Genetic Polymorphisms of NRF2-KEAP1 in Breast Cancer: a marker for prognosis and treatment

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Tese para obtenção do Grau de Doutor em
Biomedicina
(3º ciclo de estudos)

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Dedicatória

Num dia quente e chuvoso de agosto,
A vida mudou, tornou-se efémera.
Os dias passaram como uma quimera,
Cheios de incerteza que se lia no rosto.

Mas, o riso da criança não traz desgosto,
Só o tempo que passa, sem espera,
Transformando a vida numa nova era,
Valha-lhes a existência com firme imposto.

Ah, os pais, seres quase omnipotentes!
Os meus são eternos e valentes,
E, como vos estou tão agradecida!

Ó tempo, ouve a súplica na voz,
Concede a eternidade aos avós,
Para em abraço ser protegida!

Aos meus pais Dora e João

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Resumo alargado

O cancro da mama continua a ser a doença oncológica com maior impacto na morbilidade e mortalidade em mulheres em todo o mundo.

Existem diversos fatores que podem potenciar o processo carcinogénico na mama, mas a exposição prolongada a estrogénios é considerada um fator de risco elevado para o desenvolvimento do cancro da mama. A exposição a estrogénios endócrinos associa-se maioritariamente à vida reprodutiva da mulher, como menarca precoce, menopausa tardia, gravidez e amamentação. Por sua vez, a terapêutica de substituição hormonal, uma forma de exposição a estrogénios exócrinos, é considerado fator de risco de cancro da mama em mulheres pós-menopausa.

Este processo carcinogénico, pela exposição contínua a estrogénios, é potenciado por alterações como polimorfismos em genes de baixa penetrância, que podem alterar os níveis de transcrição de enzimas da via biossintética ou da via metabólica. Os polimorfismos na via biossintética podem contribuir para o aumento dos níveis de estrogénio e, os polimorfismos na via metabólica para a ineficiente destoxificação dos estrogénios.

Estudos anteriores da nossa equipa de investigação associaram o polimorfismo rs2236722 do gene *CYP19A1* e o polimorfismo "*ausente*" dos genes *GSTM1* e *GSTT1* com o aumento do risco de cancro da mama. O polimorfismo no gene *CYP19A1* está relacionado com aumento da atividade enzimática da aromatase, que resulta no aumento da biossíntese de estrogénios. O polimorfismo "*ausente*" do gene *GSTM1* e *GSTT1*, resulta ausência das enzimas de fase II glutationa S-transferase Mu1 (GSTM1) e glutationa S-transferase Theta1 (GSTT1). A GSTM1 e a GSTT1 são responsáveis pela destoxificação de formas potencialmente carcinogénicas do estrogénio, na sua ausência, ocorre o comprometimento da destoxificação dos estrogénios. A expressão destas enzimas é regulada pelo complexo fator nuclear eritróide 2 relacionado ao fator 2 - proteína Associada a Kelch-Like ECH 1 (NRF2-KEAP1 - nuclear factor erythroid 2-related factor 2 - kelch-like ECH-associated protein 1), que é o principal regulador do stress oxidativo. A desregulação deste complexo pode levar à diminuição de NRF2 no núcleo, comprometendo a indução de enzimas de fase II, o que resulta na acumulação de derivados de estrogénio com potencial para provocar dano no ácido desoxirribonucleico (ADN), como é o caso das quinonas e das semi-quinonas. Polimorfismos neste complexo, que resultem no aumento da KEAP1, com consequente

diminuição da disponibilidade de NRF2 no núcleo, ou que resultem na diminuição de NRF2, foram associados ao aumento do risco de algumas doenças, como doença coronária, hipertensão arterial, diabetes, tromboembolismo venoso e cancro do pulmão.

O funcionamento deste complexo e a sua importância na destoxificação dos estrogénios levou-nos ao objetivo da presente tese que é validar o complexo NRF2-KEAP1 como um marcador para o prognóstico e terapêutica do cancro da mama. De modo a atingir este objetivo foi analisada a influência de polimorfismos em genes de baixa penetrância na via metabólica do estrogénio. Os genótipos do polimorfismo Val432Leu do gene *CYP1B1*, polimorfismo C677T do gene *MTHFR* e polimorfismo "*ausente*" nos genes *GSTM1* e *GSTT1*, foram avaliados em mulheres com cancro da mama hormono-dependente e correlacionado com a idade aquando do diagnóstico de cancro da mama. Verificou-se que mulheres portadoras do genótipo "*ausente*" do gene *GSTT1*, ou do gene *GSTT1* em associação com o genótipo "*ausente*" do *GSTM1* foram diagnosticas com cancro da mama com idades iguais ou superiores a 50 anos. Similarmente, mulheres portadoras do alelo Val432 do *CYP1B1* e do genótipo "*ausente*" do *GSTT1* ou do *GSTM1*, assim como, portadoras do genótipo "*ausente*" do *GSTT1* e portadoras do alelo T do polimorfismo C677T do *MTHFR*, foram diagnosticadas com cancro da mama com idade igual ou superior a 50 anos. Uma vez que estes polimorfismos estão associados à destoxificação do estrogénio, os resultados indicam que polimorfismos que contribuem para uma ineficiente destoxificação do estrogénio podem predispor mulheres a desenvolver cancro da mama hormono-dependente em idades mais tardias.

O genótipo "*ausente*" dos genes *GSTM1* e *GSTT1* resulta na completa deleção dos genes com consequente ausência da enzima. No entanto, a metodologia comumente utilizada não permite distinguir o genótipo heterozigótico do genótipo homozigótico "*presente*". Assim, otimizou-se uma técnica por reação em cadeia da polimerase em tempo real (Real-Time polymerase chain reaction – Real-Time PCR), que permite distinguir estes dois genótipos no gene *GSTM1*. Esta abordagem permitiu correlacionar os genótipos "*presente*" (*GSTM1**1/1) e o genótipo heterozigótico (*GSTM1**1/0) com os polimorfismos rs35652124, rs6706649, rs6721961 do *NRF2* e o polimorfismo rs1048290 do *KEAP1* em casos de cancro da mama. Verificou-se que casos *GSTM1**1/0 (heterozigóticos) e cumulativamente heterozigóticos nos polimorfismos do *NRF2* e /ou *KEAP1* podem estar associados a cancros da mama hormono-dependente positivos para recetor do fator de crescimento epidérmico ($HER2^+$ epidermal growth factor receptor 2), um tipo molecular de cancro da mama mais agressivo.

Na literatura existem vários estudos que correlacionam polimorfismos do *NRF2* e a sua expressão com o prognóstico do cancro da mama. Pela revisão sistemática da literatura e posterior meta-análise verificou-se que a sobre-expressão de NRF2 em doentes com cancro da mama está associada a menor sobrevivência e menor sobrevivência livre de doença.

Estes resultados indicam que polimorfismos que comprometem a ação do NRF2 no núcleo podem estar associados ao comprometimento da destoxificação do estrogénio. No entanto, elevados níveis de NRF2 estão associados a um pior prognóstico. Foi por isso pertinente avaliar os genótipos dos polimorfismos, já referidos, nos genes *KEAP1* e *NRF2*; no sangue, tecido benigno circundante do tumor e tecido tumoral. Comparou-se os genótipos destes polimorfismos nos diferentes tecidos e esses dados foram correlacionados com dados clinocopatológicas. Verificou-se uma tendência para perda de heterozigotia no tecido benigno circundante, quando comparado com os genótipos do sangue e do tumor. Também se verificou maior variabilidade de genótipos em cancros da mama de grau histológico 2, quando comparado com o grau histológico 1 e 3. A aquisição de mutações somáticas e a sua diferente distribuição resultam, provavelmente, de um microambiente mais ativo e mais heterogéneo.

Estes resultados enfatizam o duplo papel do complexo NRF2-KEAP1. Polimorfismos que comprometam a disponibilidade de NRF2 no núcleo, comprometem a destoxificação do estrogénio, podendo predispor ao desenvolvimento de cancro da mama em pós-menopausa. Elevados níveis de NRF2 no núcleo promovem uma elevada destoxificação, protegendo as células saudáveis do stress oxidativo, assim como as células tumorais, do stress oxidativo promovido pela terapêutica, contribuindo para um pior prognóstico. No entanto, com a idade, os níveis de KEAP1 tendem a aumentar, este facto associado a polimorfismos no gene *KEAP1*, podem promover a desregulação no NRF2. É, por isso, pertinente fazer mais estudos no tecido benigno e no tecido tumoral, em diferentes subgrupos de participantes, de modo a compreender o papel do complexo no desenvolvimento, progressão e prognóstico do cancro da mama.

Resumo

O cancro da mama continua a ser a doença oncológica com maior impacto na morbilidade e mortalidade em mulheres em todo o mundo.

A exposição prolongada aos estrogénios é considerado um dos principais fatores de risco cancro da mama. Este processo carcinogénico é potenciado por alterações genéticas, em genes de baixa penetrância, na via biossintética e na via metabólica dos estrogénios.

As enzimas de fases II que metabolizam o estrogénio, são reguladas pelo complexo fator nuclear eritróide 2 relacionado ao fator 2 - proteína Associada a Kelch-Like ECH 1 (NRF2-KEAP1 - nuclear factor erythroid 2-related factor 2 - kelch-like ECH-associated protein 1). O funcionamento deste complexo e a sua importância na destoxificação dos estrogénios levou-nos ao objetivo da presente tese que é validar o complexo NRF2-KEAP1 como um marcador para o prognóstico e terapêutica do cancro da mama.

Para tal, estudou-se a influência de polimorfismos genéticos, em genes de baixa penetrância da via metabólica do estrogénio, no desenvolvimento de cancro da mama. O genótipo dos polimorfismos Val432Leu, C677T e "*ausente*", respetivamente dos genes *CYP1B1*, *MTHFR*, *GSTM1* e *GSTT1*, foram avaliados em mulheres com cancro da mama hormono-dependente. Verificou-se que portadoras do genótipo "*ausente*" do *GSTT1*, sozinho ou em associação com o genótipo "*ausente*" do *GSTM1*, assim como portadoras do Val432 do *CYP1B1* e do polimorfismo "*ausente*" do *GSTT1* ou do *GSTM1*, e portadoras do genótipo "*ausente*" do *GSTT1* e do alelo T do polimorfismo C677T, foram diagnosticadas com cancro da mama com idade igual ou superior a 50 anos. Os resultados indicam que polimorfismos que contribuem para uma ineficiente destoxificação do estrogénio podem predispor mulheres a desenvolver cancro da mama hormono-dependente em idades mais tardias.

Para avaliar a influência clínica dos polimorfismos rs35652124, rs6706649, rs6721961 do *NRF2* e o polimorfismo rs1048290 do *KEAP1* em casos de cancro da mama com genótipo *GSTM1* "*presente*", otimizou-se uma técnica que permitiu distinguir os genótipos heterozigótico do genótipo "*presente*". Os casos *GSTM1* heterozigóticos e cumulativamente portadores dos polimorfismos do *NRF2* e /ou *KEAP1* foram associados a cancros da mama positivos para recetor do fator de crescimento epidérmico ($HER2^+$ epidermal growth factor receptor 2).

Na literatura existem vários estudos que correlacionam polimorfismos do *NRF2* e a sua expressão com o prognóstico do cancro da mama. Após termos efetuado uma revisão sistemática da literatura, com meta-análise, verificou-se que doentes com sobre-expressão de NRF2 tinham menor sobrevivência e menor sobrevivência livre de doença.

Posteriormente os genótipos dos polimorfismos, já referidos, do gene *KEAP1* e *NRF2*, foram avaliados no sangue, tecido benigno circundante do tumor e tecido tumoral. Verificou-se uma tendência para perda de heterozigotia no tecido benigno circundante, e maior variabilidade de genótipos no grau histológico 2. A aquisição de mutações somáticas e a sua diferente distribuição resultam, provavelmente, de um microambiente mais ativo e mais heterogéneo.

Polimorfismos que comprometem a disponibilidade de NRF2 no núcleo, comprometem a destoxificação do estrogénio, podendo predispor ao desenvolvimento de cancro da mama na pós-menopausa. Elevados níveis de NRF2 no núcleo promovem uma elevada destoxificação, protegendo as células saudáveis e as células tumorais. É, por isso, pertinente fazer mais estudos no tecido benigno e no tecido tumoral, em diferentes subgrupos de participantes, de modo a compreender o papel do complexo no desenvolvimento e progressão do cancro da mama.

Palavras-chave

Cancro da mama; NRF2; KEAP1; polimorfismos; via metabólica dos estrogénios.

Abstract

Breast cancer remains the oncological disease with the greatest impact on morbidity and mortality in women worldwide.

Prolonged exposure to estrogens is considered one of the main risk factors for breast cancer. This carcinogenic process is potentiated by genetic alterations in low-penetrance genes in the estrogen biosynthetic and metabolic pathways.

Phase II enzymes responsible for estrogens detoxification, are regulated by the NRF2-KEAP1 (nuclear factor erythroid 2-related factor 2 - kelch-like ECH-associated protein 1) complex.

The value of this complex in estrogen detoxification led us to the aim of this thesis, which is to validate the NRF2-KEAP1 complex as a marker for breast cancer prognosis and therapy.

Thus, we studied the influence of genetic polymorphisms, in low penetrance genes of the estrogen metabolic pathway in breast cancer development. The genotype of Val432Leu, C677T and *null* polymorphisms, respectively of *CYP1B1*, *MTHFR*, *GSTM1* and *GSTT1* genes, were assessed in women with hormone-dependent breast cancer.

It was verified that carriers of the *null* genotype of *GSTT1*, alone or in association with the *null* genotype of *GSTM1*, as well as carriers of Val432 of *CYP1B1* and the *null* polymorphism of *GSTT1* or *GSTM1*, and carriers of the *null* genotype of *GSTT1* and the T allele of the C677T polymorphism, were diagnosed with breast cancer at the age of 50 or over. The results indicate that polymorphisms that contribute to inefficient estrogen detoxification may predispose women to developing hormone-dependent breast cancer at a later age.

In order to assess the clinical influence of the *NRF2* rs35652124, rs6706649, rs6721961 polymorphisms and the *KEAP1* rs1048290 polymorphism in breast cancer cases with a "*present*" *GSTM1* genotype, a technique was optimised which made it possible to distinguish heterozygous from *present* genotypes. Heterozygous *GSTM1* cases cumulatively carriers of the *NRF2* and/or *KEAP1* polymorphisms were associated with $HER2^{+}$ (epidermal growth factor receptor 2 positive) breast cancers.

There are several studies correlating *NRF2* polymorphisms and its expression with breast cancer prognosis. After a systematic review with meta-analysis, it was found that patients with NRF2 over-expression had lower overall survival and shorter disease-free survival. Subsequently, the genotypes of the aforementioned *KEAP1* and *NRF2* polymorphisms were assessed in blood, tumour's benign surrounding tissue and tumour tissue. There was a trend towards the loss of heterozygosity in the benign surrounding tissue and a greater variability of genotypes in histological grade 2. The acquisition of somatic mutations and their different distribution are probably the result of a more active and heterogeneous microenvironment.

Polymorphisms that compromise the availability of NRF2 in the nucleus impair estrogen detoxification and may predispose to the development of postmenopausal breast cancer. High levels of NRF2 in the nucleus promote high detoxification, protecting both healthy and tumour cells. It is therefore pertinent to carry out further studies in benign and tumour tissue, in different subgroups of participants, in order to understand the role of the complex in the development and progression of breast cancer.

Keywords

Breast cancer; NRF2; KEAP1; single nucleotide polymorphisms; estrogen metabolic pathway.

Thesis Overview

This thesis is divided in eight chapters.

After a brief description of breast cancer incidence/ mortality and classification, the biosynthetic and metabolic pathways of estrogens are revised in Chapter 1.

Chapter 2 reviews the influence of single nucleotide polymorphisms (SNPs) in biosynthetic and metabolic pathways in hormone-dependent breast cancer.

Chapter 3 establishes the main goals of the thesis.

Chapters 4 to 7 present the original research papers developed during this PhD, and are organized as follows:

- Chapter 4 presents the results of the association of SNPs in *CYP1B1*, *GSTM1*, *GSTT1*, *MTHFR*, with breast cancer risk.

- Chapter 5 presents results of the association of *GSTM1 null* polymorphism, alone or combined with *NRF2* and/or *KEAP1* polymorphisms, with clinicopathological data of breast cancer patients.

- Chapter 6 is a systematic review with meta-analysis, approaching the clinical value of NRF2 in breast cancer patient's prognosis.

- Chapter 7 presents a comparative study of blood genotypes and somatic mutations in tumour tissue and benign surrounding tumour tissue of breast cancer patients associated with clinicopathological data.

Results are discussed in Chapter 8, in an integrative approach. Herein, the impact of our results in breast cancer research and clinical practice is evaluated in order to answer the main goal of this work, which is to validate NRF2-KEAP1 as a biomarker for prognosis and therapeutic target in breast cancer.

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List of Acronyms

17α-OHP	17α-hydroxyprogesterone
17β-HSD	17β-Hydroxysteroid dehydrogenase
2-MeOE2	2-methoxyestradiol
2-OH-E2	2-hydroxyestradiol
3β-HSD	3β-hydroxysteroid dehydrogenase
4-MeOE2	4-methoxyestradiol
4-OH-E2	4-hydroxyestradiol
aa	amino acids
AF1	Activation function-1 domain
AF-2	Activation function-2
AKT	Protein kinase B
ARE	Antioxidant response elements
BRCA1 and *BRCA2*	Breast-Cancer susceptibility gene 1 and 2
BTB	Broad complex-tramtrack-bric-a-brac
bZIP	basic region-leucine zipper
CI	Confidence Interval
CNC	Cap'n'collar
COMT	Catechol-O-methyltransferase
CUL3	Cullin 3
CYP	Cytochrome P450
CYP11A1	Cytochrome P450 (CYP) 11A1 enzyme
CYP17A1	Cytochrome P450 17A1
CYP19A1	Cytochrome P450 19A1
CYP1A1	Cytochrome P450 (CYP) 1A1 enzyme
CYP1B1	Cytochrome P450 (CYP) 1B1 enzyme
DBD	DNA binding-domain
DCIS	Ductal carcinoma *in situ*
DFS	Disease-free survival
DGR	Double glycine repeat
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
E2-3,4,Q	17β-estradiol-3,4-quinone
E3	Estriol
E4	Estetrol
ER	Estrogen receptor
ER^+	Estrogen receptor positive
ER^-	Estrogen receptor negative
ERE	Estrogen response elements
ERα	Estrogen receptor alpha ERα
ERβ	Estrogen receptor beta
ESR1 gene	Estrogen receptor 1 gene
GPER	G-protein coupled estrogen receptor
GSH	Glutathione

GSTM1	Glutathione S-Transferase Mu 1
GSTP1	Glutathione S-Transferase Pi 1
GSTs	Glutathione S-transferases
GSTT1	Glutathione S-Transferase Theta 1
HER2	Human epidermal growth factor receptor 2
$HER2^-$	Human epidermal growth factor receptor 2 negative
$HER2^+$	Human epidermal growth factor receptor 2 positive
HR	Hazard ratio
HRT	Hormone replacement therapy
IARC	International Agency for research on Cancer
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IVR	Intervening region
KEAP1	Kelch-like ECH-associated protein 1
LBD	Ligand-binding domain
LCIS	Lobular carcinoma *in situ*
MAPEG	Membrane-associated proteins involved in eicosanoid and glutathione metabolism
MAPK	Mitogen-activated protein kinase
MB-COMT	Membrane-bound COMT
MTHFR	5,10-methylenetetrahydrofolate reductase
Neh	NRF2-ECH domains
NF-κB	Nuclear factor-κB
NLS	Nuclear localization signal
NOS	Not otherwise specified (it referrers to breast cancer classification)
NOS	Newcastle-Ottawa Scale (it referrers to studies quality)
NRF2	Nuclear factor erythroid 2-related factor 2
NTD	NH2-terminal domain
NTR	N-terminal region
OS	Overall survival
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PR^-	Progesterone receptor negative
PR^+	Progesterone receptor positive
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analysis
PTM	Posttranslational modifications
RE	Response element
SAM	S-adenosylmethionine
S-COMT	Soluble COMT
sMaf	Small masculoaponeurotic fibrosarcoma
SNP	Single nucleotide polymorphisms
TF	Transcription factors
TMN	T (primary tumour) N (lymph node status) M (distant metastasis)
WHR	Weighted hazard ratios

List of Scientific Publications

Papers related to this doctoral thesis

Almeida M, Ferreira CL, Tomé RM, Fonseca-Moutinho J, Polónia A, Ramalhinho AC and Breitenfeld L (2024) Somatic Mutations in KEAP1-NRF2 Complex in Breast Cancer Cancers 2024, 16(13): 2411. Doi: 10.3390/cancers16132411 IF: 4,5; Q1

Almeida M, Soares M, Ramalhinho AC, Fonseca-Moutinho J and Breitenfeld L (2021) Influence of Estrogenic Metabolic Pathway Genes Polymorphisms on Postmenopausal Breast Cancer Risk. Pharmaceuticals 2021, 14(2): 94. Doi: 10.3390/ph14020094 IF: 5,215; Q1

Almeida M, Soares M, Ramalhinho AC, Fonseca-Moutinho J and Breitenfeld L (2020) The prognostic value of NRF2 in breast cancer patients: a systematic review with meta-analysis. Breast Cancer Res Treat 179:523–532. Doi:10.1007/s10549-019-05494-4 IF: 4,872; Q2

Almeida M, Soares M, Ramalhinho AC, Fonseca-Moutinho J and Breitenfeld L (2019) Prognosis of hormone-dependent breast cancer seems to be influenced by KEAP1, NRF2 and GSTM1 genetic polymorphisms. Mol Biol Rep 2019 46(3):3213-3224. Doi:10.1007/s11033-019-04778-8. IF: 2,6; Q3

Book chapter related to this doctoral thesis

Almeida M, Soares M, Ramalhinho AC, Fonseca-Moutinho J and Breitenfeld, L (2020) "The role of estrogen receptors alpha in breast carcinogenesis: an overview" – In G. Chen, M. Tong and C.A. van Hasselt (Eds.), Estrogen Receptors: Structure, Functions and Clinical Aspects (1st ed., pp. 1-21). Nova Science Publishers, Inc. https://novapublishers.com/shop/estrogen-receptors-structure-functions-and-clinical-aspects/

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Casteleiro Alves M, **Almeida M**, Oliani AH, Breitenfeld L and Ramalhinho AC (2020) Women with polycystic ovary syndrome and other causes of infertility have a higher prevalence of GSTT1 deletion . Reproductive Biomedicine Online 41(5): 892-901 . Doi:10.1016/j.rbmo.2020.06.010. IF: 3,7; Q1

Chapter 1

Breast Cancer

Some of the contents of this chapter were originally published in:

***Chapter 1*. The Role of Estrogen Receptors Alpha in Breast Carcinogenesis: An Overview. *in* Estrogen Receptors: Structure, Functions and Clinical Aspects.**

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1.1. Worldwide and Portuguese Incidence of Breast Cancer

Breast cancer is the most diagnosed cancer in women worldwide. The last records of International Agency for research on Cancer (IARC) GLOBOCAN are related to 2022 and consist on the data registry of 185 countries [1]. Data indicates that 20 million of new cancer cases were registered (among women and men) in 2022, an increase of 700 000 new cancer cases, when compared to the record of 2020; and cancer was the death cause of 10 million people worldwide [1, 2]. Breast cancer was diagnosed in 11.5% of all population (both sexes) being surpassed by lung cancer (12.4%), which represents an inversion on the position, when compared to 2020, where breast cancer was the most diagnosed cancer [1, 2]. Specifically for female population, breast cancer represents 23.8% of new cancer cases, being the most diagnosed cancer in women in 158 countries (Figure 1) [1].

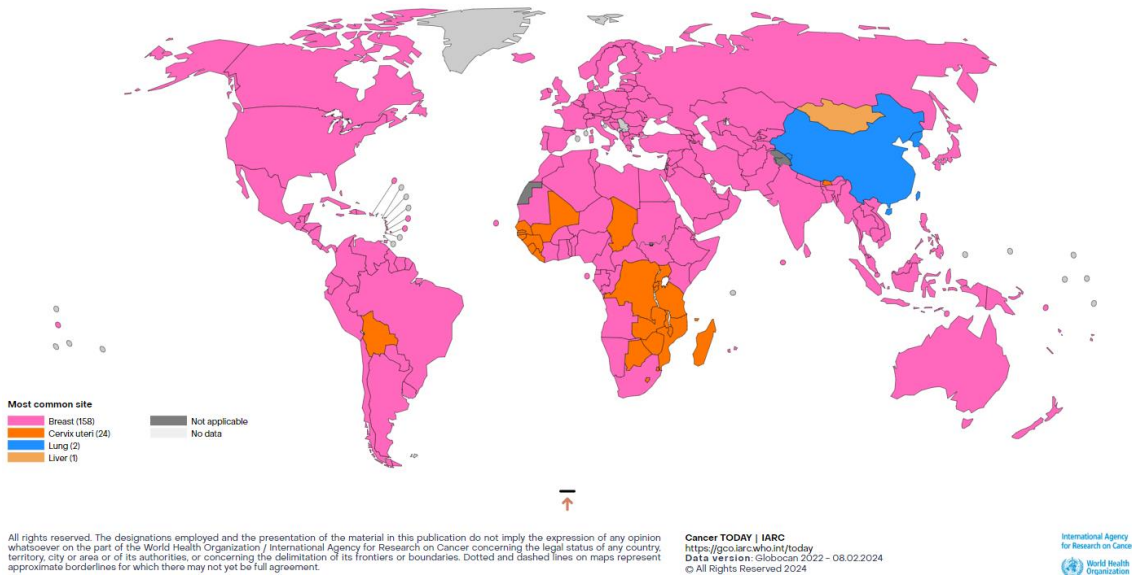

Figure 1. Females most common cancer site per country, Absolute numbers, Incidence, Females, in 2022 (excluding nonmelanoma skin cancer) [1]. Breast cancer is the most common cancer in women in the majority of the worldwide countries (158 countries of 185), with some exceptions like China where lung cancer is the more prevalent and sub-Saharan Africa were cervical cancer is the most diagnosed cancer among women.

In respect to mortality, cancer was responsible for 4 313 548 women deaths, and breast cancer is the leading cause of cancer death in women worldwide 15.4% (Figure 2), being surpassed by lung cancer in Australia, New Zealand, Northern Europe, Northern

America and China; and by cervical cancer mostly in sub-Saharan Africa and in some Latin American countries [1, 3].

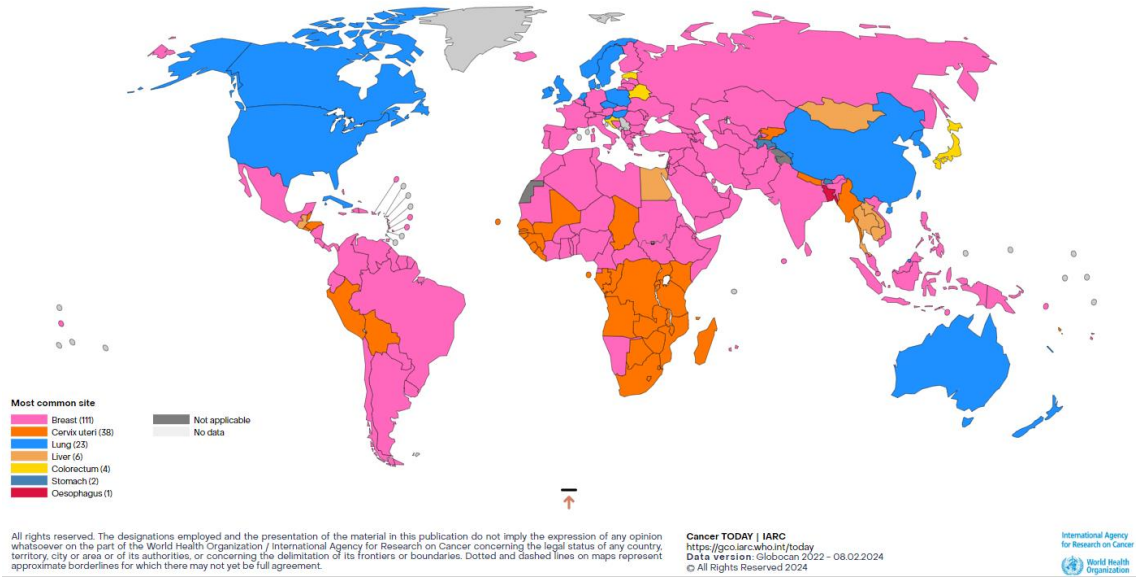

Figure 2. Most common site per country, Absolute numbers, Mortality, Females, in 2022 (excluding nonmelanoma skin cancer) [1]. Breast cancer is the leading cause of cancer in the majority of worldwide countries (111 countries). Followed by cervical cancer that is the major cause of cancer death in 38 countries and lung cancer, which is the first cause of cancer death in women in 23 countries, among them is China, North America, Australia and New Zealand.

Relatively to Portugal, taking into consideration both males and females, breast cancer is the second diagnosed cancer (8 954 new cases in 2022), being surpassed by colorectal cancer [1]. Breast cancer incidence in women follows the worldwide trends once it is the most diagnosed cancer in women (Figure 3) [1].

Breast cancer occupies the fourth position in terms of Portuguese global cancer mortality and in women is the first cause of cancer death accounting with 2 211 cases in 2022 (Figure 4) [1].

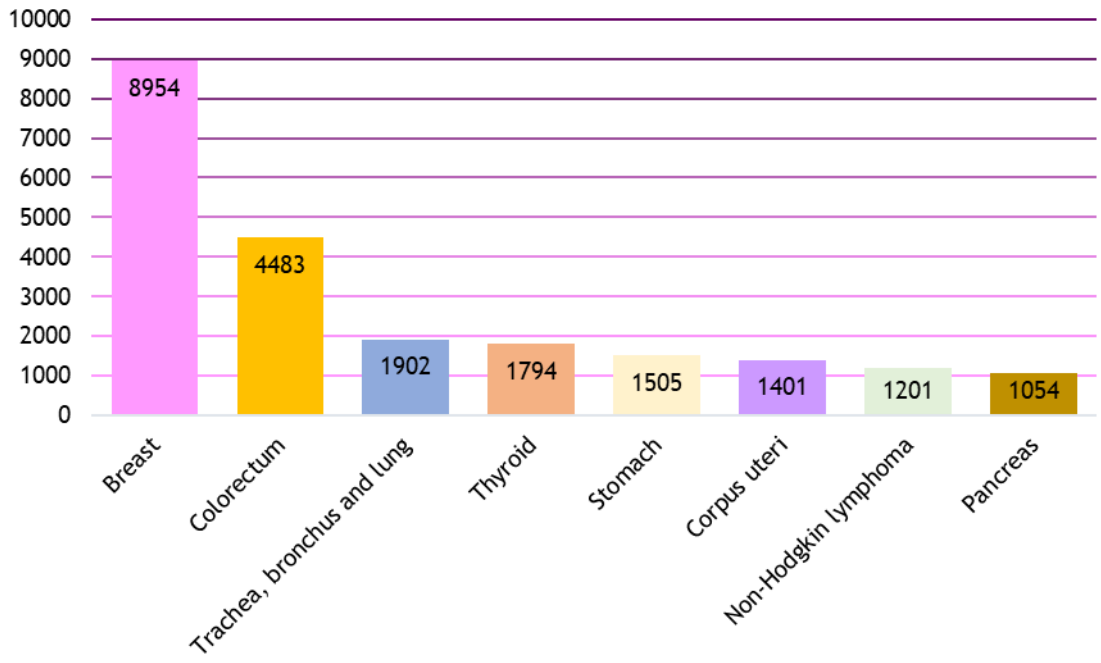

Figure 3. Absolute numbers, Incidence, Females, In 2022 Portugal; adapted from GLOBOCAN [1]. In 2022 were diagnosed 8954 cases of breast cancer in women, being the furthermost diagnosed cancer.

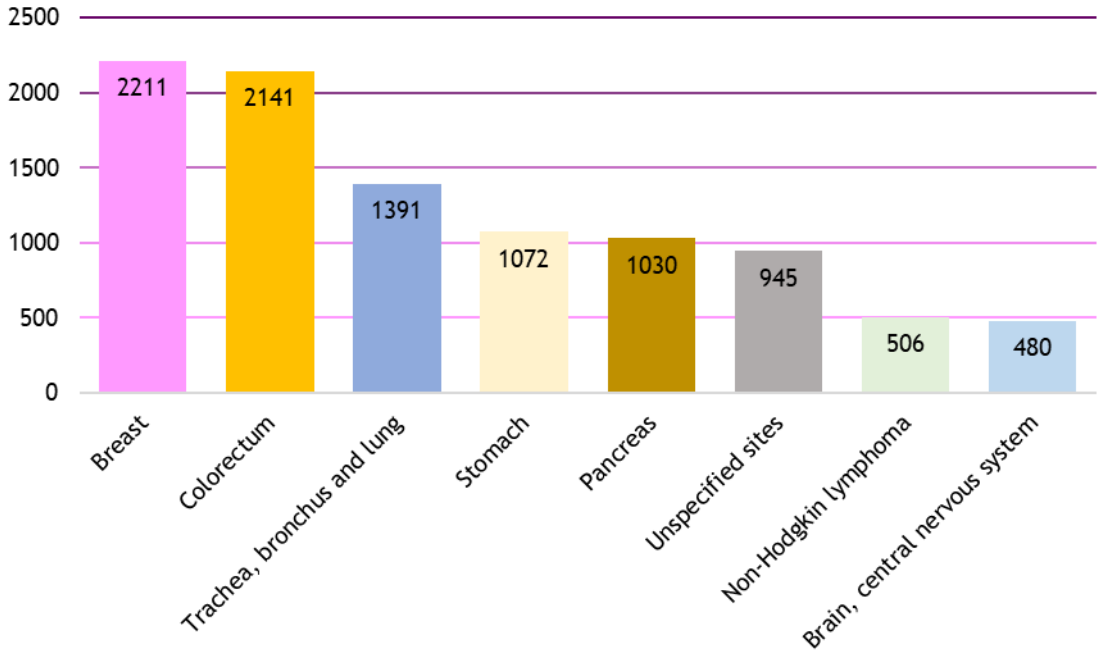

Figure 4. Absolute numbers, Mortality, Females, In 2022 Portugal; adapted from GLOBOCAN [1]. In Portugal, in 2022, breast cancer was the cause of death of 2211 women.

