with two cases of male BC and found to harbor a *BRCA2* mutation is shown in Figure 3. More complex hereditary families are shown in Figure 4 (CHEK2 1100delC) and Figure 5 (Li-Fraumeni Syndrome).

Male patients from non-HCF included 107 individuals with a personal history of neoplasia (129 cases; 1.02/patient). BC represented 77 of these cases (60%). Figure 2 displays both HCF and non-HCF cancer diagnoses registered before testing.



	Breast	Prostate	Colorectal	Melanoma	NHL	Sarcoma	HNC	Renal Cell	Gastric	Pancreas	Other *
HCF	18	13	4	1	1	1	0	0	0	0	1
Non-HCF	76	20	1	8	5	2	4	3	1	1	7
Total	94	33	5	9	6	3	4	3	1	1	8

Figure 2 - Previous cancer diagnoses: HCF – Hereditary cancer family; Non-HCF–Non-hereditary cancer family; NHL – Non-Hodgkin Lymphoma; HNC–Head and Neck Cancer. *Other sites: Chronic Lymphocytic Leukemia (0:1); Urothelium (0:1); Wilms tumor (0:1); Thyroid (0:1); Parathyroid (0:1); Testicular (0:1); Squamous cell skin cancer (0:1); Basal cell carcinoma (1:0).

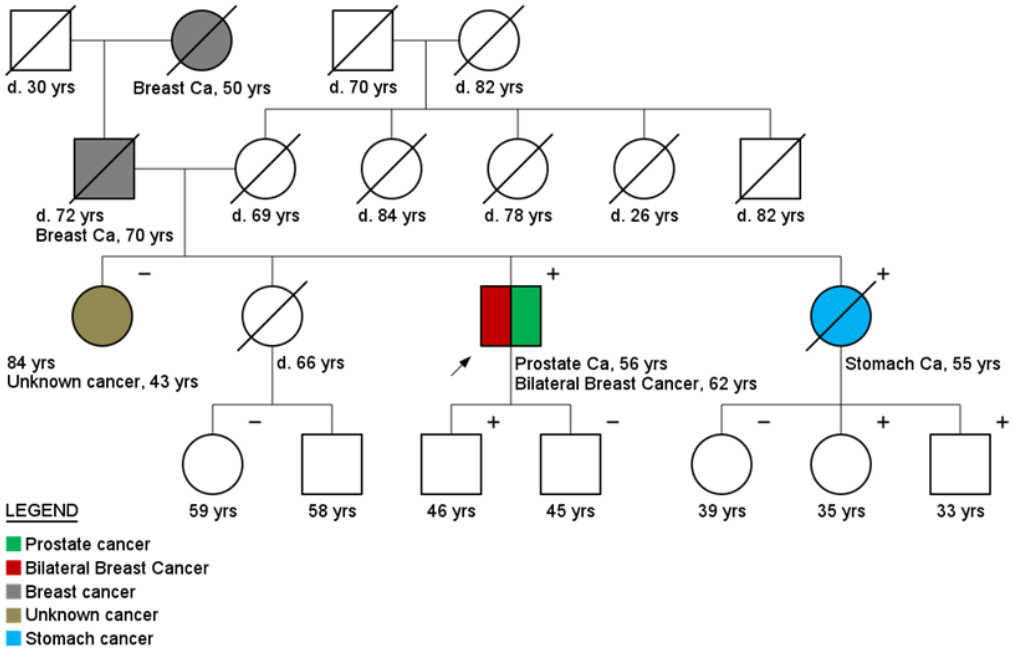


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Figure 3 - Pedigree of a bilateral breast cancer and prostate cancer patient (arrow), carrier of a *BRCA2* mutation (c.9098_9099insA). Plus (+) and minus (−) signs represent the carrier status of tested family members.

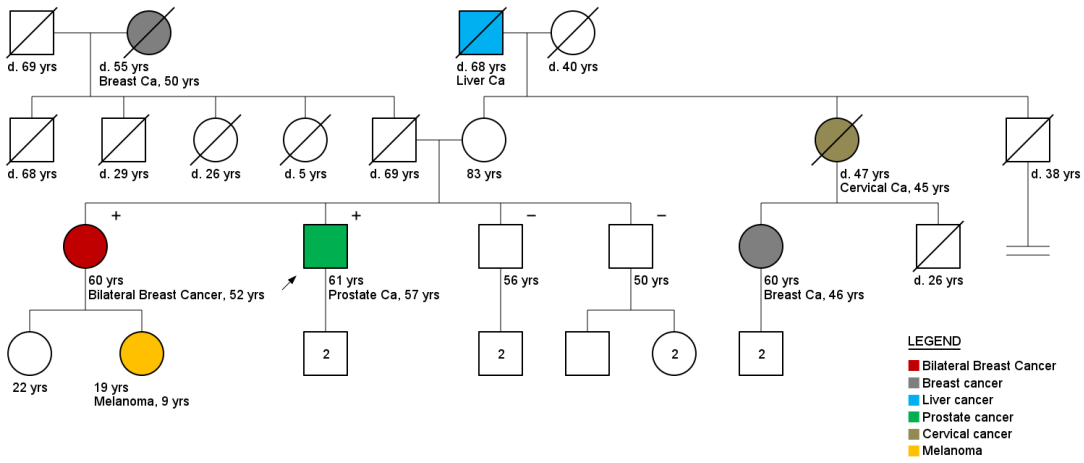

Figure 4 - Pedigree of a prostate cancer patient (arrow), carrier of a *CHEK2* mutation (1100delC). Plus (+) and minus (−) signs represent the carrier status of tested family members.

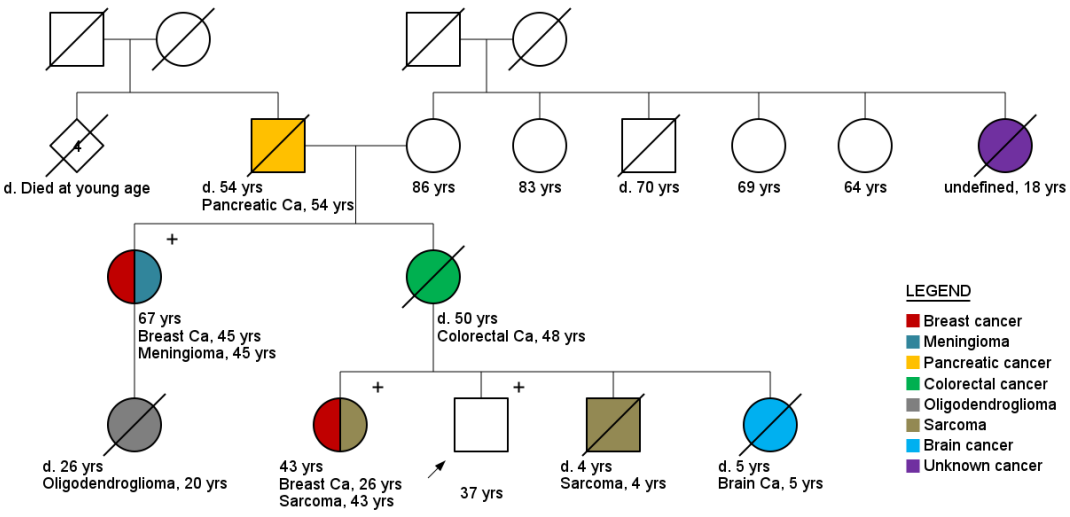

Figure 5 - Pedigree of a male healthy carrier (arrow) of pathogenic TP53 variant (c.481G > A). Plus (+) and minus (−) signs represent the carrier status of tested family members.

Prospective follow-up: Ninety-seven male MC were included in this prospective surveillance program, 19 of which were cancer survivors. With a median follow-up of 36.9 months (range 0–131,1mo), 14 patients were diagnosed with 17 new cancer cases (Table 2). For those 14 patients the median follow-up was 82.6 months). PC was the most frequently diagnosed neoplasia (5 cases). Eleven patients (78.6%) are alive, all but one without relapse (this one is a patient diagnosed with advanced pancreatic cancer alive after 36 months after his diagnosis). Three patients were diagnosed with advanced cancer and died of the disease (two with gastric cancer (GC) and one with disseminated adenocarcinoma).

An example of the importance of the surveillance programs for MC is the case of a 76-year-old male BC patient (index patient indicated with an arrow in Figure 3) that started follow up in April 2005, after being diagnosed with a *BRCA2* germline mutation (c.9098_9099insA). Three new early cancers were diagnosed and treated during follow up (PC, contralateral BC and Bladder cancer). The patient is alive and in complete remission of all cancers.

Table 2 - Cancer diagnoses during follow-up.

FUP Diagnoses	***T* = 17**
Prostate cancer	5
Gastric cancer	3
Breast cancer	2
Colorectal cancer	2
Pancreas cancer	1
Kidney cancer	1
Urothelium	1
Thyroid	1
Occult primary	1

Discussion

In this study, we analyzed the characteristics of male patients seeking counselling in our clinic and reviewed data from prospective follow up of MC at risk for hereditary cancer. Most of our high-risk patients are carriers of *BRCA1/2* pathogenic variants and adhered to a surveillance plan that allowed for cancer detection and treatment in 14 patients. In 10 of these patients (71%) early detection led to successful treatment. In four patients (GC (2), metastasis of unknown origin (1), pancreatic cancer (1)), their cancer diagnosis was made in an advanced stage and, with the exception of the patient diagnosed with advanced pancreatic cancer (alive 36 months after his initial diagnosis), they all died of progressive disease. The unexpected survival of the patient with advanced pancreatic cancer may be related with the known platinhyper sensitivity of *BRCA* cancers [31, 32].

The predominance of *BRCA1/2* male carriers in our study (contrasting with rarer *TP53* and *CHEK2* carriers) is related to the predicted prevalence of these different genetic events in the general population. Also, multigene panels started to be regular practice in molecular diagnosis only recently [14], and our cohort started in 2000. It is likely that the number of male carriers of other pathogenic non-*BRCA1/2* mutations will increase in the future.

The significant underrepresentation of *BRCA1* MC in our sample is due to several factors. First, there was a delay in testing and counselling male individuals from *BRCA1* families, since *BRCA2* men seemed to be at higher cancer risks and there was no clear recommendation to test men [19]. More recently, however, not only *BRCA2* but also *BRCA1* status has been considered one of the factors to take into account before deciding on PC screening [16], and we started including men from *BRCA1* families in the counselling process.