Evaluating the incidence and mortality due to breast cancer in women with ages between 10 and 49 years old (values chosen for menarche and menopause), in Portugal

there were registered 2 135 new cases of breast cancer and 212 deaths [1]. On what concerns to epidemiological data in Portugal in postmenopausal women (50 to +85 years old) the numbers of incidence and mortality were, respectively, 6 819 and 1 999 [1]. These data follow the worldwide trends, were incidence and mortality in reproductive ages was of 668 556 and 141 373, respectively; and after menopause the incidence of new diagnosis was of 1 628 190 and 524 723 deaths [1].

Breast cancer burden is estimated to increase in the next 20 years. Projections for year 2040 indicates that new diagnoses of breast cancer will increase 40% worldwide and deaths 50% [1, 4]. In Portugal it is predicted that more 100 cases per year will be diagnosed in 2040, however breast cancer mortality is predicted to increase to 2 210, which represents an increase of 346 more deaths due to breast cancer [1]. However, these data are projections that are dependent on lifestyle, that can influence risk factors and from public health policies, namely the ones related to early diagnosis and new therapeutics.

1.2. Carcinogenesis and it's Risk Factors

Breast cancer is a heterogeneous disease with several molecular subtypes that lead to different treatment and prognosis [5-7]. Breast carcinogenesis is a complex multistep process triggered by the cooperation of several intrinsic and extrinsic factors [8]. Among the risk factors that contribute to tumour initiation there is age, reproductive history, endogenous and exogenous estrogen levels, previous benign breast disease, lifestyle, ionizing radiation and mutations [9-11]. Hereditary breast cancers account from 5 to 10% and might be due to single nucleotide polymorphisms (SNP), that are the most common type of genetic variations [12]. SNPs might be in high penetrance genes such as *BRCA1* and *BRCA2* (Breast-Cancer susceptibility gene 1 and 2) or in low penetrance genes such as estrogen receptor 1 gene (*ESR1* gene) also, mutations can occur *de novo* [12]. Genetic (inherited or acquired) and epigenetic alterations allow the escape from protective regulatory mechanisms, allowing an uncontrolled proliferation (Figure 5) [5]. The mutated cells can acquire new genetic alterations giving rise to precancerous lesions or benign tumours [7]. The critical turning point occurs when mutated cells acquire the ability to invade surrounding tissues, bloodstream or lymphatic system [5, 7]. The invasion process leads to breast cancer development and later, due to invasion of distant tissues, to metastasis development [5, 7].

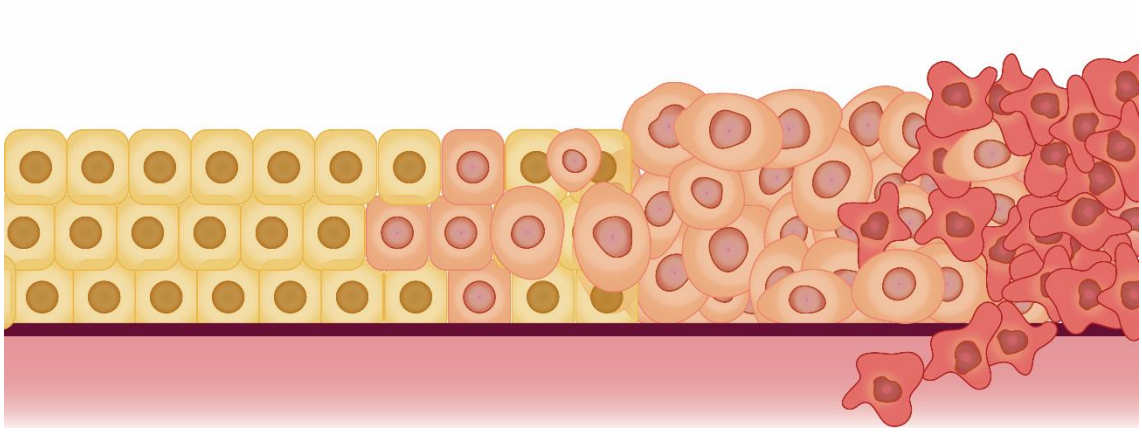

Figure 5. Schematic representation of the carcinogenic process. Genetic or epigenetic alterations may occur in a normal cell (initiation). These altered cells escape from protective mechanisms and multiply (promotion), uncontrolled proliferation (progression) leads to tumour development and, finally, cells acquire the ability to invade other tissues, leading to tumour dissemination (metastization).

Although several risk factors have been related to breast cancer, prolonged exposure to endocrine or exocrine estrogens and its subsequent level of detoxification is being considered one of the main risk factors for breast cancer development [8].

Throughout their lives, woman have different exposure to endogenous estrogen, being age at menarche one of the focal points of estrogen exposure [13]. In a median value, menarche occurs at the age of 12 years old and its occurrence 2 years earlier is related with 11% increased breast cancer risk [13, 14]. Also, a year old at menopause, being 50 years of age considered as a median value, was related to a 1.029 increase of the relative risk of breast cancer development [13].

Parity and lactation are events also related to endogenous estrogen levels once breast tissue suffers differentiation from pregnancy to lactation cessation and undifferentiated breast has increased risk of carcinogenesis [15]. Moreover, during pregnancy and lactation, ovulation inhibition occurs, decreasing the secretion of hormones of the menstrual cycle, with a consequent diminished exposure of breast tissue to estrogens. Thus, breastfeed exerts a protective effect counting with less 11% risk of breast cancer development; if lactation is performed during 12 months or more the protective effect increases to 28% [16]. The impact of breast tissue exposure to estrogen levels is of such greatness that the Collaborative Group on Hormonal Factors in Breast Cancer estimated that in developed countries if women increased the breastfeeding period, breast cancer incidence would be reduced by 42% [17]. This study also verified a relative risk reduction of 7% for each full term pregnancy [17].

On what concerns to exogenous estrogens hormone replacement therapy (HRT) has a high impact in cancer risk. In menopause women's estrogen levels decrease rapidly, being, as previously referred, protective against breast cancer development. However, due to menopause side effects, HRT is widely prescribed in order to moderate the side effects, but this exposure to exogenous estrogens confers a higher risk of 23% for breast cancer development, when compared to women that never performed HRT [16].

1.3. Breast Cancer Classification

Breast cancer classifications arise from the heterogeneity of breast cancer and due to the need to use a standardize language.

One of the classifications is the morphological study, that takes into account if the tumour is limited to epithelial tissue or has invaded the stroma and if the tumour arises from the lobules or the ducts [18]. This classification is standardized by the International Agency for Research on Cancer, World Health Organization.

As previously referred the tumour might arise from the ducts and if the stroma has not been invaded is defined as ductal carcinoma *in situ* (DCIS) and is divided in five subtypes: comedo, solid, cribiform, papillary and micropapillary [18, 19]. DCIS evolution to invasive ductal carcinoma (IDC) depends on the grade and type [18]. The most common IDC (75%) is invasive ductal carcinoma, not otherwise specified (NOS) [18]. Other types of IDC are: tubular, invasive cribiform, mucinous, medullary, invasive papillary, invasive micropapillary, apocrine, neuroendocrine, metaplastic, lipid-rich, secretory, oncocytic, adenoid cystic and acinic cell [18, 19].

If the tumour has an intralobular proliferation is designed as lobular carcinoma *in situ* (LCIS) may also evolve to an invasive form, defined as invasive lobular carcinoma (ILC), representing 5 to 15% of invasive breast carcinomas [18, 19].

The TNM classification of tumour staging defines categories taking into account the clinical size of the primary tumour (T), lymph node status (N) and distant metastasis (M) [20]. The Nottingham combined histologic grade assesses and combines the molecular features of the tumour [20].

The previous referred classifications together with the immunohistochemistry study of the tumour (St. Gallen Classification), that allows to define the subtype of breast cancer will contribute to define treatment and prognosis.

1.3.1. Molecular Subtypes of Breast Cancer

Breast cancer is conventionally classified, based on molecular subtypes through immunohistochemical analysis of the estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), with an additional measure of the proliferation marker Ki-67 [21, 22]. The molecular classification is based on St. Gallen International Consensus Guidelines [23]. This classifications surrogates molecular breast cancer subtypes as: luminal A (ER positive (ER^+)/ PR positive (PR^+) and HER2 negative ($HER2^-$), with low Ki-67), luminal B ($ER^+/HER2^\pm$, high Ki-67 or PR negative (PR^-)), HER2-enriched (ER^-/PR^-/ HER2 positive ($HER2^+$)), basal-like $ER^-/PR^-/HER2^-$ (Table 1) [23].

Luminal A breast cancer is the molecular subtype with better prognosis, with a good response to endocrine therapies [20]. It accounts for 50% of the invasive breast cancers and typically has low grade [18].

Unlike luminal A, the molecular subtype luminal B, that represents around 20% of breast cancer, responds better to conventional chemotherapy instead of endocrine therapies [20, 23]. It has a good prognosis, but worse than luminal A, with higher incidence for distant metastasis [18, 20, 22, 24, 25].

The subtype HER2-enriched is one of the less common subtypes, accounting for 15% [18, 26]. The absence of ER expression and HER2 high expression is characteristic of a tumour with poor prognosis; it is also related to *TP53* gene mutations, high histological grade and lymph node metastasis [18, 20]. A combined treatment with Trastuzumab and chemotherapy has shown to reduce the risk of recurrence and mortality in half, compared with chemotherapy alone [22, 27].

The molecular subtype with worse prognosis is triple-negative breast cancer ($ER^-/PR^-/HER2^-$) [7, 20, 23]. This subtype is common in women with *BRCA1* gene mutations and due to its aggressive biologic characteristics, lack of well-defined targets (usually treated with conventional chemotherapy) this subtype has a poor prognosis, with higher risk of recurrence and death in a 5 years follow-up [20, 23, 26, 28, 29].

Table 1. Intrinsic molecular subtypes of breast cancer – St. Gallen Guidelines [7, 18, 23, 26]

Molecular subtypes		**% of the molecular subtype**	**Prognosis**
Luminal A	$ER^+/PR^+/HER2^-$, low Ki-67	50%	Best prognosis
Luminal B	$ER^+/HER2^-$, high Ki-67 or PR^-	20%	Low survival than Luminal A
	$ER^+/PR^{\pm}/HER2^+$, any Ki-67		
HER2-enriched	ER-/PR-/HER2+	15%	Poor prognosis
Triple-negative	ER-/PR-/HER2-	15%	Worse prognosis
			More common in *BRCA1* gene mutations

As previously referred, the majority of breast cancers (70%) express estrogen receptors [24, 27]. Although the good prognosis of luminal breast cancer, it is a worldwide interest to further study this subtype of breast cancer, hereinafter referred as hormone-dependent breast cancer.

1.4. Hormone-dependent Breast Cancer: Estrogens as a source

Estrogens are steroid hormones that plays a critical role in the development of sexual secondary characteristics and in the development and maintenance of female reproductive tissues.

There are four main forms of estrogen: estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4). The high levels of E2 are present predominantly during reproductive women life being mainly produced by the gonads and in lower quantities by adrenal cortex, breast, brain, vessels, being the adipose tissue the main source of E1 in postmenopausal women [30]. During pregnancy E3 is the primary circulating estrogen, being produced by placenta; also during pregnancy, the human fetal liver produces E4 [31, 32].

Although their importance is predominantly related to reproduction, estrogens are considered carcinogens and exposure to estrogens is a comproved risk factor for breast cancer development [33]. Estrogens were firstly linked to breast cancer in 1896, when surgeon *Sir* George Thomas Beatson performed an oophorectomy on a breast cancer

patient verifying that the procedure led to a remarkable regression of the breast cancer [34]. This was the first step to better understand the hormone-dependent breast cancer.

Thus, high exposure to endogenous or exogenous estrogen levels are critical for the development of the most frequent molecular subtype of breast cancer [20]. Menarche, menopause, pregnancies, full-term pregnancies, breast feeding, obesity, alcohol consumption are related to endogenous estrogen exposures, once these factors are directly related to estrogen production by ovaries, or in case of diet and alcohol consumption, to the conversion of cholesterol in estrogen [14, 35-38]. However, modern life led to the exposure to high levels of exogenous estrogens. Oral contraceptives and HRT are great sources of exogenous estrogens; also, xenoestrogens like bisphenol A present in plastics, and phytoestrogens like gynestein and daidzaeine share structural characteristics with E2, thus being considered endocrine disruptors [39-41].

1.4.1. Estrogens Biosynthetic Pathway

Cholesterol is the precursor of all steroid hormones. Through the action of cytochrome P450 (CYP) 11A1 enzyme (CYP11A1), located in the inner membrane of the mitochondria, converts cholesterol in pregnenolone. This is the first step of steroidogenesis [42, 43]. Further, pregnenolone exits the mitochondria, enters in the endoplasmatic reticulum and is converted in 17α-hydroxypregnenolone or in progesterone, through the action of Cytochrome P450 17A1 (CYP17A1) and 3β-hydroxysteroid dehydrogenase (3β-HSD), respectively [44]. After 17α-hydroxypregnenolone is converted in 17α-hydroxyprogesterone (17α-OHP) by CYP17A1. The dehydroepiandrosterone (DHEA) and 17α-OHP through the lyase activity of CYP17A1 give rise to androstenedione [45]. Androstenedione is the subsequent product of CYP17A1 lyase activity [44]. Androstenedione is the precursor of testosterone and estrogen. 17β-Hydroxysteroid dehydrogenase (17β-HSD) has androgenic and estrogenic activity, being responsible for testosterone and estradiol formation [46]. Finally, Cytochrome P450 19A1 (CYP19A1), also known as Aromatase, identified by André S. Meyer, catalyses testosterone in estradiol and androstenedione in estrone [47]. While in premenopausal women estrogen synthesis occurs mainly in the ovary, in postmenopausal women adipose tissue is the great source of estrogens [48]. The described pathway is the general biosynthetic model of estradiol production

by the human body (Figure 6), that starts in the mitochondria but most of the process is developed in the endoplasmatic reticulum. After its production, estrogen passively through the membranes, binds to estrogen receptors and becomes biologically active [33].

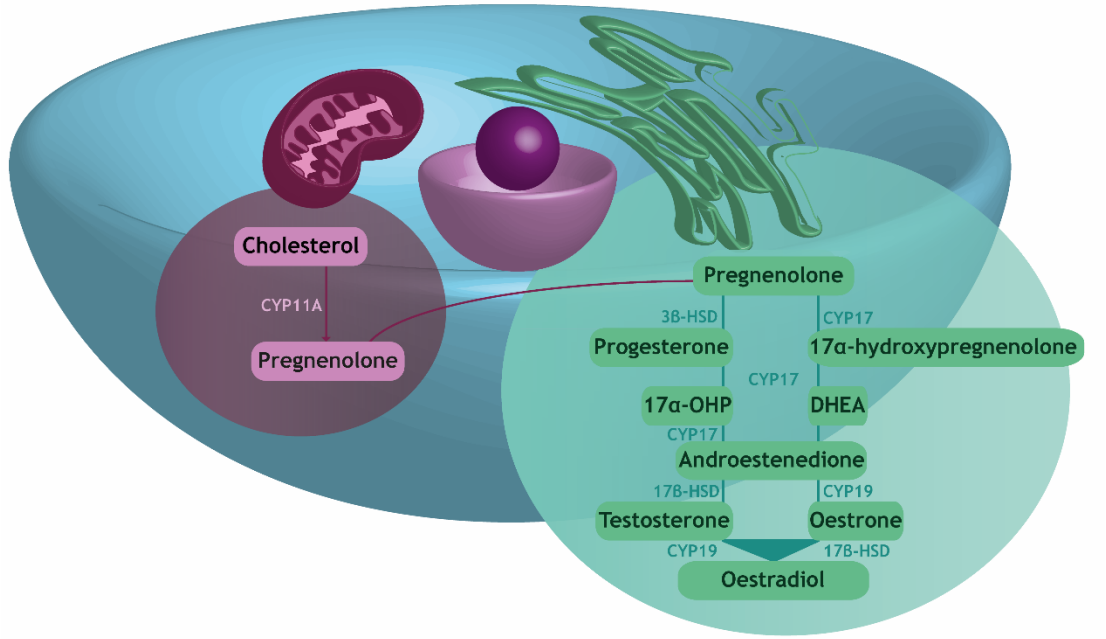

Figure 6. Biosynthetic pathway of estrogen. The first step in the steroidogenesis pathway is the conversion of cholesterol into pregnenolone catalysed by CYP11A1 in the mitochondria. Pregnenolone leaves the mitochondria and enters the endoplasmic reticulum, where, through a series of enzymatic reactions, that end in the aromatization of androstenedione, estradiol (E2) is formed.

Several factors lead to estradiol increased production, like SNPs in genes that codify the enzymes involved in this pathway. The metabolic pathway of estrogens is the next critical step, once it detoxifies estrogens preventing deoxyribonucleic acid (DNA) damage.

1.4.2. Estrogens Metabolic Pathway

Estrogens metabolization (Figure 7) is a critical step for hormone-dependent breast cancer. Two major phase I enzymes are involved in the first step of estrogen metabolism. Cytochrome P450 (CYP) 1A1 enzyme (CYP1A1) converts estradiol in 2-hydroxyestradiol (2-OH-E2) and Cytochrome P450 (CYP) 1B1 enzyme (CYP1B1) is responsible for the conversion of estradiol into 4-hydroxyestradiol (4-OH-E2) [8, 33, 49, 50]. The hydroxylation performed by these two enzymes lead to two distinct

metabolites, with different biologic activities. The metabolite 4-OH-E2 has an increased reactivity when compared to 2-OH-E2, contributing to DNA adducts formation and ultimately leading to carcinogenesis [8, 33, 49, 50].

These estrogen metabolites are methylated by catechol-O-methyltransferase (COMT), a phase II enzyme, in 2-methoxyestradiol (2-MeOE2) and in 4-methoxyestradiol (4-MeOE2), from 2-OH-E2 and 4-OH-E2, respectively. Methyl group addition to the catechol estrogens prevents the electron oxidation into semiquinones and quinones.

S-adenosylmethionine (SAM) is the methyl donor and results from remethylation of homocysteine to methionine by 5,10-methylenetetrahydrofolate reductase (MTHFR). The folate metabolism is then an indirect intervenient in estrogens metabolism, contributing to catechol estrogens inactivation by COMT.

COMT and MTHFR contribute to the equilibrium of estrogens, nevertheless if there is an imbalance due to oversynthesis of estrogens or due to low activity of MTHFR and COMT, due, for example, to SNPs, semiquinones and quinones levels increase, needing detoxification [8].

Glutathione S-transferases (GSTs) are a superfamily of Phase II enzymes and consist in three members: cytosolic GSTs, mitochondrial GSTs, and membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) [51]. The cytosolic GSTs are predominant in mammals, are widespread in human tissues and are classified in the structure and amino acid sequences, constituting seven classes of cytosolic GSTs: the class alpha (α), mu (μ), pi (π), theta (θ), omega (ω), sigma (σ) and zeta (ζ) [52, 53]. The detoxifying enzymes catalyse the conjugation of glutathione (GSH) with electrophilic compounds like xenobiotics and endogenous substances leading to its detoxification [53]. These phase II enzymes conjugate estrogen quinones with GSH making them easier to excrete due to a solubility increase [53]. The role developed by GSTs is a key factor for hormone-breast cancer prevention, once low levels of quinones are related to a lower carcinogenic effect of estrogens [53]. However, SNPs in the genes that codify these enzymes, are related with alterations in the risk of developing hormone-dependent breast cancer [54, 55].

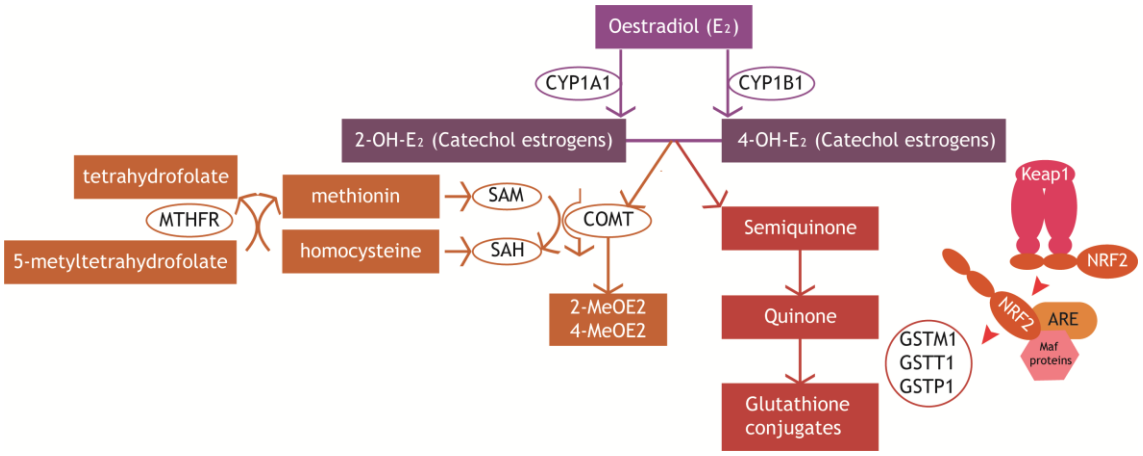

Figure 7. Metabolic pathway of estrogens. The metabolization of estrogens occurs in two phases. Firstly, is catalysed by phase I enzymes, namely CYP1A1 and CYP1B1. Cathecol estrogens formed in phase I can be methylated, or conjugated with glutathione, in order to be easily excreted.

The expression of these Phase II enzymes is regulated by the nuclear factor erythroid 2-related factor 2 - kelch-like ECH-associated protein 1 (NRF2-KEAP1) complex [56].

NRF2 is a transcription factor, belongs to the cap'n'collar (CNC)–basic region-leucine zipper (bZIP) family and is the master regulator of the cellular metabolism [57]. Under basal circumstances NRF2 resides in the cytoplasm linked to Keap1, forming the NRF2-KEAP1 complex (Figure 8) [56]. NRF2 has seven conserved regions NRF2-ECH (Neh) domains, among which the Neh2 region that comprises two motifs: DLG (low affinity binding) and ETGE (high affinity binding), responsible for binding to KEAP1 [58]. Both motifs of NRF2 binds to DC domain of KEAP1 [59].

When the oxidative stress levels increase KEAP1 frees NRF2 that enters in the nucleus [59]. The master regulator binds to DNA, to antioxidant response elements (ARE) and to small masculoaponeurotic fibrosarcoma (sMaf) proteins inducing the expression of Phase II enzymes, like Glutathione S-Transferase Mu 1 (GSTM1), Glutathione S-Transferase Theta 1 (GSTT1) and Glutathione S-Transferase Pi 1 (GSTP1) [60]. When basal state is achieved, KEAP1 translocates to the nucleus, binds NRF2 and takes it back to the cytoplasm to suffer proteasome degradation [59].

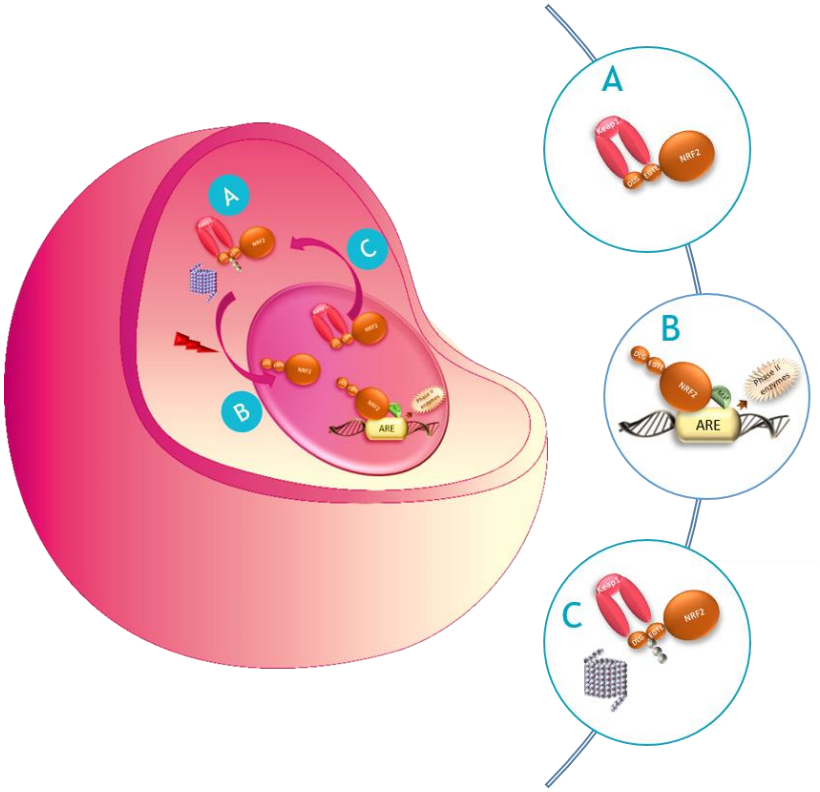

Figure 8. NRF2-KEAP1 pathway. A – NRF2 resides in the cytoplasm and forms a complex with KEAP1. **B** – In the presence of oxidative stress, KEAP1 frees NRF2 that, in the nucleus induces the expression of cytoprotective genes. **C** - When basal state is achieved KEAP1 binds NRF2 and take it back to the cytoplasm.

Thus, the complex NRF2-KEAP1 is the major regulator of oxidative stress, once NRF2 acts in order to promote a basal state and KEAP1 acts as a sensor of NRF2 actions [59].

The *modus operandi* of the complex protects the cell; however, the behaviour of the complex is the same in cancer cells. On this regard, it can be consider a "dark side" of NRF2-KEAP1 complex [61]. Malignant cells will be protected from oxidative stress promoted by treatment, leading to cancer progression. Moreover, SNPs in the complex may lead to an increased cytoprotection leading to progression of cancer cells [62]. Moreover, NRF2-KEAP1 complex is more active in estrogen receptor positive breast cancers [63, 64].

1.5. Estrogen Receptors

Part of a book chapter in Appendix and published as:

Almeida M, Soares M, Fonseca-Moutinho J, Ramalhinho AC, Breitenfel L Chapter 1. The Role of Estrogen Receptors Alpha in Breast Carcinogenesis: An Overview. *in* Estrogen Receptors: Structure, Functions and Clinical Aspects. Nova Publishers. 2020; ISBN: 978-1-53618-228-6.

Estrogens are steroidal hormones that regulate several physiological processes in normal cells including maintenance of reproductive organs, regulation of cardiovascular system, homeostasis of central nervous system, maintenance of bone density and cholesterol levels [65, 66]. Estrogens also play an important role in several pathological processes like cancer, metabolic diseases and osteoporosis [65-67]. The endogenous estrogens are estrone (E1), estradiol (E2) and estriol (E3), being E2 the more predominant estrogen in non-pregnant and pre-menopausal women [68, 69].

The biological effects of E2 are mostly mediated by its binding and activation to estrogen receptors [66-69]. There are two different classes of estrogen receptors: nuclear estrogen receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and membrane estrogen receptors, the G-protein coupled estrogen receptor (GPER also known as GPR30) [70-72].

The distribution of steroid receptors varies among tissues being ERα mainly expressed in uterus, ovarian theca cells, breast (being the most expressed ER in breast epithelium), prostate stroma, Leydig cells in testis, epididymis, pituitary, kidney, bone, white adipose tissue and liver [67, 69, 72, 73]. ERβ is mainly expressed in ovarian granulose cells, central nervous system, cardiovascular system, lung, male reproductive organs, prostate epithelium, colon, kidney, bone marrow and immune system [67, 69, 72, 73].

1.5.1. Estrogen Receptors Structure

The estrogen receptors ERα and ERβ are encoded by two distinct genes. The ESR1 gene located at 6q25.1 was cloned for the first time by Walter et al. (1985) and the cDNA was first sequenced by Green et al. (1986) [74, 75]. ESR1 gene contains eight exons that encode ERα, a 66kDa protein, composed by 595 amino-acids [74-76]. The ESR2 gene

located at 14q23.2 was first identified by Mosselman et al. 1996 and cDNA was first cloned by Kuiper et al. 1996, ESR2 contains eight exons that encode ERβ, a 60kDa protein, composed by 530 amino-acids [66, 77, 78].

The ERs are composed by six structural domains (Figure 9) and they share structural characteristics, will focus on describing ERα structure.

The A/B region, encoded by exon 1, shares 17% of homology between ERs, the ligand-independent activation function-1 domain (AF1) part of the NH2-terminal domain (NTD) is involved in the transcriptional activation of target genes and is an essential domain of interactions with co-regulators [66, 67, 73].

The DNA binding-domain (DBD) in the C region is encoded by exons 2, 3 and a part of exon 4, is highly conserved between ERs (97%) and is essential for the specific binding to DNA sequences of target genes, regulating their expression [66, 67, 73].

The D domain, flexible hinge finger, shares 36% of homology, is encoded by exon 4 and links the C domain to E domain, contains the nuclear localization signal (NLS), resulting in the activation of ER signalling in cells, aiding in the translocation of ERα to the nucleus [66, 73, 79].

The exons 4-8 encode for E and F domains, the domain E and F domains shares 56% and 18% of homology, respectively [66]. These domains also called ligand-binding domain (LBD) is a globular region that contains a hormone-binding-se, a dimerization interface and a ligand-dependent activation function-2 (AF-2), that has conformational alterations depending on the presence of different ligands [66, 73].

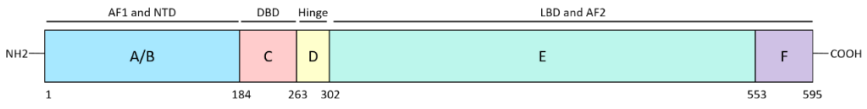

Figure 9. ERα structure. The ERα is a 66kDa protein, with 595 amino-acids, composed by six structural domains. A/B region is involved in the transcriptional activation of target genes, through the ligand-independent activation function-1 domain (AF1) part of the NH2-terminal domain (NTD). The C and D regions have the DNA binding domain and nuclear localization signal, respectively. The E and F regions have the ligand binding domains (LBD) and ligand-dependent activation function-2 (AF-2).

1.5.2. ERα Signalling

Estrogen plays an important role in cell proliferation and differentiation, mainly by binding to its receptors, among them ERα, leading to their activation [80, 81].

In breast cancer, ERα mediates E2 action in the nucleus by two distinct pathways: the genomic and non-genomic pathways (Figure 10) [66, 73, 82, 83]. The genomic pathway can be divided into estrogen and estrogen response elements (ERE) dependent pathway ("classical" genomic pathway), and estrogen-dependent/ERE-independent pathway ("nonclassical" genomic pathway) and estrogen-independent/ERE-dependent pathway [82].

In the "classical" pathway, E2 binds to ERs, through LBD in the cytoplasm, inducing a conformational change in ERα, enabling ERα dimerization and translocation to the nucleus [73, 83, 84]. The complex ERα-E2, through the DBD of ERα binds to estrogen response elements (ERE), activated ERα triggers the recruitment of co-regulators, through AF-1 and AF-2 domains, that modulates the transcriptional activity [7, 73, 80, 82-86]. Alternatively, activated ERα, by E2, can recruit and bind, through protein-protein interactions, to transcription factors (TF) such as SP1, nuclear factor-κB (NF-κB) and AP1, at their respective response element (RE), stimulating the transcription of growth and survival genes [70, 80, 82, 83, 85, 86].

There is also a third pathway, E2-independent/ERE-dependent ERα, a crosstalk between ERα and growth factors induces the activation of kinases, like mitogen-activated protein kinase (MAPK) pathway [82, 84, 86, 87]. Phosphorylated ERα translocates to the nucleus and initiates its transcriptional activity, binding to EREs and recruiting co-regulators [83, 84, 86].

The non-genomic pathway, is not well understood as the genomic pathway, but is largely studied for ERα [73, 86]. In this signalling pathway, E2 acting at the membrane, stimulates the ERα located in the cytoplasm, after that, it initiates a signalling cascade, leading to rapid physiological responses [73, 83, 86, 88]. The ERα-E2 complex activates kinases, such as Src kinase and phosphatidylinositol 3-kinase (PI3K) pathway, activating the signalling cascade of MAPK and protein kinase B (AKT), these pathways enable the activation of other transcription factors, and the facilitators of cell growth and proliferation [7, 80, 82-84, 86, 89].

These genomic and non-genomic mechanisms of ERα signalling are presented separately, however the signalling pathways are strongly related, enforcing the central role of ERα in cell development and differentiation [84, 86].