In a universe focused on female breast and ovarian hereditary cancers [15], it is important to understand how these men were referred for counselling. Besides the increased awareness for the risk of inherited cancer, the main reason for male patient referral is a previous BC diagnosis. This is particularly true in our study population, in which BC was the most frequent cancer observed among previously affected patients. It is estimated that BRCA mutations are responsible for nearly 10% of male BC [33], with a reported lifetime risks of inherited male BC ranging from 5% to 10% in *BRCA2* MC and 1% to 5% in *BRCA1* MC [34]. Also, it seems that the probability of a *BRCA* mutation

(especially *BRCA2*) increases if there is a significant family history of breast and ovarian cancer [33, 35]. For PC, 2% of the cases are attributable to *BRCA* mutations and the estimated lifetime risk for PC is 20% in *BRCA1* MC and 40% in *BRCA2* MC [35, 36]. This data helps to explain why BC (94) and PC (33) were the most frequently observed cancers in previously affected patients. Either through their physician or through self-referral, these men felt at risk of belonging to a hereditary cancer family. Counselling for individuals in Hereditary Prostate Cancer Families (HPCF) is very complex, since other known and unknown genetic factors besides *BRCA1/2* mutations seem to be implied [37]. In our cohort 28 of all 4,320 families reviewed met criteria for HPCF (three or more cases of PC in first degree relatives; patients with PC in each of three successive generations or two first degree relatives with PC before the age of 55). Of these, 18 PC patients consented on *BRCA1/2* testing after counselling but *BRCA2* pathogenic variants were only observed in six cases, leaving all other HPCF without any genetic cause identified (data not shown).

Increased surveillance of healthy *BRCA1/2* male carriers is still controversial, even if higher cancer risks are recognised [19, 36, 38, 39]. Data are accumulating however that, at least for *BRCA2* male carriers, early detection is advisable: *BRCA2* male BC displays pathologic characteristics related with greater biological aggressiveness, in comparison either with the sporadic male counterpart or *BRCA1/2* female cases [40]. Also, *BRCA2* PC displays an aggressiveness that justifies treatment [41]. We observed an unexpected good compliance with follow up (82% of all carriers kept regular surveillance) and the neoplasia most frequently diagnosed was PC, with five cases of Gleason 6–9 (data not shown). Early treatment has allowed, so far, survival without relapse in all PC patients. As previously mentioned, two deaths attributable to GC were observed. On the other hand, some studies include GC in the *BRCA2* phenotype [19, 42, 43], others did not confirm this [36]. Other factors may contribute for the GC cases observed in our *BRCA2* carriers, since Portugal has a high incidence and mortal- ity of this cancer [42]. There are no clear guidelines regarding screening of digestive cancers in *BRCA2* carriers and even for pancreatic cancer screening (part of both the *BRCA1* and *BRCA2* phenotype [33]) the recommendation is to include those carriers in prospective studies [44].

With the progressive inclusion of multigene panels in molecular testing, the number of male carriers of non-*BRCA* mutations is likely to increase, adding to the

complexity of counselling and surveillance. *TP53* carriers pose a complex challenge to cancer risk manage- ment clinics [45]. This patient population not only is at high risk for multiple primary neoplasia since childhood [46], as they also have an increased susceptibility to DNA damage by ionising radiation, limiting the use of radiologic exams in surveillance [45–47]. Most surveillance recommendations for Li-Fraumeni patients and their families focus mainly on breast and colorectal cancer surveillance, which may be insufficient given the high-risk for other types of cancer such as bone and soft tissue sarcoma (diagnosed in 25% of all germline *TP53* MC [48]), brain tumours, leukaemia, adrenocortical carcinoma among others less frequently observed (gastric, prostate, pancreas, melanoma, lymphoma) [45, 47, 49–51]. Interest in the concept of "whole-body imaging" is emerging, and a number of trials assessing the role of whole-body MRI (WB-MRI) in high-risk cancer patients are ongoing [52, 53]. Early this year, the first results of the SIGNIFY study were published [54]. This was a small UK trial, aiming to evaluate the role of WB-MRI in *TP53* MC screening. Eighty-eight patients were enrolled, 44 were MC and the other 44 patients were healthy control individuals. During the study, six patients in the experimental arm had a can- cer diagnosed, but only four were a direct result of WB-MRI screening. One of the questions raised by this study was the false-negative findings leading to further investigation and radiation, and the potential distress caused by these [54]. More robust data is needed before recommendations can be made.

The clinical management of patients harboring moderate penetrance gene variants (like *CHEK2* gene) is challenging. The *CHEK2* 1100delC mutation may explain up to 5% of BC families with a *BRCA1/2* phenotype but with a *BRCA1/2* negative test result [55, p. 2]. This particular mutation has been shown to increase female BC risk by 2- to 3-fold and a 10-fold increase risk in male BC [10]. It has also been associated with PC [56]. According to NCCN guidelines, male MC for a *CHEK2* gene mutation lack specific medical management guide- lines for BC risk. Nevertheless, this risk has increased the concern about male BC screening, patient breast awareness education, clinical breast examinations, and mammography [49, 57]. To include or not, prostate and colorectal cancer screening is still debatable.

For high-risk couples where the male is a *BRCA1*, *BRCA2* or a *TP53* carrier, counselling should include discussion regarding the possibility of preimplantation genetic diagnosis (PGD) [46, 58]. PGD uses fertilization in vitro technology and allows embryos

to be tested prior the transfer to the uterus, according to their mutational status [46, 58]. Although PGD implies some ethical issues that are not in the framework of this study, several couples are interested in pursuing it, and proper counselling should be made available.

Several questions remain to be answered, and one of them is the management of high-risk patients with variants of unknown clinical significance [59]. Although 18% of all BC cases tested by our group were found to be carriers of a *BRCA1/2* pathogenic mutation, nearly 30% of our male referrals because of a cancer diagnosis (including multiple previous diagnoses) had an inconclusive test result. This can be a source of distress [60] for patients and their families and poses unique problems in management and follow up since there is a lack of predictive cancer models for these men.

Conclusions

Men from HBOCS families actively seek counselling concerning their risk for hereditary cancer, and the great majority of carriers with pathogenic variants are compliant with increased surveillance. Management of cancer risk is complex, not only for *BRCA1/2* and *TP53* MC but also for men at high risk of hereditary cancer without identified pathogenic variants. It is likely that the current use of panel testing will identify new genes responsible for Breast, Prostate and other male cancers in HBOCS like families. This may clarify the genetic contributor but will add to the complexity of clinical management. Genetics Clinics need to adapt to the needs of a growing male population and more clinical research is also needed to develop guidelines for the management of complex phenotypes.

Conflicts of interest

The authors have no conflicts of interest to declare.

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4.4.3 - In the three abstracts we also found association of male BC with family history, bilaterality and other primary malignancies. The case of invasive lobular carcinoma with familiar history of BC and a g*BRCA* mutation was also characterized.

Abstracts Published in international peer-review journals

1. Santos F, Félix A, Carvalho A, Machado P, Vaz F, André S.

***BRCA2* Mutation in Male Breast Cancer.**

Mod Pathol. 28 (s2), 4-573, **2015**.

Background: Male breast cancer (MBC) is a rare entity and its management has been extrapolated from female breast cancer. *BRCA2* mutations confer an increased risk of breast carcinoma, bilateral carcinoma (BC) and other malignancies. Several founder mutations have been identified, including the previously reported c.156_157insAlu of Portuguese origin. We aim to characterize a cohort of MBC, with studied *BRCA2* gene, and analyze its clinicopathological features.
Design: We studied 25 non-related MBC cases, diagnosed in our institution, with *BRCA2* gene evaluation. Medical charts and histological slides were reviewed. "Intrinsic" subtype was classified using immunohistochemical staining for hormonal receptors, HER2 and Ki67.
Results: Clinicopathological features and *BRCA2* gene mutations presented in tables I and II. No significant differences in age, family history, histological type and grade and "intrinsic" subtype were found between male patients with and without *BRCA2* gene mutation. Bilateral carcinomas and other neoplasia are associated with *BRCA2* mutation (p=0.01 and p=0.02). All patients presenting bilateral tumors and *BRCA2* mutations also developed other malignancies. In our cohort, Portuguese founder mutation (c.156_157insAlu) was encountered in two male patients (20%).

BRCA2 Mutation	MBC n (%)	Bilateral carcinoma n (%)	Other malignancies n (%)
c.156_157insAlu	2 (20.0)	2 (20.0)	1 (10.0)
c.1786G>C/2	1 (10.0)	0	0
c.2808_2811del4	2 (20.0)	1 (10.0)	1 (10.0)
c.4380_4381del2	1 (10.0)	0	0
C.6468_6469del2	1 (10.0)	0	0
c.9098_9099insA	2 (20.0)	1 (10.0)	3 (30.0)
p.E475X	1 (10.0)	0	0

Clinicopathological Features	*BRCA2 Gene*	
	Non Mutated (n=15)	Mutated (n=10)
Median Age (years)	62	64.50
Family history (n, %)	4 (26.7)	7 (70.0)
Histological Type (n, %)		
Invasive carcinoma, NOS	13 (86.7)	8 (80.0)
Invasive lobular carcinoma	1 (6.7)	1 (10.0)
Invasive papillary carcinoma	1 (6.7)	1 (10.0)
Histological Grade		
Well differentiated (G1)	2 (13.3%)	0
Moderately differentiated (G2)	11 (73.3%)	6 (60.0%)
Poorly differentiated (G3)	2 (13.3%)	4 (40.0%)
"Intrinsic" subtype (n, %)		
Luminal A	7 (46.7)	7 (70.0)
Luminal B HER2-	6 (40.0)	3 (30.0)
Luminal B HER2+	1 (6.7)	0
Triple negative	0	0
Bilateral carcinoma (n, %)	0	4 (40.0)
Other malignancies (n, %)		
Prostate carcinoma	1 (6.7)	3 (30.0)
Gastric carcinoma	0	1 (10.0)
Bladder carcinoma	0	1 (10.0)
Colorectal carcinoma	1 (6.7)	0

Conclusions: *BRCA2* gene mutation status, although common in male breast cancer patients (40%), does not affect histological features. Bilateral carcinomas and other malignancies are frequently found in male patients harboring *BRCA2* mutations.

2. Canas Marques R, Cunha F, Machado P, Vaz F, André S.

Male breast invasive lobular carcinoma: report of two cases

Virchows Arch. 463 (2); 101-352, **2013** (PS-17-066).