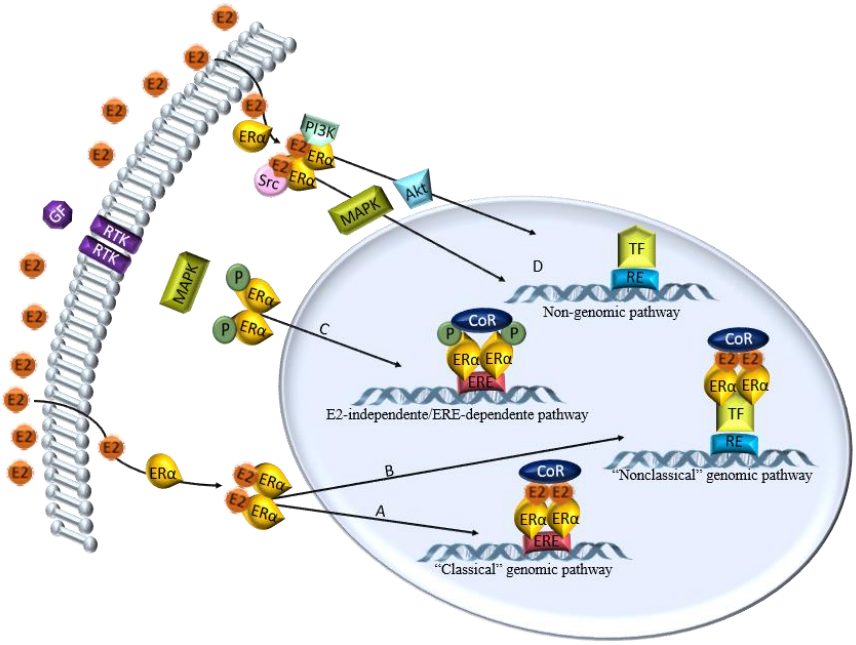

Figure 10. ERα signalling pathways. A) is the "classical" genomic pathway, E2 binds to ERs, the complex binds to ERE and co-regulators are recruited. B) "nonclassical" genomic pathway, E2 binds to ERs, through protein-protein interactions the complex binds to transcription factors (TF) at their respective response element (RE). C) estrogen-independent/ERE-dependent pathway, a crosstalk between ERα and growth factors induces the activation of kinases, phosphorylated ERα binds to EREs and recruiting co-regulators. D) non-genomic pathway, ERα-E2 complex activates kinases, through MAPK and AKT signalling cascades, transcription factors and their regulators enable the activation of other transcription factors.

Chapter 2

Genetics Underlying Hormone-dependent Breast Cancer

Some of the contents of this chapter were originally published in:

***Chapter 1*. The Role of Estrogen Receptors Alpha in Breast Carcinogenesis: An Overview. *in* Estrogen Receptors: Structure, Functions and Clinical Aspects.**

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The genetic landscape of breast cancer is complex, with several genes contributing to breast cancer risk and outcome. Paul Pierre Broca, in 1866, was the first to relate genetic predisposition with breast cancer development [90]. Although, hereditary factors are related only to 5-10% of breast cancers [12]. Carriers of mutations in high penetrance genes, like *BRCA1* and *BRCA2*, have an approximately 70% risk, by the age of 70 years, of developing breast cancer [91, 92].

BRCA1 and *BRCA2* genes, mutations and correlations with an increased risk of breast cancer development were identified during the 90's decade of the 20th century [91, 93-97]. *BRCA1* gene locates on chromosome 17q21 and *BRCA2* on 13q12-13 chromosome [91, 92], codify BRCA1 and BRCA2 protein, respectively, which are involved in DNA damage repair and transcriptional regulation [98].

Mutations in *BRCA1* and *BRCA2* genes can lead to proteins loss of function, taking to genomic instability, compromising DNA repair, leading to a higher likelihood of DNA damage and ultimately increasing breast cancer risk [99, 100]. The pathogenic variants of BRCA1 and BRCA2 are considered the major genetic risk factors for breast cancer and current guidelines for genetic testing comprise their genotyping [23, 101]. Nevertheless, only a small percentage of breast cancer is due to high penetrance genes polymorphisms; other polymorphisms in moderate or low penetrating genes have been studied in respect to breast cancer risk and prognosis [102]. One of the approaches, due to breast cancer heterogeneity, is the identification and the weight of low penetrance genes polymorphisms in each molecular subtype of breast cancer.

The most common molecular subtype of breast cancer is the ER^+ (hormone-dependent breast cancer). Many of the enzymes involved in estrogen synthesis and metabolism have a polymorphic distribution in the human population and many SNPs have been studied in the last decades [54, 55, 72, 103].

2.1. Single Nucleotide Polymorphisms in Estrogens Pathway

The reproductive life of a woman is characterized by the production of high levels of estrogens, mainly by the ovaries. After, during climacteric and later in the menopause, estrogens mostly result from peripheral enzymatic conversion of cholesterol in estrogen in the liver, kidney, brain, adrenal, and peripheral adipose tissue [104].

In this regard, SNPs in genes responsible for encoding enzymes related to estrogens biosynthetic and metabolic pathways (Figure 1) may alter estrogens synthesis and detoxification with possible relation with breast cancer risk and clinical outcome.

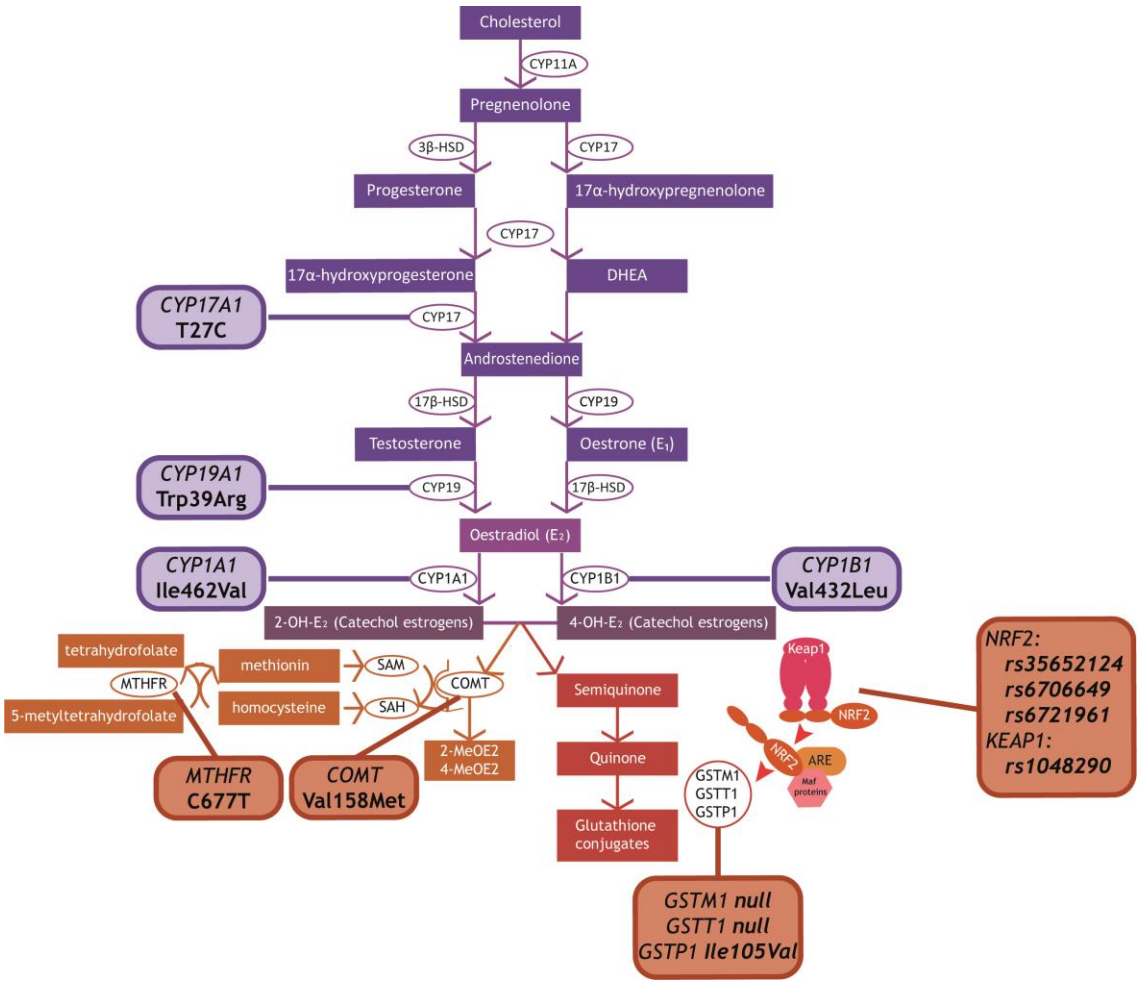

Figure 1. SNPs in biosynthetic and metabolic estrogen pathways.

2.1.1. *CYP17A1*

CYP17A1 gene located on chromosome 10q24.32 codifies CYP17A1 enzyme. CYP17A1 plays an important role in steroids synthesis through catalysis, giving rise to androstenedione (testosterone and oestrone precursor), as previously referred [45].

C*YP17A1* gene is expressed in several tissues but mainly in gonads and adrenals [105]. Genetic alterations in *CYP17A1* can have an important impact in estrogens biosynthesis. *CYP17A1* rs743572 polymorphism (also known as T27C or A1/A2) is a T→C substitution [45, 106]. The C allele was correlated with increased *CYP17A1* transcription, leading to an increased activity of CYP17A1 protein [107]. The C allele was also associated with

increased progesterone transcription and estradiol in premenopausal women [108]. The C (A2) variant has been related with elevated risk of polycystic ovary syndrome and with ovarian cancer risk [109, 110]. On what concerns to breast cancer, it was associated with increased breast cancer risk in postmenopausal women [108]. The genotype profile in breast cancer might be of main importance once increased activity of CYP17A1, in C allele carriers, leads to increased estrogens levels. Once estrogens are considered carcinogens and risk factors for breast cancer, a higher activity of the estrogens biosynthetic pathway enzymes might lead to estrogens accumulation during lifetime.

2.1.2. *CYP19A1*

CYP19A1 gene is located at 15q21.1 and encodes CYP19A1 (aromatase), that catalyses the conversion of androgens to estrogens. CYP19A1 is highly expressed in adipose tissue, being the enzymatic aromatization a large source of estrogens [111].

One of the studied polymorphisms in *CYP19A1* in breast cancer is rs2236722 (39 Trp/Arg or T/C) [112]. Previously, our research team correlated this polymorphism with breast cancer risk, verifying that carriers of the C allele have higher risk for breast cancer development [55]. However, the literature shows conflicting results when associating the genotypes of this polymorphism and breast cancer risk [113]. There is also no consensus in the literature as to the effect of this polymorphism on the enzymatic activity of CYP19A1. These conflicting results might be due to the fact that this polymorphism is rare in some populations [113, 114]

2.1.3. *CYP1A1*

CYP1A1 gene is located at 15q24.1 and encodes CYP1A1, a phase I enzyme expressed in breast tissue [115]. CYP1A1 is responsible for catalysing the 2-hydroxylation of estradiol in 2-OH-E2 [115].

rs1048943 polymorphism (Ile462Val) in exon 7 (T→C substitution), has been correlated with increased catalytic activity of CYP1A1 [116]. Carriers of the C allele present a higher risk to develop breast cancer due to increased levels of the catechol estrogen 2-OH-E2 [117]. The Ile462Val polymorphism was also found to increase the risk of other cancers such as lung cancer, leukemia, esophageal carcinoma and prostate cancer [117].

2.1.4. *CYP1B1*

CYP1B1 gene codifies cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) and is located on chromosome 2p21–p22 [118]. The phase I enzyme, CYP1B1, plays a significant role in estrogens hydroxylation, converting estradiol in 4-hydroxyestradiol (4-OH-E2) [119]. The 4-OH-E2 reacts with quinones and forms a catechol estrogen, 17β-estradiol-3,4-quinone (E2-3,4,Q), that can form adducts and damage DNA. CYP1B1 was described as being highly expressed in breast tumours with a possible association to tumorigenesis [120].

A highly studied polymorphism in *CYP1B1* is the Val432Leu or rs1056836, located in exon 3 [121-123]. This polymorphism leads to the substitution of leucine to valine and the Val432 variant has been associated with an increased enzymatic activity of CYP1B1 [119].

The association of this polymorphism with breast cancer risk is still unclear. Three meta-analysis found no association between carriers of the altered allele and breast cancer diagnosis [121-123]. However, the polymorphic variants were found to have a three-fold increase in the catalytic activity of CYP1B1 when compared to the wild-type allele [119]. This may lead to catechol estrogen accumulation, and through lifetime, can eventually have influence in breast cancer risk and prognosis.

2.1.5. *MTHFR*

Estrogens detoxification is also dependent of the folate metabolism pathway. MTHFR plays a role in the conjugation and inactivation of catechol estrogens by COMT [124, 125]. MTHFR is a key enzyme in folate metabolism, catalysing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate[126]. This reaction is essential for the re-methylation of homocysteine to methionine, a precursor of SAM. SAM is the methyl donor of the reactions catalysed by COMT, in order to inactivate the cathecol estrogens formed by phase I enzymes, such as CYP1B1 [127]. Therefore, alterations in MTHFR activity can affect the inactivation of catechol estrogens *via* COMT.

The *MTHFR* gene is located on chromosome 1p36.3 and codifies MTHFR protein [128]. A well-studied polymorphism in *MTHFR* is the rs1801133, commonly referred as C677T, which results in an alanine to valine substitution in MTHFR [129]. This substitution has been correlated with a lower activity of MTHFR, leading to a poor detoxification of catechol estrogens, once re-methylation of SAM is compromised,

leading to a deficient supply of the methyl group to COMT, and ultimately compromising the catechol estrogens detoxification [129].

Several researches have associated C677T polymorphism with breast cancer risk. Case-control studies and meta-analysis associated TT genotype with increased risk of breast cancer development [130-132]. Also, breast cancer patients carriers of the T allele had tumours with worse clinicopathological factors, such as higher tumours, lymph node metastasis, advanced stage and molecular subtypes with lower prognosis [133].

2.1.6. *COMT*

COMT is localised at 22q11.21 and encodes two proteins: the soluble COMT (S-COMT) and the membrane-bound COMT (MB-COMT), isoforms with similar amino acids sequences, except in the N-terminal [134]. These phase II enzyme isoforms receive a methyl group from SAM, that will allow the catalysis by COMT leading to the inactivation of catechol estrogens [135]. Both S-COMT and MB-COMT are expressed in breast tissue and the rs4680, or Val158Met, or Val108Met polymorphisms, for MB-COMT and S-COMT, respectively, have been related with breast cancer risk [136]. It was found that the mutated allele leads to COMT lower activity [136]. Catechol estrogens derived from CYP1A1 and CYP1B1 activity, 2-OH-E2 and 4-OH-E2, respectively, suffer a lower conversion into 2-MeOE2 and 4-MeOE2. This deficient detoxification leads to DNA adducts formation and DNA damage, highly contributing to breast cancer risk [137].

2.1.7. *GSTs*

GSTs are a superfamily of phase II enzymes. GSTs play a key role in cytoprotection through the conjugation reaction of reduced glutathione forming GSH conjugates, allowing the detoxification of toxic compounds such as catechol estrogens [53].

2.1.7.1. *GSTM1* and *GSTT1*

GSTM1 gene, responsible for GSTM1 protein codification is located on chromosome 1p13.3 and the *GSTT1* gene, responsible for GSTT1 protein codification, is located on chromosome 22q11.23 [138, 139]. Two main polymorphisms have being studied in these genes, the *null* polymorphisms of *GSTM1* and *GSTT1 – deletion of the entire genes* -, once they imply the total absence of the enzymes, compromising detoxification and cytoprotection [140].

The homozygous deletion of GSTM1 and/or GSTT1 *null* polymorphism of GSTM1 has been related with several diseases such as coronary heart disease, higher COVID-19 mortality, female infertility, risk of several cancers such: lung cancer, colorectal cancer and breast cancer [54, 55, 141-146].

However, the studies reflect the result of double allele deletion *GSTM1**0/0 and *GSTT1**0/0, not distinguish the *GSTM1**1/1 and *GSTT1**1/1 from *GSTM1**1/0 and *GSTT1**1/0, respectively. A possible approach will be further performed in the present thesis.

2.1.7.2. GSTP1

GSTP1 gene localised at chromosome 11q13.2 codifies GSTP1 protein. Similar to the previously referred GSTs, the phase II enzyme GSTP1 catalyses the conjugation of GSH with endogenous and exogenous electrophilic compounds, increasing their solubility and excretion [53].

rs1695 (also known as Ile105Val), A→G substitution, leads to a lower activity pf GSTP1 [147]. Ile105Val has been correlated, in a meta-analysis by Miao et al. 2020, with increased risk of breast cancer, alone or in association with *GSTM1* and *GSTT1 null* polymorphisms [148]. Thus, carriers of the Val allele have a compromised estrogens detoxification leading to quinones accumulation and possible to carcinogenesis events [53].

2.1.8. *KEAP1*

NRF2-KEAP1 complex is considered the master regulator of oxidative stress. KEAP1 is a cytoplasmatic protein that under basal circumstances binds to NRF2, in the cytoplasm, and targets NRF2 for ubiquitination and proteasomal degradation [57, 149]. KEAP1 protein is encoded by the *KEAP1* gene located at chromosome 19p13.2 and consists in six exons [56]. *KEAP1* protein has a full-length of 624 amino acids (aa) [56].

KEAP1 is rich in cysteine residues. It is constituted by the N-terminal region (NTR) (1-60 aa), a broad complex-tramtrack-bric-a-brac (BTB) (61-179 aa), a intervening region (IVR) (180-314 aa), a double glycine repeat (DGR) (315-498 aa) and a C-terminal region (499-624 aa) (Figure 2) [150].

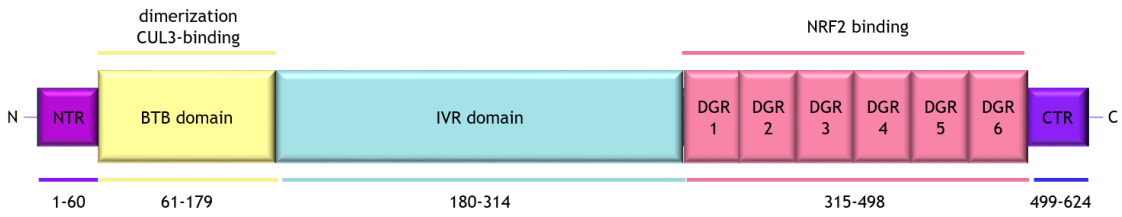

Figure 2. KEAP1 domain structures.

The BTB domain is responsible for interaction with cullin 3 (CUL3). In basal circumstances, KEAP1 binds NR2 and through the KEAP1-CUL3 complex promotes ubiquitination and proteasomal degradation of NRF2 [151]. The DGR domain, constituted by six Kealch motifs, is responsible for NRF2 binding leading to its degradation. In the presence of oxidative stress, KEAP1 cysteine modifications arises leading to NRF2 degradations' decrease, which allows the translocation of NRF2 to the nucleus [58].

Among the several polymorphisms identified in *KEAP1*, three of them have been related with breast cancer: rs34197572 has been associated with breast cancer risk and with poor overall survival, rs9676881 and rs1048290 with higher KEAP1 expression and shorter relapse free survival [152].

The rs1048290 polymorphism has previously been associated with several diseases such as the risk of chronic obstructive pulmonary disease, recurrence free survival in breast cancer patients and progression free-survival in endometrioid endometrial adenocarcinoma [152-154].

Polymorphism rs1048290, located in the fourth exon, codifies the DGR domain of the protein. This polymorphism is related with a higher expression of KEAP1; the increase availability of NRF2 negative's regulator leads to a compromised detoxification due to lower availability of NRF2 in the nucleus. In addition, the DGR domain is responsible for the molecular interaction between Keap1 and NRF2 [155]. The rs1048290 polymorphism was related with higher cytosolic levels of NRF2, probably due to a higher affinity in DGR domain to bind NRF2 [156]. Thus, lower levels of NRF2 in the nucleus lead to lower expression of phase II enzymes, which in turn highly compromise the response to aggressions, both internal and external.

2.1.9. *NRF2*

NRF2 is a transcription factor with 605 amino acids length, encoded by *NRF2* or *NFE2L2* gene located at locus 2q31.2 of chromosome 2, constituted by five exons and 4 introns [157]. NRF2 has seven Neh [58]. Neh1 contains the domains to attach sMaf proteins, promoting the binding of NRF2 to ARE. Neh2 domain is in the N-terminal region and possesses the motifs DLG and ETGE that binds to KEAP1 (Figure 3) [58]. Neh3/Neh4/Neh5 act as transactivation domains [158]. Neh6 contributes to NRF2 degradation under stress conditions, when NRF2 is in the nucleus [159]. Neh7 is responsible for DNA binding domain [160].

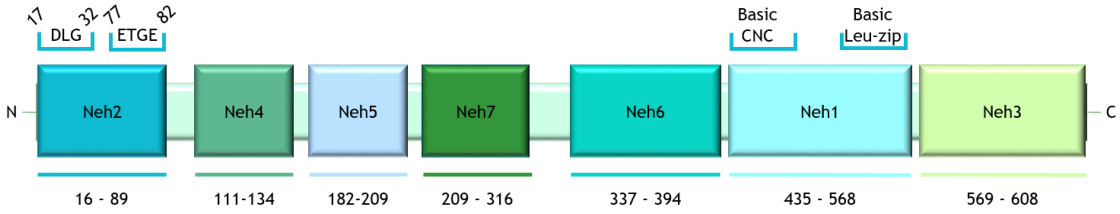

Figure 3. NRF2 domain structures

Among all the *NRF2* polymorphisms identified and studied, three main polymorphisms (rs35652124, rs6706649 and rs6721961) in the promoter region have been correlated with several clinical implication.

The polymorphisms rs35652124 (A→G), rs6706649 (G→A), rs6721961 (C→A) seem to be related with low transcriptional activity of *NRF2* leading to low available levels of NRF2 [161, 162]. Less available levels of NRF2 highly compromise the induction of phase II enzymes, resulting with several physiopathological consequences.

This polymorphisms have been associated with several pathologies: rs35652124 and rs6721961 were related with cardiovascular mortality in haemodialysis patients [163] and rs35652124 was associated with Parkinson's disease, increased risk of vitiligo and as a risk factor for chronic kidney disease [164-166]. The A allele of rs6721961 was correlated with lung cancer incidence, Alzheimer's disease and mild cognitive impairment, risk of venous thromboembolism in users of oral estrogens [167-169]. rs6706649 was related with susceptibility of chronic obstructive pulmonary disease and associated with gastric inflammation [170, 171].

In what concerns to breast cancer, few studies have been performed. rs6721961 was associated with increased risk of breast cancer and the AA (homozygous mutant) genotype of rs6721961 was correlated with breast cancer incidence in pre-menopausal women [172, 173].

2.1.10. *ESR1* mutations

Part of a book chapter in Appendix and published as:

Almeida M, Soares M, Fonseca-Moutinho J, Ramalhinho AC, Breitenfel L Chapter 1. The Role of Estrogen Receptors Alpha in Breast Carcinogenesis: An Overview. *in* Estrogen Receptors: Structure, Functions and Clinical Aspects. Nova Publishers. 2020; ISBN: 978-1-53618-228-6.

The risk for the development of breast cancer is long been associated to lifetime exposure to E2 or estrogenic compounds, mainly through E2/ERα dysregulation in the genomic and non-genomic pathways, that might lead to breast carcinogenesis [7, 84, 86, 174-178]. The genetic component should not be discarded once mutations in ERα gene (*ESR1*) may alter the protein expression and function.

Several mutations in *ESR1* have been described, among them, single nucleotide polymorphisms (SNPs). The most studied SNPs in *ESR1* are rs2234693 (PvuII), rs9340799 (XbaI) and rs2228480 (T594T) (Table 1) [174, 179, 180].

Other SNPs studied in *ESR1* are rs1062577, rs2046210, rs2077647, rs2273206, rs2881766, rs926778 and rs9479118 (Table 1) [179, 181, 182].

On what concerns to the SNPs rs2234693 (PvuII), rs9340799 (XbaI)), both polymorphisms are located at intron 1 and are thought to be functional polymorphisms [174, 179, 180, 183]. The possible mechanism of action is a change in *ESR1* transcription activity, contributing to carcinogenesis [174, 179, 180, 183].

The PvuII [c.453-397T>C] polymorphism has been suggested to increase ERα transcription or produce ERα isoforms [184]. Several studies have been performed in order to understand the correlation of PvuII and breast cancer risk, however there are conflicting results. Gonzalez-Mancha et al. (2008) performed a study on which 1148 samples of Spanish women were genotyped and PvuII T allele (altered allele) was moderately correlated with the increased risk of developing breast [185]. Ramalhinho

et al. (2013) performed a similar study in 228 Portuguese women from Central Eastern Portugal and PvuII genotypes were not associated with breast cancer variation risk, the same research team found similar results during a research performed in 2019 [174]. Carrillo-Moreno et al. (2019) also studied PvuII polymorphism in Mexican population, correlating positively breast cancer risk with PvuII and similar results were found by Wang et al. (2014) [186, 187].

The polymorphism XbaI [c.454-351A>G], was studied by Ladd et al. (2008) in Dutch post-menopausal women and no correlation with breast cancer risk was found, similar results were found by Shen et al. (2006) in a Chinese case-control study [188, 189]. However, Wang et al. (2014) associated XbaI polymorphism with increased risk of breast cancer. In the opposite, Ramalhinho et al. (2013) associated the homozygous mutant genotype with a reduced risk of breast cancer [174, 187].

As previously referred, others found out that polymorphism in *ESR1* is the polymorphism T594T [c.1782G>A], located at exon 8, although the exact functionality is unknown, probably has a role on co-regulators recruitment [179]. The T594T polymorphism was correlated with breast cancer risk in Tunisian, Chinese and Australian women [187, 190, 191].

Other SNPs have been studied in breast cancer, among them, the rs1062577 located in the 3'-UTR of *ESR1* is thought to promote higher ERα expression, leading to higher proliferation rate in breast cancer cells [192, 193]. This polymorphism was studied in Chinese and Iranian population by Chen et al. (2016) and Dehghan et al. (2017), respectively, being correlated with the increased risk of breast cancer in both populations [181, 192]. The rs2046210, located upstream *ESR1* was studied in the Chinese, Vietnamese, Japanese population and European population, all studies correlated rs2046210 with an increased risk of breast cancer [187, 194-196].

Son et al. 2015 also studied SNPs in *ESR1* and their correlation with breast cancer risk, and the research group found a positive correlation between rs2881766, rs2077647, rs926778, and rs2273206, and breast cancer risk in Korean population [182]. The polymorphism rs2881766 was also studied by Chen et al. (2016) and was found positively correlated with breast cancer risk in Chinese population, this research group also found an association between breast cancer risk and the polymorphism rs9479118 [181].

All these results emphasize the need to better understand the risk potentiated by *ESR1* SNPs, the impact on ERα, and consequently in breast cancer and therapeutic outcome.

Along with SNPs, other *ESR1* mutations have been studied, mainly posttranslational modifications (PTM) that affect ER signalling, creating an imbalance between ER degradation and stability, that in turn might have impact in therapeutic resistance and clinical outcome [84, 86, 197, 198].

The most common point mutations in *ESR1* are Y537N, Y537S, D538G and K303R, these mutations are related with constant ER activity [84, 197, 199, 200].

The Y537S mutation was first described by Weis et al. (1996) and has been shown to confer hormone independent activation [86, 201, 202]. The Y537N mutation, first described by Fuqua lab in 1997, has found to promote a constitutive transactivation function to ER [86, 201, 203].

Merenbakh-Lamin et al. (2013) first described the mutation D538G that occurs in LBD and results in higher levels of ERα, which is independent of the ligand [197, 204]. Toy et al. (2017) found Y537S, Y537N and D538G in patient samples with metastatic and endocrine-refractory breast cancer, however these mutations were not found in the primary samples, these mutations probably promote hormone-independent growth and activity, and are acquired after endocrine treatment [197].

On what concerns to K303K mutation, it was first described by Fuqua lab in 1997 [203]. The mutation leads to polyubiquitination of ERα in response to antiestrogens, and to an altered recruitment of co-regulators, which is associated with lymph node metastasis and enlarged tumour size, and consequently lead to poor prognosis [84, 86, 205].

It seems that all these point mutations on ERα affects its functional role and lead to endocrine resistance [86]. Moreover, these mutations are possibly more frequently acquired after treatment, when they were not identified in the corresponding sample of the primary tumour but were present in metastatic ER^+ tumours after treatment [197, 206]. Besides the biochemical and clinical implications of these point mutations, there is another challenge, Ross et al. (2019) refer that ER immunohistochemistry is not predictive of *ESR1* mutation status, in this regard, not only the *ESR1* mutations should be analysed in clinical practice, but therapeutics should also be more personalized regarding the result of the SNPs and point mutations [207].

Table 1. Studies correlating ESR1 polymorphisms and the risk of breast cancer, worldwide.

SNPs	Authors	Year	Population	Relation to Breast Cancer
rs1062577	Chen et al. [181]	2016	Chinese	Increased risk of breast cancer
	Dehghan et al. [192]	2017	Iranian	
rs2046210	Wang et al. [187]	2014	Chinese	Increased risk of breast cancer
	Thanh et al. [194]	2018	Vietnamese	
	Mizoo et al. [195]	2013	Japanese	
	Campa et al. [196]	2011	European	
rs2077647	Son et al. [182]	2015	Korean	Increased risk of breast cancer
rs2234693	Wang et al. [187]	2014	Chinese	Increased risk of breast cancer
(PvuII)	Carrillo-Moreno et al. [186]	2018	Mexican	Increased risk of breast cancer
	Gonzalez-Mancha et al. [185]	2008	Spanish	Moderately correlated with breast cancer risk
	Ramalhinho et al. [174]	2013	Portuguese	No association with breast cancer risk
rs2228480	Wang et al. [187]	2014	Chinese	Increased risk of breast cancer
(T594T)	Kallel et al. [190]	2009	Tunisian	
	Curran et al. [191]	2001	Australian	
rs2273206	Son et al. [182]	2015	Korean	Increased risk of breast cancer
rs2881766	Son et al. [182]	2015	Korean	Increased risk of breast cancer
	Chen et al. [181]	2016	Chinese	Increased risk of breast cancer
	Dai et al. [208]	2019	Chinese	Associated with lymph node metastasis and ER expression and decreased breast cancer risk
rs926778	Son et al. [182]	2015	Korean	Increased risk of breast cancer
rs9340799	Wang et al. [187]	2014	Chinese	Increased risk of breast cancer
(XbaI)	Ladd et al. [188]	2008	Dutch	No correlation with breast cancer risk
	Shen et al. [189]	2006	Chinese	No correlation with breast cancer risk
	Ramalhinho et al. [174]	2013	Portuguese	Reduced risk of breast cancer for xx (homozygous mutant) carriers
rs9479118	Chen et al. [181]	2016	Chinese	Increased risk of breast cancer

Chapter 3

Aims

The aim of this thesis is to validate NRF2-KEAP1 as biomarker for prognosis and therapeutic target for breast cancer.

In order to reach the main aim, we defined specific goals:

1. To analyse the influence of polymorphic low penetrating genes of the estrogen biosynthetic and metabolic pathways.
2. To perform an exhaustive literature review related to the NRF2 influence in breast cancer outcome.
3. To study single nucleotide polymorphisms in NRF2-KEAP1 complex in blood, tumour tissue and benign surrounding tumour tissue.

Chapter 4

Influence of Estrogenic Metabolic Pathway Genes Polymorphisms on Postmenopausal Breast Cancer Risk

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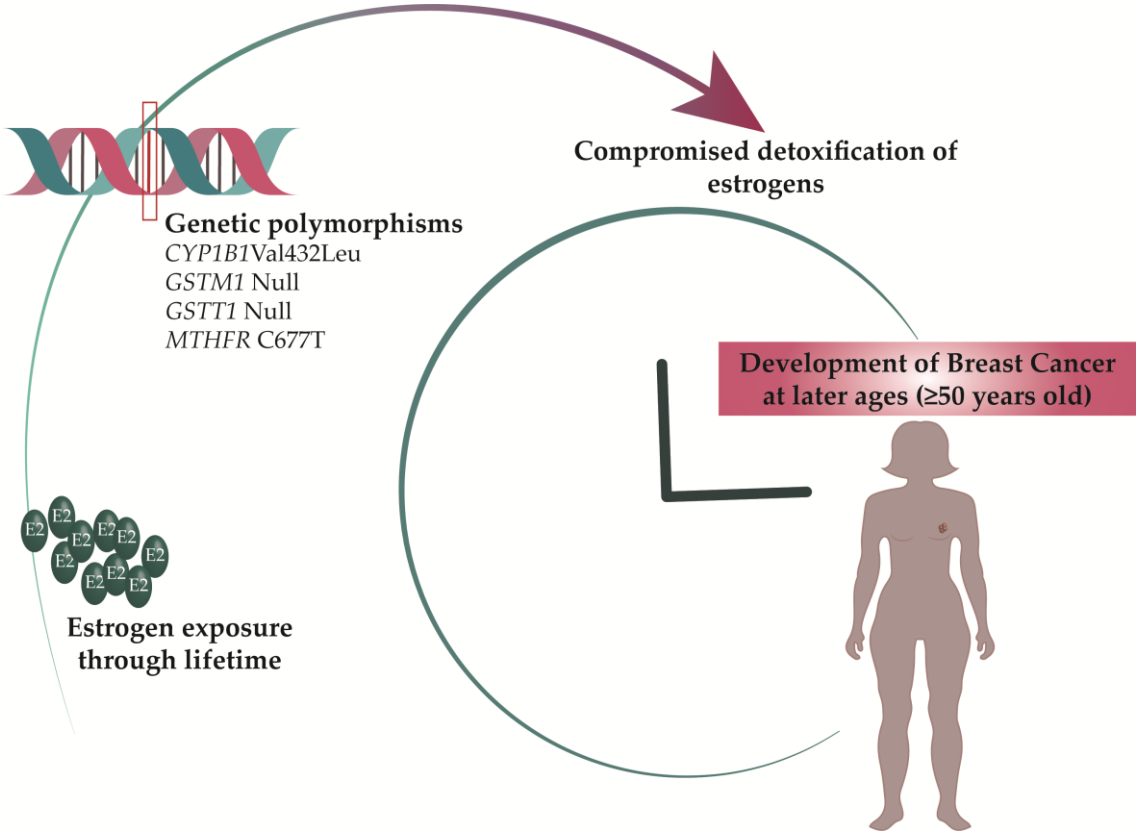
Graphical Abstract



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Article

Influence of Estrogenic Metabolic Pathway Genes Polymorphisms on Postmenopausal Breast Cancer Risk

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Abstract: Estrogen metabolism plays an important role in tumor initiation and development. Lifetime exposure to high estrogens levels and deregulation of enzymes involved in estrogen biosynthetic and metabolic pathway are considered risk factors for breast cancer. The present study aimed to evaluate the impact of mutations acquisition during the lifetime in low penetrance genes that codify enzymes responsible for estrogen detoxification. Genotype analysis of *GSTM1* and *GSTT1* null polymorphisms, *CYP1B1* Val432Leu and *MTHFR* C677T polymorphisms was performed in 157 samples of women with hormone-dependent breast cancer and correlated with the age at diagnosis. The majority of patients with *GSTT1* null genotype and with both *GSTM1* and *GSTT1* null genotypes were 50 years old or more at the diagnosis (p-value = 0.021 and 0.018, respectively). Older women with *GSTM1* null genotype were also carriers of the *CYP1B1*Val allele (p-value = 0.012). As well, *GSTT1* null and *CYP1B1*Val genotypes were correlated with diagnosis at later ages (p-value = 0.022). Similar results were found associating *MTHFR* C677T and *GSTT1* null polymorphism (p-value = 0.034). Our results suggest that estrogen metabolic pathway polymorphisms constitute a factor to be considered simultaneously with models for breast cancer risk assessment.