Objective: Male breast cancer (MBC) is infrequent and occurs in older age than female breast cancer (FBC). Invasive lobular carcinoma (ILC) is the second most frequent subtype of FBC (with high rate of multicentricity and bilaterality and increased incidence since 1980s) but is exceptional in men. In FBC, the occurrence of ILC and invasive carcinoma of no special type was not different between carriers of *BRCA2* mutations and controls. *BRCA2*-related MBC has been reported at earlier age compared with non-*BRCA2*-related, with no differences in other clinicopathological features. Our aim is to report the clinicopathological characteristics of two cases of male ILC, one with BRCA mutation.

Method: Clinical data was obtained from medical records. Histopathologic characteristics were recorded.

Results: Table 1 summarizes the clinicopathological data. Table 1 FH: familiar FBC history; Ie: in evaluation; NED. No evidence of disease.

Conclusions: The two cases of ILC in males patients occur in relatively young patients with familiar FBC history, being one BRCA2 related. Multicentricity or bilaterality were not present. The immunoprofile is the usual in ILC of FBC.

Age (years)	FH	Stage	Grade	ER/PR	ERBB2	Ki67	*BRCA2*	E-cadherin	Follow-up (months)
51	Yes	3B	2	+	-	5.6%	Ie	-	NED 7
64	Yes	3B	3	+	-	18.9%	+	-	NED 14

3. Santos F, Cunha F, Machado P, Vaz F, André S.

Bilateral Male Breast Cancer and *BRCA2* Mutation.

Virchows Arch. 463 (2); 101-352, **2013** (PS-17-078).

Objective: Bilateral Male Breast Cancer (BMBC) is exceptionally rare. Several risk factors have been proposed, including BRCA2 mutations. In women, *BRCA1/2* mutation carriers have a high risk of developing bilateral breast cancer, but in men this risk is less well studied. Also, *BRCA2* mutation is associated with increased risk of other malignancies. The BMBC cases of our institution were reviewed.

Method: We report 4 cases of BMBC. Clinical data was obtained from medical records. Histopathological characteristics were recorded.

Results: First breast malignancies presented at a mean age of 60 years (median 57.5 years). One patient had synchronous tumors (ST). All patients underwent modified radical mastectomy. All tumors were invasive carcinomas of no special type, positive estrogen receptors (ER). The ST was ERBB2 positive; all the others were ERBB2 negative. All patients had a family history of breast cancer and all carried *BRCA2* mutations. Other malignancies developed in all cases: prostate cancer occurred in three, one of them also had vesical urothelial carcinoma, and a neuroendocrine gastric neoplasia in the patient with ST.

Conclusions: All the BMBC had *BRCA2* mutations and a positive family history. Also, all developed other malignancies. BMBC data relies on institutional case series, and multi-institutional investigation may improve the current knowledge.

Chapter V

Figure 12 - Virchow, Rudolf. Cellular pathology. Twenty lectures delivered in the Pathological Institute of Berlin during February, March and April, 1858. (1st Edition 1860 Publisher: New York: Robert M. de Witt (Internet archive – open library.org). Access: 1st September 2019.

Discussion

Male BC accounts for particular clinicopathologic features, most likely resultant from a distinct carcinogenesis that integrates specific genetic and epigenetic signatures. It was been considered an entity with rising incidence, usually late detected and associated high risk for adverse outcomes. These characteristics elected male BC as a practical example of how personalized analysis should be also warranted in rare neoplasms, aiming to the comprehensive identification of their specifiers and the consequent research of additional biomarkers. This will allow an innovative clinical management directed to optimal care and cure (5, 11, 12, 54, 56, 65, 74, 90, 92).

As a way to contribute to this aim, we began to identify all male BC cases (n=196) diagnosed and treated in IPOLFG, since March 1970 to March 2018, to describe the clinical and pathological profile currently used for classification and therapy, with the intention of obtaining a solid basis for additional research.

Regarding the clinicopathological profile of all the series, we would like to emphasize that most our results were globally similar to the previously reported in literature, confirming that studies involving smaller, but well-characterized series, may be representative and contribute to the improvement of disease knowledge.

Several relevant points are further discussed, emphasizing the most particular clinicopathologic features in the all cohort of patients in IPOLFG (Chapter IV - 4.1). In addition, we debate some specific genetic and also epigenetic signatures involved in male BC carcinogenesis, and present a preliminary immunohistochemical identification of potential biomarkers that may permit new therapeutic approaches that warrant innovation in the control of this disease.

Particular clinicopathologic features in the all cohort of patients in IPOLFG

The number of patients included in all series in IPOLFG are similar as the one reported in IPOPFG by Abreu *et al*. (54) and confirms the low incidence of this condition in Portuguese men. The annual registered incidence by ROR-Sul in 2012 was 29/100.000 habitants in South Portugal and Madeira (21). In that year, 9 patients were diagnosed and treated in IPOLFG, corresponding to a very small percentage of the diagnosed cases in South Portugal and Madeira. This low number raises the question if reference centers, like IPOFG centers, should aim to threat all male BC patients.

Curiously, a rising number of cases of male BC was found in our cohort in the last decade, as registered in Figure 13. But this could be just a bias towards a better referenciation of rare cancers to the specialized institutions.

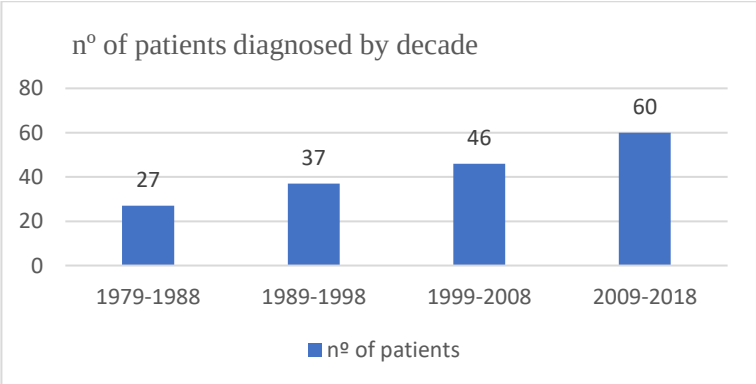

Figure 13 – Graphic of male BC incidence found in our cohort by decades.

Among the major risk factors recognized to be associated with BC, advancing age is the higher ranked, followed by a positive family history linked to gene mutations, and also hormonal imbalance and radiation exposure (8, 34, 36).

Aging is considered to be a risk factor, a clinical characteristic and a prognostic feature of male BC.

Aging of the population seems to be one important reason for the rising incidence of male BC (8). In the present cohort, the high incidence in older males is confirmed by the percentage of the patients aged ≥70 years old (41.2%).

The mean and median age of patients at diagnosis (65.2 and 66.5 years respectively) found in this cohort agree with the statement that average age at diagnosis is approximately 5-10 years more compared with the average age at diagnosis in female BC (1, 35). Moreover, the reported pattern in age distribution, with only one peak due to the absence of early onset cancers, and a gradual cumulative increase in incidence with aging, is one of the specifiers of male BC and it was also found in our series (Figure 14).

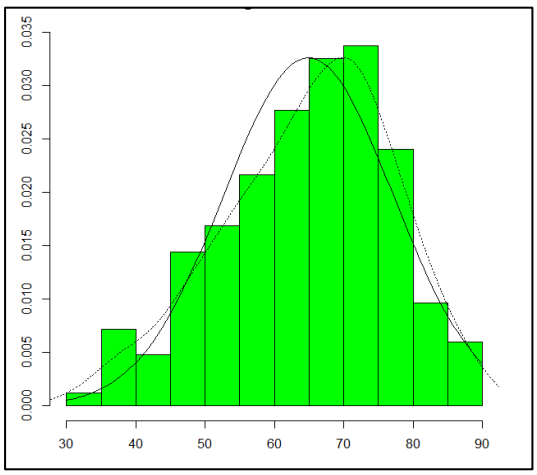

Figure 14 – Histogram (age and frequency of patients in the present study – chapter 4.1) - The mean and median age of patients at diagnosis was 65.2 and 66.5 years (range, 31–89 years).

In our series, elderly patients exhibited larger carcinomas compared with younger patients, and old age was a prognostic factor significantly associated with low 5 and 10-year OS in Kaplan-Meier estimates. A longer OS was associated with young patients (<40

years; p=0.010), which is concordant with data from previous studies (8, 12, 14, 41, 42, 75). The lower OS in older patients may be related with tumor biology, but also with late diagnosis, less treatment and comorbidities. In male BC, aging is also a critical factor for the therapeutic approach and some comorbidities in the elderly population may result in variable palliative treatment (101).

Obesity is a major health problem and is associated with an incremental risk of developing BC and with a concomitant worse survival (101). In males, obesity is one of the most common cause of high estrogen levels due to peripheral aromatization of testosterone in the adipose tissue (27). The increasing obesity trend in the population has been also associated to the rising incidence of male BC (1, 23).

We found that the distribution of this risk factor in the population of male BC patients was similar to the distribution in the incidence of obesity of Portuguese elderly men. In fact, after reviewing all the clinical records, obesity was reported in approximately 20% of the patients in the total series, being 22.5% in the cohort of the 40 patients used in the IHC study. This incidence is similar to the reported in the general population where obesity was found in 22% and 14.9% in men between 50-59 and 60-64 years old, respectively (29). These unexpected results may result of incomplete data in patient files, and should be specifically further evaluated in prospective studies.

In addition, obesity in these cancer patients may also have impact on response to hormonal therapy. Furthermore, especially in old patients, obesity is commonly associated with comorbid conditions such as diabetes, hypertension, and heart disease, and may result in difficult treatment decisions (101).

In literature, some data suggest **radiation exposure** to increase the risk of male BC (8, 23). This risk factor was only observed in one patient in this series, who was treated by radiation to Hodgkin lymphoma 13 years before the diagnosis of male BC.

Beyond these clinical factors, **anatomic stage** is one of the most important features in the profile of male BC, as determinant in therapy and prognosis (56).

The common high anatomic stage at presentation is a very poor prognostic factor and associates with high number of deaths for male BC.

In this cohort, similar to the literature (12, 15, 18, 43-46, 50), a low percentage of patients (3.6%) was diagnosed with *in situ* carcinomas (pT0), while 40.3% presented with pT4 carcinomas. Metastatic disease at presentation was present in more than half of

patients (56.4%) with axillary lymph node metastasis and distant metastasis in 9.2%, confirming that male BC is usually diagnosed as advanced disease.

The value of **histological type** of invasive carcinomas to identify specific groups of carcinomas as distinctive entities, according to the literature, is is not significant. In our cohort, 90.3% of the cases were classified as invasive carcinoma of no specific type. This histological type is very heterogeneous in itself and does not have distinctive morphological nor clinical characteristics. Other morphologies with more homogeneous characteristics, such as lobular, mucinous or papillary, were recognized in less than 10% of cases. We also did not find a correlation of this parameter with all variables studied.

On contrary, **clinically defined subtypes** classification is much more useful to stratify carcinomas and is currently used to guide therapeutic decisions (48). In our series, as in literature (5,12), male BC was almost always a **Luminal-like** disease (ERα and PR positive and ERBB2 negative). We found a poorer outcome for Luminal B-like subtype cases (low or absent PR and high cell proliferation), that was significantly associated with low OS ($p<0.001$), in comparison to Luminal A-like subtype and in accordance to the literature (12, 13).

As previously described (9, 54, 55), the number of cases belong to other molecular subtypes in our series was low. We identified a small percentage of ERBB2-positive cases (6.8%), that were all Triple positive. Positive ERBB2 expression /Triple positive carcinomas were significantly associated with M1 carcinomas at presentation, high AS and high Ki-67 expression, and also with low OS in univariate analysis, confirming the advantage of this subtyping over the histological classification.

Male BC Triple-negative carcinoma cases were also few (7.4%) in our series. In contrast with other subtypes, this category was not confirmed to be significantly associated with a dismal prognosis in our series. We are convinced that the absence of significant association between Triple-negative cases and OS in our series ($p=0.0556$), may be related with the low number of the patients associated with the number of censored observations (5 patients died for other causes) and the number of patients lost for follow-up (4 cases).

In general, **cell proliferation** in cancer is a main biological factor that can be used as a measure of disease aggressiveness and a predictor factor for chemotherapy response. High cell proliferation is usually associated with poor outcome (56).

In the present study, we evaluate cell proliferation using different approaches and obtained a confirmation in all our studies that proliferation is an important parameter in the evaluation of these tumors. **Poorly differentiated carcinomas (G3** - low duct formation, high pleomorphism, high mitotic count) were significantly associated with DFS in univariate and multivariate Cox regression analyses. The immunohistochemistry evaluation of Ki67 showed that the elevated frequency of **high Ki-67 values** (58.9% of the cases) is statistically significant associated (in univariate Cox regression analysis) with G3 neoplasms, but also with old age, positive FH, pN1, high AS, and also with DFS and OS, confirming the prognostic value of cell proliferation in male BC. In addition, flow cytometry DNA ploidy showed a high percentage of **aneuploidy** (88.6% of the 79 evaluated cases) corroborating the results found in our previous comparative study between genders (62), and those reported in the study of Bezié *et al.* (61). A very interesting finding was the significant association of aneuploidy with bilateral male BC in the present series. As bilaterality is also associated gBRCA2 mutations, the high percentage of aneuploid cases seems to be related to the genomic instability in the carcinogenesis of male BC.

In the long time period of this study, **therapeutic procedures** for male BC followed the evolution of the recommendations for BC in women and variable protocols were used. Tamoxifen is the currently the most frequent employed systemic treatment in the curative and palliative settings. The relatively low rate of hormonotherapy used in the treatment of patients compared with the high percentage of ER-positive carcinomas identified in the present study and in previous studies, may be related with the fact that the use of tamoxifen in males was only recently standardized (12).

Different chemotherapy agents and regimens have been used and the introduction of taxane chemotherapy marked a significant advance in the treatment of metastatic disease in female BC. In this series, the patients diagnosed in the years following the introduction of this therapy exhibited significantly increased 5 and 10-year DFS (p=0.030) and OS (p=0.050) compared with those diagnosed prior to the introduction of taxane chemotherapy. This improvement may be also associated with the therapeutic effects but also with standardized clinicopathological evaluation and improved follow-up observed in recent years. However, comorbidities and therapy collateral consequences remain important, metastatic disease persists incurable and male BC therapy is still a challenge (101, 102)

To study **survival** we evaluated in 145 patients after excluding patients with M1 carcinomas, patients also with non-primary breast neoplasms and patients with only *in situ* carcinomas. We found low 5 and 10 year OS rates (77.5 and 59.2%, respectively) and low 5 and 10-year DFS rates (65.9 and 58.2%) respectively. Nevertheless, the OS survival in our cohort was slightly longer compared with those demonstrated by Tural *et al.* (75.2 and 52.5%) (42) and Chen *et al.* (72.9 and 53.9%, respectively) (78), perhaps because these series included patients diagnosed until 2011 and 2013, respectively.

Amongst the 18 patients (almost 10% of the cases) with distant metastasis at presentation (M1), only 1 was alive with bone metastasis after 34 months of follow-up. All other 17 patients succumbed to the disease, with the mean and median survival times of 18.7 and 15.5 months (range, 1–38 months), underlining the fact that M1 disease is still incurable.

Specific genetic and also epigenetic signatures involved in male BC carcinogenesis

Family history of BC and germline mutations have been underlined in male BC in last decades and are indubitably associated.

In spite of the association between FH and germline mutations, the American Society of Clinical Oncology (ASCO) recommends that all male BC should be offered genetic counseling and testing, regardless of FH (12).

In the study of Freitas *et al*. (Chapter IV - 4.4), 51.3% of the male patients identified in hereditary cancer families were mutation carriers: *BRCA2* – 102 (84.3%), *BRCA1* – 16 (13.2%), *CHEK2* – 1 (0.8%) and *TP53* – 2 (1.7%). In addition, about 14% of male patients belonging to hereditary cancer families had been previously diagnosed with a total of 39 cancer cases (0.17 case/ patient), being BC the most frequent diagnosed neoplasm, followed by prostate and colorectal cancer in these families.

In literature, **g*BRCA2*** is the best characterized high penetrance predisposition gene and the most frequently mutated in male BC. In high-risk families, *gBRCA2* mutations are estimated to correspond to 60% a 76% of male BC cases, while *gBRCA1* mutations account for 10 to 16% of male BC cases (103).

As our study included patients diagnosed from 1970 onwards, the majority of patients had no information in their clinical records regarding family history or g*BRCA*

mutational status. However, a confirmed FH of BC was significantly associated with the presence of g*BRCA2* mutations and poor OS.

Like aging, g*BRCA2* mutations are a risk factor, are associated with clinical characteristics and are a prognostic feature of male BC.

In our cohort, as reported by Ottini *et al*. (104), g*BRCA2* mutations were associated with carcinomas without progesterone receptors and high proliferation rates (Luminal B-like subtype).

It was been estimated that personal history of male BC in one breast is associated with a 16% increased risk of developing a second primary contralateral BC in comparison with the general male population, and a second primary tumor has been reported in more than 11% of BC in male patients (8, 105).

In our cohort, bilaterality and non-breast malignant neoplasms were also associated with family history and gBRCA2 mutations. In the all series, synchronous bilateral tumors are uncommon (1 patient/ 0.5% of the cases), metachronous bilaterality occurs in 3.1% of the patients and non-breast malignant neoplasms occurs in 14.3% of the patients. From the patients with a confirmed g*BRCA2* mutation, 30.8% had bilateral carcinomas and 38.5% had non-breast malignant neoplasms.

The incidence of bilateral male BC and the occurrence of non-breast malignant tumors, most frequently prostate carcinoma, justifies a careful follow-up of these patients and raise the question whether men with BC and a g*BRCA2* mutation should carry out a contralateral prophylactic mastectomy. We also highlight the relevance of investigate the genetic background in a male patient with multiple neoplasms, even in elderly and upon an unknown FH.

In addition, the significant association with shorter DFS and OS in univariate analysis qualifies g*BRCA2* mutations as a prognostic factor and also underlines the clinical relevance of evaluating g*BRCA2* status in all patients in male BC.

However, male BC carcinogenesis is complex and multifactorial, and in addition to the accumulation of genetic changes, epigenetic somatic alterations may also have an important role (79).

The availability of a larger amount of biological data about somatic mutations in male BC and their investigation as predictive biomarkers may provide a better therapeutic approach, namely in the context of therapy resistance.

Malignant cells are characterized not only by genomic instability but also by other alterations such as **Homologous recombination DNA repair system** (HRR) defects that allows proliferation despite DNA damage (106).

The Homologous recombination DNA repair system (HRR) is an essential tool in the preservation of genome integrity and in suppression of carcinogenesis (87, 89) and it is not restricted to *BRCA* mutations. Either germline or somatic *BRCA2* mutations and aberrant promotor methylation are factors causing homologous recombination deficiency and inability to repair DNA (87). Owing to the lack of systematic studies on altered methylation patterns of HRR genes in male BC, and because *BRCA2* promoter methylation evaluation is still controversial as *BRCA2* promoter methylation seems to be very rare, we explored the epigenetic signature of the HRR genes *ATM, BRCA1, PALB2, and RAD51* gene paralogs, *RAD51B* and *XRCC3*.

Epigenetics is described as genome heritable alterations that are not due to changes in DNA sequence. The best characterized epigenetic changes in tumors consists on **aberrant methylation**, occurring mostly at gene promoter regions, and associated with transcription repression (87). Both DNA hypermethylation and hypomethylation may be directly associated with carcinogenesis (79, 82).

In our second study (Chapter IV - 4.2), we concluded that no significant associations were found between epigenetic alterations in the tested HRR genes and male BC clinicopathological parameters, favoring their role in male BC carcinogenesis but not in prognosis. However, our cohort is too small to allow definitive conclusions.

But, assembled in a panel, ***RAD51B* & *XRCC3* promoter methylation** discriminated male BC from gynecomastia with 91.5% sensitivity, 89.5% specificity and 91.2% accuracy. Moreover, promoter methylation levels were lower in paired non-tumor tissues (breast normal adjacent tissue and homolateral non-metastatic lymph nodes), comparing to male BC samples. Although requiring further investigation and validation, quantitative promoter methylation of the panel *RAD51B* & *XRCC3* constitutes a promising biomarker for male BC monitoring settings.

We didn´t found statistically significant associations between epigenetic alterations in the tested HRR genes and male BC clinicopathological parameters, favoring their role in male BC carcinogenesis but not in prognosis. However, our cohort is too small to allow definitive conclusions.