Keywords: breast cancer; *GSTM1*; *GSTT1*; *CYP1B1*; *MTHFR*

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1. Introduction

Breast cancer is the most common cancer in women, counting 2.1 million cases diagnosed in 2018 [1]. Breast cancer risk has long been associated with reproductive and hormonal history, lifestyle and hereditary [2]. Mainly, these risk factors are related to exposure to high levels of endogenous or exogenous estrogens and to mutations inherited or acquired during lifetime [3].

Estrogens metabolism plays an important role in tumor initiation and development, estrogen being considered a carcinogen [4]. This fact is not only due to the exposure to high levels of estrogen and to estrogen receptor (ER) status, to which estrogen binds to exerts its actions, but also to the possible deregulation of the enzymes involved in the estrogen biosynthetic and metabolic pathway, such as Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1), glutathione S-transferases (GSTs) and 5,10-methylenetetrahydrofolate reductase (MTHFR) [5–8].

CYP1B1, codified by the *CYP1B1* gene, located on chromosome 2p21–p22, is a Phase I enzyme, responsible for generating 4-hydroxyestradiol (4-OH-E2), a catechol estrogen metabolite (Figure 1) [9].

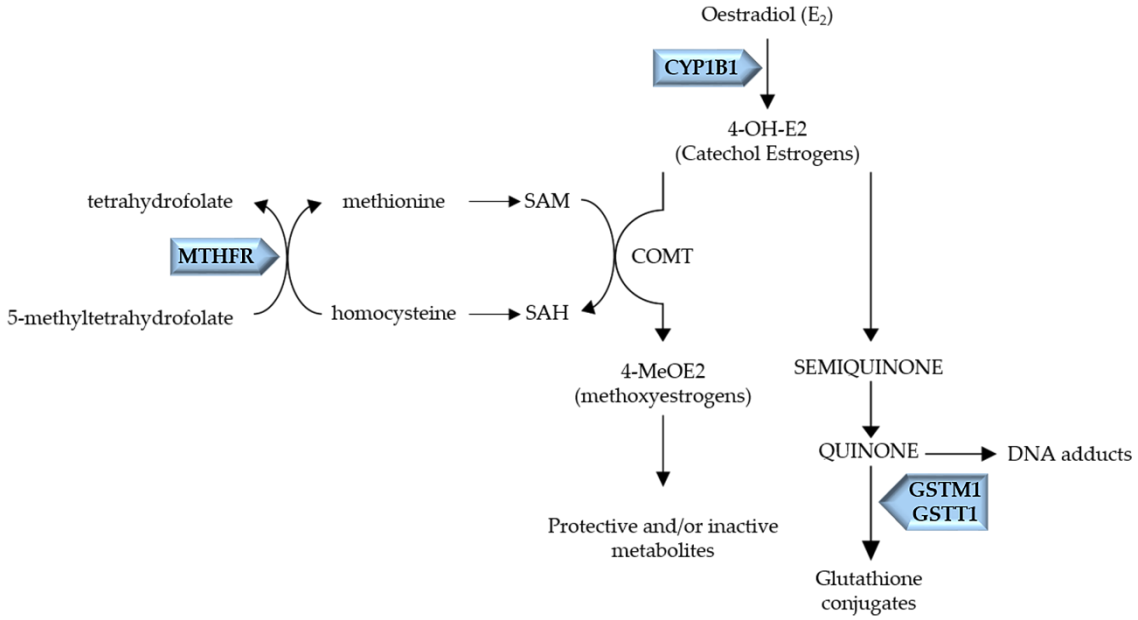

Figure 1. Schematic representation of the metabolic pathway of Oestradiol. CYP1B1, a Phase I enzyme, codified by the *CYP1B1* gene, leads to 4-OH-E2 production. The Phase II enzymes, COMT, codified by the *COMT* gene, and GSTM1/GSTT1, codified by *GSTM1* and *GSTT1* genes, respectively, inactivate the estrogen catechol, semiquinone and quinone, diminishing DNA adducts formation. MTHFR, an enzyme of the folate metabolism, catalyzes 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which allows the remethylation of homocysteine to methionine, a precursor of S-adenosylmethionine (SAM). SAM is the methyl donor for COMT catalyzed reactions, allowing the inactivation of catechol estrogens.

An important polymorphism in *CYP1B1* is Val432Leu polymorphism, in exon 3 (rs1056836; location chromosome 2:38071060) [10]. This polymorphism leads to an amino acid substitution of Leucine to Valine, Val432 allele was found to increase the 4-hydroxilase activity of CYP1B1 [11]. A lack or low level of Phase II detoxifying enzymes, such as catechol-O-methyltransferase (COMT) and GSTs, might lead to quinones accumulation, resulting in DNA adducts formation and tumor initiation [3]. Also, MTHFR is involved in the conjugation and inactivation of catechol estrogens by COMT. MTHFR is the key enzyme of folate metabolism, catalyzing 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which allows the remethylation of homocysteine to methionine, a precursor of S-adenosylmethionine (SAM) [12]. In turn, SAM is the methyl donor for COMT catalyzed reactions, which allow inactivation of catechol estrogens [12]. In this regard, MTHFR activity alterations will affect indirectly the inactivation of catechol estrogens by COMT. MTHFR is codified by the *MTHFR* gene, located on chromosome 1p36.3 [13]. One of the well-studied polymorphisms of *MTHFR* is the polymorphism C677T (rs1801133, location chromosome 1:11796321), which leads to a substitution of alanine with valine, being correlated with lower activity of MTHFR [13,14]. Thus, this polymorphism possibly affects COMT catalyzed reactions, compromising the catalyzation of 4-OH-E2 to 4-methoxyestrogens (4-MeOE2) [12].

The GSTs, a superfamily of Phase II enzymes, are responsible for metabolic detoxification of estrogen, playing a key role in the catalysis of glutathione (GSH) conjugation with catechol estrogen quinones, which are rapidly excreted by the cell [3]. There are seven classes of cytosolic GSTs: alpha (α), mu (μ), kappa (κ), pi (π), theta (θ), omega (Ω) and zeta (ζ) [15]. Among the polymorphisms studied in GSTs, Glutathione S-transferase Theta1 (*GSTT1*) and Glutathione S-transferase Mu1 (*GSTM1*) null polymorphisms, the homozygous genotype of which implies the total absence of the enzyme, has previously been related to breast cancer risk by our research team [16,17]. GSTM1 and GSTT1 enzymes are codified by the *GSTM1* gene located on chromosome 1p13.3 and by the *GSTT1* gene

located on chromosome 22q11.2, respectively [18–20]. The presence of the homozygous null polymorphisms results in the total absence of the enzymes' activity; consequently, in estrogen metabolism, it will compromise the detoxification of catechol estrogens, known to contribute to hormone-induced carcinogenesis through DNA adducts formation [3,19,20].

As previously referred, mutations acquisition during a lifetime, particularly in low penetrance genes, are a risk factor for breast cancer and probably have a higher impact in breast cancer carcinogenesis in older women.

The polymorphism Val432Leu (*CYP1B1*) increases CYP1B1 activity, contributing to higher levels of catechol estrogens that are detoxified by Phase II enzymes. However, C677T polymorphism of *MTHFR* possibly affects 4-OH-E2 catalyzation by COMT; in addition, the total absence of GSTM1 and GSTT1, due to the null polymorphisms, will highly compromise the detoxification of catechol estrogens. Thus, we suppose that mutations acquisition during a lifetime might be related to breast cancer development at later ages.

In this regard, the present study was designed to investigate the impact of mutations acquisition in low penetrance genes during the lifetime in breast cancer development—more specifically, the impact of the null polymorphisms in *GSTM1* and *GSTT1* in breast cancer development at later ages.

2. Results

In the present study, 157 patients diagnosed with breast cancer were included, with a mean age of 63.71 years, as shown in Table 1. The majority of the patients were more than 50 years old (80.25%), and the mean ages for patients less than 50 years old was 43.65 years and 68.65 years for patients 50 years old or more.

Table 1. Age characteristics of the 157 breast cancer patients included in the study.

	Patients *n* (%)	**Mean (SD, Min–Max)**
Total	157	
Age		63.71 (13.777, min 34–max 95)
<50	31 (19.75%)	43.65 (4.270, min 34–max 49)
≥50	126 (80.25%)	68.65 (10.395, min 50–max 95)

Genotypes distribution of *GSTT1* and *GSTM1* according to breast cancer patient's age are summarized in Table 2. The majority of breast cancer patients with the null genotype of *GSTT1* were 50 years old or more, and only 4 of the 47 cases identified with the null genotype were diagnosed with breast cancer before 50 years old (OR 3.497; 95% CI 1.149–10.641; *p*-value = 0.021). Concerning the correlation of *GSTM1* genotypes with the age of the patients, the majority of the cases 50 years old or more presented the null genotype; however, the distribution of present and null genotypes in patients less than 50 years old was similar (OR 1.973; 95% CI 0.892–4.363; *p*-value = 0.090).

Table 2. Association of *GSTT1* and *GSTM1* genotypes with breast cancer patients' age.

Genotype	**Age, *n* (%)**		**OR (95% CI) [a]**	***p*-Value**
	<50	**≥50**		
GSTT1				
Present	27 (17.2)	83 (52.9)		1
Null	4 (2.5)	43 (27.4)	3.497 (1.149–10.641)	0.021 *
GSTM1				
Present	17 (10.8)	48 (30.6)		1
Null	14 (8.9)	78 (49.7)	1.973 (0.892–4.363)	0.090

[a] OR, odds ratio; CI, confidence interval; *, indicates a significant result.

A two-way combination of *GSTT1* and *GSTM1* genotypes with the age of the patients at breast cancer diagnosis was also performed (Table 3). The majority of those with the *GSTT1* null genotype and *GSTM1* present genotype were diagnosed at ages equal to or greater than 50 years old (OR 6.588; 95% CI 0.796–54.558; *p*-value = 0.050), and 29 of the 32 cases with both null genotypes were also identified in patients at later ages (OR 4.549; 95% CI 1.204–17.181; *p*-value = 0.018).

Table 3. Association between *GSTT1* and *GSTM1* genotypes combination and patient age at diagnosis of breast cancer.

GSTT1	*GSTM1*	Age, *n* (%)		OR (95% CI) [a]	*p*-Value
		<50	≥50		
Present	Present	16 (10.2)	34 (21.7)		1
Present	Null	11 (7)	49 (31.2)	2.096 (0.866–5.072)	0.097
Null	Present	1 (0.6)	14 (8.9)	6.588 (0.796–54.558)	0.050 *
Null	Null	3 (1.9)	29 (18.5)	4.549 (1.204–17.181)	0.018 *

[a] OR, odds ratio; CI, confidence interval; *, indicates a significant result.

The null polymorphism of *GSTT1* was also combined with the polymorphism Val432Leu of *CYP1B1*. As represented in Table 4, the combination of the null genotype of *GSTT1* with the altered allele of *CYP1B1* were identified in 39 patients; of those, 35 were 50 years old or more at the diagnosis of breast cancer (OR 4.167; 95% CI 1.159–14.979; *p*-value = 0.022).

Table 4. Association between *GSTT1* and *CYP1B1* Val432Leu genotypes combination and patient age at diagnosis of breast cancer.

GSTT1	*CYP1B1* Val432Leu	Age, *n* (%)		OR (95% CI) [a]	*p*-Value
		<50	≥50		
Present	Leu/Leu (WT)	10 (6.4)	21 (13.4)		1
Present	Leu/Val + Val/Val	17 (10.8)	62 (39.5)	1.737 (0.689–4.378)	0.239
Null	Leu/Leu (WT)	0	8 (5.1)	NA [b]	0.062
Null	Leu/Val + Val/Val	4 (2.5)	35 (22.3)	4.167 (1.159–14.979)	0.022 *

[a] OR, odds ratio; CI, confidence interval; [b] Not applicable; *, indicates a significant result.

The combined analysis of *GSTM1* null polymorphism with Val432Leu of *CYP1B1* was also performed.

In Table 5, there were identified 47 patient carriers of the altered allele of *CYP1B1* with the genotype of *GSTM1* present; of those, 38 were 50 years old or more (OR 3.378; 95% CI 1.038–10.992; *p*-value = 0.038). The number of patients with null genotype of *GSTM1* and homozygous wild type genotype of *CYP1B1* diagnosed at later ages was 19 (OR 7.6; 95% CI 1.350–42.799; *p*-value = 0.013). Concerning patients with the presence of both polymorphisms, *GSTM1* null genotype and carriers of the altered allele of *CYP1B1*, 59 were 50 years old or more at diagnosis and only 12 were younger ages (OR 3.933; 95% CI 1.286–12.029; *p*-value = 0.012).

Table 5. Association between *GSTM1* and *CYP1B1* Val432Leu genotypes combination and patient age at diagnosis of breast cancer.

GSTM1	*CYP1B1* Val432Leu	Age, *n* (%)		OR (95% CI) [a]	*p*-Value
		<50	≥50		
Present	Leu/Leu (WT)	8 (5.1)	10 (6.4)		1
Present	Leu/Val + Val/Val	9 (5.7)	38 (24.2)	3.378 (1.038–10.992)	0.038 *
Null	Leu/Leu (WT)	2 (1.3)	19 (12.1)	7.600 (1.350–42.799)	0.013 *
Null	Leu/Val + Val/Val	12 (7.6)	59 (37.6)	3.933 (1.286–12.029)	0.012 *

[a] OR, odds ratio; CI, confidence interval; *, indicates a significant result.

Additionally, the combination of *GSTT1* with the polymorphism C677T of *MTHFR* was performed, and results are summarized in Table 6. Among the 32 patients with both null genotype of *GSTT1* and altered allele of *MTHFR*, only 2 patients were diagnosed with breast cancer before 50 years old (OR 5; 95% CI 1.009–24.773; *p*-value = 0.034).

Table 6. Association between *GSTT1* and *MTHFR* C677T genotypes combination and patient age at diagnosis of breast cancer.

GSTT1	*MTHFR* C677T	Age, *n* (%)		OR (95% CI) [a]	*p*-Value
		<50	≥50		
Present	CC (WT)	10 (6.4)	30 (19.1)		1
Present	CT+TT	17 (10.8)	53 (33.8)	1.039 (0.422–2.557)	0.933
Null	CC (WT)	2 (1.3)	13 (8.3)	2.167 (0.415–11.302)	0.351
Null	CT+TT	2 (1.3)	30 (19.1)	5 (1.009–24.773)	0.034 *

[a] OR, odds ratio; CI, confidence interval; *, indicates a significant result.

All these results indicate that the cumulative presence of polymorphisms related to catechol estrogens metabolism might be related with breast cancer diagnosis at later ages.

3. Discussion

Breast cancer has long been associated with estrogens exposure, lifestyle and genetic conditions [3,4,8]. Besides the inherited genetic alterations, acquired polymorphisms and genomic instability during life might predispose women to breast cancer [21]. High levels of estrogen have been associated with breast cancer risk, contributing to cellular proliferation of mutated cells and eventually increasing the opportunity for new mutations, leading to tumor progression [22]. In this regard, in breast cancer the estrogen metabolic pathway is of main importance due to estrogens detoxification [23]. Lifetime estrogen exposure and alterations in the enzymes involved in estrogens detoxification might influence cellular hormone-dependent growth [23,24]. Thus, due to mutations in genes that codify enzymes of the estrogen metabolic pathway, it is pertinent to identify subgroups of individuals more susceptible to the exposure of high levels of estrogens [10]. We performed an evaluation of the age at diagnosis of hormone-dependent breast cancer associated with mutations that might compromise the estrogen metabolic pathway.

Genetic mutations in GSTs, mainly the null genotype of *GSTT1* and *GSTM1* are considered a risk factor for breast cancer [16,17], but little is known about the presence of these polymorphisms and age at the diagnosis of breast cancer.

The age at breast cancer diagnosis was correlated with the null polymorphism of *GSTM1* and *GSTT1*, both alone or in association. As well, age at breast cancer diagnosis was correlated with *GSTM1* and *GSTT1* genotypes, together with other polymorphisms in the estrogen metabolic pathway, namely with *CYP1B1* Val432Leu and *MTHFR* C677T polymorphisms. The genotyping of these polymorphisms was performed in 157 women with histologically confirmed hormone-dependent breast cancer from Hospital Centre of Cova da Beira.

GSTM1 and GSTT1 are phase II enzymes that detoxify catechol estrogen quinones through the conjugation of GSH [3]. The absence of these enzymes, due to the null polymorphism of *GSTM1* and *GSTT1*, compromises the detoxification and allows the accumulation of catechol estrogens, leading to DNA adducts formation [17]. In the present study, we verified that the majority of breast cancer patients with the null genotype of *GSTT1* were 50 years old or more (*p*-value = 0.021), and in a two-way association of *GSTT1* and *GSTM1* genotypes with age, we verified similar results: the majority of the elderly patients had both polymorphisms (*p*-value = 0.018). We suppose that prolonged exposure to estrogen levels combined with an inefficient detoxification due to *GSTM1* and *GSTT1* null genotype are related to breast cancer development at later ages. This fact can be explained by the accumulation of catechol estrogens and DNA adducts formation during a lifetime, which culminate in breast cancer development.

Furthermore, there is the cumulative factor of other polymorphisms in the metabolic pathway of estrogens. It was verified that women over 50 years old with *GSTM1* null polymorphism are also carriers of the Val allele of *CYP1B1* Val432Leu polymorphism (p-value = 0.012), and similar results were found for the presence of *GSTT1* null polymorphism and Val allele of *CYP1B1* (p-value = 0.022). The *CYP1B1* Val432 allele promotes higher activity of CYP1B1, leading to higher levels of 4-OH-E2 and a consequent increase of carcinogenic catechol estrogen quinones [3,10,23]. The absence of *GSTM1* and *GSTT1* compromise the detoxification of these high levels of catechol estrogens, which eventually will contribute to tumor development in later ages.

The polymorphism of *MTHFR* C677T promotes a lower activity of MTHFR and a consequent decrease of detoxification via COMT. A two-way association of *MTHFR* C677T and *GSTT1* null genotype was performed and we verified that the majority of women carriers of both altered T allele of *MTHFR* C677T and *GSTT1* null genotype were 50 years old or more at the age of diagnosis (p-value = 0.034). These results might be explained by the fact that the metabolic pathway is extremely compromised due to inexistent GSTT1 and low COMT activity; low levels of Phase II enzymes highly compromise 4-OH-E2 detoxification and eventually will contribute to tumor development due to inefficient estrogens detoxification during reproductive life.

The four low penetrating genes analyzed in the present study indicate that mutations in enzymes that lead to an inefficient detoxification associated with exposure to endogenous or exogenous estrogens during life, might be a trigger to hormone-dependent breast cancer development at later ages. Once estrogens exert their biological activity by binding to their receptors, the referred inefficient detoxification might predispose women to the development of estrogen receptor positive (ER+) breast cancer.

These results are not only pertinent to understanding the influence polymorphisms in the metabolic pathway of estrogens but are also of main importance when considering hormone replacement therapy (HRT). Women with these genotypes are at higher risk of developing breast cancer; continuing the exposure to estrogens through therapy might increase the risk, and once these polymorphisms lead to inefficient estrogens detoxification, these estrogens might turn biologically active by binding to ER, contributing to the development of hormone-dependent breast cancer. This study indicates that it would be pertinent to evaluate in clinical practice the genotypes of each woman when considering HRT.

Once it was expected that by 2035 the number of new cancer cases would double among the older population (65 years old or more) [25]; it is important to identify the mechanisms that might contribute to this tendency. In the particular case of breast cancer development in older women, this study indicates that SNPs in low penetrance genes might have a profound impact on tumor development. Thus, genetic evaluation will contribute to identifying women at higher risk of breast cancer development at later ages and also women who are candidates for HRT. If preventive measures are taken, in the future, it will eventually be possible to forestall the expected increase of breast cancer cases in older women.

4. Materials and Methods

4.1. Study Population

The study group consisted of a total of 157 women with histologically confirmed hormone-dependent (ER positive) breast cancer diagnosed at Child and Women Health Department, Gynaecologic Oncology Division of Hospital Centre of Cova da Beira, Covilhã, Portugal. Informed consent was obtained from all individual participants included in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã, Portugal.

4.2. DNA Extraction

Blood was collected by venous puncture to EDTA tubes and genomic DNA was isolated using Wizard Genomic DNA purification kit (Promega) according to the instructions of the manufacturer and stored at 4 °C.

4.3. Genotyping

Genotyping of *GSTM1* and *GSTT1* (present or null polymorphism) was performed by multiplex polymerase chain reaction (PCR) with the co-amplification of β-globin gene as positive control, as previously described by our group [16].

Regarding to *CYP1B1* polymorphism and *MTHFR* polymorphism, the genotyping was performed by PCR-restriction fragment length polymorphism (RFLP). For both *CYP1B1* Val432Leu and *MTHFR* C677T polymorphisms, the amplification of the fragments containing the polymorphism in study was carried out in a total volume of 50 µL, and contained 10 pmol of each primer, 1.5 mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of DreamTaq DNA polymerase and 100 ng of genomic DNA, using a MyCycler thermal cycler (Bio-Rad).

4.3.1. CYP1B1

The genotyping of *CYP1B1* Val432Leu was performed with slight changes to the protocol of Zheng et al. [9].

The primers set used for *CYP1B1* Val432Leu genotyping were:
Forward primer: 5′-TCACTTGCTTTTCTCTCTCC-3′
Reverse primer: 5′-AATTTCAGCTTGCCTCTTG-3′.

The reaction mixtures were pre-incubated for 1 min at 94 °C. PCR conditions were 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C for 35 cycles. The final extension was at 72 °C for 7 min. The amplified DNA fragment had 650bp. The PCR product was digested by Eco57I (Fermentas, St. Leon-Rot, Germany) restriction endonuclease for 16 h. Digested fragments were electrophoresed through 3% agarose gels stained with GreenSafe Premium (NZYTech, Lisbon, Portugal).Genotypes were distinguished by the pattern of fragments created by the digestion. Homozygous wild type Val/Val genotype was identified by the non-digested fragment of 650 bp; homozygous Leu/Leu genotype was identified by two digested fragments of 340 bp and 310 bp; heterozygous Val/Leu genotype was defined by presence of all fragments 650 bp, 340 bp and 310 bp.

4.3.2. MTHFR

The genotyping of *MTHFR* C677T was performed with slight changes to the protocol of Reljic et al. [26].

The primers set used for *MTHFR* C677T genotyping were:
Forward: 5′- TGAAGGAGAAGGTGTCTGGGGGA-3′
Reverse: 5′- AGGACGGTGCGGTGAGAGTG-3′.

The PCR conditions were 30 s at 94 °C, 30 s at 61 °C and 1 min at 72 °C, for 30 cycles. The final extension was at 72 °C for 2 min. The amplified DNA fragment had 198 bp. The PCR product was digested by 1 U of HinfI (Fermentas, St. Leon-Rot, Germany) restriction endonuclease for 16 h. Digested fragments were electrophoresed through 3% agarose gels stained with GreenSafe Premium (NZYTech, Lisbon, Portugal). Homozygous wild type CC genotype was identified by the non-digested fragment of 198 bp; homozygous TT genotype was identified by two digested fragments of 175 bp and 23 bp; heterozygous CT genotype was defined by presence of all fragments 198 bp, 175 bp and 23 bp.

4.4. Statistical Analysis

In order to examine the association between genotypes in women with breast cancer, statistical analysis was performed using SPSS, version 23. Chi-squared tests were used, considering a statistical significance when p-value was <0.05.

Author Contributions: Conceptualization, L.B.; Data curation, M.A.; Funding acquisition, L.B.; Investigation, M.A. and M.S.; Methodology, M.A., M.S., J.F.-M., A.C.R. and L.B.; Project administration, L.B.; Resources, A.C.R. and L.B.; Supervision, J.F.-M., A.C.R. and L.B.; Validation, J.F.-M., A.C.R. and L.B.; Writing—original draft, M.A.; Writing—review and editing, M.A., M.S., J.F.-M., A.C.R. and L.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All the procedures were performed in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã, Portugal.

Informed Consent Statement: Informed consent was obtained from all the individual participants included in the study.

Data Availability Statement: The data presented in this study are available within the article or on request from the corresponding author.

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

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Chapter 5

Prognosis of Hormone-dependent Breast Cancer seems to be influenced by *KEAP1*, *NRF2* and *GSTM1* Genetic Polymorphisms

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ORIGINAL ARTICLE

Prognosis of hormone-dependent breast cancer seems to be influenced by *KEAP1*, *NRF2* and *GSTM1* genetic polymorphisms

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Abstract

Influence of Glutathione S-transferase Mu1 (GSTM1) has long been studied in breast cancer and *GSTM1* null genotype was correlated with breast cancer risk. Nuclear factor-erythroid 2-related factor-2 (NRF2) is a transcription factor that forms a complex with Kelch-like ECH-associated protein-1 (KEAP1). Recent studies have demonstrated that expression of these proteins is deregulated in several malignancies. Thus, in the present study we aim to distinguish *GSTM1* heterozygous from wild type genotype in breast cancer patients and evaluate the presence and clinical significance of *NRF2* and *KEAP1* polymorphisms, alone or in association, with breast cancer prognosis, in cases confirmed to have *GSTM1*-present genotype. Study population consisted in 52 patients with breast cancer. Genomic DNA was extracted, *GSTM1* was genotyped through multiplex PCR and gene dose was evaluated through real-time PCR. All cases were sequenced, through Sanger sequencing, for specific regions of *NRF2* and *KEAP1*. Genotyping and clinicopathological data were correlated and statistical analysis was performed. *GSTM1* wild type was identified in 1 case and 26 cases were identified as heterozygous, these data were correlated with Human Epidermal growth factor Receptor 2 (HER2) status (p value = 0.017). We also verified that most cancers diagnosed at younger ages had the presence of *KEAP1* and/or *NRF2* polymorphisms. The association of *GSTM1* heterozygous genotype with rs1048290 and rs35652124 seems to be associated with $HER2^+$ ($p < 0.05$). Our results suggest that *GSTM1* * 1/0 genotype and the cumulative presence of at least one allele mutated in *KEAP1* and/or *NRF2* polymorphisms might be associated with worse prognosis for breast cancer patients.

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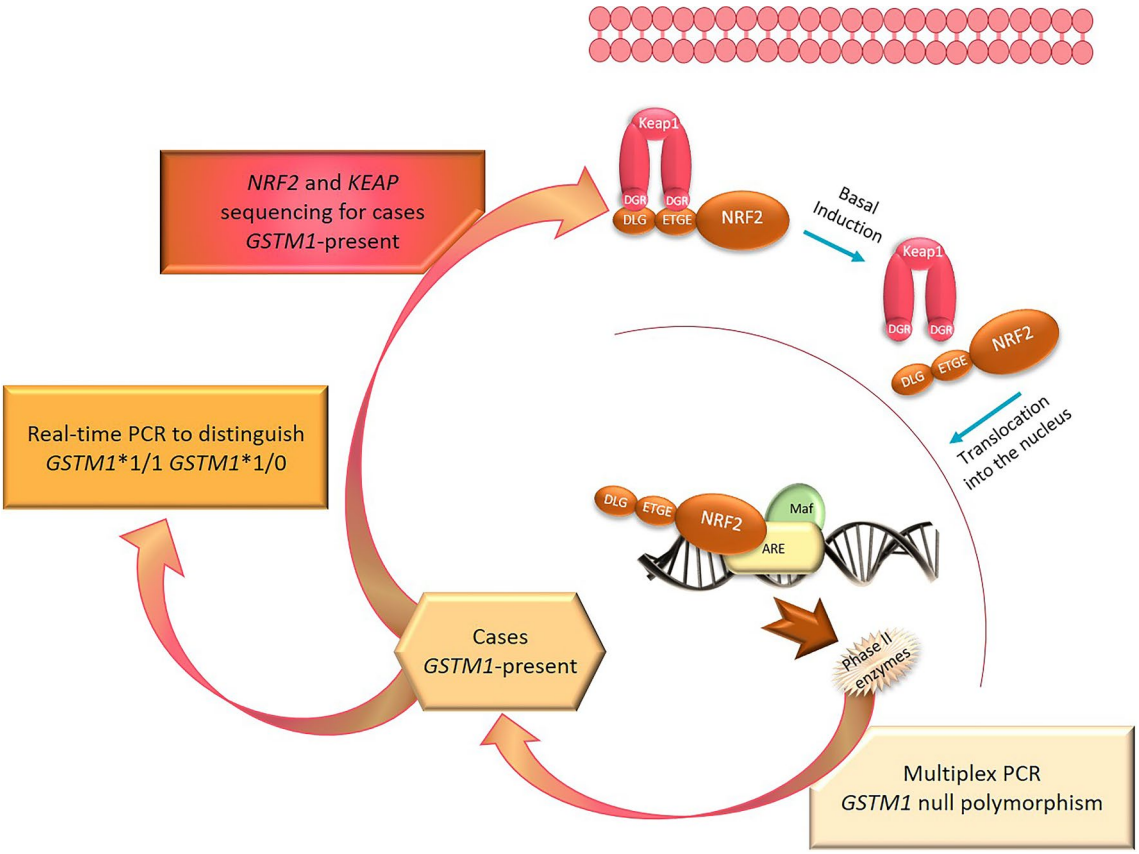
Graphical abstract



Keywords Breast cancer · NRF2 · KEAP1 · GSTM1

Introduction

Breast cancer is one of the most common malignancies in women, being the leading cause of women cancer death worldwide [1, 2].

Several risk factors have been linked to breast cancer and metabolic imbalance is known to play a vital role in breast cancer etiology. Disease progression, continuous therapeutic, exposure to oxidants, leads to the augment of reactive oxygen species creating genomic instability and resistance, resulting in disease progression and poor outcome [3].

Glutathione S-transferases (GSTs) are a superfamily of multifunctional phase II enzymes, induced under oxidative stress conditions and play a key role in cellular detoxification [4]. Several polymorphisms in GSTs have been related to breast cancer, mainly Glutathione S-transferase Mu1 (*GSTM1)* null polymorphism [5–7]. The homozygous deletion of *GSTM1* was previously studied by our research team and it was positively related with the increased risk of breast cancer development [6, 7]. However, there is a lack of studies evaluating the impact of *GSTM1* heterozygous genotype (*GSTM1* * 1/0), once most common techniques only distinguish between present genotype (*GSTM1* * 1/1) and null genotype (*GSTM1* * 0/0).