In the study of Freitas *et al.* in IPOLFG (Chapter IV - 4.4), almost 30% of the men referrals with a male BC diagnosis (and sometimes multiple neoplasms) had an inconclusive test result, with consequent difficulties in the management and follow-up. Since DNA methylation is reflected within circulating DNA, detection of quantitative promoter methylation of the panel *RAD51B* & *XRCC3* in the plasma may be feasible, and the development of a commercial blood-based test in male BC could be implemented in genetic risk consultation of male patients and families, with special interest in those without detected g*BRCA1/2* mutations and with FH of BC.

Homologous recombination deficiency is currently tested in clinical trials, in particular as a response marker for DNA-damaging agents and PARP inhibitors (PARPi). Poly (ADP-ribose) polymerases (PARPs) proteins are enzymes whose functions include to help repair damaged DNA single strand breaks. PARP inhibitors (PARPi) are effective in carcinomas harboring dysfunctional HRR, including g*BRCA1/BRCA2* related BC, and were approved by the Food and Drug Administration (106). It was been purposed that the use of PARPi might be extended to a wider group of BC patients with defective HRR, beyond detected g*BRCA* mutations. The availability of a larger amount of biological data in the investigation of *RAD51B* & *XRCC3* as predictive biomarkers to the response to PARPi and/or immune-checkpoint inhibitors may provide a better therapeutic approach for male BC (107, 108).

In this setting, the evaluation of the immunohistochemical expression as a surrogate marker of *RAD51B* & *XRCC3* functionality could also be important and deserves further investigation.

Preliminary immunohistochemical identification of potential biomarkers

Other important subject to discuss in male BC is the concept of **homogeneous versus heterogeneous characteristics** of a particular neoplasm.

As previously discussed, almost all of our cases were histological classified as invasive carcinoma of no specific type, a very heterogeneous morphological and clinical category.

Intratumor heterogeneity, defined as the coexistence, in a neoplasm, of subpopulations of cells differing in genetic, epigenetic, phenotypic or behavioral features, has emerging as an unavoidable issue regarding BC, mainly due to the resistance to chemotherapy and target therapies (93, 109).

The technological advances have significantly increased our understanding of intratumor heterogeneity. However, current genomics can`t distinguish mutations drivers shared by the cells of the entire tumor from drivers only for small clones, or passengers not sufficient to drive tumor proliferation, and the targeting of genetic aberrations of small clones may not have impact on the growth of the whole tumor. Consequently, only few single genetic, epigenetic, or structural genomic alterations can provide clinically relevant useful biomarkers. In addition, a single biopsy of a large tumor may induce biomarker results, and metastatic carcinomas remain incurable, with no permanent and definitive response to chemotherapy or targeted therapies (93, 109, 110, 111).

The intratumor variability of somatic *BRCA1/2* mutation, *BRCA1* promoter methylation status, and the overall homologous recombination deficiency score were evaluated in small samples from different areas of the same tumor. The results indicate that homologous recombination, when present, is a homogeneous feature of BC, conferring to homologous repair deficiency assay a promising tool with low susceptibility to sampling error (112).

Homogenous genomic alterations in the malignant cells of an entire tumor may provide selective advantage, but their identification in early carcinogenesis may be a strategy for an effective treatment (93).

The **immunohistochemistry technique** allows the identification of gene products, and can be used for assessing biomarkers able to identify subsets of patients with different response to treatments and diverse outcomes. While with recognized limitations in quantitative analysis, IHC is a simple and cost-effective method to approach the heterogeneous *versus* homogeneous phenotype of gene expressed proteins in malignant epithelial and stromal associated cells and, when combined with other techniques for assessing molecular biology, may have translational relevance.

In a recent study, immunohistochemical evaluation of ER intratumor heterogeneity was reported to be an independent prognostic factor related with long-term risk of fatal BC, mainly for patients with Luminal A subtype (113).

We further analyzed the IHC pattern of a panel of biomarkers, previously studied in female BC and some in male BC, related to gene expression, tumor proliferation, differentiation, invasion, migration, metastasis and survival, and considered potential tools for target therapy. We used TMAs, evaluated three or four 1.5 mm diameter cores

for from different morphologic areas of each case to account for the heterogeneity of the lesions.

We implemented a different approach to evaluate immunostaining. Our intention was to evaluate positivity as a marker of widely expression "all or not all" for that our results underline the presence of homogeneous versus heterogeneous positivity. Malignant epithelial cells positivity was divided in "homogeneous phenotype" and "heterogeneous phenotype". The staining pattern was also recorded in stromal fibroblasts and interstitial stroma, as described in Chapter IV - 4.3.

The evaluated cohort is characterized by clinicopathologic features classically associated with "good prognosis" such as Luminal-like subtype and anatomic stage I/II. The identified high percentage of AR receptors and Cyclin D1 positivity have also been associated with male BC favorable prognosis in previous studies (114, 115). Accordingly, we found a low percentage of disease recurrence and death in this cohort (17.5%).

However, these conditions are commonly found in the current clinical management of male and female BC, emphasizing the importance of the results obtained.

The molecular markers **p16, ATF3, β6 integrin, FASN and FATP1 are significantly intercorrelated, and significantly correlated to high cell proliferation, if homogenous phenotype of epithelial staining is present**.

All these biomarkers but ATF3 have been previously studied in BC. This transcription factor is considered crucial in cellular stress response, with roles in epithelial cell proliferation and in the promotion of tumor progression as a "breast stroma related gene" (27-29) and has not been evaluated in male BC before. Its positive homogeneous phenotype in these male BC cases is significantly associated with important clinicopathological prognostic factors such as g*BRCA2* mutations, Luminal B subtype, pN1 and AS III.

p16 has important roles as tumor suppressor and in cell cycle regulation. In female BC, p16 overexpression was yet been reported as related to high proliferation and unfavorable prognosis (116).

β6 integrin was been associated with unfavorable prognosis in different cancer types (117). In this series, probably due to the relatively small cohort of cases, β6 integrin is not directly associated with prognosis, although its homogeneous membrane cell staining in male BC is associated with ATF3 and FATP1 homogeneous phenotype, which

are related to worse prognosis. This result points out to the need to further investigation of β6 integrin.

Furthermore, the study highlights the **significant associations of homogeneous malignant epithelial staining of FASN with DFS and OS, as well as ATF3 with OS and Collagen IV with DFS.**

FASN and FATP1 have been chosen for evaluation because of the multifunctional involvement in fatty acids metabolism, which is essential in malignant cells proliferation (118). In a previous study (118) we tested fatty acids metabolism, using a cell line derived from a female BC patient (MDA-MB-231: ATCC® HTB-26TM) and skin fibroblasts. In that study, FASN was expressed in MDA-MB-231 and in fibroblasts and FATP1 seems to play a pivotal role on lipids uptake by breast cancer cells. In the presented series, FASN and FATP1 were expressed in fibroblasts, their homogeneous intratumoral epithelial cells staining is significantly intercorrelated, and the uniform distribution of FATP1 in malignant epithelial cells was significantly associated with AS III and with Luminal B intrinsic subtype. The significant associations between the homogeneous immunoexpression of these biomarkers and high proliferation as assessed by Ki67 immunoexpression should also be highlighted to understand the carcinogenesis of a subgroup of poor prognosis male BC.

Collagens are the major structural component of the stroma and essential in male BC microenvironment. In the present series, the intensity of stromal staining in Collagen I and Collagen IV seems to be important in revealing structural differences with a probably role in the interaction between epithelial cells and stroma. Collagen I strong diffuse intensity is significantly associated with ATF3 epithelial homogeneous phenotype and the intense diffuse Collagen IV stromal immunophenotype is significantly associated with a shorter DFS. Stromal diffuse intense staining may be consistent with stromal intense remodulation in aggressive male BC carcinomas. This finding supports previous studies of the potential value of inhibiting collagen IV synthesis or deposition to control female BC progression (61).

These results allow us to hypothesize that, in male BC, the homogeneous immunophenotype may correspond to a clonal high proliferative population related with poor prognosis, but also with the potential to respond to a combined target therapy against the identifiable biomarkers. The prognostic relevance of the epithelial homogeneous

phenotype found in these biomarkers points out to their eligibility for further investigation with potential application in the clinical management of Luminal-like male BC.

Gynecomastia cases were included in this series aimed to use them as benign comparative control model. The study of these cases displayed interesting results.

Although gynecomastia is very common and not considered a pre-malignant entity, in the cohort selected for the IHC study, we found that male BC was associated with gynecomastia in 22.5% of the cases. In the literature, this association has been stated as nearly inexistent to be present in 20-40 % of the cases (5). The complex and multifactorial etiopathogenesis and sharing of same risk factors by both conditions are a cumbersome to clarify the relation between these two entities, especially in those cases as one example in our series, which was clinically associated with androgen deprivation therapy for a prostate carcinoma in a patient with a gBRCA2 mutation.

Gynecomastia also constitutes a complex clinical scenario in which specific biomarkers might aid in differential etiologic diagnosis and monitoring.

In tissue samples, the methylation panel combining *RAD51B and XRCC3* accurately discriminated male BC from gynecomastia. Surprisingly, however, higher RAD51B & XRCC3 promoter methylation levels were found in gynecomastia compared to male BC. Promoter methylation acts in concert with other epigenetic mechanisms (e.g., histone post-translational modifications and chromatin remodeling) to achieve effective gene silencing. Thus, higher promoter methylation RAD51B & XRCC3 levels may be related with histone-related factors that might preclude effective gene silencing, contrarily to BC. Nevertheless, this interesting finding might also be related with proliferation, high estrogen levels or other yet unknown risk factors, and deserves to be investigated in larger series.

In the IHC evaluation, a characteristic heterogeneous phenotype was present in the epithelial cells of all ducts with Cyclin D1, ATF3 and FATP1. The homogeneous pattern of some markers highlighted in male BC as well as the intense stromal staining with Collagen I and/ or Collagen IV were not found in gynecomastia.

A better characterization of the genetic and epigenetic features of gynecomastia as well as an extensive IHC study of the balance of biomarker receptors between epithelial and mesenchymal components, may be important factors to elucidate any possible relation of this common entity with male BC and for the clinical management of gynecomastia.

Conclusions

At the end of this thesis, we summarize and reflect on our research that aimed to study male BC carcinogenesis by the characterization of clinical, pathological, genetic and epigenetic particular features that can be used to improve the scientific knowledge of male BC as a specific entity.

1. The general clinical and pathological features in our series are consistent with those of large and/or multi-institutional studies, confirming that studies involving smaller, but well-characterized clinicopathological and molecular subgroups, diagnosed and followed in multidisciplinary departments within single institutions, are important in improving the understanding of this disease.

a) The results of the first study were obtained in a representative cohort of the male BC entity, regarding risk factors and particular clinicopathologic parameters.

b) Male BC is more likely to be diagnosed in older patients and exhibited poorer prognosis in elderly.

c) Male BC is characterized by the high predominance of ERα/ PR positive, moderately differentiated (G2), high Ki67 expression, Luminal B-like and aneuploid tumors.

d) Germline *BRCA2* mutations are a dominant genetic feature in male BC, a risk factor and a prognostic marker.

e) Male BC patients with g*BRCA2* mutations frequently develop contralateral carcinomas and non-breast malignant neoplasms.

2. Epigenetic analysis identified significant differences between male BC and gynecomastia tissue samples, and we purpose the quantification of a promoter methylation panel - *RAD51B* & *XRCC3* as a promising biomarker for male BC monitoring settings.

3. Homogeneous immunophenotype of several biomarkers have potential to define particular male BC subgroups that could be eligible for further investigation, attending the possible application in the clinical management of male BC:

4. Prognostic biomarkers in male BC identified in this thesis:

In univariate analysis:

a) Older age is associated with poorer OS

b) g*BRCA2* mutations, high anatomical stage, high Ki67 expression and Luminal B subtype are associated with shorter DFS and OS

c) FASN (an enzyme for endogenous synthesis of fatty acids) homogeneous epithelial phenotype is significantly associated with poor DFS and OS

d) ATF3 (a mediator of cellular stress response) homogenous epithelial phenotype is significantly associated with poor OS

e) Collagen IV (a network-forming collagen) diffuse and intense staining of stroma is significantly associated with shorter DFS.

In multivariate analysis:

a) Family history, high anatomical stages, and Luminal B subtype are associated with poor OS

b) Collagen IV diffuse and intense staining of stroma is significantly associated with shorter DFS.

These conclusions highlight the value of the heredo-familiar/ genetic context, of early detection, of *RAD51B* & *XRCC3* promoter levels and of pathologic/ phenotypical characterization in male BC, as valuable tools to contribute to improve the scientific knowledge of male BC as a specific entity.

Chapter VI

Figure 15 - Previous studies within the scope of this thesis and future perspectives

Abstracts of other previous Publications and Communications within the scope of this thesis

Publications in International peer-review journals

1. Ferreira M, Mesquita M, Quaresma M, André S.

Prolactin receptor expression in gynecomastia and male breast carcinoma. Histopathology. 53(1):56-61, 2008. doi: 10.1111/j.1365-2559.2008.03059.x. PMID:18613925

Aims: Despite the well-established function of prolactin (PRL) in normal breast development, its role in breast cancer pathogenesis is still controversial. PRL activity is dependent on the activation of a transmembrane protein, the PRL receptor (PRLR). The aim was to evaluate and compare PRLR expression in gynaecomastia and male breast carcinoma (MBC).

Methods and results: PRLR expression was detected immunohistochemically in 30 cases of gynaecomastia and 30 cases of MBC. The whole series was also assessed for oestrogen

receptors (ER), progesterone receptors (PR) and androgen receptors (AR). A cut-off of 10% was used as the criterion for positivity. Histological type and tumour differentiation were evaluated. Pathological stage was assessed [Tumour Node Metastasis (TNM)-International Union Against Cancer system]. Statistical analysis was performed with Fisher's exact test. PRLR positivity was seen in 20% of gynaecomastia cases and in 60% of MBC cases (P = 0.003). In gynaecomastia immunoreactivity was predominantly observed in luminal cell borders, whereas in MBC the reactivity was heterogeneous and mainly cytoplasmic. There was no statistically significant correlation between PRLR expression and ER, PR, AR, pTNM or histological grade.

Conclusions: PRLR is significantly more expressed in MBC than in gynaecomastia, and with different patterns of reactivity, suggesting a role for PRL in male breast carcinogenesis.

2. André S, Pinto AE, Laranjeira C, Quaresma M, Soares J.

Male and female breast cancer--differences in DNA ploidy, p21 and p53 expression reinforce the possibility of distinct pathways of oncogenesis.

Pathobiology. 74(6):323-7, 2007. PMID:18087196. doi: 10.1159/000110025.

AIM: The purpose of this study was to compare the immunohistochemical profile of cell cycle inhibitors of G1/S phase transition (p21, p53 and pRb), Ki-67 proliferation marker and DNA ploidy in male (MBC) and female breast cancer (FBC).

MATERIAL AND METHODS: One hundred patients (50 non-consecutive cases of FBC and an equal number of MBC) were selected according to homogeneous features regarding age, histological type, tumour grading, nodal status and absence of neoadjuvant therapy. The expression of p21, p53, pRb and Ki-67 was assessed by immunohistochemistry, and DNA ploidy was analyzed by flow cytometry. Correlations between variables were evaluated using the chi(2) test.

RESULTS: The incidence of DNA aneuploid, p21-positive and p53-negative tumours was significantly higher in MBC than in FBC; pRb and Ki-67 revealed no statistically significant differences between the two entities. In MBC, high tumour grade correlated with aneuploidy, Ki-67 and pRb positivity; ploidy and p53 were also associated. In FBC, only ploidy and grade showed a strong correlation.

CONCLUSION: The significant dissimilarities regarding DNA ploidy, p21 and p53 in these quite homogeneous groups of FBC and MBC point to different genomic instability and to differences in cell cycle proliferative control, reinforcing the view of somewhat distinct tumour oncogenesis.

3. Fonseca RR, Tomás AR, André S, Soares J.

Evaluation of ERBB2 gene status and chromosome 17 anomalies in male breast cancer.

Am J Surg Pathol. 30(10):1292-8, 2006. PMID:1700116. doi: 10.1097/01.pas.0000213354.72638.bd

Male breast cancer (MBC) is an uncommon neoplasm that shares several biologic characteristics with its female counterpart. In the latter, abnormalities in the expression and/or copy number of the ERBB2 gene are present in 10% to 30% of invasive carcinoma and behave as poor prognostic markers. ERBB2 abnormalities have also been reported in MBC, yet at lower frequency, but their prognostic significance remains controversial. Furthermore, no study has addressed the impact of chromosome 17 abnormalities in MBC survival. In this study, the ERBB2-gene status (overexpression and amplification) and chromosome 17 numerical abnormalities were investigated in a series of 50 archival cases of MBC. The results, together with patient's age, histologic grade, pathologic stage, and estrogen receptor status were correlated with overall survival. ERBB2-protein overexpression was present in 7 cases (14%), ERBB2-gene amplification in 4 (8%), and aneuploidy of chromosome 17 in 12 cases (33.3%). The pathologic stage, ERBB2 overexpression and ERBB2 amplification were significantly correlated with overall survival (P=0.002, 0.016, and 0.009, respectively). No correlation was observed between chromosome 17 aneuploidy and overall survival. Therefore, despite their low incidence in MBC, expression abnormalities of ERBB2 behave, together with the pathologic stage of the tumor, as predictors of overall survival, akin to what has been reported for its female counterpart.

4. Fonseca R, Tomás AR, André S.

Absence of Epstein-Barr virus EBER transcripts in male breast cancer.

Virchows Arch. 447(1):113-4, 2005. No abstract available. PMID:15926072. doi:10.1007/s00428-005-1230-6.

Letter to the Editor

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus infecting more than 90% of the adult population worldwide. Since its discovery in 1964, EBV DNA has been demonstrated to be associated with several malignancies (e.g. Burkitt lymphoma, Hodgkin lymphoma, lymphomas occurring in immunodeficient individuals, and nasopharyngeal carcinoma) where it

is almost always found in the latent state. Recently, it has been proposed that breast carcinomas should also be included in the list of EBV-related neoplasms [4]. Infection with EBV results in the establishment of a life-long carrier state, associated with the expression of a restricted set of viral antigens which encode two non-translated nuclear RNAs (EBER-1 and -2), six nuclear antigens (EBNAs), three integral membrane proteins (LMPs), and a polypeptide (RK-BARF0). Three forms of latent infection, referred to as latency types I–III, have been demonstrated in EBV-carrying B-cell lines and EBV-carrying tumour biopsy samples.

The viral EBER-1 and EBER-2 transcripts persist throughout the EBV infection cycle and accumulate to very high copy numbers in the nucleus, making them ideal candidates for identification of latent EBV infection. Male breast cancer is a rare entity (less than 1% of cases of breast cancer), and most of the knowledge about its biology has been extrapolated from the female counterpart. It occurs predominantly in old men (average 60 years of age, 10 years older than female breast cancer patients). The oestrogen receptor is expressed in most cases (80-93%), and it has been associated with an unfavorable outcome. In male breast cancer an eventual association with EBV would have implications for both prevention and therapy. We studied a sample of 23 invasive male breast carcinomas from southern Portugal for the presence of a latent EBV infection by detecting the EBER transcripts using chromogenic in situ hybridization (CISH). ISH was performed on paraffin-embedded sections using the INFORM EBER assay (Ventana Medical Systems, Franffurt, Germany) according to the manufacturer`s instructions (regarding positive and negative controls, and RNA preservation controls with samples). A nasopharyngeal carcinoma was used as a positive control. The patient`s ages ranged from 39-87 years (mean 64.3 years). All samples were invasive ductal carcinomas of not otherwise specified (NOS) type. Tumor differentiation was assessed using the Elston and Ellis grading system: 3 (13.1%) samples were classified as grade 1, 13 (56.5%) as grade 2 and 7 (30.4%) as grade 3.

No EBER transcripts were found in our 23 male breast cancers. No EBV-positive lymphocytes were detected. The presence of EBV transcripts has been studied in female breast cancer using immunohistochemistry (IHC), PCR and ISH. In studies using IHC/PCR, EBV DNA was detected in 10-51% of female breast cancers, while in studies using ISH no EBV was detected in female breast cancers. The disparity in the results may have been due to differences in the sensitivity of the probes used for the detection of EBV infection. Herrman and Niedobitek have suggested that EBV DNA detected by PCR in breast carcinoma tissues is likely to be related to the presence of EBV-infected lymphocytes in the tumor stroma, and thus does not indicate infection of the tumor cells by the virus. The study of DNA extracts by PCR is clearly unsatisfactory since it does not indicate the cellular source of any viral genome detected. In recent studies, laser capture microdissection combined with real-time quantitative BAMHIC PCR has

been used in an attempt to overcome this problem, but even with such techniques the results are controversial.