Another prominent example of oxidative pathway imbalance is the nuclear factor-erythroid 2-related factor 2 (NRF2), a transcription factor that plays an essential role in cytoprotection. NRF2, under basal circumstances, resides in the cytoplasm and forms a complex with Kelch-like ECH-associated protein 1 (KEAP1). When exposed to oxidative stress, KEAP1 frees NRF2 that moves to the nucleus where it binds to antioxidant response elements (ARE) and associates with Maf proteins, inducing the expression of cytoprotective genes like phase-II detoxification enzymes (Fig. 1) [8–11]. When basal state is achieved KEAP1 moves to the nucleus, binds to NRF2 and take it back to the cytoplasm to suffer proteasome degradation [8, 12]. Thus, NRF2 was consider a cancer inhibitor, preventing DNA damages and mutagenic events. However, further studies suggest that this complex is dysregulated in some cancers and might have a protumorigenic function that can lead to chemoresistance [9, 13, 14]. In addition NRF2

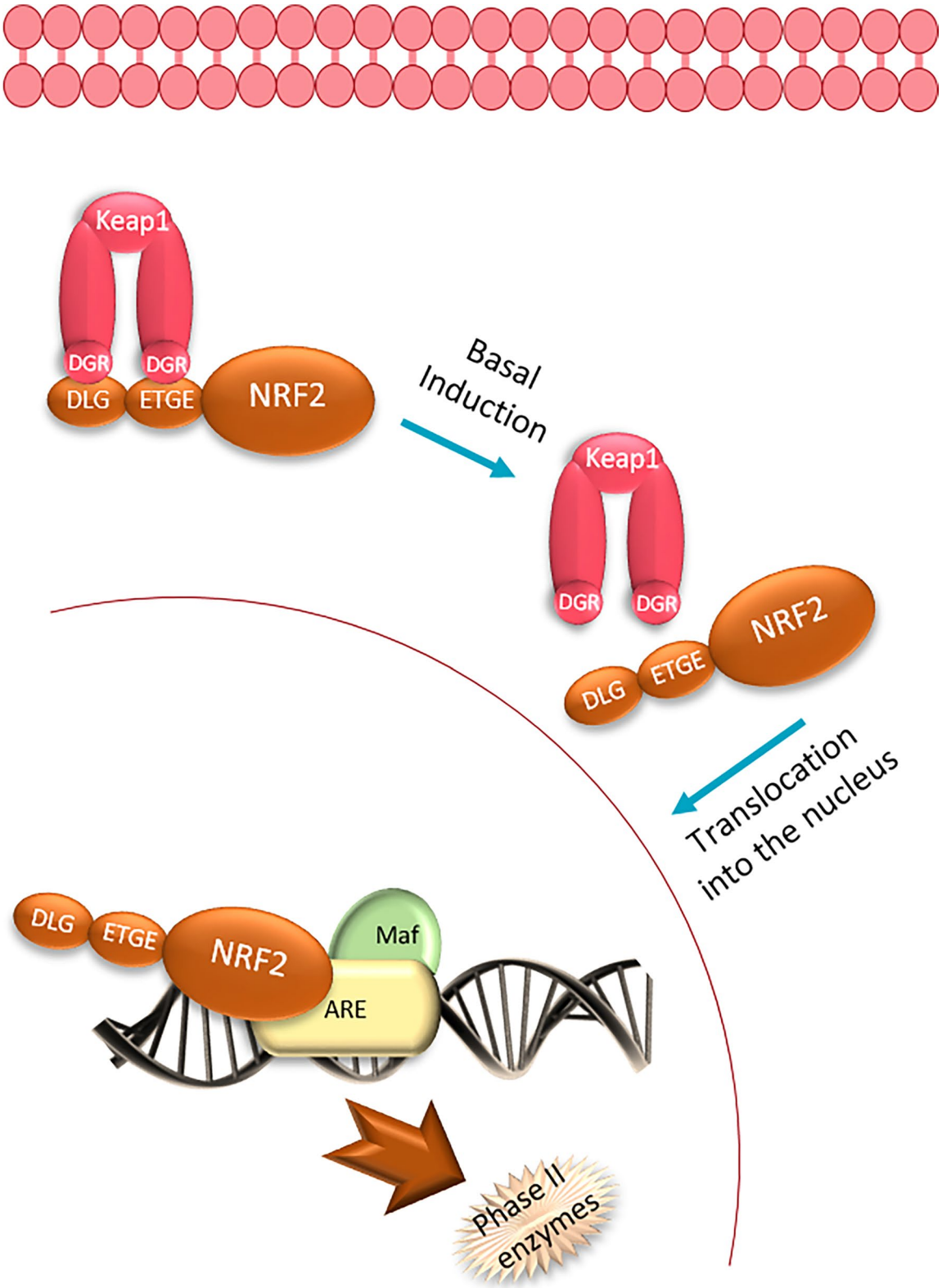


Fig. 1 NRF2-KEAP1 pathway. NRF2, under basal circumstances, resides in the cytoplasm and forms a complex with KEAP1. Being DLG/ETGE and DGR the binding domains of NRF2 and KEAP1, respectively. When exposed to oxidative stress KEAP1 frees NRF2, that moves to the nucleus where it associates with Maf proteins and binds antioxidant response elements, inducing the expression of cytoprotective genes like phase-II detoxification enzymes. When basal state is achieved KEAP1 moves to the nucleus, binds NRF2 and take it back to the cytoplasm to suffer proteasome degradation. (Author: Micaela Almeida, presented at XIII Symposium in Health Research Centre—UBI)

is encoded by *NRF2* or *NFE2L2* gene located on chromosome 2q31.2, that has been shown to be highly polymorphic [8]. Single nucleotide polymorphisms (SNPs) of *NRF2*, namely rs35652124 (−653, A > G; location chromosome 2:177265345), rs6721961 (−617, C > A; location chromosome 2:177265309) and rs6706649 (−651, G > A; location chromosome 2:177265343) polymorphisms, located in the promoter region of *NRF2*, have been studied and related to several diseases, from cardiovascular and neurodegenerative diseases to several types of cancers [8, 15–19]. Hartikainen et al. in 2015 related for the first time *KEAP1* (gene located on chromosome 19p13.2) polymorphisms with breast cancer risk, and identified the tagging SNP rs1048290 (location chromosome 19:10489766) with higher expression of KEAP1 protein [20]. This SNP is located in the genomic region (exon 4) that encodes for double-glycine repeat domain (DGR domain) of KEAP1 [21]. This domain interacts directly with NRF2, thus interfering with NRF2-KEAP1 linkage and consequently with NRF2 protein levels [22]. Once KEAP1 is the negative regulator of NRF2, which in turn regulates the expression of phase-II enzymes, we question if the association of several SNPs in *KEAP1*, *NRF2* and *GSTM1* are related with breast cancer prognosis.

Thus, in the present study we aim to distinguish *GSTM1* heterozygous from wild type genotype in breast cancer patients and evaluate the presence and clinical significance of *NRF2* and *KEAP1* polymorphisms, alone or in association, with breast cancer prognosis, in cases confirmed to have *GSTM1*-present genotype.

Materials and methods

Study population

The study group consisted in a total of 52 women with histologically confirmed breast cancer diagnosed at Child and Women Health Department, Gynaecologic Oncology Division of Hospital Centre of Cova da Beira, Covilhã—Portugal. Informed consent was obtained from all individual participants included in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã—Portugal.

DNA extraction

Blood samples were collected to EDTA tubes and genomic DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega) according to the instructions of the manufacturer.

Genotyping

Genotyping of *GSTM1* (present or null) was performed by multiplex PCR with the co-amplification of *β-globin* gene as positive control, as previously described by our group [6].

The real-time PCR assay was performed for every samples with *GSTM1* present genotype, in CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers sets for *GSTM1* and *β-globin* were:

GSTM1 forward: 5′- GAACTCCCTGAAAAGCTAAAG-3′.

GSTM1 reverse: 5′- GTTGGGCTCAAATATACGGTGG-3′.

β-globin forward: 5′-CAACTTCATCCACGTTCACC-3′.

β-globin reverse: 5′-GAAGAGCCAAGGACAGGTAC-3′.

The master Mix, for *GSTM1* and *β-globin*, had a total volume of 20 µL, with 0.3 µM of each primer, 10 µL of SYBR Green qPCR Master Mix and 100 ng of genomic DNA (gDNA) [23, 24]. Efficiency of the amplification was determined for all primer sets using serial dilutions of gDNA. The reaction mixture was incubated at 95 °C for 10 min and PCR consisted in 40 cycles at 95 °C for 10 s, 58 °C for 10 s and at 72 °C for 20 s, finally the temperature of the reaction mixture was increased up to 95 °C at a rate of 0.1 °C/s, starting at 68 °C for 15 s.

The gene dose of *GSTM1* was calculated as previously described by Girault et al. [24] *GSTM*1gene dose = $2^{(Ct\beta Glob - CtGSTM1)}$, the value of the threshold cycle considered for our study was the point where PCR product was first detected (Fig. 2) enabling us to distinguish *GSTM1* wild type genotype (*GSTM1* *1/1) from heterozygous (*GSTM1* *1/0).

Sequencing

Briefly, each PCR reaction mixture for amplification of *NRF2* and *KEAP1* fragments was carried out in a total volume of 50µL and contained 10 pmol of each primer, 1.5 mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of DreamTaq DNA polymerase and 100 ng of genomic DNA, using MyCycler™ Thermal Cycler (Bio-Rad).

The region containing the polymorphism rs1048290 of *KEAP1* was amplified with the primers designed with the programme Primer3web version 4.1.0:

Forward primer: 5′-TGTCCCCATTTTTCTTACGC-3′.

Reverse primer: 5′- AGCAAAAGCAAAAGCAGTCC-3′.

with the following conditions: 95° for 5 min for initial denature followed by 35 cycles at 95° for 30 s, at 53° for 30 s, at 72° for 30 s and additionally 72° for 10 min.

The region containing the polymorphisms rs35652124, rs6721961 and rs6706649 of *NRF2* was amplified by PCR with the primers [16]:

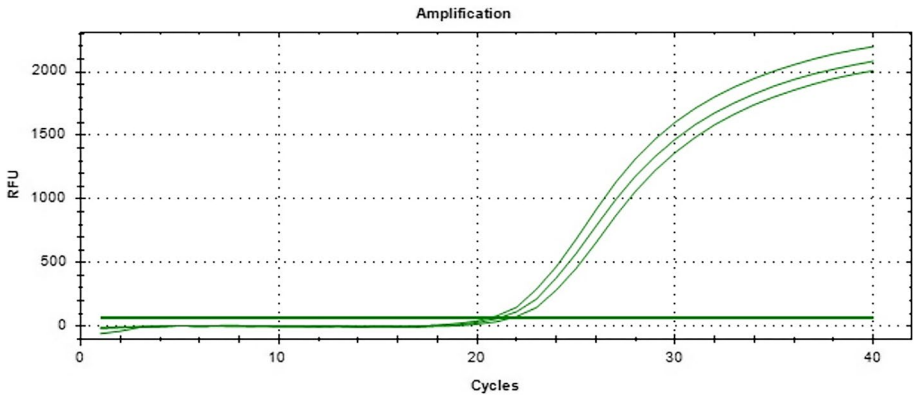


Fig. 2 *GSTM1* real-time PCR. *GSTM1* standard curve obtained by real-time PCR were the value of the threshold cycle considered for our study was the point where PCR product was first detected

Table 1 Clinicopathological characteristics of the 52 breast cancer patients examined in this study

	Patients *n* (%)	Mean (min–max)
Total	52	
Age		65.96(36–89)
< 50	14 (26.9)	
≥ 50	38 (73.1)	
Histological grade		
Grade 1 or 2	39 (76.5)	
Grade 3	12 (23.5)	
ER status		
Positive	52 (100)	
Negative	0	
PR status		
Positive	40 (78.4)	
Negative	11 (21.6)	
HER2 status		
Positive	36 (70.6)	
Negative	15 (29.4)	

Forward primer: 5′-CCCTGATTTGGAGGTGCA GAACC-3′.

Reverse primer: 5′-GGGGTTCCCGTTTTTCTCCC-3′.

with the following conditions: 95° for 10 min for initial denature followed by 35 cycles at 95° for 30 s, at 53° for 30 s, at 72° for 30 s and additionally 72° for 10 min.

The PCR amplified fragments of *KEAP1* and *NRF2* were sequenced through Sanger sequencing (performed by STABVIDA). Sequenced data were analysed in ChromasPro programme.

Statistical analysis

Statistical analysis was performed using SPSS, version 23. Chi Squared tests were used, considering a statistical significance when p-value was < 0.05.

Results

In the present study, 52 women were diagnosed with breast cancer. The clinicopathological data of the patients are summarized in Table 1. The mean age was 65.96 years, and the majority of the patients was diagnosed with breast cancer with 50 years old or more (73.1%). The histological grade of the majority of the breast cancers was grade 1 or 2 (76.5%), all positive for estrogen receptor (ER) and the majority positive for progesterone receptor (PR) (78.4%) and for Human Epidermal growth factor Receptor 2 (HER2) (70.6%). For one patient the only data we could access was age and ER immunostaining once the patient did not withdraw consent, although did not appear for further follow-up.

The women diagnosed with breast cancer were genotyped for the null polymorphism of *GSTM1*, through multiplex PCR. *GSTM1* deletion occurred in 25 cases (48.1%) and we identified the presence of *GSTM1* in 27 cases (51.9%) (Table 2). The majority of the patients diagnosed with breast cancer before 50 years old have a GSTM1 present genotype and the majority of women with this genotype are positive for HER2 (43.1%), p-value = 0.025.

Through real-time PCR we identified 1 case homozygous (*GSTM1* *1/1) and 26 cases heterozygous (*GSTM1* *1/0) for *GSTM1* polymorphism. *GSTM1* *1/1 and *GSTM1* *1/0 genotypes were correlated with clinicopathological data (Table 3). All women diagnosed with breast cancer with an age under 50 years old have a genotype *GSTM1* *1/0 and the only case with *GSTM1* *1/1 genotype was diagnosed after 50 years old (p-value = 0.434). All the cases with high histological grade and PR^- are *GSTM1* *1/0 genotype. When correlating *GSTM1* genotypes with HER2 status we verified that the only case with the wild type genotype for *GSTM1* null polymorphism is $HER2^-$, and 84.6% of cases are *GSTM1* * 1/0 and $HER2^+$ (p-value = 0.017).

Through Sanger sequencing we confirmed the presence of the SNPS of *KEAP1* (rs1048290) and of *NRF2* (rs35652124, rs672961 and rs6706649), as shown in Fig. 3. We also performed a correlation between *KEAP1* rs1048290 and *NRF2*

Table 2 Correlation between present and null genotypes of GSTM1 with clinicopathological data

	GSTM1		
	Present *n* (%)	Null *n* (%)	*p* value
Total	27 (51.9)	25 (48.1)	
Age			
< 50	10 (19.2)	4 (7.7)	0.087
≥ 50	17 (32.7)	21 (40.4)	
Histological grade			
Grade 1 or 2	19 (37.3)	20 (39.2)	0.560
Grade 3	7 (13.7)	5 (9.8)	
ER status			NA
Positive	27 (51.9)	25 (48.1)	
Negative	0	0	
PR status			0.679
Positive	21 (41.2)	19 (37.3)	
Negative	5 (9.8)	6 (11.8)	
HER2 status			**0.025**
Positive	22 (43.1)	14 (27.5)	
Negative	4 (7.8)	11 (21.6)	

Bold values means that have statistical significance ($p < 0.05$)

Table 3 Correlation between wild type and heterozygous GSTM1 genotypes with clinicopathological data

	GSTM1		*p* value
	GSTM1 * 1/1 *n* (%)	*GSTM1* * 1/0 *n* (%)	
Total	1 (3.7)	26 (96.3)	
Age			
< 50	0	10 (37)	0.434
≥ 50	1 (3.7)	16 (59.3)	
Histological grade			
Grade 1 or 2	1 (3.8)	18 (69.2)	0.536
Grade 3	0	7 (26.9)	
ER status			NA
Positive	1 (3.7)	26 (96.3)	
Negative	0	0	
PR status			0.619
Positive	1 (3.8)	20 (76.9)	
Negative	0	5 (19.2)	
HER2 status			**0.017**
Positive	0	22 (84.6)	
Negative	1 (3.8)	3 (11.5)	

Bold values means that have statistical significance ($p < 0.05$)

rs35652124, rs6721961 and rs6706649, with clinicopathological data for the population in study (Table 4). The majority of the patients have at least one altered allele for *KEAP1* polymorphism (73.1%) and 12 of the 14 cases diagnosed before 50 years old have the presence of the altered allele (Fig. 4a). Most of cases with high histological grade (Fig. 4b), PR^- and $HER2^+$ also have the altered allele of *KEAP1*. The samples were also sequenced for polymorphisms in the promoter region of *NRF2*, and we identified the genotypes AG or GG of the polymorphism rs35652124 in 23 cases (44.2%), the heterozygous genotype of the polymorphism rs6721961 in 12 cases (23.1%) and the heterozygous genotype of the polymorphism rs6706649 in 10 case (19.2%).

The polymorphisms of *KEAP1* and *NRF2* were also correlated in the cases with *GSTM1* present genotype (*GSTM1* *1/1 and *GSTM1* *1/0). In Table 5 it can be verified

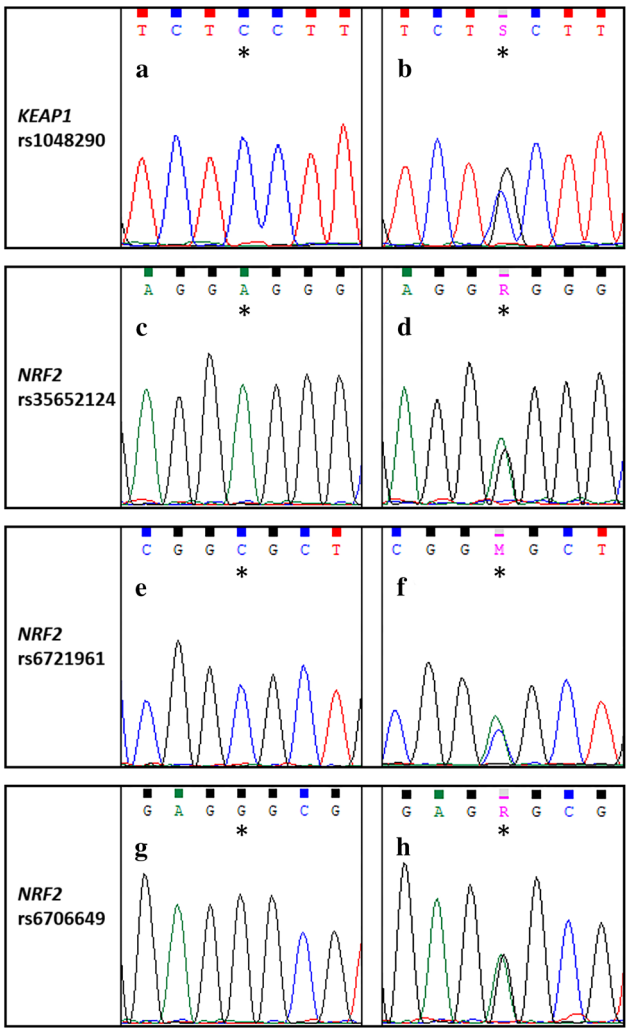


Fig. 3 Sanger sequencing results for the SNPs found for *KEAP1* and *NRF2,* where asterisk represents the ancestral or altered allele. The colour of the lines represent: green—adenine, blue—cytosine, black—guanine and red—thymine. **a** *KEAP1* rs1048290 CC (wild type); **b** *KEAP1* rs1048290 CG genotype; **c** *NRF2* rs35652124 AA (wild type); **d** *NRF2* rs35652124 AG genotype; **e** *NRF2* rs6721961 CC (wild type) genotype; **f** *NRF2* rs36721961 CA genotype; **g** *NRF2* rs6706649 GG (wild type) genotype; **h** *NRF2* rs6706649 GA genotype. (Color figure online)

Table 4 Correlation of *KEAP1* rs1048290, *NRF2* rs35652124, rs6721961 and rs6706649 with clinicopathologic data, for the study population

Genotype	Total	Age *n* (%)			Histological Grade *n* (%)			ER status *n* (%)			PR status *n* (%)			HER2 status *n* (%)		
		<50	≥50	*p* value	1 or 2	3	*p* value	+	−	*p* value	+	−	*p* value	+	−	*p* value
KEAP1 rs1048290																
CC	14 (26.9)	2 (3.8)	12 (23.1)	0.212	11 (21.6)	2 (3.9)	0.423	14 (26.9)	0	NA	11 (21.6)	2 (3.9)	0.530	7 (13.7)	6 (11.8)	0.125
CG/GG	38 (73.1)	12 (23.1)	26 (50)		28 (54.9)	10 (19.6)		38 (73.1)	0		29 (56.9)	9 (17.6)		29 (56.9)	9 (17.6)	
NRF2 rs35652124																
AA	29 (55.8)	9 (17.3)	20 (38.5)	0.453	24 (47.1)	4 (7.8)	0.086	29 (55.8)	0	NA	23 (45.1)	5 (9.8)	0.477	19 (73.3)	9 (17.6)	0.637
AG/GG	23 (44.2)	5 (9.6)	18 (34.6)		15 (29.4)	8 (15.7)		23 (44.2)	0		17 (33.3)	6 (11.8)		17 (33.3)	6 (11.8)	
NRF2 rs6721961																
CC	40 (76.9)	12 (23.1)	28 (53.8)	0.361	29 (56.9)	10 (19.6)	0.522	40 (76.9)	0	NA	31 (60.8)	8 (15.7)	0.741	29 (56.9)	10 (19.6)	0.287
CA	12 (23.1)	2 (3.8)	10 (19.2)		10 (19.6)	2 (3.9)		12 (23.1)	0		9 (17.6)	3 (5.9)		7 (13.7)	5 (9.8)	
NRF2 rs6706649																
GG	42 (80.8)	10 (19.2)	32 (61.5)	0.300	33 (64.7)	8 (15.7)	0.171	42 (80.8)	0	NA	33 (64.7)	8 (15.7)	0.470	29 (56.9)	12 (23.5)	0.964
GA	10 (19.2)	4 (7.7)	6 (11.5)		6 (11.8)	4 (7.8)		10 (19.2)	0		7 (13.7)	3 (5.9)		7 (13.7)	3 (5.9)	

that the majority of cases diagnosed before 50 years old, have the presence of at least one altered allele of *KEAP1* polymorphism. The majority of the cases with high histological grade, PR^- negative and $HER2^+$ also have the presence of *KEAP1* and of *NRF2* rs35652124 polymorphisms.

We also evaluated the association of *KEAP1* rs1048290 polymorphism with the *NRF2* polymorphisms and correlated them with clinicopathological data (Table 6). The majority of the cases with genotype wild type for *NRF2* polymorphisms and *KEAP1* rs1048290 were diagnosed at a later age (≥ 50 years old). Most of the cases with high histological grade, PR^- and $HER2^+$ have the cumulative presence of the polymorphism of *KEAP1* and rs35652124 of *NRF2*. Although, When correlating these clinical data with *KEAP1* polymorphism and with rs6721961 and rs6706649 of *NRF2*, the cases with high histological grade, PR^- and $HER2^+$ have the polymorphism of *KEAP1* but are wild type for these polymorphisms of *NRF2*.

KEAP1 and *NRF2* polymorphisms were correlated with *GSTM1* genotypes and with HER2 status. In Table 7a) it can be verified that 35.3% of the cases have the presence of *KEAP1* polymorphism and are *GSTM1* present (p-value = 0.029). When comparing the three genotypes of *GSTM1* with *KEAP1* and with HER2, 35.3% of $HER2^+$ cases have the presence of *KEAP1* polymorphism and are *GSTM1* *1/0, 21.6% have the presence of *KEAP1* polymorphism and are *GSTM1* *0/0 (Table 7b) and the only case *GSTM1* *1/1 is $HER2^-$ (p-value = 0.014). Similar results were found for rs35652124. Thus, we correlated *KEAP1* and *NRF2* polymorphisms with cases *GSTM1* *1/1 and *GSTM1* *1/0 (Table 7c). All the cases $HER2^+$ are *GSTM1* *1/0. 69.2% of the cases with *KEAP1* and *GSTM1* polymorphism are $HER2^+$, p-value = 0.044, similar results were found for rs35652124 (p-value = 0.043). In opposite 73.1% of the cases $HER2^+$ are *GSTM1* *1/0 and are wild type for rs6721961 and for rs6706649, with p-values of 0.042 and 0.047, respectively.

Discussion

Polymorphisms in the metabolic pathway of breast cancer, namely in genes that codify for GSTs, like *GSTT1* null polymorphism and *GSTP1* (A/G) polymorphism have been associated, alone or in combination, with breast cancer risk [6, 7]. Among them is *GSTM1* null genotype, in which the null genotype results in the total absence of GSTM1 expression, and has previously been related, by our team, with the augmented risk of breast cancer [6, 7]. In the present study we genotyped *GSTM1* null polymorphism in 52 women, patients of Hospital Centre of Cova da Beira, diagnosed with breast cancer. *GSTM1* was present in 27 cases of the breast cancer patients. The widely

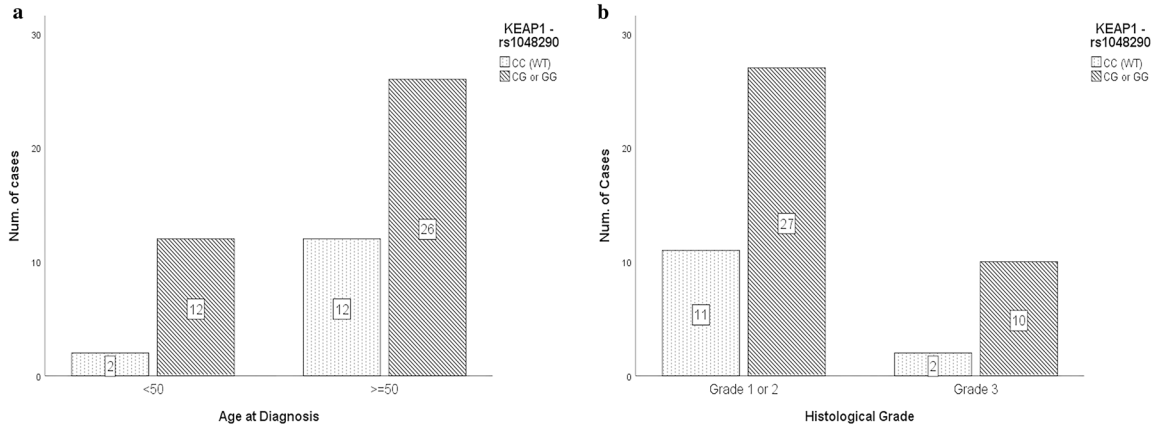


Fig. 4 **a** Correlation between KEAP1 rs1048290 with age at diagnosis, were it can be observed that the majority of cases diagnosed before 50 years old had the presence of at least one altered allele of KEAP1 rs1048290. **b** Correlation between KEAP1 rs1048290 and histological grade, were we observe that 10 of the 12 cases with Grade 3 are CG or GG genotypes

used technique to genotype this polymorphism is multiplex PCR. However, this technique allows us to identify the presence or absence of *GSTM1*, resulting in the loss of information about the heterozygous genotype. Thus, we might be losing the predictive value of the heterozygosity of *GSTM1* null SNP. To avoid this bias, we performed a real-time PCR, as described by other teams [23, 24], in all cases identified as *GSTM1*-present, in order to evaluate the dose of *GSTM1* gene. This procedure enabled us to distinguish between *GSTM1* *1/1 (wild type genotype—1 case identified) and *GSTM1* *1/0 (heterozygous genotype—26 cases identified). To evaluate the predictive value of the heterozygous genotype in breast cancer prognosis, *GSTM1* gene dose was correlated with clinicopathologic data. The case with *GSTM1* *1/1 genotype was diagnosed with breast cancer with an age over 50 years old, and it is a $ER^+/PR^+/HER2^-$ (Luminal A subtype of breast cancer), which is the subtype with better prognosis. Besides, the most pertinent result (p-value < 0.05) is the correlation between heterozygous and wild type genotypes of *GSTM1* with HER2 status. Only the case *GSTM1* *1/1 and 3 cases *GSTM1* *1/0 are $HER2^-$, being the majority of the heterozygous cases (84.6%) $HER2^+$, suggesting a more aggressive and a worse prognosis for breast cancer cases with *GSTM1* *1/0 genotype. Also all PR^- cases have a *GSTM1* *1/0 genotype. Our results, that evaluate the predictive value of the heterozygous genotype of *GSTM1* null polymorphism, suggest that *GSTM1* *1/0 might be related with more aggressive breast cancers and with poor prognosis. The simple evaluation of the presence/absence of *GSTM1* might be hiding the predictive value of the heterozygous genotype of *GSTM1* in breast cancer patients and our results suggest that there is the need to study the influence of the three genotypes of *GSTM1* null polymorphism in breast cancer in more detail and with larger samples.

Once GSTM1 is a phase II detoxifying enzyme related with the augment of breast cancer risk, we considered pertinent to analyse the upstream pathway, in particular NRF2 and KEAP1, in cases genotyped as *GSTM1*-present, in order to withdraw the risk potentiated by *GSTM1* homozygous null polymorphism.

We sequenced, through Sanger Sequencing, a fragment of the promoter region of *NRF2*, known to be highly polymorphic [8] and the region of *KEAP1* that encodes for DGR domain of KEAP1, domain responsible for binding NRF2 [21, 22]. Sequencing data of *KEAP1* and of *NRF2* were correlated with clinicopathological data, in a single or associated way.

For *KEAP1* we identified 38 women with CG/GG genotypes for rs1048290 polymorphism. When *KEAP1* polymorphism was correlated with clinical data in cases with *GSTM1* present genotype we verified the altered allele is present in the majority of the cases diagnosed before 50 years of age, with high histological grade, PR^- and $HER2^+$. Although no statistical significance was found, these are pertinent results, once cases heterozygous (CG) or homozygous (GG) have worst clinical characteristics. For *NRF2* we found the SNPs rs35652124, rs6721961 and rs6706649, and the results of rs35652124 were similar to the ones verified for *KEAP1*.

We also performed a combined evaluation of clinical data with the genotypes of the SNPs of *KEAP1* and *NRF2*. We verify that all cases with high histological grade are wild type for *NRF2* rs6721961 and rs6706649 polymorphisms and are heterozygous or homozygous for the rs1048290 polymorphism of *KEAP1*. Correlating rs35652124 of *NRF2* with rs1048290 of *KEAP1*, we verify that 6 of the 7 cases with high histological grade have the presence of both SNPs and 46.2% of the cases with both SNPs are $HER2^+$. Thus, we suppose that rs1048290 might change the normal function of KEAP1-NRF2 complex, compromising the antioxidant

Table 5 Correlation between *KEAP1* rs1048290, *NRF2* rs35652124, rs6721961 and rs6706649 with clinicopathologic data, for patients with *GSTM1* * 1/1 or *GSTM1* * 1/0 genotypes

Genotype	Total	Age *n* (%)			Histological Grade *n* (%)			ER status *n* (%)			PR status *n* (%)			HER2 status *n* (%)		
		< 50	≥ 50	*p* value	1 or 2	3	*p* value	+	–	*p* value	+	–	*p* value	+	–	*p* value
KEAP1 rs1048290																
CC	5 (18.5)	1 (3.7)	4 (14.8)	0.382	4 (15.4)	0	0.187	5 (18.5)	0	NA	4 (15.4)	0	0.289	4 (15.4)	0	0.354
CG/GG	22 (81.5)	9 (33.3)	13 (48.1)		15 (57.7)	7 (26.9)		22 (81.5)	0		17 (65.4)	5 (19.2)		18 (69.2)	4 (15.4)	
NRF2 rs35652124																
AA	12 (44.4)	6 (22.2)	6 (22.2)	0.212	10 (38.5)	1 (3.8)	0.079	12 (44.4)	0	NA	10 (38.5)	1 (3.8)	0.261	9 (34.6)	2 (7.7)	0.735
AG/GG	15 (55.6)	4 (14.8)	11 (40.7)		9 (34.6)	6 (23.1)		15 (55.6)	0		11 (42.3)	4 (15.4)		13 (50)	2 (7.7)	
NRF2 rs6721961																
CC	23 (85.2)	8 (29.6)	15 (55.6)	0.561	15 (57.7)	7 (26.9)	0.187	23 (85.2)	0	NA	17 (65.4)	5 (19.2)	0.289	19 (73.1)	3 (11.5)	0.562
CA	4 (14.8)	2 (7.4)	2 (7.4)		4 (15.4)	0		4 (14.8)	0		4 (15.4)	0		3 (11.5)	1 (3.8)	
NRF2 rs6706649																
GG	24 (88.9)	8 (29.6)	16 (59.3)	0.260	17 (65.4)	6 (23.1)	0.790	24 (88.9)	0	NA	18 (69.2)	5 (19.2)	0.369	19 (73.1)	4 (15.4)	0.432
GA	3 (11.1)	2 (7.4)	1 (3.7)		2 (7.7)	1 (3.8)		3 (11.1)	0		3 (11.5)	0		3 (11.5)	0	

Table 6 Correlation between *NRF2* rs35652124, rs6721961 and rs6706649 with *KEAP1* rs1058290 and with clinicopathologic data, in patients with *GSTM1* Present genotype

Genotype		Age *n* (%)			Histological grade *n* (%)			ER status *n* (%)			PR status *n* (%)			HER2 status *n* (%)		
		< 50	≥ 50	*p* value	1 or 2	3	*p* value	+	–	*p* value	+	–	*p* value	+	–	*p* value
NRF2 rs35652124	*Keap1 rs1048290*															
AA	CC	1 (3.7)	3 (11.1)	0.322	3 (11.5)	0	0.248	4 (14.8)	0	NA	3 (11.5)	0	0.578	3 (11.5)	0	0.727
AA	CG/GG	5 (18.5)	3 (11.1)		7 (26.9)	1 (3.8)		8 (29.6)	0		7 (26.9)	1 (3.8)		6 (23.1)	2 (7.7)	
AG/GG	CC	0	1 (3.7)		1 (3.8)	0		1 (3.7)	0		1 (3.8)	0		1 (3.8)	0	
AG/GG	CG/GG	4 (14.8)	10 (37)		8 (30.8)	6 (23.1)		14 (51.9)	0		10 (38.5)	4 (15.4)		12 (46.2)	2 (7.7)	
NRF2 rs6721961	*Keap1 rs1048290*															
CC	CC	1 (3.7)	3 (11.1)	0.579	3 (11.5)	0	0.317	4 (14.8)	0	NA	3 (11.5)	0	0.516	3 (11.5)	0	0.689
CC	CG/GG	7 (25.9)	12 (44.4)		12 (46.2)	7 (26.9)		19 (70.4)	0		14 (53.8)	5 (19.2)		16 (61.5)	3 (11.5)	
CA	CC	0	1 (3.7)		1 (3.8)	0		1 (3.7)	0		1 (3.8)	0		1 (3.8)	0	
CA	CG/GG	2 (7.4)	1 (3.7)		3 (11.5)	0		3 (11.1)	0		3 (11.5)	0		2 (7.7)	1 (3.8)	
NRF2 rs6706649	*Keap1 rs1048290*															
GG	CC	1 (3.7)	4 (14.8)	0.416	4 (15.4)	0	0.418	5 (18.5)	0	NA	4 (15.4)	0	0.320	4 (15.4)	0	0.419
GG	CG/GG	7 (25.9)	12 (44.4)		13 (50)	6 (23.1)		19 (70.4)	0		14 (53.8)	5 (19.2)		15 (57.7)	4 (15.4)	
GA	CC	0	0		0	0		0	0		0	0		0	0	
GA	CG/GG	2 (7.4)	1(3.7)		2 (7.7)	1 (3.8)		3 (11.1)	0		3 (11.5)	0		3 (11.5)	0	

Table 7 Correlation between the polymorphisms of *KEAP1* and *NRF2* in study with *GSTM1* genotypes

Genotype		HER2 status *n* (%)		
		+	–	*p* value
(a)				
KEAP1 rs1048290	*GSTM1*			
CC	Present	4 (7.8)	0	**0.029**
CC	Null	3 (5.9)	6 (11.8)	
CG/GG	Present	18 (35.3)	4 (7.8)	
CG/GG	Null	11 (21.6)	5 (9.8)	
NRF2 rs35652124	*GSTM1*			
AA	Present	9 (17.6)	2 (3.9)	0.151
AA	Null	10 (19.6)	7 (13.7)	
AG/GG	Present	13 (25.5)	2 (3.9)	
AG/GG	Null	4 (7.8)	4 (7.8)	
NRF2 rs6721961	*GSTM1*			
CC	Present	19 (37.3)	3 (5.9)	0.142
CC	Null	10 (19.6)	7 (13.7)	
CA	Present	3 (5.9)	1 (2)	
CA	Null	4 (7.8)	4 (7.8)	
NRF2 rs6706649	*GSTM1*			
GG	Present	19 (37.3)	4 (7.8)	0.144
GG	Null	10 (19.6)	8 (15.7)	
GA	Present	3 (5.9)	0	
GA	Null	4 (7.8)	3 (5.9)	
(b)				
KEAP1 rs1048290	*GSTM1*			
CC	1/1	0	0	**0.014**
CC	1/0	4 (7.18)	0	
CC	0/0	3 (5.9)	6 (11.8)	
CG/GG	1/1	0	1 (2)	
CG/GG	1/0	18 (35.3)	3 (5.9)	
CG/GG	0/0	11 (21.6)	5 (9.8)	
NRF2 rs35652124	*GSTM1*			
AA	1/1	0	0	0.057
AA	1/0	9 (17.6)	2 (3.9)	
AA	0/0	10 (19.6)	7 (13.7)	
AG/GG	1/1	0	1 (2)	
AG/GG	1/0	13 (25.5)	1 (2)	
AG/GG	0/0	4 (7.8)	4 (7.8)	
NRF2 rs6721961	*GSTM1*			
CC	1/1	0	1 (2)	0.056
CC	1/0	19 (37.3)	2 (3.9)	
CC	0/0	10 (19.6)	7 (13.7)	
CA	1/1	0	0	
CA	1/0	3 (5.9)	1 (2)	
CA	0/0	4 (7.8)	4 (7.8)	
NRF2 rs6706649	*GSTM1*			
GG	1/1	0	1 (2)	0.065
GG	1/0	19 (37.3)	3 (5.9)	
GG	0/0	10 (19.6)	8 (15.7)	
GA	1/1	0	0	
GA	1/0	3 (5.9)	0	
GA	0/0	4 (7.8)	3 (5.9)	
(c)				
KEAP1 rs1048290	*GSTM1*			
CC	1/1	0	0	**0.044**
CC	1/0	4 (15.4)	0	
CG/GG	1/1	0	1 (3.8)	
CG/GG	1/0	18 (69.2)	3 (11.5)	
NRF2 rs35652124	*GSTM1*			
AA	1/1	0	0	**0.043**
AA	1/0	9 (34.6)	2 (7.7)	
AG/GG	1/1	0	1 (3.8)	
AG/GG	1/0	13 (50)	1 (3.8)	
NRF2 rs6721961	*GSTM1*			
CC	1/1	0	1 (3.8)	**0.042**
CC	1/0	19 (73.1)	2 (7.2)	
CA	1/1	0	0	
CA	1/0	3 (11.5)	1 (3.8)	
NRF2 rs6706649	*GSTM1*			
GG	1/1	0	1 (3.8)	**0.047**
GG	1/0	19 (73.1)	3 (11.5)	
GA	1/1	0	0	
GA	1/0	3 (11.5)	0	

Bold values means that have statistical significance ($p < 0.05$)

(a) Correlation between HER2 and the association of *KEAP1* and *NRF2* polymorphisms with *GSTM1* Present/Null genotypes; (b) Correlation between HER2 and the association of *KEAP1* and *NRF2* polymorphisms with the three genotypes of *GSTM1*; (c) Correlation between HER2 and the association of *KEAP1* and *NRF2* polymorphisms in cases with the genotype Present of *GSTM1*

response and contributing to metabolic imbalance. Cumulative presence of *GSTM1* *1/0 with *KEAP1* rs1048290 polymorphism seems to be related with worse prognosis once breast cancers were diagnosed at younger ages, with high histological grade with and with $HER2^+$ (p-value < 0.05). As well, the majority of the cases $HER2^+$ have the cumulative presence of *GSTM1* *1/0 genotype and are heterozygous (AG) or homozygous (GG) for rs35652124 of *NRF2* (p-value = 0.043). However, when correlating *GSTM1* *1/0 genotype with rs6721961 and with rs6706649 the majority of the cases $HER2^+$ is wild type for these polymorphisms of *NRF2*, p-value of 0.042 and 0.047, respectively. The polymorphism of *KEAP1* rs1048290 is correlated with higher expression of KEAP1 protein, and consequently with augmented levels of cytoplasmic NRF2 and diminished levels of nuclear NRF2 [21]. Cases with GSTM1*1/0 once have a deletion in one allele of GSTM1 might have reduced levels

of GSTM1. The cumulative presence of this *GSTM1* genotype with lower nuclear NRF2, due to the polymorphism rs1048290 of *KEAP1* might result in metabolic imbalance, compromising the antioxidant response.

These results, are the first, to our knowledge, to combine *GSTM1* * 1/1, *GSTM1* * 1/0, *KEAP1* and *NRF2* genotyping in breast cancer patients, and suggest that these polymorphisms of *KEAP1* and of *NRF2* might be compromising the normal function of the NRF2-KEAP1 complex, and associated *GSTM1* * 1/0 genotype might be related with worse prognosis for breast cancer patients. However, further studies with a larger population should performed in order to understand if KEAP1 might be consider a target for breast cancer therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Chapter 6

The Prognostic Value of NRF2 in Breast Cancer Patients: a Systematic Review with Meta-Analysis

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REVIEW

The prognostic value of NRF2 in breast cancer patients: a systematic review with meta-analysis

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Abstract

Purpose Nuclear factor E2-related factor 2 (NRF2) is a transcription factor that plays a major role in the regulation of intracellular antioxidant response. The effect of NRF2 overexpression in many malignancies is still unclear and recent meta-analysis correlated NRF2 overexpression with poor prognosis in a variety of human cancers. However, the effect of NRF2 overexpression in breast cancer is still unclear. Thus, the main goal of this work was to clarify the role of NRF2 expression in survival and relapse of breast cancer patients by performing a systematic review according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement, followed by a meta-analysis.

Methods The electronic search was conducted in PubMed, Scopus, SciELO, Web of Science and Embase between November of 2017 and September of 2018. To be included, studies should evaluate NRF2 expression in breast cancer tissue, through immunohistochemistry and/or mRNA and had to report one or more of the following outcomes: overall survival (OS), disease-free survival (DFS), mean survival and median survival.

Results For the meta-analysis, seven studies were included and NRF2 expression was correlated with OS and DFS. It was observed that compared to patients with low NRF2 expression, patients with NRF2 overexpression had poorer OS with a hazard ratio of 1.82 (95% CI 1.32–2.50; p value < 0.0001), and poorer DFS, with a hazard ratio of 1.79 (95% CI 1.07–3.01; p value $= 0.03$).

Conclusions These results suggest that tumours that overexpress NRF2 have a worse clinical outcome. Thus, NRF2 expression could be a marker for the prognostic of breast cancer patients and, in the future, it would be pertinent to focus on improving treatment efficacy for patients with NRF2 overexpression.

Keywords NRF2 · Breast cancer · Systematic review · Meta-analysis

Introduction

The nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF2) is a transcription factor that belongs to the cap n collar (CNC) subfamily and is considered the major regulator of cellular defence mechanism [1, 2]. Under basal circumstances, NRF2 resides in the cytoplasm bound to its negative regulator Kelch-like ECH-associated protein 1 (KEAP1), forming the KEAP1-NRF2 complex [3–5]. When cells are exposed to stress conditions, KEAP1 frees NRF2 that translocate to the nucleus, binds to antioxidant response elements (ARE) and associates with Musculoaponeurotic fibrosarcoma (Maf) proteins, inducing the expression of stress-related genes like phase II detoxification enzymes [1, 6, 7]. When basal homoeostasis is achieved KEAP1 moves to the nucleus and binds NRF2, the complex KEAP1-NRF2

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travels to the cytoplasm and NRF2 is degraded through proteasome pathway [1, 5–8]. Therefore, NRF2 has been considered a major regulator of the oxidative stress, protecting normal cells from toxic insults and preventing carcinogenesis [1, 3, 5, 6]. However, NRF2 is considered to have a dual role in cancer. NRF2 is responsible for inducing the expression of several cytoprotective genes; however, mutations in *NRF2* gene or in *KEAP1* gene might result in high amounts of unbound NRF2, resulting in a metabolic imbalance, contributing to cancer cell proliferation, antisenescence and antiapoptosis [7, 9]. Moreover, NRF2 deregulation may be associated with a less oxidative stress, which explains chemo- and radiotherapy resistance, leading to cancer cell protection and higher proliferation [7, 9].

The controversial role of NRF2 in cancer was studied in two meta-analyses, one by Guo and Shen [10] and other by Wang et al. [11], where the role of NRF2 in several types of cancers was evaluated [10, 11]. Both meta-analyses concluded that NRF2 overexpression was correlated with lower overall survival (OS) and lower disease-free survival (DFS), considering that NRF2 expression is a poor prognostic factor in solid cancers [10, 11]. However, these studies evaluated NRF2 expression in different types of cancers, as a whole.

Once breast cancer is the most frequently diagnosed cancer in women worldwide and the leading cause of cancer death in most countries, including less developed regions, it is pertinent to evaluate the role of NRF2 expression in this type of cancer [12]. Therefore, the main goal of this work was to clarify the role of NRF2 expression in breast cancer patients' survival and relapse by performing a systematic review according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement, followed by a meta-analysis.

Methods

Search strategy, inclusion criteria and study selection

The electronic search for this systematic review with meta-analysis was conducted between November of 2017 and September of 2018 in PubMed, Scopus, SciELO, Web of Science and Embase. These databases were queried with Boolean operator tools, specifically using the terms: NRF2 OR NFE2L2 OR Nrf2, in combination with the terms: breast carcinoma OR breast cancer. The advanced setting option "related articles" was also employed to obtain even broader results. Language restrictions, date of study, blinding or publication status were not used during the search. Furthermore, the reference lists of the relevant studies were checked to find additional articles. Following the PRISMA recommendations [13], titles and abstracts of retrieved records were initially screened and the full texts of those considered relevant were then downloaded and analysed in detail. The corresponding authors of the articles that had restricted access were contacted to kindly send the manuscript files. The literature selection process was conducted by two independent reviewers, with a third reviewer voting in case of discrepancies. To be included in this systematic review, studies should evaluate NRF2 expression in breast cancer tissue, through immunohistochemistry and/or mRNA and had to report at least one of the following outcomes: OS, DFS, mean survival and median survival.

Data extraction and synthesis

The following data were extracted from each included study: the first author's last name, year of publication, country, number of cases with NRF2 overexpression and low expression, mean age of patients, follow-up time and detection method. Two authors independently extracted data using an assigned protocol. The results that were extracted from the outcomes OS and DFS were HRs with the corresponding 95% confidence intervals (CI) or p values. Two authors independently reviewed each study to ensure the validity of the extracted data. In cases of discordance, a third reviewer was consulted to analyse discrepancies in data extraction.

Quality of studies

The quality of each included study was assessed using the Newcastle–Ottawa Scale (NOS) taking into consideration the selection, comparability and ascertainment of outcome. The studies with scores ≥ 7 were defined as high quality. This classification was independently assigned by two authors and discrepancies in assessment were resolved through discussions between the authors or by consultation with a third investigator.

Statistical analysis

A meta-analysis was conducted to determine the prognostic value of NRF2 in breast carcinoma patients. It was performed an assessment on the pooled effect of OS and DFS in terms of weighted hazard ratios (WHR) (overexpression of NRF2 versus low expression of NRF2). The values of the outcomes were extracted as HRs and 95% CIs. Data statistical analysis was performed using Comprehensive Meta-Analysis software (Version 2.0). Forest plots were generated to illustrate the study-specific effect sizes along with a 95% CI. The random-effects model was used, however, when a fixed-effects model would be more useful, i.e. when there was little variance in effect sizes, the software automatically converted the random-effects into a fixed-effects model [14].

Heterogeneity between study results was tested using the *Q*-statistic (also referred to as "Cochrane *Q*"). A *p* value of less than 0.05 leads to the conclusion that some (undetermined) degree of heterogeneity exists. The statistic I^2 of Higgins et al. [15] was used as a measure of inconsistency across the findings of the included studies. The scale of I^2 has a range of 0 to 100% and values on the order of 25%, 50% and 75% are considered low, moderate and high heterogeneity, respectively [15]. I^2 reflects the proportion of observed dispersion which was due to the heterogeneity.

Three different analyses were used to assess the potential impact of publication bias on the present meta-analysis. One analysis was a funnel plot in which the log of HR was plotted against the corresponding SE [16, 17]. In the absence of publication bias, the studies were symmetrically distributed in relation to the WHR. Given that the interpretation of a funnel plot is largely subjective, Egger's regression test was performed to test for potential publication bias [18]. Finally, the Trim and Fill approach [19, 20] was applied, which uses an iterative procedure to remove the most extreme small studies from the positive or negative side of the funnel plot and recalculate the HRs to yield an unbiased estimate of WHR. This approach allows the best estimate of the unbiased pooled effect size to be obtained and lends itself an intuitive visual display as it creates a funnel plot that includes both the observed studies and the imputed studies. As a result, this visual display shows how the pooled effect size shifted when the imputed studies are included.

The sensitivity analysis was also performed by removing each study one at a time to evaluate the stability of the results.

Results

Search and selection of studies

The search strategy and the subsequent analysis of the studies were performed taking into consideration the PRISMA statement [13]. The flow diagram (Fig. 1) describes the steps that were applied and resulted in the selected articles. The search produced the identification of 2751 articles. After removal of duplicates, 221 articles were screened, and 199 were excluded due to the fact of being reviews, or having irrelevant content for the present meta-analysis. The resulting 22 articles were full-screened, nine met the inclusion criteria for qualitative synthesis and from those nine articles, seven met the inclusion criteria for quantitative analysis

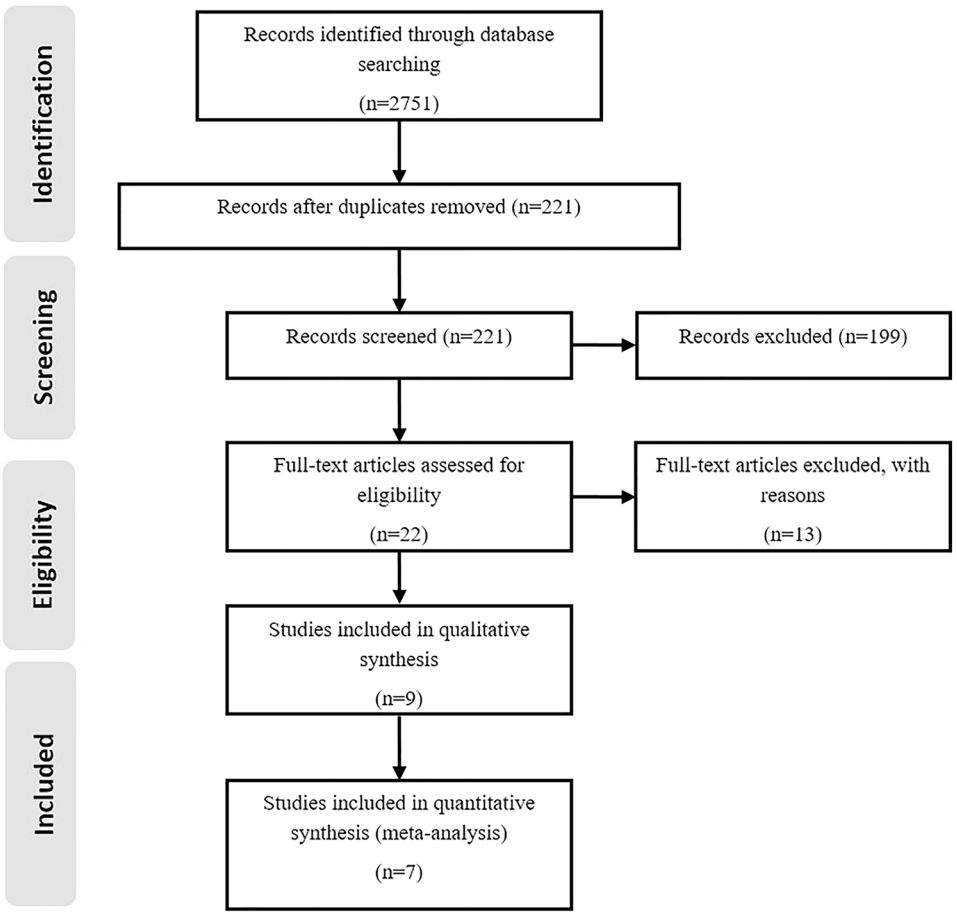

Fig. 1 Flow diagram of database search, trial selection and articles included in this meta-analysis

(Fig. 1). The main reason for article exclusion was insufficient data or incomplete outcome data. From the seven articles that met the inclusion criteria for the quantitative analysis (articles 1 to 7, Fig. 2), three considered more than one study (studies ii, iii, v, vi, ix, x and xi, from Fig. 2), performing a total of 11 studies. In two of the 11 studies, NRF2 expression was correlated with both OS and DFS (studies f, g, j and k, from Fig. 2), which gives a total of 13 studies included in the present review with meta-analysis (Fig. 2).

Included studies and their characteristics

The studies included in the analysis were published from 2014 to 2018 and all studies included evaluation of NRF2 expression in breast cancer patients and correlation of NRF2 expression with patient survival (OS and/or DFS). From the seven articles included in the present meta-analysis, four correlated NRF2 expression with OS and/or DFS in breast cancer patients recruited by the research team (non-public datasets) [21–24]. Wolf et al. [25] correlated NRF2 expression with OS and/or DFS in patients recruited for the study and also with data from public datasets [25]. Zhang et al. [26] and Bocci et al. [27] correlated NRF2 expression with OS and/or DFS using data from public datasets [26, 27].

The principal characteristics of the included studies that performed their own recruitment are outlined in Table 1a), namely author, year of publication, country, number of patients, sample size, mean age, stage/grade, follow-up time, method used for the evaluation of NRF2 expression, outcomes (OS and/or DFS) and NOS score. The data of studies that used public datasets are outlined in Table 1b), namely author, year of publication, country, number of patients, dataset, platform, dataset reference, outcomes (OS and/or DFS) and NOS score.

Risk of publication bias

The 11 eligible studies were evaluated, independently, by two investigators, according to NOS. In the present meta-analysis, six studies obtained a score of 7 and five obtained a score of 8, indicating a high quality for the majority of the studies. The NOS score found in the assessment of the risk of publication bias from the included studies are given in Table 1. It is important to note, however, that the assessment of quality of studies is a subjective task because it is based on the personal judgments of the review authors.

NRF2 and DFS

Concerning the meta-analysis results for DFS, seven studies were included with a total of 4852 patients enrolled. The forest plot for this outcome is represented in Fig. 3a). Compared to patients with low NRF2 expression, patients with overexpression of NRF2 had poorer DFS (HR = 1.79, 95% CI 1.07–3.01, p value = 0.03) and a high heterogeneity (Q value = 29.73; p value < 0.001; $I^2 = 79.57\%$) was observed for this outcome.

NRF2 and OS

The meta-analysis results for OS are summarized in Table 2 and graphically reported on Fig. 3b). For this outcome, six studies were considered, with a total of 2569 patients. It was possible to verify that a higher expression of NRF2 would have worse impact on OS (HR 1.82,

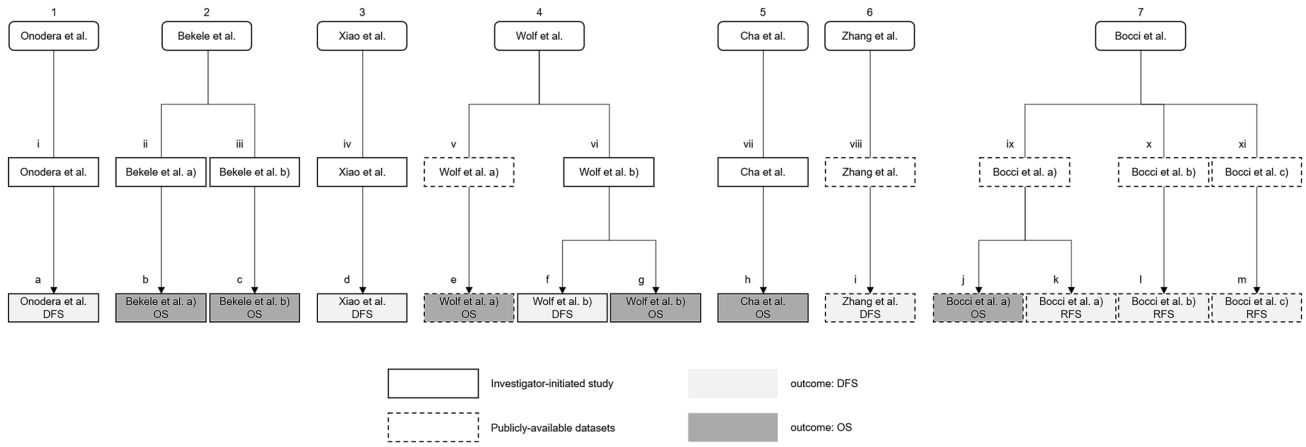

Fig. 2 Diagram of the included studies. In the meta-analysis, 7 studies were included , Bekele et al., Wolf et al. and Bocci et al. considered more than one study, studies ii, iii, v, vi, ix, x and xi, respectively, making a total of 11 studies. The studies of Wolf et al. b) and Bocci et al. a) evaluated both OS and DFS, studies f, g, j and k, respectively, performing a total of 13 different evaluations (from a to m)

Table 1 Characteristics of the studies included in this meta-analysis. a) Characteristics of the studies with recruitment of participants (non-public datasets) and b) characteristics of the studies that used public available datasets

a)

Study	Year of publication	Country	No. of patients	Sample size (NRF2 overexpression/NRF2 low expression)	Mean age	Stage/grade	Follow-up time mean (months)	Detection method	Outcomes of interest	NOS
Onodera et al.	2014	Japan	106	47/59	57 (31–81)	I–III	103	Immunohistochemical nuclear NRF2	DFS	8
Bekele et al. a)	2016	Canada	82	27/55	NR	I–III	NR	mRNA levels through microarray	OS	7
Bekele et al. b)	2016	Canada	94	42/52	NR	I–III	NR	mRNA levels through microarray	OS	7
Xiao et al.	2016	China	72	64/8	50 (28–75)	I–III	72	Immunohistochemistry	DFS	7
Wolf et al. b)	2016	Austria	176	62/114	NR	I–III	NR	mRNA expression levels by quantitative reverse-transcription PCR	DFS,OS	8
Cha et al.	2017	South Korea	126	16/110	NR	NR	NR	Immunohistochemistry	OS	8

b)

Study	Year of publication	Country	No. of patients	Dataset	Platform	Dataset Ref	Outcomes of interest	NOS score
Wolf et al. a)	2016	Austria	1942	METABRIC	NA	Curtis, et al. [30]	OS	8
Zhang et al.	2016	China	4142	Kmplot, 2015 version	NA	Győrffy et al. [31, 32]	DFS	8
Bocci et al. a)	2018	India	149	GSE10893	GPL1390	Weigman et al. [33]	OS RFS/DFS	7
Bocci et al. b)	2018	India	160	GSE18229	GPL1390	Weigman et al. [33]	RFS/DFS	7
Bocci et al. c)	2018	India	47	GSE2607	GPL1390	Weigman et al. [33]	RFS/DFS	7

NR not reported, *DFS* disease-free survival, *RFS* relapse-free survival, *OS* overall survival, *NOS* Newcastle–Ottawa Scale, *NA* not applicable

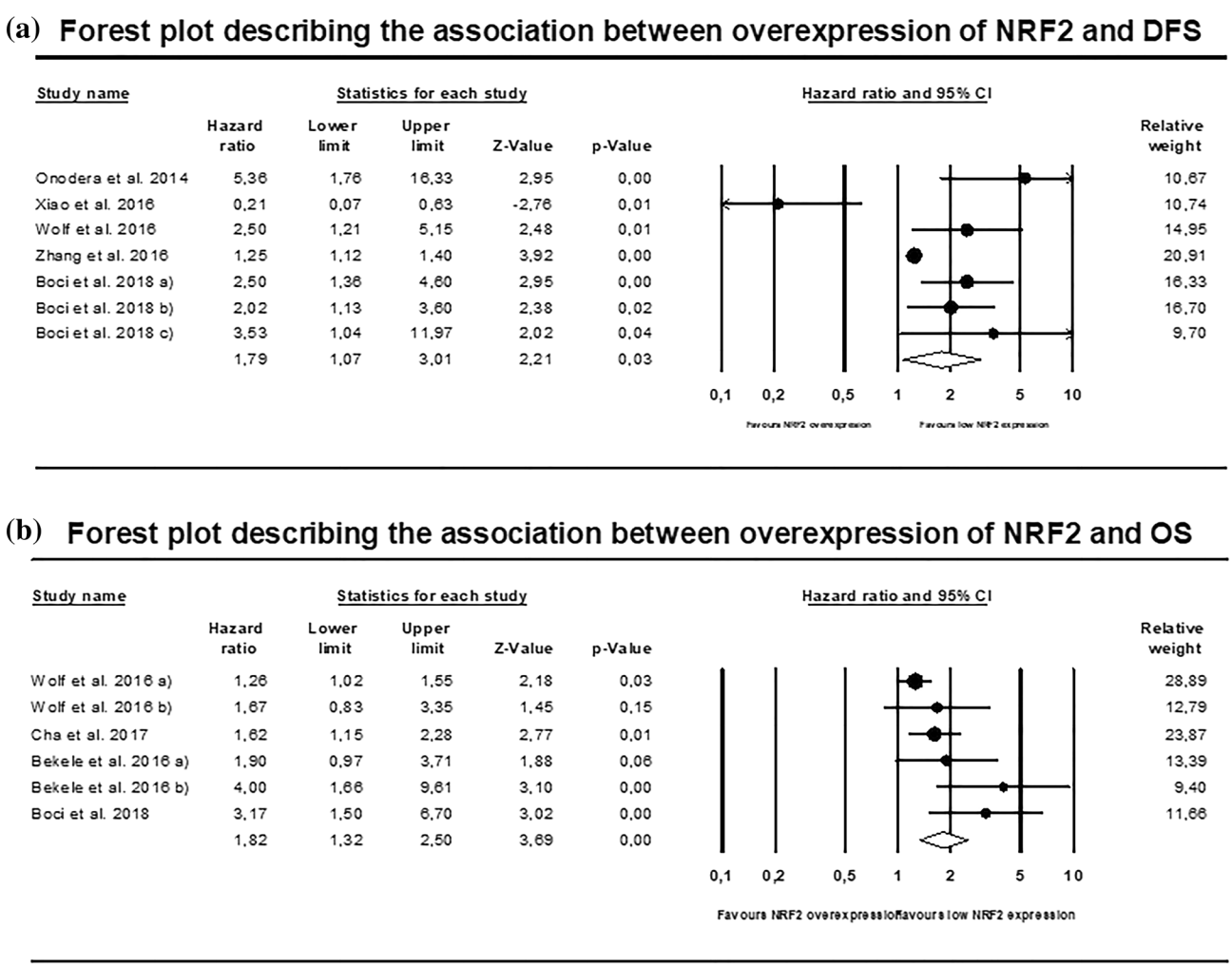

Fig. 3 **a** Forest plot for the association between NRF2 and disease-free survival. Heterogeneity: $\tau^2 = 0.33$; Q value = 29.73; df = 6; p value < 0.001; $I^2 = 79.57$. Test for overall effect: $z = 2.21$; p value = 0.03. **b** Forest plot for the association between NRF2 and overall survival. Heterogeneity: $\tau^2 = 0.08$; Q value = 12.11; df = 5; p value = 0.03; $I^2 = 58.72$. Test for overall effect: $z = 3.69$; p value < 0.001

Table 2 Meta-analysis results

Outcomes	Number of studies	I^2 (%)	WHR		
			Observed		Adjusted
			95% CI	p value	95% CI
DFS	7	79.57	1.79 (1.07–3.01)	0.03*	1.43 (0.89–2.30)
OS	6	58.72	1.82 (1.32–2.50)	< 0.0001*	1.41 (1.02–1.96)

WHR weighted hazard ratio, *CI* confidence interval, *DFS* disease-free survival, *OS* overall survival

*Indicates a significant result

95% CI 1.32–2.50, p value < 0.0001). There is a moderate inconsistency across the findings of the studies (Q value = 12.11; p value = 0.03; $I^2 = 58.72\%$), in other words, 58.72% of the observed dispersion is real, not spurious.

Sensitivity analyses

The sensitivity analysis was performed by excluding one or more studies from the analysis to evaluate how this affected

the results. The results showed that the pooled effect of NRF2 expression on outcomes did not change substantially if a single or few studies were omitted (data not shown). Overall, the sensitivity analysis demonstrated that this meta-analysis findings on the effect of NRF2 expression in breast cancer outcomes are robust.

Publication bias

In order to evaluate the publication bias, there were generated funnel plots for DFS (Fig. 4a) and OS (Fig. 4b), considering the Trim and Fill adjustment. In the funnel plots (Fig. 4), it can be identified in blue, the observed studies, and in red, the necessary imputed studies to obtain absence of bias. It can be observed that for both DFS and OS, Fig. 4a and b), respectively, there are more studies on the right than on the left, so for each one of these measures, several studies are imputed on the left to "adjust"

Table 3 Assessment of publication bias for the impact of NRF2 expression on OS and DFS

Outcomes	Egger's regression test			
	95% CI	*t*	df	*p* value
DFS	− 1.52 to 3.99	1.16	5	0.30
OS	0.86 to 4.14	4.23	4	0.01

CI confidence interval, *df* degrees of freedom, *DFS* disease-free survival, *OS* overall survival

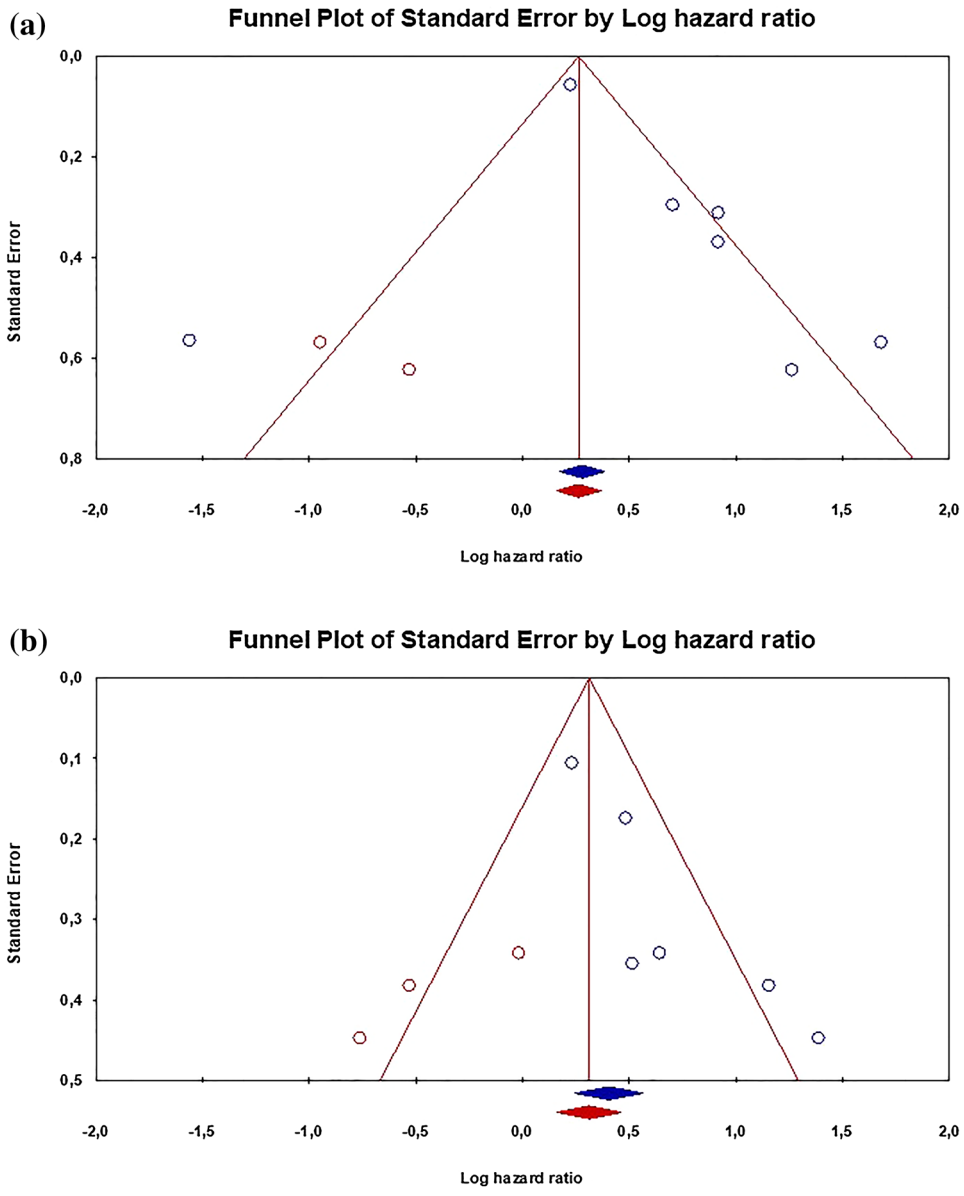

Fig. 4 Funnel plots were generated for (**a**) disease-free survival and (**b**) overall survival, considering the Trim and Fill adjustment

the funnel plots for the absence of publication bias. The "adjusted" HR estimates shifted (Table 2).

The presence of publication bias was also explored using Egger's regression test (Table 3). This test did not indicate evidence of publication bias for the effect of NRF2 expression for the DFS (p value = 0.30): however, there is evidence of publication bias for OS (p value = 0.01).