In conclusion, our findings corroborate those of Glaser et al. and do not support an association between an EBV latent infection and the pathogenesis and/or progression of male breast cancer. Although we cannot totally exclude an association of EBV within particular histological subtypes (e.g. medullary carcinoma/ lymphoepithelial-like carcinoma) that were not represented in our series of male breast cancer, the results of studies of these histological subtypes of breast cancer in females argue against this association.

5. André S, Fonseca I, Pinto AE, Cardoso P, Pereira T, Soares J.

Male breast cancer - a reappraisal of clinical and biologic indicators of prognosis.

Acta Oncol. 40(4):472-8. 2001. PMID:11504306. doi: 10.1080/028418601750288190

Between 1970 and 1998, 90 cases of male breast cancer with available pathological material were retrieved. The disease often presented in aged patients (median--66 years) and as advanced stage (stage III/IV-51%). Excluding stage IV disease, the neoplasia were predominantly ductal invasive carcinomas. NOS (not otherwise specified) (92%), grade 1 and grade 2 (94%), positive for estrogen and progesterone receptors (72% and 74%), negative for androgen receptors (100%), p53 negative (95%), c-erbB-2 negative (88%) and DNA aneuploid (73%). Assessment of disease outcome is determined by stage at time of diagnosis, and axillary lymph node status was the only parameter found to have a statistically significant correlation with either disease-free interval or overall survival ($p < 0.001$) by multivariate analysis. Clinically useful information on the probability of relapse can be added by determining c-erbB-2 ($p = 0.02$) and progesterone receptors ($p = 0.04$) in stage III and tumor ploidy ($p = 0.04$) in pN1 subgroups of patients.

6. Soares J, Pinto AE, Cunha CV, André S, Barão I, Meneses e Sousa J, Cravo M.

Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression.

Cancer. 1;85(1):112-8, 1999. PMID: 9921982

The global DNA methylation of 136 breast lesions (117 primary invasive carcinomas, 5 benign phyllodes tumors, 11 fibroadenomas, and 3 sclerosing adenosis) and their respective adjacent parenchyma was analyzed using an in vitro enzyme assay.

In the group of patients with breast carcinoma, DNA hypomethylation was correlated with clinical and pathologic parameters known to affect disease prognosis. Histopathologic type, disease stage, and tumor grade were evaluated according to the World Health Organization classification, the TNM system, and the criteria of Elston and Ellis' criteria, respectively. DNA flow cytometry was performed in fresh/frozen samples stained with propidium iodide. Hormone receptor (estrogen and progesterone receptor) status was determined by immunocytochemistry.

The comparative study of DNA methylation showed that the DNA of breast carcinomas was statistically significantly less methylated than the DNA of the respective adjacent parenchyma (P=0.0001), the DNA of breast benign lesions (P=0.0002), and the DNA of normal parenchyma (P < 0.0001). A statistically significant correlation was found between the global DNA hypomethylation and the disease stage (P=0.0009), tumor size (P=0.0026), and histologic grade (P=0.0097) of malignant neoplasms. A trend for DNA from breast carcinomas with positive axillary lymph nodes (N1) to be more hypomethylated than those without nodal involvement (NO) (P=0.055) was verified. In contrast, no significant association was found between DNA methylation and histologic type of tumors, hormone receptors, DNA ploidy, and S-phase fraction.

The current shows that DNA hypomethylation is increased in breast carcinomas, playing a potentially important role in tumor development. These findings also suggest that DNA methylation status may be a biologic marker with prognostic significance in this group of neoplasms.

7. Cunha F, André S, Soares J.

Morphology of male breast carcinoma in the evaluation of prognosis.

Pathol Res Pract. 186(6):745-50, 1990. PMID:1964731. doi: 10.1016/S0344-0338(11)80265-7

We studied a series of 44 consecutive cases of male breast carcinoma over a 14-year period in order to evaluate the clinico-pathological characteristics and the impact of some morphologic factors on prognosis. The age of the patients ranged from 38 to 84 years (mean 62 +/- 10.8). All the patients presented a painless mass, associated with nipple retraction in 13 cases (29.4%), skin ulceration in 12 cases (27.2%) and nipple discharge in 6 (13.6%). Microscopically all the tumors were infiltrating ductal carcinomas, 42 being of the NOS type. A better survival was associated with low mitotic index, T1 tumors and absence of peritumoral lymphatic permeation. However, only these two parameters had statistical significance and were found to have predictive value on the prognosis of the disease. The degree of differentiation assessed according to Bloom and Richardson's classification showed no influence on prognosis. Post-surgical radiotherapy did not seem to influence the outcome of the disease.

Abstracts Published in International peer-review journals

1. André S, Pereira T, Silva F, Machado P, Aparício M, Silva G, Pinto AE.

Independent predictors of survival in male breast cancer: unfavorable impact of older age in a multivariate analysis of 166 patients.

Mod Pathol. 17: 35A, 2014

Background: Male breast cancer (MBC) is a rare disease with unpredictable outcome. The aim of this study was to investigate the independent prognostic value of clinico-pathological characteristics and biomolecular markers in relation to overall (OS) and disease-free survival (DFS) in MBC.

Design: the study involved 166 men with MBC and mean follow-up time of 81.9 months (1-396). Age, family history, bilateral growth, histological type and grading, tumor size (pT), nodal status (pN), distant metastasis (M), and disease staging were evaluated. Estrogen (ER) and progesterone receptors (PR) and c-erbB-2 oncoprotein were assessed by immunohistochemistry.

HER-2 gene amplification was determined by FISH, DNA ploidy by flow cytometry, and BCRC2 mutations by PCR. A Cox regression model was used for statistical analysis of the prognostic variables.

Results: Fifty-seven (33.7%) patients died of disease and 62 (37.3%) experienced disease recurrence. The 5- and 10-year OS and DFS were 70.1 and 56.7% and 62.9% and 52.5%, respectively. The Kaplan-Meier (K-M) curves of age, pT, pN, M, disease stage, histological grade and PR showed statistical differences for DFS. In univariate analysis, all the previous variables, with the exception of HER-2 and BRCA2 (excluded to few data) for OS, and age, which was added, for DFS, were significantly associated with clinical outcome. In multivariate analysis, older age (≥70 years) and advanced disease staging (pT, pN e M) for OS, as well as the same previous parameters, together with high grade tumors and lack of PR for DFS, retained statistically significant association with worse prognosis.

Conclusions: The results show the unfavorable prognostic value of older age and disease extension (pT, pN, and M) in male breast cancer. High grade of tumor differentiation and lack of PR are also predictors of worse DFS.

2. Ferreira M, Mesquita M, Quaresma M, André S.

Prolactin receptor expression in benign and malignant breast lesions of male and female patients.

Virchows Arch. 447(2): 129-551, 2005

Background: Despite the well established function of prolactin (PRL) in normal breast development, the role of this pituitary hormone in breast cancer is still controversial. PRL activity is dependent on the activation of a transmembrane protein, the PRL receptor (PRLR). The aim was to evaluate and compare PRLR expression in gynaecomastia and male breast carcinoma (MBC).

Objective: Evaluate and compare PRLR expression in male and female breast carcinoma, as well as in gynecomastia and female breast lesions.

Methods: PRLR expression was detected immunohistochemically, using a monoclonal antibody (clone B6.2), in formalin-fixed paraffin-embedded tissue of 30 cases of gynaecomastia, 30 cases of benign female breast cancer lesions (including ductal hyperplasia, fibroadenoma, sclerosing adenosis, papilloma and radial scar), 30 cases of

MBC and 30 cases of FNC (all invasive ductal carcinomas NOS). The whole series was also assessed for oestrogen receptors (ER), progesterone receptors (PR) and androgen receptors (AR). A cut-off of 10% was used as the criterion for positivity. Histological type was evaluated according to the WHO classification. Tumor differentiation and pathological stage were assessed using the Elston and Ellis grading system and TNM-UICC system, respectively. Statistical analysis was performed with Fisher´s Exact Test.

Results: PRLR positivity was seen in 20% of gynaecomastia cases, in 20% of benign female breast lesions, in 60% of MBC cases and in 50% of FBC. PRLR expression was significantly higher in MBC than in gynaecomastia (p=0.003). Likewise, it was significantly higher in FBC than in benign female breast pathology (p=0.029). There was no statistically significant correlation between PRLR expression and ER, PR, AR, pTNM, histological grade or patients´ age.

Conclusions: The results indicate that: 1) there is no difference in PRLR expression between male and female benign breast lesions; 2) there is no difference in PRLR expression between MBC and FBC; 3) PRLR is significantly more expressed in invasive breast carcinoma than in benign male and female breast lesions, suggesting a role for PRL in breast carcinogenesis.

3. André S, Laranjeira C, Quaresma M, Pinto AE.

Different biological characteristics in female and male breast cancer.

Modern Pathol. 17: 22A, 2004

Background: Female and male breast cancer are heterogeneous and complex diseases that appear to share many biological characteristics. The purpose of this study was to compare the imunohistochemical profile of some cell cycle inhibitors of G1/S-phase transition (p53, p21 and pRb), hormone receptors (ER and PR) and proliferation markers (Ki-67 and ploidy) between breast cancer in female and male, and to correlate data with overall survival.

Design: We retrieved 50 cases of female breast cancer (FBC) and 50 of male breast cancer (MBC) with no significant differences in age, nodal status, stage and

histological type. We collected the clinicopathological data, and performed an immunohistochemistry study (StrepABC system and DAB) using monoclonal antibodies (ER, PR, Ki-67, p53, p21, pRb) and flow cytometric analysis (frozen samples stained with propidium iodide). Association between variables (Pearson Qui-Square Test or the Fisher's Exact Test), overall survival (Kaplan-Meier) and differences between curves (Log-rank Test) were evaluated.

Results: The frequency of ER and PR positive tumors, aneuploidy, p53 negative, p21 positive and unaffected pRb neoplasms was significantly higher in MBC than in FBC ($p<0.001$; $p=0.001$; $p<0.001$; $p=0.014$; $p<0.001$ and $p=0.059$, respectively). In MBC, Ki-67 was significantly associated with grade and stage ($p=0.042$ and $p=0.040$, respectively). In FBC, Ki-67 positivity was higher in older patients ($p=0.025$) and lower in p21 positive tumors ($p=0.042$); ploidy was associated with grade ($p<0.001$), ER ($p=0.001$) and PR ($p=0.012$), and grade was also associated with ER ($p=0.002$) and PR ($p=0.022$). In FBC, nodal status was the only parameter related with overall survival ($p=0.027$), and in MBC both nodal status ($p=0.044$) and stage ($p=0.048$) were related to overall survival.