Subgroup analysis

In the present meta-analysis, as already referred, two types of data were included: data from public available datasets and data from patients recruited by the research team. Thus, we performed an analysis of NRF2 expression with DFS and OS independently in studies with data from public datasets and with no public datasets, as represented in Table 4. For DFS, four studies from public available datasets were included and data of NRF2 expression and DFS were correlated and the results were of HR 2.02, 95% CI 0.98–4.16, p value = 0.06, $I^2 = 69\%$. For DFS, with data from non-public datasets, three studies were included and the results were of HR 1.49, 95% CI 0.59–3.79, p value = 0.40, $I^2 = 89.41\%$. For OS, two studies of public datasets and four studies from non-public datasets were included. Correlating NRF2 expression with OS, the results obtained were of HR 1.38, 95% CI 1.08–1.76, p value = 0.01, $I^2 = 34.09\%$ and of HR 2.38, 95% CI 1.62–3.49, p value < 0.0001, $I^2 = 10.14\%$, respectively, for public and non-public datasets.

Table 4 Subgroup analysis

Outcome	WHR			
	Number of studies	95% CI	p value	I^2 (%)
DFS	7	1.79 (1.07–3.01)	0.03*	79.57
Data type				
DFS–Non-public dataset	3	1.49 (0.59–3.79)	0.40	89.41
DFS–Public dataset	4	2.02 (0.98–4.16)	0.06	69.00
OS	6	1.82 (1.32–2.50)	< 0.0001*	58.72
Data type				
OS–Non-public dataset	4	2.38 (1.62–3.49)	< 0.0001*	10.14
OS–Public dataset	2	1.38 (1.08–1.76)	0.01*	34.09

WHR weighted hazard ratio, *CI* confidence interval, *DFS* disease-free survival, *OS* overall survival

*Indicates a significant result

Discussion

NRF2 is known for its essential role in cytoprotection, under the presence of aggressions like oxidative stress and xenobiotics, and it protects cells, inducing the expression of several genes, among them phase II enzymes [9]. On this regard, NRF2 is considered a main regulator of oxidative stress, and its overexpression protects cells and prevents the initiation of carcinogenesis, being considered a tumour suppressor [7]. However, NRF2 overexpression on cancer cells may lead to cancer cells protection, protecting them from oxidative stress and ultimately leading to resistance to therapy [7, 9], being urgent to understand the clinical outcomes of NRF2 expression in breast cancer tissue. Thus, we performed a major literature review, in order to identify studies that correlated NRF2 expression in breast cancer tissue and correlated it with clinical outcomes (DFS and OS).

The electronic search on the role of NRF2 expression on breast cancer resulted in the identification of seven publications, corresponding to a total of 7096 patients [21–27]. The seven identified publications consisted in different approaches: four of the publications studied NRF2 expression in breast cancer tissue from patients recruited for the study [21–24]; Wolf et al. [25] used not only data from patients recruited for the study, but also from public available datasets [25] and two publications used data from publicly available datasets [26, 27]. Due to the fact that the included studies consisted in the use of public datasets and non-public datasets, the results were analysed in two ways: correlation of NRF2 expression with DFS and OS was performed taking into account all the studies, independently of using public or non-public datasets (Fig. 3; Table 2), and on Table 4, the correlation of NRF2 expression with DFS and OS was performed separately, for studies with public datasets and for studies of non-public datasets.

The statistical treatment of data, taking into account all the studies, indicates that NRF2 overexpression in breast cancer is correlated with low DFS (HR 1.79, 95% CI 1.07–3.01, p value = 0.03) and with low OS (HR 1.82, 95% CI 1.32–2.50, p value < 0.0001). Observing the funnel plots (Fig. 4), it is possible to verify that publication bias cannot be completely excluded. These combined results indicate that some factors may be influencing the results of this meta-analysis. On what concerns the sensitivity analysis, the pooled effects of the NRF2 expression on the defined outcomes of this study did not change substantially if a single or a few studies were omitted. This indicates that the findings now achieved, regarding the prognostic value of NFR2 expression in breast cancer patients, are robust.

Posteriorly, the statistical treatment of data was performed separately for studies with public datasets and

non-public datasets (Table 4). Data from four studies of public datasets were evaluated for NRF2 expression and DFS with the results of HR 2.02, 95% CI 0.98–4.16, p value = 0.06, $I^2 = 69\%$, and data from three studies of non-public datasets were evaluated for NRF2 expression and DFS with the results of HR 1.49, 95% CI 0.59–3.79, p value = 0.40, $I^2 = 89.41$. These results differ from the ones where the studies were evaluated together, independently of the origin of the database, probably due to a bias, because the follow-up time differs from study to study. On what concerns to the correlation of NRF2 expression and OS, two studies of public datasets were evaluated with the results of HR 1.38, 95% CI 1.08–1.76, p value = 0.01, $I^2 = 34.09\%$ and four studies of non-public datasets were evaluated HR 2.38, 95% CI 1.62–3.49, p value < 0.0001, $I^2 = 10.14\%$. These results for the correlation between NRF2 expression and OS are in accordance with the results of the combined studies.

Our results are in accordance with the ones obtained by Wang et al. [11] and by Guo and Shen [10]: both meta-analyses verified that NRF2 overexpression, in several types of cancers, is correlated with poor outcomes [10, 11]. These results are of main importance due to the need to understand the role of NRF2 in cancer. Activation of NRF2 and its translocation to the nucleus leads to the induction of phase II detoxifying enzymes and antioxidants, resulting in cytoprotection and acting as a tumour suppressor [5]. In tumour environment, NRF2 might become excessively activated, NRF2 saturation in the nucleus might compromise the complex NRF2-KEAP1 and the consequently proteasome degradation of NRF2 [28]. Excessive amounts of NRF2 in the nucleus may lead to tumour cells protection due to the transcription of its downstream genes, protecting cancer cells from oxidative stress [5]. One of the main problems in breast cancer is therapeutic resistance, Bekele et al. [21] found that NRF2 overexpression in breast cancer patients treated with Tamoxifen is related with lower OS [21]. These results might be explained by the excessive presence of NRF2 in the nucleus, resulting in cytoprotection of cancer cells, leading to poor outcomes.

Our results might also be explained by this assumption, excessive NRF2 in breast cancer cells nucleus protects cancer cells, leading to tumour progression and resistance [5, 29]. In the present meta-analysis, we conclude that breast cancer patients with overexpression of NRF2 had poorer survival, irrespective to overall survival and disease-free survival, once compared to breast cancer patients whose tumour cells express low NRF2 expression. Thus, optimizing treatment for patients with NRF2 overexpression may improve their survival. To our knowledge, it is the first time that a meta-analysis correlating NRF2 overexpression in breast cancer patients with OS and DFS is performed. Overall, this systematic review with meta-analysis shows that NRF2 overexpression is related with worse prognostic for breast cancer. Future research should focus on establishing NRF2 as a marker for breast cancer prognosis and improving treatment efficacy for patients with NRF2 overexpression, eventually taking into account the different breast cancer's subtypes, since this may improve their survival.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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Chapter 7

Somatic Mutations in KEAP1-NRF2 complex in Breast Cancer

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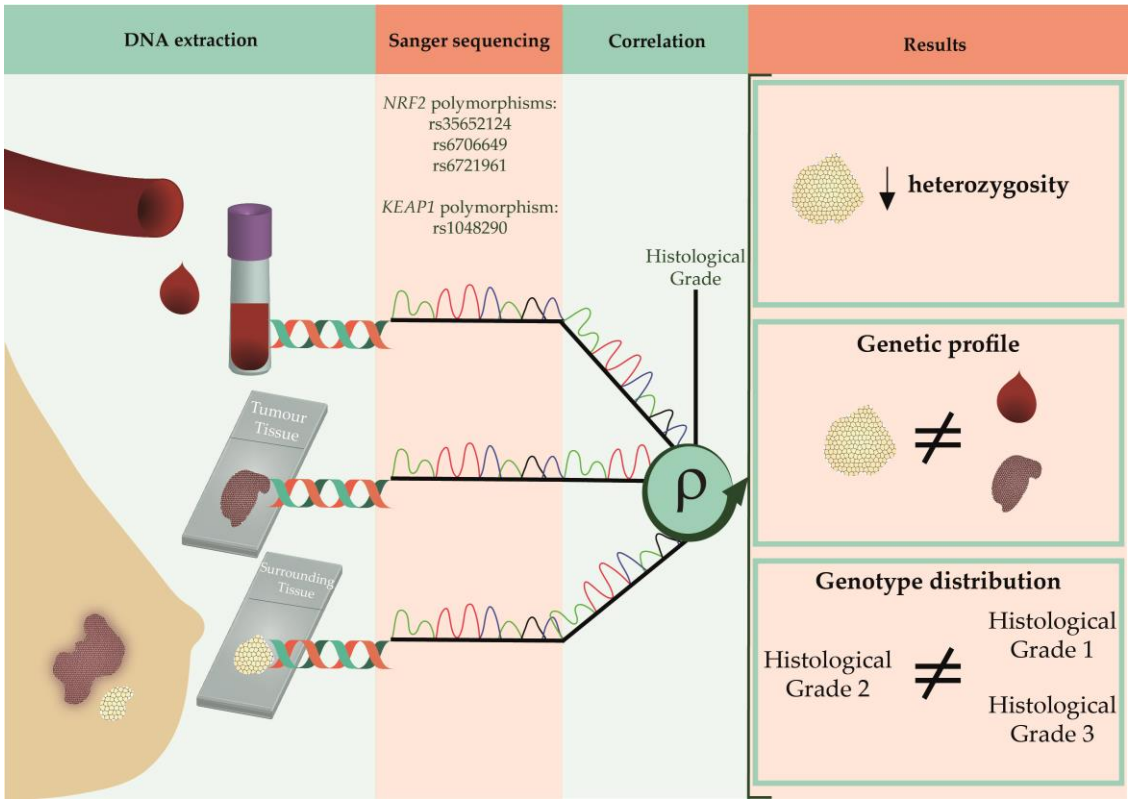
Graphical Abstract



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Article

Somatic Mutations in KEAP1-NRF2 Complex in Breast Cancer

Micaela Almeida [1,2,3,*], Catarina L. Ferreira [1,2], Rosa Maria Tomé [2,4], José Fonseca-Moutinho [1,2,4], António Polónia [3,5], Ana Cristina Ramalhinho [1,2,4] and Luiza Breitenfeld [1,2]

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Simple Summary: Breast cancer remains a burden for women worldwide. Among the factors that contribute to breast cancer development is estrogen exposure during one's lifetime, both endocrine and exocrine. Polymorphisms in KEAP1-NRF2 complex, the master regulator of oxidative stress metabolism, have been correlated with a poorer outcome. The aim of our study was to identify and correlate polymorphisms in this complex, in breast cancer tissue, benign surrounding tissue and in peripheral blood. We verified a tendency towards the loss of heterozygosity in the benign surrounding tissue when compared to blood and to tumour tissue. Correlating with clinical data, histologic grade 2 has a higher variability of genotypes. The results indicate a heterogeneous and active microenvironment. Therefore, clinical approaches would benefit from the evaluation of somatic mutations in this complex. Moreover, further studies should be developed in order to evaluate the predictive value of the histologic grades, taking into consideration the genetic profile.

Abstract: Breast cancer remains the leading cause of cancer deaths for women. Long-term estrogen exposure is considered carcinogenic due to semiquinone production and to compromised detoxification. Metabolic regulator polymorphisms, such as *KEAP1* (rs1048290) and *NRF2* (rs35652124, rs6721961, rs6706649), can be valuable in understanding the individual cytoprotection profile. Thus, we aim to genotype these polymorphisms in blood, tumours and surrounding tissue, to identify somatic mutations and correlate it to prognoses. A total of 23 controls and 69 women with histological confirmed breast cancer were recruited, and DNA from blood/surrounding/tumour tissue was genotyped. Genotyping and clinicopathological data were correlated. We verified that rs35652124 presents different genotype distribution between the blood/surrounding tissue (p-value = 0.023) and tumour/surrounding tissues (p-value = 0.041). Apart from rs35652124 and considering the histological grade, the other four polymorphisms have different distributions among different tissues. There is a tendency towards the loss of heterozygosity in the surrounding tissue when compared to blood and tumour tissues, and higher genotype variability in histologic grade 2. These somatic mutations and different distribution patterns may indicate a heterogeneous and active microenvironment, influencing breast cancer outcome. Additionally, it would be pertinent to evaluate the predictive value of the histologic grade 2 considering somatic mutation profiles and distributions.

Keywords: breast cancer; NRF2; KEAP1

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1. Introduction

Breast cancer remains the type of cancer with the highest incident rate in women worldwide and the leading cause of cancer death in women in 123 countries [1]. The development of breast cancer is due to several factors, among them endogenous and exogenous hormones exposure, compromised immunological system, metabolic imbalance, oxidative stress and genetic alterations [2,3].

Estrogen exposure is widely described as a carcinogenic factor for breast cancer development, which may be due to endocrine or exocrine estrogens [4]. Genomic instability and somatic mutation acquisition might allow for the carcinogenic effect of estrogen, whether through increased estrogen levels or through inefficient estrogens detoxification due to a compromised metabolism [5,6].

The single nucleotide polymorphisms (SNPs) in the metabolic pathway of estrogen might compromise the induction of phase II detoxifying enzymes, responsible for the transformation of endogenous and exogenous compounds into a more excretable form [7]. Low levels of phase II enzymes result in an estrogen increase, contributinsg to quinone accumulation and eventually leading to carcinogenesis [7]. Previous studies performed by our research team indicate that the metabolic imbalances due nuclear factor Erythroid 2-related Factor 2 (NRF2) and Kelch-Like ECH Associated Protein 1 (KEAP1) lead to poor outcomes in breast cancer patients [8,9].

The transcription factor NRF2 is encoded by the *NF2EL2* gene, commonly known as the *NRF2* gene, located at chromosome 2 (2q31.2) [10]. The negative regulator of NRF2 is KEAP1 encoded by the *KEAP1* gene, located at chromosome 19 (19p13.2) [11]. The complex KEAP1-NRF2 is a major regulator of phase II metabolism. In the presence of radicals produced by internal or external aggressions, the NRF2 that normally binds to KEAP1 in the cytoplasm translocates to the nucleus and binds to antioxidant responsive elements (AREs), small Maf proteins and DNA, leading to the release of phase II enzymes [12]. The reactive oxygen species are then eliminated and a basal state is achieved. KEAP1 acts as a negative regulator of NRF2. Once it enters the nucleus it binds NRF2 and takes it back to the cytoplasm to suffer proteasome degradation [13].

However, SNPs in *NRF2* and/or *KEAP1* might interfere with the function of the complex. The mutated allele of rs1048290 (C > G) polymorphism in *KEAP1* increases KEAP1 expression and increases the capability of KEAP1 to bind NRF2, leading to decreased levels of NRF2 in the nucleus, with a consequent decrease in detoxification enzymes and to a metabolic imbalance due to a compromised metabolism [9,14,15]. The rs35652124 (A > G), rs6721961 (C > A) and rs6706649 (G > A) polymorphisms of *NRF2* also exert a similar effect on the metabolism. These polymorphisms are related to a lower expression of NRF2 levels, leading to a decrease in the expression of phase II enzymes and consequently compromising cytoprotection [9,16]. The effects of these polymorphisms in genomic DNA extracted from peripheral blood have been described in several pathologies [17,18]. However, on what concerns breast cancer, fewer studies have been performed. Hartikainen et al., in 2015, associated the carriers of the altered allele of rs1048290 with a higher KEAP1 expression and shorter relapse-free survival [14]. Relative to *NRF2*-selected polymorphisms, women with a homozygous (AA) genotype of rs6721961 had an increased risk of breast cancer development [16]. These polymorphisms are related to a higher expression of KEAP1, increasing the binding of KEAP1 to NRF2, and to low levels of NRF2. Both polymorphisms, studied by Hartikainen and colleagues, might be related to breast cancer development once cytoprotection is compromised due to low levels of NRF2 in the nucleus, leading to lower induction of phase II enzymes [14,16].

Nevertheless, there is a lack of studies evaluating the presence/absence of the altered allele of these polymorphisms in tumour tissue and in the surrounding tissue. In this regard, we aim to evaluate and compare the genotype of these SNPs of *KEAP1* (rs1048290) and of *NRF2* (rs35652124, rs6721961, rs6706649) in blood, surrounding tissue and tumour tissue, and correlate it with clinicopathological data to evaluate a possible relation with breast cancer prognosis.

2. Materials and Methods

2.1. Study Population

The participants recruited for the study were women followed by the Child and Women Health Department, Gynaecology Oncology Division of Cova da Beira Local Health Unit, Covilhã, Portugal, who agreed to take part in the study and signed the informed consent form. The recruitment period was from December of 2016 until June of 2020. The study was approved by the Institutional Review Board of Cova da Beira Local Health Unit with the numbers n.º 28/2008 and an addendum in 17 August 2016; further approval for new data acquisition, n.º 20/2023.

A total of 92 women were recruited. The inclusion criteria for the control group were women 18 years old or older, with no previous history of breast cancer and without family history of cancers. The exclusion criteria were women with amenorrhea, previous history of hysterectomy or oophorectomy.

For the patient group, 69 women were recruited. The inclusion criteria were women 18 years old or older that underwent fine needle biopsy and had a breast cancer diagnosis. The exclusion criteria were women with amenorrhea, previous history of hysterectomy or oophorectomy. After the tumour was surgically removed and analysed by pathology, it was found that six cases were not hormone-dependent breast cancers (ER^+) and were therefore excluded from the study. Thus, the total number of breast cancer patients included in the study for further analysis was 63.

2.2. Sample Collection

The samples of the patients consisted of peripheral whole blood and slides from the tumour and from the surrounding tissue of the breast cancer patients.

The peripheral whole blood was collected by venous puncture to further DNA extraction.

Through hematoxyline–eosine slides, the medical pathologist of the research team identified the blocks with tumour tissue and with surrounding tissue. For each patient there were selected two blocks, one with tumour tissue and another one with surrounding tissue.

At the Anatomic Pathology Service of Local Health Unit of Cova da Beira, 10 μm slides from each block were cut and assembled.

2.3. DNA Extraction from Peripheral Blood

The Wizard Genomic DNA purification kit (Promega) was used to isolate DNA from peripheral blood collected on EDTA tubes. The manufacturer's instructions were followed, and the DNA extraction sample was stored at 4 ºC.

2.4. DNA Extraction from Paraffin-Embeded Slides

Prior to DNA extraction, hematoxylin and eosin (H&E) staining was performed in order to evaluate the integrity of the morphology. H&E staining allowed us to carry out a morphological control, verifying the integrity of the morphology and the presence of tumour tissue and of benign surrounding tissue. For DNA extraction from paraffin-embedded slides, an extraction protocol was optimized. Firstly, in order to deparaffinizate, the slides were fully submerged in xylene on a 50 mL falcon. Incubation was performed at room temperature for 7 min. After, the slides were submerged in absolute ethanol for 5 min at room temperature. The slides were left to air-dry for 5 to 10 min. All the material was carefully manipulated in order to avoid cross-contamination. The tissue was then scraped from the slide.

The tissue from each slide was scraped off with a new scalpel and placed in previously identified 1.5 mL microtubes. After paraffin removal, the samples were ready for genomic DNA extraction, which was performed using the QIAmp DNA FFPE Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions). The

DNA extraction from paraffin-embedded slides was performed on an extraction cabinet and filtered tips were used to avoid contamination.

Following the protocol, the DNA samples were stored at 4 °C once the real-time PCR was performed, since the real-time PCR was carried out within the time period in which the samples could be stored at 4 °C. They were subsequently stored at −20 °C.

2.5. NRF2 and KEAP1 Sequencing

The amplification of the regions of interest of *NRF2* and of *KEAP1* from genomic DNA extracted from peripheral blood were amplified by conventional PCR as described by Almeida, M. et al., 2019 [9].

The amplification of the fragments of *NRF2* and *KEAP1* were performed using Real-Time PCR and each master Mix had a total volume of 20 μL, containing 0.3 μM of each primer, 10 μL of Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and 100 ng of genomic DNA.

For the polymorphisms rs35652124, rs6721961 and rs6706649 of *NRF2* the primer set was the same one used for conventional PCR. The Real-Time PCR conditions were as follows: initial incubation at 95 °C for 10 min, followed by 70 cycles at 95 °C for 10 s, 60 °C for 10 s and at 72 °C for 20 s, finally the temperature of the reaction mixture was increased up to 95 °C at a rate of 0.1 °C/s, starting at 68 °C for 15 s.

For *Keap1* rs1048290 polymorphism the primer sets were designed with Primer3Plus:

Forward primer: 5′-TTGCAGGTATGAGCCAGAGC-3′;
Reverse primer: 5′-GATGGTAGGGGGTGTTCCTG-3′.

The Real-Time PCR conditions were 95 °C for 10 min, 70 cycles at 95 °C for 10 s, 57 °C for 10 s and at 72 °C for 20 s. The reaction mixture temperature was increased up to 95 °C at a rate of 0.1 °C/s, starting at 68 °C for 15 s.

The amplified fragments of *NRF2* and *KEAP1* were sequenced through Sanger sequencing (performed by STABVIDA), and the sequencing results were analysed in ChromasPro version 2.1. Three researchers evaluated the sequencing results independently and 10% of the samples was randomly selected and re-evaluated. All results were in accordance.

2.6. Statistical Analysis

The statistical analysis was performed using IBM SPSS statistics version 25. A Chi-squared test was performed, considering a statistical significance when p-value was <0.05.

3. Results

For the present study, a total of 23 controls and 63 breast cancer patients were included. The characteristics of the study population are described in Table 1.

Table 1. Characteristics of the studied population.

Parameter		*n* (%)
	Controls	
Total		23 (100)
Age		
	Mean	52.70
	Minimum	24
	Maximum	87
	Breast Cancer Patients	
Total		63 (100)
Age		
	Mean	65.08
	Minimum	36

Maximum	95
ER status	
Positive	63 (100)
Negative	0
PR status	
Positive	48 (76.2)
Negative	14 (22.2)
Missing	1 (1.6)
HER2 status	
Positive	8 (12.7)
Negative	54 (85.7)
Missing	1 (1.6)
Histological Grade	
1	12 (19)
2	36 (57.1)
3	15 (23.8)

All genomic DNA, extracted from peripheral blood, was sequenced for rs35652124, rs6706649 and rs6721961 polymorphisms of *NRF2* and for rs1048290 of *KEAP1*.

The Hardy–Weinberg equilibrium was calculated for the control and patient genotypes. Breast cancer risk was also calculated (Table 2).

Table 2. Hardy–Weinberg equilibrium (HWE) and breast cancer risk for the polymorphisms under study in blood samples.

SNP	Controls *n* (%)	HWE	Cases *n* (%)	HWE	OR (95% CI) [1]	*p*-Value
	23 (100)		63 (100)			
NRF2						
rs35652124						
AA (wt)	13 (56.52)		33 (52.38)		Ref.	
AG	8 (34.78)	0.895	26 (41.27)	0.932	1.280 (0.462–3.550)	0.634
GG	2 (8.70)		4 (6.35)		0.788 (0.128–4.837)	0.796
rs6706649						
GG (wt)	19 (82.61)		50 (79.37)		Ref.	
GA	3 (13.04)	0.293	12 (19.05)	0.961	1.520 (0.386–5.988)	0.547
AA	1 (4.35)		1 (1.59)		0.380 (0.023–6.386)	0.486
rs6721961						
CC (wt)	18 (78.26)		48 (76.19)		Ref.	
CA	5 (21.74)	0.843	14 (22.22)	1	1.050 (0.331–3.336)	0.934
AA	0		1 (1.59)		NA	0.541
KEAP1						
rs1048290						
CC (wt)	8 (34.78)		18 (28.57)		Ref.	
CG	12 (52.17)	0.901	36 (57.14)	0.417	1.333 (0.463–3.843)	0.594
GG	3 (13.04)		9 (14.29)		1.333 (0.283–6.279)	0.715

[1] OR, odds ratio; CI, confidence interval.

The Hardy–Weinberg equilibrium is similar for the populations in the study (controls and cases, p-value ≥ 0.05). In terms of assessing the risk for the carrier allele, it was found that none of the genotypes under study had an increased risk value for developing breast cancer (p-value ≥ 0.05).

In addition to peripheral blood, we obtained samples of tumour surrounding tissue and tumour tissue for each woman . Sequencing was performed for each (blood, surrounding and tumour tissues), although we were not able to obtain DNA from all

tissues of each sample. For DNA extracted from paraffin-embedded slides the success rate was not the same. For *NRF2*: 26 (92.9%), samples of surrounding tissue were successfully amplified and 22 (78.6%) samples of tumour tissue were amplified (Table 3). The *KEAP1* fragment was successfully amplified in 23 (82.1%) and in 19 (67.9%) surrounding and tumour tissue samples, respectively. Therefore, from a total of 28 breast cancer cases, 16 were successfully genotyped for the three types of tissue (blood, surrounding and tumour tissue) for *NRF2* SNPs (rs35652124, rs6706649 and rs6721961) and for rs1048290 of *KEAP1*.

The study of the allele and genotype frequencies lead us to include only cases that have been simultaneously amplified in blood, surrounding tissue and tumour tissue for this analysis. Therefore, 21 cases were successfully amplified, for all tissues, for rs35632124, rs6706649 and rs6721961 of *NRF2*. The Hardy–Weinberg equilibrium was calculated for each polymorphism for each population of tissues, taking into account the genotype frequencies (Table 4) and allele frequency (Table 5).

Table 3. Hardy–Weinberg equilibrium for each genotype in the different tissues, for the polymorphisms in the study of *NRF2* and *KEAP1*.

SNP	Blood *n* (%)	HWE	Surrounding Tissue *n* (%)	HWE	Tumour Tissue *n* (%)	HWE
	21 (100)		21 (100)		21 (100)	
NRF2						
rs35652124						
AA (wt)	11 (52.39)		14 (66.67)		12 (57.14)	
AG	9 (42.85)	0.884	4 (19.05)	0.094	8 (38.09)	0.974
GG	1 (4.76)		3 (14.29)		1 (4.76)	
rs6706649						
GG (wt)	19 (90.48)		15 (71.43)		16 (76.19)	
GA	2 (9.52)	0.974	1 (4.76)	<0.001 *	4 (19.05)	0.595
AA	0		5 (23.81)		1 (4.76)	
rs6721961						
CC (wt)	17 (80.95)		19 (90.48)		18 (85.71)	
CA	4 (19.05)	0.890	2 (9.52)	0.974	3 (14.29)	0.940
AA	0		0		0	
KEAP1	16 (100)		16 (100)		16 (100)	
rs1048290						
CC (wt)	5 (31.25)		7 (43.75)		5 (31.25)	
CG	9 (56.25)	0.802	8 (50)	0.807	9 (56.25)	0.802
GG	2 (12.5)		1 (6.25)		2 (12.5)	

* Indicates a significant result.

As can be seen in Table 3, there is a significant difference (p-value < 0.001) concerning rs6706649. This polymorphism has a different frequency distribution in the surrounding tissue (Hardy–Weinberg equilibrium is not observed).

Table 4 allows us to compare the allele frequency of the three tissues studied (blood, surrounding tissue and tumour tissue).

Table 4. Allele frequencies for the polymorphisms in study of *NRF2* and *KEAP1*.

	Blood ***n*** **(%)**	**Surrounding Tissue** ***n*** **(%)**	***p*-Value OR (95% CI)**	**Surrounding Tissue** ***n*** **(%)**	**Tumour Tissue** ***n*** **(%)**	***p*-Value OR (95% CI)**	**Blood** ***n*** **(%)**	**Tumour Tissue** ***n*** **(%)**	***p*-Value OR (95% CI)** [1]
NRF2									
rs35652124									
A	31 (36.9)	32 (38.1)	0.801	32 (38.1)	32 (38.1)	>0.999	31 (36.9)	32 (38.1)	0.801
G	11 (13.1)	10 (11.9)	0.881 (0.328–2.367)	10 (11.9)	10 (11.9)	1 (0.366–2.730)	11 (13.1)	10 (11.9)	0.881 (0.328–2.367)
rs6706649									
G	40 (47.6)	31 (36.9)	0.007 *	31 (36.9)	36 (42.9)	0.175	40 (47.6)	36 (42.9)	0.137
A	2 (2.4)	11 (13.1)	7.097 (1.465–34.384)	11 (13.1)	6 (7.1)	0.470 (0.156–1.418)	2 (2.4)	6 (7.1)	3.333 (0.632–17.574)
rs6721961									
C	38 (45.2)	40 (47.6)	0.397	40 (47.6)	39 (46.4)	0.645	38 (45.2)	39 (46.4)	0.693
A	4 (4.8)	2 (2.4)	0.475 (0.082–2.746)	2 (2.4)	3 (3.6)	1.538 (0.244–9.714)	4 (4.8)	3 (3.6)	0.731 (0.153–3.485)
KEAP1									
rs1048290									
C	19 (29.7)	22 (34.4)	0.434	22 (34.4)	19 (29.7)	0.434	19 (29.7)	19 (29.7)	>0.999
G	13 (20.3)	10 (15.6)	0.664 (0.238–1.857)	10 (15.6)	13 (20.3)	1.505 (0.539–4.207)	13 (20.3)	13 (20.3)	1 (0.369–2.712)

[1] OR, odds ratio; CI, confidence interval; * indicates a significant result.

As we might observe, the only significant difference observed is related to the rs6706649 of *NRF2* (p-value = 0.007). There is a great number of A allele carriers (mutated allele) when compared with the wild-type allele (G).

When the distribution of the alleles is compared with tumour tissue no significant differences are verified.

The genotypes frequencies were correlated for the different tissues in order to verify if the genotype distribution is the same between the different types of tissue.

The genotypes frequency between the surrounding tissue and the blood is significantly different for rs35652124 (p-value = 0.023) (Table 5). There is a decrease in the heterozygous genotype frequency from blood (42.9%) to the surrounding tissue (19%). There is also a significant difference between the genotype frequency of the tumour and of the surrounding tissues (p-value = 0.041). There is a decrease in the homozygous genotype frequency and an increase in the heterozygous genotype in tumour tissue, which we can also observe in blood tissue.

For the remaining polymorphisms under study, no statistical significance was found. However, there seems to be a trend: the surrounding tissue has a different genotypes distribution when compared to other tissues (blood and tumour).

Table 5. Genotype frequency Hardy–Weinberg equilibrium for each genotype in different tissues of origin, for the polymorphisms in study of *NRF2* and *KEAP1*.

Type of Tissue	Genotypes Frequency *n* (%)			*p*-Value	
	NRF2—rs35652124				
	AA	AG	GG		
Blood	11 (52.40)	9 (42.90)	1 (4.80)	Ref.	
Surrounding Tissue	14 (66.70)	4 (19)	3 (14.30)	0.023 *	Ref.
Tumour Tissue	12 (57.10)	8 (38.10)	1 (4.80)	0.950	0.041 *
	NRF2—rs6706649				
	GG	GA	AA		
Blood	19 (90.5)	2 (9.5)	0	Ref.	
Surrounding Tissue	15 (71.4)	1 (4.8)	5 (23.8)	0.055	Ref.
Tumour Tissue	16 (76.2)	4 (19)	1 (4.80)	0.382	0.105
	NRF2—rs6721961				
	CC	CA	AA		
Blood	17 (81)	4 (19)	0	Ref.	
Surrounding Tissue	19 (90.5)	2 (9.5)	0	0.378	Ref.
Tumour Tissue	18 (85.7)	3 (14.3)	0	0.679	0.634
	KEAP1—rs1048290				
	CC	CG	GG		
Blood	5 (31.3)	9 (56.3)	2 (12.5)	Ref.	
Surrounding Tissue	7 (43.8)	8 (50)	1 (6.3)	0.696	Ref.
Tumour Tissue	5 (31.3)	9 (56.3)	2 (12.5)	> 0.999	0.696

* Indicates a significant result.

Based on the above results, it was considered pertinent to study the relationship between the distributions of the different genotypes in the tissues with clinicopathological factors, such as histologic grade (Table 6).

Table 6. Genotype frequency distribution in each one of the tissues and correlation with the breast cancer histological grade.

Type of Tissue	Overall % in Each Histological Grade			*p*-Value	
Blood	*NRF2*—rs35652124				
Histological Grade	AA	AG	GG		
1	3 (42.9)	4 (57.1)	0	Ref.	
2	6 (54.5)	4 (36.4)	1 (9.1)	0.288	Ref.
3	2 (66.7)	1 (33.3)	0	0.390	0.307
Surrounding Tissue					
Histological Grade					
1	3 (42.9)	2 (28.6)	2 (28.6)	Ref.	
2	9 (81.8)	1 (9.1)	1 (9.1)	0.393	Ref.
3	2 (66.7)	1 (9.1)	0	0.188	<0.001 *
Tumour Tissue					
Histological Grade					
1	4 (57.1)	3 (42.9)	0	Ref.	
2	6 (54.5)	4 (36.4)	1 (9.1)	0.809	Ref.
3	2 (66.7)	1 (33.3)	0	0.023 *	0.307
Blood	*NRF2*—rs6706649				
Histological Grade	GG	GA	AA		
1	6 (85.7)	1 (14.3)	0	Ref.	
2	11 (100)	0	0	<0.001 *	Ref.
3	2 (66.7)	1 (33.3)	0	<0.001 *	<0.001 *
Surrounding Tissue					
Histological Grade					
1	5 (71.4)	1 (14.3)	1 (14.3)	Ref.	
2	9 (81.8)	0	2 (18.2)	0.114	Ref.
3	1 (33.3)	0	2 (66.7)	<0.001 *	<0.001 *
Tumour Tissue					
Histological Grade					
1	5 (71.4)	1 (14.3)	1 (14.3)	Ref.	
2	9 (81.8)	2 (18.2)	0	0.002 *	Ref.
3	2 (66.7)	1 (33.3)	0	0.043 *	<0.001 *
Blood	*NRF2*—rs6721961				
Histological Grade	CC	CA	AA		
1	5 (71.4)	2 (28.6)	0	Ref.	
2	10 (90.9)	1 (9.1)	0	<0.001 *	Ref.
3	2 (66.7)	1 (33.3)	0	<0.001 *	<0.001 *
Surrounding Tissue					
Histological Grade					
1	6 (85.7)	1 (14.3)	0	Ref.	
2	10 (90.9)	1 (9.1)	0	<0.001 *	Ref.
3	3 (100)	0	0	<0.001 *	<0.001 *
Tumour Tissue					
Histological Grade					
1	6 (85.7)	1 (14.3)	0	Ref.	
2	10 (90.9)	1 (9.1)	0	<0.001 *	Ref.
3	2 (66.7)	1 (33.3)	0	<0.001 *	<0.001 *
Blood	*KEAP1*—rs1048290				
Histological Grade	CC	CG	GG		
1	1 (16.7)	5 (83.3)	0	Ref.	