Conclusions: 1) FBC and MBC show significant biological differences regarding ER and PR expression, DNA ploidy, and cell cycle inhibitors, which in addition to the different associations found between parameters within each group, suggests distinct tumor pathogenesis. 2) In both groups of neoplasias, nodal status remains the most important predictor of overall survival.

4. Fonseca R, André S, Laranjeira C, Tomás AR, Pinto AE, Fonseca I.

Male breast cancer: Correlation between clinicopathologic features and c-erbB2 status.

Modern Pathol. 16, 2003 and Lab Invest vol 84:115A, 2004

Background: Male breast cancer is an uncommon disease that shares with its female counterpart many biological characteristics and the treatment is identical in both. Alterations in c-erbB-2 are seen in 20% to 30% of invasive female breast cancer, have prognostic relevance and may be predictive of therapy, but their role in male breast cancer

remains controversial. The aim of this study was to evaluate and correlate clinico-pathologic features and c-erbB-2 status in male breast cancer.

Design: We retrieved 50 cases of male breast cancer, collected the clinicopathological data, performed an immunohistochemistry study using monoclonal antibodies (ER, Ki-67, p53, p21, Rb, c-erbB-2 (StrepABC system and DAB)), assessed each case for c-erbB-2 amplification by fluorescence in-situ hybridization (INFORM®HER2/neu gene detection kit) and performed flow cytometric analysis (Epics Profile II flow cytometer). Overall survival (Kaplan-Meier) and differences between curves (log-rank test) were evaluated.

Results: Patients age ranged from 39 to 87 years old (mean=63.6 yrs). Follow-up was obtained in all cases with a median period of 120 months. Twenty-nine (58%) patients died of the disease. All cases were invasive ductal carcinomas, NOS; stage I (n=10), stage II (n=23) and III/IV (n=17). The positive immunohistochemical results were ER-80% (n= 40) positive> 10% cells, Ki-67 - 64% (n= 32) positive> 10% cells, p53 - 4% (n= 2) positive> 10% cells, p21 - 70% (n= 35) positive> 10% cells, Rb - 70% (n= 35), c-erbB-2 - 12% (n= 6) (graded 2 and 3+ on a 0 to 3+ scale). There were 80% (n=40) aneupoloid cases and 8% (n=4) c-erbB-2 amplified cases (>10 gene copies/cell). All amplified cases belonged to patients >50 yrs and pathological stage II. Three c-erbB-2 amplified cases were ER and Rb positive. All amplified cases were Ki-67 positive, p53 negative, p21 positive and aneuploid. In univariate analysis, stage (p=0.002), c-erbB-2 overexpression (p=0.016) and c-erbB-2 amplification (p=0.009) were significantly related with overall survival.

Conclusions: 1) The percentage of c-erbB-2 amplified carcinomas in male breast is low. 2) Amplified c-erbB-2 tumors share some "aggressive" biological characteristics. 3) In this series, c-erbB-2 abnormalities, together with pathological stage are, statistically significant, related with overall survival

Oral Communications in Scientific Meetings

1. André S, Pinto A, Laranjeira C, Quaresma M, Soares

Male and female breast cancer- differences in DNA ploidy, p21 and p53 immunoexpression suggest distinct pathways of oncogenesis.

Congresso da la Sociedad Ibérica de Citometria. Coimbra, 2007

2. Ferreira M, Fonseca R, André S.

Expression of mismatch repair proteins in sporadic male breast carcinoma.

X Congresso Nacional de Anatomia Patológica, Porto, 2005

3. Ferreira M, Mesquita M, Quaresma M, André S.

Prolactin receptor expression in benign and malignant pathology of male and female.

IX Congresso Nacional de Anatomia Patológica and XII Reunião Anual da Sociedade Portuguesa de Citologia. Luso, 2004

4. Fonseca R, Tomás AR, Quaresma M, André S, Soares J

Invasive ductal Carcinoma of the male breast – gene c-erbB2 amplification and hiperploidy of chromosome 17.

IX Congresso Nacional de Anatomia Patológica/XII Reunião Anual da Sociedade Portuguesa de Citologia. Luso, 2004

5. Fonseca R, Tomás AR, André S, Soares J.

Gene HER2/neu in invasive ductal carcinoma of the male breast.

VII Congresso Nacional de Anatomia Patológica. Aveiro, 2002

6. André S, Fonseca I, Pinto A, Cardoso P, Pereira T, Soares J.

Male breast carcinoma – biological characteristics and clinical evolution- study of 90 cases.

4º Congresso Nacional de Anatomia Patológica. Cascais, 1999

7. Soares J, André S, Fonseca I.

Male breast carcinoma: morphologic characteristics and their impact on prognosis.

USCAP 85th Annual Meeting, Washington DC, 1996

8. Soares J, André S, Fonseca I.

Estudo imunohistoquímico de 51 casos de carcinoma da mama masculina.

II Encontro de Patologistas Portugueses. Luso, 1990.

9. Cunha F, André S, Soares J.

Male breast cancer. Study of prognostic factors in a series of 44 cases.

VII Reunião Luso-Espanhola de Anatomia Patológica, Lisboa, 1987

III Congresso Nacional de Oncologia. Lisboa, 1987

Poster Presentations in Scientific Meetings

1. Santos F, Cunha F, Machado P, Vaz F, André S.

Bilateral male breast cancer and BRCA2 mutation.

25th European Congress of Pathology. Lisboa, 2013

2. Marques RC, Cunha F, Machado P, Vaz F, André S.

Male breast invasive lobular carcinoma: report of two cases

25th European Congress of Pathology. Lisboa, 2013

3. André S, Laranjeira C, Quaresma M, Pinto A.

Different biological characteristics in female and male breast cancer.

93th Annual Meeting of the United States and Canadian Academy of Pathology. Vancouver, 2004

4. Fonseca R, André S, Laranjeira C, Tomás AR, Pinto A, Fonseca I.

Male breast cancer: Correlation between clinicopathologic features and c-erbB2 status.

92th Annual Meeting of the United States and Canadian Academy of Pathology. Washington DC, 2003

5. Fonseca R, Tomás AR, André S, Soares J.

Gene HER2/neu in invasive ductal carcinoma of the male breast.

VII Congresso Nacional de Anatomia Patológica. Aveiro, 2002

6. Soares J, André S, Fonseca I.

Male breast carcinoma: morphologic characteristics and their impact on prognosis.

Annual Meeting, US and Canadian Academy of Pathology. Washington DC, 1996

AWARD

1. Saudade André

Male Breast Cancer – clinical, morphologic, genomic and metabolic characterization.

Prémio NOVARTIS | EXCELLENCE in Fundamental Medical Research da NOVA (Prémio Novartis | NOVA).

Future Perspectives

1. To immortalize gynecomastia and male BC cell lines to be used *in vitro* research

This was one of the specific aims of this thesis. After unsuccessful procedure with 2D cell cultures for gynecomastia and male BC, we extended a previous collaboration with Instituto of *Biologia Experimental e Tecnológica em Oeiras (IBET)* in female BC, where we contribute with samples collection for cell culture, immunohistochemistry of the cell culture samples and the comparison of those results with the original tumor biopsy.

We collected cases of female BC for the study "Long-term cultures of patient-derived breast cancer organoids as a platform for the evaluation of tumor-stromal interaction and resistance mechanisms to clinically approved drugs" (completed PhD thesis - Marta Estrada). The aim of that study was to perform *ex vivo* cultures of female BC explants, and optimize the system in order to retain tissue architecture and cell composition in culture for long periods, with maintenance of the BC markers and phenotype identified at diagnosis. The strategy combined cell microencapsulation in an inert scaffold (alginate) and bioreactor technology, to generate tumor micro tissues composed of tumor spheroids, fibroblasts and/or monocytes. After one month in culture, Marta and collaborators verified that the morphological and molecular features of the explants were consistent with the original tumor, maintaining cell viability, the epithelial and stromal components and the expression of ER and PR. Hormone receptor functionality was being assessed by evaluation of PR, one of ER downstream effectors, after fulvestrant treatment. Following validation, this model can be used as a platform to study the interaction between the different cell populations and also to provide mechanistic knowledge on the pathways involved in disease progression.

We added to this study one case of gynecomastia and 5 cases of male BC, with variable success in terms of cellularity but without success in immortalizing cell lines. Many cell lines have been used in the study of female BC *in vitro*, allowing standardized

examination of a variety of cellular biologic processes ranging from gene and protein function to drug effect *in vitro* and this method, in our opinion, would be particularly useful in examining rare gene variants or rare diseases such as male BC.

As yet, to our knowledge, a single Japanese study by Maeda *et al*. described an unregistered human male BC cell line KBC2 (8).

Our project is to continue with this collaboration to particularly evaluate male BC characteristics in 3D cultures and to immortalize cell lines of both male BC and gynecomastia.

2. Promoter methylation levels of *RAD51B* and *XRCC3*

IPO of Porto had published, in 2016 (Abreu et al), a series of 111 cases of male BC. I intend to propose to joint their cases for the validation of the results of promoter methylation levels of *RAD51B* and *XRCC3* and also the evaluation of all series by immunohistochemistry to check the expression of RAD51B and XRCC3 genes and to correlate them with methylation levels of their promoters.

In addition, further collaboration in liquid biopsies analyses may be crucial to assess the potential of this panel for early detection, in high risk populations, and in disease monitoring as well.

3. Male BC microenvironment

Some of the components of breast stroma have been identified as new biological markers in BC. The results obtained in this series with β1, β3 and β4 integrin and collagens I and IV are particularly interesting, and may be validated in larger series of male BC pathways of intercellular communication able to respond to therapeutic targeting. I would like to continue the collaboration with Professor Ana Félix, regarding her experience in this area.

4. Male BC metabolism

The collaboration with Jacinta Serpa in the study of microenvironment and metabolism in female BC produced two papers related to FATP1. I intend to go on with this collaboration with emphasis for male BC and gynecomastia, in order to better understand the influence of microenvironment in the metabolic remodeling in male breast carcinogenesis. New insights on this issue can pave the path for new therapeutic

strategies, not only based on new targets but also in patient stratification according to molecular features, predicting a better adjusted therapeutic regime.

Chapter VII

Figure 16 - Spring at IPOLFG in front of Cytopathology Laboratory (2019)

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Figure 17 - Saudade André, 2019