2	4 (44.4)	3 (33.3)	22.2	<0.001 *	Ref.
3	0	1 (100)	0	<0.001 *	<0.001 *
Surrounding Tissue					
Histological Grade	CC	CG	GG		
1	1 (16.7)	5 (83.3)	0	Ref.	
2	6 (66.7)	2 (22.2)	1 (11.1)	0.029 *	Ref.
3	0	1 (100)	0	<0.001 *	<0.001 *
Tumour Tissue					
Histological Grade	CC	CG	GG		
1	1 (16.7)	5 (83.3)	0	Ref.	
2	4 (44.4)	3 (33.3)	2 (22.2)	<0.001 *	Ref.
3	0	1 (100)	0	<0.001 *	<0.001 *

* Indicates a significant result.

With the exception of the rs35652124 polymorphism, the genotype distribution for each histologic grade is different among the tissues.

In this particular study, only the cases that were genotyped for the three tissue types were analysed. However, due to the limited size of the sample, it was considered pertinent to analyse the variation in genotypes for each polymorphism, in each histological grade (Figure 1).

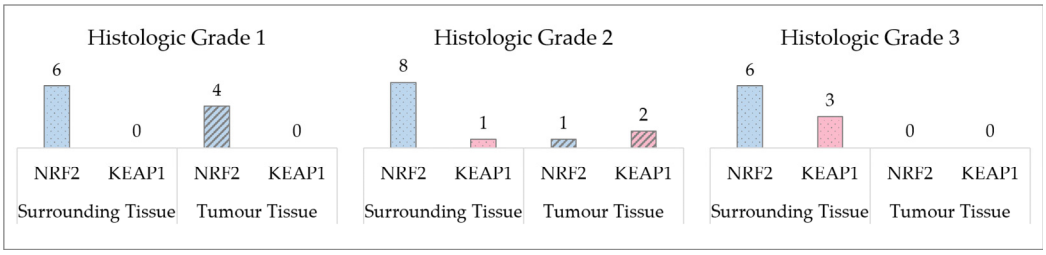

Figure 1. Number of acquired mutations in the surrounding and tumour tissues, when compared to the genotype profile of the blood.

In Figure 1 we can observe the count of genotypes in the tissues that are different when compared to blood genotypes. In histologic grade 1 the number of genotypes that are different from the blood genotypes is higher in the surrounding tissue. In histologic grade 2 we can observe a greater difference in the *NRF2* polymorphism count, as in histologic grade 1. In addition, there are genotypes differences in *KEAP1*. In histologic grade 3 there is no difference in tumour tissue genotypes, however, surrounding tissue has a greater number of genotypes.

4. Discussion

Metabolic imbalance has long been identified as one of the factors for poor prognosis, increased recurrence, decreased disease-free survival and resistance to therapy. Among the factors that may play a leading role in breast cancer metabolism is the KEAP1-NRF2 complex, which regulates the metabolism and, therefore, the response to oxidative stress. The *NRF2* rs35652124, rs6706649 and rs6721961 polymorphisms are related to a decrease in NRF2 transcription, which may compromise the response to oxidative stress. The *KEAP1* rs1048290 minor allele has also been associated with impaired response to oxidative stress, since it is related to higher KEAP1 protein expression and with an increased association between KEAP1 and NRF2, with the NRF2 becoming less available to enter the nucleus and promote the transcription of phase II enzymes [14,15].

It is therefore considered pertinent to evaluate the genotypes of these polymorphisms in the tumour and in the benign tissue surrounding the tumour. Starting from the genotype of the peripheral blood, the evaluation of somatic mutations in tumour tissue

and in the benign tissue that surrounds the tumour might be pertinent to better understand if they have a pathological effect in breast cancer development and aggressiveness [19].

In the present study women with diagnosed breast cancer (n = 63) and women with no history of breast cancer (n = 23) were included. Genomic DNA from peripheral blood was sequenced for rs35652124, rs6706649, rs6721961 of *NRF2* and rs1048290 of *KEAP1*. The Hardy–Weinberg equilibrium was evaluated and it was not verified as deviating from the HWE (p-value > 0.05) for both controls and patients, as expected for low-penetrance genes. After, the risk for developing breast cancer was assessed and an increased risk for breast cancer development was not found in the population under study (p-value > 0.05). These results are not in accordance with the ones verified by Hartikainen et al. for rs6721961 and for rs1048290 [14,16]. This is possibly due to the effect of a lower sample size. Nevertheless, there is a lack of studies correlating all these polymorphisms of the metabolic pathway with the risk of breast cancer development. Similarly, there is a lack of studies assessing the genotypes of the referred polymorphisms and tumour tissues, as described in the present study. Genomic DNA was extracted from peripheral blood from 56 paraffin-embedded tissue slides (28 slides of surrounding benign tissue and 28 slides of tumour tissue). Most likely, the lack of studies using these source materials is due to the difficulty in extracting genomic DNA from paraffin-embedded tissues. The long-term storage and the possibility of DNA degradation might compromise the results. Moreover, the quality and quantity of the genomic DNA extracted might not be ideal. In the present study, the DNA was extracted from 10 µm slides and instead of a conventional PCR, a real-time PCR with a high number of cycles was performed (70 amplification cycles) in order to guarantee enough template DNA for Sanger sequencing. Even so, not all samples were amplified and genotyped. For the polymorphisms of *NRF2*, a total of 21 cases were properly sequenced for the tree types of tissue (blood, surrounding benign tissue and tumour tissue). For *KEAP1*, a total of 16 cases were sequenced for the three types of tissue. This is a limitation of the study, however, due to the source of the material it is a successful rate.

This step led to the study of the HWE for all tissues and for each polymorphism. The principle of the HWE was verified for the majority of the polymorphisms, with the exception of the rs6706649 in the surrounding benign tissue, which was in linkage disequilibrium (LD) (p-value < 0.001). Assessing the allele frequencies, it was verified that the mutated allele (A) frequency is higher in the surrounding benign tissue when compared to blood, suggesting somatic mutations in the surrounding benign tissue.

Therefore, a correlation between the genotype distribution of rs35652124, rs3706649, rs6721961 of *NRF2* and rs1048290 of *KEAP1* in the three different types of tissues (blood, surrounding benign and tumour tissues) was performed. For rs35652124, a significant difference in the genotype distribution was found between blood and surrounding benign tissue (p-value = 0.023) and between tumour tissue and surrounding benign tissue (p-value = 0.041). The surrounding benign tissue had somatic mutations that reflect a tendency towards a lower heterozygous genotype (nine cases in the blood and four cases in the surrounding tissue) and increased homozygous genotypes (wild-type and mutant). Although this difference in the profile of the surrounding benign tissue is more prevalent in rs35652124, all the polymorphisms studied show genotypic differences between the tissues, highlighting the loss of heterozygosity in the surrounding tissue. The loss of heterozygosity in benign breast tissue was associated with an increased risk for breast cancer development by Euhus et al., 2002 [20].

The increase in somatic mutations in benign tissue has long been correlated with age and consequently with cancer development [21–23]. Thus, a correlation between genotype alterations among the tissues and the histologic grade of the breast cancer was performed. It was verified that, in addition to the tendency already referred to for the loss of heterozygosity, the histologic grade 2 tends to have a different genetic profile from histologic grade 1 and 3.

These results lead to several questions. Why are there no variations in *KEAP1* polymorphism in histologic grade 1 cases? Why is the variability in genotype profiles greater in surrounding benign and tumour tissues in breast cancer with histologic grade 2? Why were there no verified alterations in the tumour tissue, when compared to the surrounding benign tissue genotypes, for the four polymorphisms in histologic grade 3 cases?

NRF2 is responsible for the regulation of several cytoprotective genes, like phase II enzymes. The altered alleles of the *NRF2* polymorphisms in this study are related to a lower activity of NRF2 and *KEAP1* rs1048290 is related to an increased affinity to bind NRF2, leading to its lower availability in the nucleus. A possible answer to the first question is that although the transcription of NRF2 is lower or normal, it is still available in the nucleus to trigger the metabolic response. Thus, this protects cells and eventually contributing to a less aggressive development.

The majority of cases had a histological grade 2 and, in this histological grade, we verified a greater variability in genotypes, both in surrounding benign and in tumour tissues. The histological classification, through Nottingham histological grade, results from the evaluation of tubular formation, nuclear pleomorphism and mitosis [24]. Therefore, the Nottingham scale is a prognostic factor that aids clinicians to identify the best approach for each patient. A histological grade 2 is consider a moderate grade [24,25]. However, this classification does not take into account the characteristics of the benign surrounding tissue.

Field cancerization is an assumption that the cells of the surrounding benign tumour tissue have molecular alterations that might not alter the morphology of the tissue and that can develop into malignant cells [26]. Somatic mutations in this surrounding tissue can be acquired throughout one's life or result from the microenvironment and, in either case, somatic mutations can lead to the development of cancer or they can be alterations that do not trigger cancer [27,28].

Thus, histologic grade 2 breast cancer tumours might have an increased instability than observed, once field cancerization occurs without morphological alterations [29]. The polymorphisms in *NRF2* and *KEAP1* might be indicative of a more heterogeneous and active microenvironment that might confer a survival advantage to cells with compromised cytoprotection, also, higher recurrences might be related to the changes in the surrounding tissue [28,30].

The histologic grade 3 is the grade with a lower prognosis. Considering that cancerization can confer survival advantages to altered cells, it is possible that histologic grade 3 breast cancers are a well-established population of cells that suffered a cancerization process, and on the other hand the surrounding benign tissue is suffering the cancerization process and therefore contributing to a future cancer progression [29].

These findings are of great concern once they could affect the therapeutic approach. In a meta-analysis, our research team verified that breast cancer patients that overexpressed NRF2 had a lower survival compared to the ones that expressed lower levels of NRF2 [8]. The dual role of the complex NRF2-KEAP1 is of main importance. On what concerns breast cancer development, lower levels of NRF2 due to polymorphisms in *NRF2* or *KEAP1* alone or in association with polymorphisms in estrogen biosynthesis and metabolism might contribute to inefficient estrogen detoxification. After the disease develops, high levels of NRF2 will protect cancer cells from oxidative stress and eventually promote resistance to therapeutic approaches. Low levels of NRF2 are related to a better response to therapeutic approaches; however, healthy cells present a low response to oxidative stress, being at risk. This study, in the future, might have an impact on the therapeutic approach once loss of heterozygosity in the surrounding tissue, leading to lower levels of NRF2, may be a worse prognosis. In opposition, a well-stablished population of tumour cells whose genotype is related to increased levels of NRF2 might resist therapeutic approaches.

Our findings are of main interest in increasing the knowledge about somatic mutations in *KEAP1* and *NRF2* in breast tissue (benign and malignant), however, the

study was performed in a small sample population. Further studies should be performed on a larger population and follow-up should be performed in order to understand the impact of these mutations in breast cancer overall survival, disease-free survival, relapse-free survival and therapeutic responses. Also, reproductive data should be evaluated such as age at menarche, menopause, full-term pregnancies, abortions, time of breast feeding and hormone replacement therapy.

5. Conclusions

Cancerization processes have been debated over the years, and the present study provides evidence that the surrounding tissue might demonstrate more instability, having a genotype profile different from that of tumour tissue.

Similarly, the predictive value of the histologic grade 2 should be re-evaluated considering the metabolic imbalance and somatic mutation profiles.

Overall, the KEAP1-NRF2 complex, in addition to the possible dual role it plays in breast cancer, can also be an important factor for clinical evaluation and surgical approaches.

To our knowledge, there are no similar studies published. It is urgent to replicate this study in a larger population in order to contribute to the mitigation of breast cancer incidence and mortality. This is pertinent, since NRF2 has been associated with a worse prognosis and therapeutic resistance, and limiting genetic evaluation to peripheral blood does not provide a real insight into the acquisition of somatic mutations in tissues [8]. In the future, a more in-depth genetic evaluation could help clinicians achieve a better prognosis for breast cancer patients.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Cova da Beira Local Health Unit, Covilhã, Portugal of Cova da Beira Local Health Unit (approval numbers: n.º 28/2008 and its addendum of 17 August 2016; further approval for new data acquisition n.º 20/2023, 23 March 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available within the article or on request from the corresponding author.

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

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Chapter 8

General Discussion and Main Conclusions

Breast cancer continues to promote high mortality and morbidity among women worldwide. Although the efforts for early detection and treatment improvement in the last decades, the heterogeneity of the disease makes it a challenge, demanding more research for carcinogens identification, biomarkers for prognosis and new therapeutic approaches.

On what concerns to carcinogens, lifetime exposure to estrogens is a well-stablished carcinogenic factor [20]. The biological effects of E2 are predominantly mediated by its binding to estrogen receptor [74]. Approximately 70% of breast cancers express estrogen receptors [24]. An excessive estrogens exposure and an inefficient estrogens detoxification results, among others, in metabolites, such as quinones and semiquinones, which can damage DNA and lead to carcinogenesis.

Several factors contribute to this. Lifestyle factors like diet, smoke and alcohol consumption, and also hormonal history, namely early menarche, late menopause, pregnancy, hormone replacement therapy, as also inherited or acquired polymorphisms involved in the biosynthetic and metabolic pathways of breast cancer [9].

Previous studies of our research team identified *CYP19A1* rs2236722, *GSTM1* and *GSTT1 null* polymorphisms as risk factors for breast cancer [54, 55]. These results suggest that oxidative stress is involved in this process. Thus, we considered pertinent to evaluate in this work if NRF2-KEAP1 complex could be a biomarker for breast cancer prognosis, and a therapeutic target for breast cancer. Thus, in order to continue our previous research, and evaluate possible associations among SNPs in both biosynthetic and metabolic pathways, and its possible relation with *NRF2-KEAP1*, several SNPs were studied in a more restrict group of women in which was possible to obtain peripheral blood, but most important, paraffin blocks of breast cancer and surrounding tissue from breast cancer patients that underwent surgery.

BRCA1 185delAG and 5382insC and *BRCA2* 6174delT polymorphisms were evaluated in DNA extracted from peripheral blood for all the participants and all were negative.

Afterwards, in estrogenic biosynthetic pathway were evaluated *CYP17A1* rs743572 and *CYP19A1* rs2236722 polymorphisms. In what concerns to detoxification, *CYP1A1* rs1048943, *CYP1B1* rs1056836, *MTHFR* rs1801133, *COMT* rs4680, *GSTM1* and *GSTT1 null* polymorphisms, *GSTP1* rs1695, *KEAP1* rs1048290 and *NRF2* rs35652124,

rs6706649, rs6721961 SNPs were evaluated. In addition, *ESR1* XbaI and PvuII were evaluated due to the role in the estrogenic response pathway.
For this work, 23 women without breast cancer and 69 women with histologically confirmed breast cancer were recruited. After tumour histological analysis, it was verified that six patients had ER^- breast cancers; therefore, the final dimension of the population was of 23 controls and 63 patients with hormone-dependent breast cancer.

In previous studies of our research team, women carriers of the polymorphisms *CYP19A1* rs2236722, *GSTM1* and *GSTT1 null* polymorphisms seemed to have higher risk for breast cancer development. In the present work, none of the fifteen polymorphisms studied was correlated with increased breast cancer risk, possibly due to low number of controls and patients.

To counteract the limitation of the low number of controls and patients, the genotyping and clinical data of breast cancer patients recruited in the ambit of the present work, were aggregated to the main database of our research group (with exception of *KEAP1* and *NRF2* SNPs genotyping), resulting in a total of 157 breast cancer patients. It was verified that breast cancer diagnosis after 50 years old was correlated with *GSTM1* and *GSTT1 null* polymorphisms, alone or in association with carriers of *CYP1B1* rs1056836 and/or *MTHFR* rs1801133. These results emphasize the effect of compromised estrogen detoxification pathway leading to breast cancer development at later ages (Chapter 4).

Our previous results indicated that carriers of *GSTM1* deletion had higher risk of breast cancer development. Once GSTM1 induction is regulated by NRF2, that binds to DNA, sMAF proteins and AREs in order to promote the induction of phase II enzymes, is relevant to evaluate the association of *GSTM1* deletion with *NRF2-KEAP1* complex polymorphisms. As mentioned in Chapter 2, we found important to optimize a methodology that distinguished between heterozygous "*present/null*" and wild-type "*present /present* " genotype of *GSTM 1 null* polymorphism ("*null /null* "). By implementing a new approach to *GSTM1* genotyping (Chapter 5), we were able to verify that it is pertinent to distinguish the three genotypes and not just classify them as *present* or *null*. When we correlated heterozygous *present/null* genotypes with clinical data, it was verified that the majority of cases with this genotype had a more aggressive molecular subtype of breast cancer, luminal B (HER2 expression). If the previous methodology was used, all heterozygous cases would have been classified has wild-type ("*present/present*"), leading to misinformation. However, when distinguishing between wild-type and heterozygous, only one patient had a wild-type genotype. Thus, *GSTM1*

null polymorphism in heterozygoty was correlated with a worse prognosis. In fact, only $HER2^+$ cases were carriers of GSTM1 deletion in heterozygosity. Also, $HER2^+$ cases are carriers of *KEAP1* rs1048290 and *NRF2 rs35652124* mutated alleles, also reinforcing a worse prognosis due to a compromised estrogens detoxification pathway.

Besides the risk for breast cancer development and the outcome considering the clinicopathological parameters, the dual-role of NRF2 is ambivalent. On one hand, the regulated action of the NRF2-KEAP1 complex protects cells from oxidative stress (when there are decreased levels of NRF2 in the nucleus, due to polymorphisms in *KEAP1* and/or *NRF2*, there is an impairment in the antioxidant response). One the other hand, high levels of NRF2 are also associated with lower DFS and lower OS in breast cancer patients (Chapter 6). In addition, the overall survival of patients treated with Tamoxifen was lower in tumours with NRF2 overexpression. In truth this is not a dual role, it is NRF2 behaving in tumour cells as it behaves in healthy cells: protects them from oxidative stress.

Considering the results obtained in the systematic review and meta-analysis, it became evident that it would be pertinent to evaluate the polymorphic profile of the tumour itself, by assessing the genetic profile of the tumoural tissue and of the benign tissue surrounding the tumour, once the cells morphology can be apparently normal, but mutations might be present. Thus, *KEAP1* rs1048290 and *NRF2* rs35652124, rs6706649, rs6721961 polymorphisms were evaluated in peripheral blood, tumour surrounding tissue and tumour tissue (Chapter 7). It was verified that genotypes distribution is different on the tumour surrounding tissue when compared to blood and tumour. Concretely, it was verified a tendency to the loss of heterozygosity in the surrounding tissue, and histological grade 2 tumours have a different genetic profile from grade 1 and grade 3 tumours.

Somatic mutations in benign tumour surrounding tissue may be related to increased age (mutations acquisition throughout life) and with breast cancer development. Somatic mutations, particularly, in these genes, will lead to an inefficient detoxification, due to the consequent lower expression of phase II enzymes. Moreover, estrogens exposure over time is itself a carcinogen, associated with a compromised detoxification, probably creates a critical microenvironment, contributing to breast cancer development, progression and poor outcome. Thus, the so-called “dual-role” of NRF2, postulated by several authors, might be conferred by the role of *NRF2* SNPs in tumour early phases of development *versus* its role when tumour has already developed. Also, polymorphisms that decrease NRF2 expression and consequently diminish its

availability in the nucleus, associated with polymorphisms in estrogenic biosynthetic and metabolic pathways, might contribute to tumour development due to estrogens over-synthesis and inefficient detoxification. In this case, NRF2 low availability in the nucleus does not properly protect cells. When tumour has already developed, the effects of NRF2-*KEAP1* complex SNPs are of main importance once they protect cancer cells from oxidative stress.

In conclusion, the main achievements of this work, that aimed to validate the complex NRF2-KEAP1 as a biomarker for prognosis and therapeutic target for breast cancer were:

- Polymorphisms in estrogens metabolic pathway, namely *CYP1B1* rs1056836, *GSTM1* and *GSTT1* deletion, and *MTHFR* rs1801133, should be considered along with breast cancer risk assessment models, once women with these polymorphisms are in increased risk of developing breast cancer in postmenopause. In this regard, these polymorphisms will contribute to identify women that are candidates for hormone replacement therapy, as carriers of the mutant allele will poorly detoxify estrogens contributing to breast cancer development.
- Women carriers of rs35652124 *NRF2*, rs1048290 *KEAP1* and *GSTM1* heterozygous ("*present* / *null*") genotype seem to have breast cancer with worse prognosis. These polymorphisms are related with KEAP1 higher affinity to bind NRF2, low availability of NRF2 in the nucleus leading to lower induction of phase II enzymes and cumulatively with a poorer detoxification due heterozygosity of *GSTM1*.
- NRF2 expression might be a marker for breast cancer prognosis. Breast cancer patients whose tumours overexpress NRF2 have lower disease-free survival and lower overall survival.
- Somatic mutations in *NRF2-KEAP1* complex should be evaluated both in surrounding benign tissue and in tumour tissue. We verified a tendency to the loss of heterozygosity in the surrounding tissue that may lead to lower levels of NRF2, and probably to a worse prognosis, due to a compromised detoxification. In opposite if there is no impairment of NRF2 expression in the tumour, there may be resistance to therapy. Moreover, throughout life KEAP1 expression levels tends to increase [209] emphasizing that KEAP1 is the negative regulator of NRF2, somatic mutations acquisition over time and less NRF2 in the nucleus might lead to a poor prognosis in postmenopausal women.

Concluding, *NRF2-KEAP1* polymorphisms evaluation in blood, surrounding tissue and tumour tissue allow the identification of somatic mutations acquisition, and an inference of the breast cancer prognosis and response to therapeutic could be performed. These results indicates that NRF2-KEAP1 complex can be used as predictive marker of breast cancer prognosis and resistance to therapeutic.

8.1. Future Perspectives

In the future, it is necessary to replicate this study in a larger sample and perform the follow-up of the patients to better understand the role of the complex in cancer development and prognosis. During the development of the present work several constraints due to SARS-CoV-2 pandemic were present. Also, the recruitment of the expected 150 controls and 150 breast cancer patients was not possible due to two major factors: the referral criteria for breast cancer patients changed and the majority of breast cancer patients were no longer followed up at the collaborating hospital. These factors led to a lower recruitment rate. Besides sample increase, also studies in pre and postmenopausal breast cancer patients subgroups can provide further information.

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APPENDIX

The Role of Estrogen Receptors Alpha in Breast Carcinogenesis: An Overview.

in Estrogen Receptors: Structure, Functions and Clinical Aspects.

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Chapter 1

THE ROLE OF ESTROGEN RECEPTORS ALPHA IN BREAST CARCINOGENESIS: AN OVERVIEW

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ABSTRACT

Estrogen receptors are divided in two different classes: nuclear estrogen receptors, which can occur in two different isoforms, ERα and ERβ, and membrane estrogen receptors (mERs) (GPER also known as GPR30, ER-X, and Gq-mER), which are mostly G protein-coupled receptors. Nuclear ERα and ERβ mediate the biological effects of estrogens in different tissues. The distribution of these receptors differs from tissues to tissues. ERα is predominantly expressed in uterus, ovarian theca cells, liver and is the most expressed ER isoform in breast epithelium.

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ERα protein is codified by ERα gene (ESR1), which is located on chromosome 6q25.1. The eight exons of ESR1 encode the 530 amino acids of ERα, with a molecular size of 54 kDa. ERs are composed by six functional domains: A, B, C, D, E and F. Among these functional domains is H2-terminal domain (NTD), DNA-binding domain (DBD) and ligand-binding domain (LBD). Through LBD, Estradiol (E2) binds to ERs and induces the conversion of the inactive ER to the active form. Estrogens have been clearly identified as carcinogens, the main effects of estrogens are thought to be via stimulating breast-cell proliferation, which increases the chances of proliferation of a potentially mutated cell. ERα activation by estrogens is generally considered responsible for enhanced proliferation in breast cancer in which ERα is expressed in 50 to 80% of cases. Also, several polymorphisms and point mutations in ERα may alter the protein expression and function. Thus, ERα has been considered as an important breast cancer risk factor and as a relevant therapeutical prognostic factor. This chapter provides an overview and discusses the challenges of ERα role in breast carcinogenesis.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women and the most frequent cause of death in women in the majority regions of the world [1]. Breast cancer initiation is generally a consequence of cumulative risk factors, such as single nucleotide polymorphisms (SNPs), acquired mutations, family history, reproductive history and lifestyle habits [2, 3]. The exact etiology of breast cancer is still unknown, however there is a strong evidence that genes of the estrogen pathway may influence breast cancer risk and that estrogens play a critical role in the development, progression of the disease [2, 4]. Endogenous estrogen levels might be critically involved in the progression of breast cancer, once about 70-75% of breast cancers express estrogen receptors (ERs) and respond well to endocrine therapy [2-6]. On this regard, we will further discuss the effect of estrogen receptors, mainly of estrogen receptor alpha (ERα) in human breast cancer.

ESTROGEN RECEPTORS

Estrogens are steroidal hormones that regulate several physiological processes in normal cells including maintenance of reproductive organs, regulation of cardiovascular system, homeostasis of central nervous system, maintenance of bone density and cholesterol levels [7, 8]. Estrogens also play an important role in several pathological processes like cancer, metabolic diseases and osteoporosis [7-9]. The endogenous estrogens are estrone (E1), estradiol (E2) and estriol (E3), being E2 the more predominant estrogen in non-pregnant and pre-menopausal women [6, 10].

The biological effects of E2 are mostly mediated by its binding and activation to estrogen receptors [6, 7, 9, 10]. There are two different classes of estrogen receptors: nuclear estrogen receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and membrane estrogen receptors, the G-protein coupled estrogen receptor (GPER also known as GPR30) [2, 5, 11].

The distribution of steroid receptors varies among tissues being ERα mainly expressed in uterus, ovarian theca cells, breast (being the most expressed ER in breast epithelium), prostate stroma, Leydig cells in testis, epididymis, pituitary, kidney, bone, white adipose tissue and liver [2, 6, 9, 12]. ERβ is mainly expressed in ovarian granulose cells, central nervous system, cardiovascular system, lung, male reproductive organs, prostate epithelium, colon, kidney, bone marrow and immune system [2, 6, 9, 12].

Estrogen Receptors Structure

The estrogen receptors ERα and ERβ are encoded by two distinct genes. The ESR1 gene located at 6q25.1 was cloned for the first time by Walter et al. (1985) and the cDNA was first sequenced by Green et al. (1986) [13, 14]. ESR1 gene contains eight exons that encode ERα, a 66kDa protein, composed by 595 amino-acids [13-15]. The ESR2 gene located at 14q23.2 was first identified by Mosselman et al. 1996 and cDNA was first cloned by

Kuiper et al. 1996, ESR2 contains eight exons that encode ERβ, a 60kDa protein, composed by 530 amino-acids [7, 16, 17].

The ERs are composed by six structural domains (Figure.1) and they share structural characteristics, will focus on describing ERα structure.

The A/B region, encoded by exon 1, shares 17% of homology between ERs, the ligand-independent activation function-1 domain (AF1) part of the NH2-terminal domain (NTD) is involved in the transcriptional activation of target genes and is an essential domain of interactions with co-regulators [7, 9, 12].

The DNA binding-domain (DBD) in the C region is encoded by exons 2, 3 and a part of exon 4, is highly conserved between ERs (97%) and is essential for the specific binding to DNA sequences of target genes, regulating their expression [7, 9, 12].

The D domain, flexible hinge finger, shares 36% of homology, is encoded by exon 4 and links the C domain to E domain, contains the nuclear localization signal (NLS), resulting in the activation of ER signalling in cells, aiding in the translocation of ERα to the nucleus [7, 12, 18].

The exons 4-8 encode for E and F domains, the domain E and F domains shares 56% and 18% of homology, respectively [7]. These domains also called ligand-binding domain (LBD) is a globular region that contains a hormone-binding site, a dimerization interface and a ligand-dependent activation function-2 (AF-2), that has conformational alterations depending on the presence of different ligands [7, 12].

Figure 1. ERα structure. The ERα is a 66kDa protein, with 595 amino-acids, composed by six structural domains. A/B region is involved in the transcriptional activation of target genes, through the ligand-independent activation function-1 domain (AF1) part of the NH2-terminal domain (NTD). The C and D regions have the DNA binding domain and nuclear localization signal, respectively. The E and F regions have the ligand binding domains (LBD) and ligand-dependent activation function-2 (AF-2).

ERα Signalling

Estrogen plays an important role in cell proliferation and differentiation, mainly by binding to its receptors, among them ERα, leading to their activation [19, 20].

In breast cancer, ERα mediates E2 action in the nucleus by two distinct pathways: the genomic and non-genomic pathways (Figure. 2) [7, 12, 21, 22]. The genomic pathway can be divided into estrogen and estrogen response elements (ERE) dependent pathway ("classical" genomic pathway), and estrogen-dependent/ERE-independent pathway ("nonclassical" genomic pathway) and estrogen-independent/ERE-dependent pathway [21].

In the "classical" pathway, E2 binds to ERs, through LBD in the cytoplasm, inducing a conformational change in ERα, enabling ERα dimerization and translocation to the nucleus [12, 22, 23]. The complex ERα-E2, through the DBD of ERα binds to estrogen response elements (ERE), activated ERα triggers the recruitment of co-regulators, through AF-1 and AF-2 domains, that modulates the transcriptional activity [12, 20-26]. Alternatively, activated ERα, by E2, can recruit and bind, through protein-protein interactions, to transcription factors (TF) such as SP1, nuclear factor-κB (NF-κB) and AP1, at their respective response element (RE), stimulating the transcription of growth and survival genes [4, 20-22, 24, 25].

There is also a third pathway, E2-independent/ERE-dependent ERα, a crosstalk between ERα and growth factors induces the activation of kinases, like mitogen-activated protein kinase (MAPK) pathway [4, 21, 23, 25]. Phosphorylated ERα translocates to the nucleus and initiates its transcriptional activity, binding to EREs and recruiting co-regulators [22, 23, 25].

The non-genomic pathway, is not well understood as the genomic pathway, but is largely studied for ERα [12, 25]. In this signalling pathway, E2 acting at the membrane, stimulates the ERα located in the cytoplasm, after that, it initiates a signalling cascade, leading to rapid physiological responses [12, 22, 25, 27]. The ERα-E2 complex activates kinases, such as Src kinase and phosphatidylinositol 3-kinase (PI3K) pathway, activating the

signalling cascade of mitogen-activated protein kinase (MAPK) and protein kinase B (AKT), these pathways enable the activation of other transcription factors, facilitators of cell growth and proliferation [20-23, 25, 26, 28].

These genomic and non-genomic mechanisms of ERα signalling are presented separately, however the signalling pathways are strongly related, enforcing the central role of ERα in cell development and differentiation [23, 25].

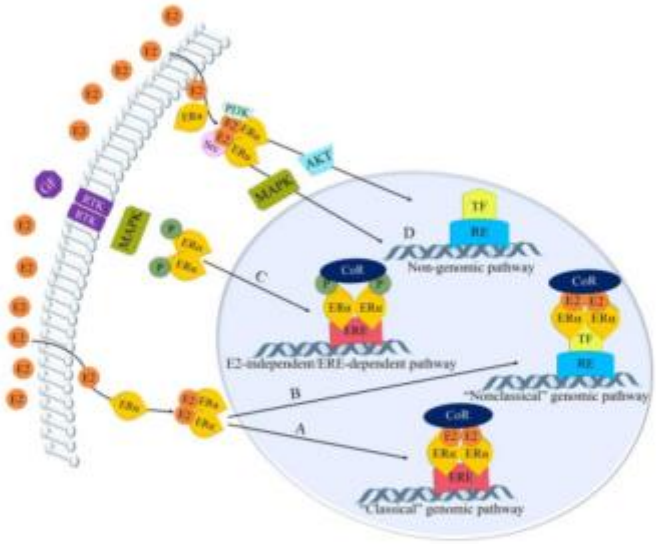

Figure 2. ERα signalling pathways. A) is the "classical" genomic pathway, E2 binds to ERs, the complex binds to ERE and co-regulators are recruited. B) "nonclassical" genomic pathway, E2 binds to ERs, through protein-protein interactions the complex binds to transcription factors (TF) at their respective response element (RE). C) estrogen-independent/ERE-dependent pathway, a crosstalk between ERα and growth factors induces the activation of kinases, phosphorylated ERα binds to EREs and recruiting co-regulators. D) non-genomic pathway, ERα-E2 complex activates kinases, through MAPK and AKT signalling cascades, transcription factors and their regulators enable the activation of other transcription factors.

ESR1 Mutations

The risk for the development of breast cancer is long been associated to lifetime exposure to E2 or estrogenic compounds, mainly through E2/ERα dysregulation in the genomic and non-genomic pathways, that might lead to breast carcinogenesis [2, 4, 21, 23, 25, 26, 29, 30]. The genetic component should not be discarded once mutations in ERα gene (*ESR1*) may alter the protein expression and function.

Table 1. Studies correlating *ESR1* polymorphisms and the risk of breast cancer, worldwide

SNPs	Authors	Year	Population	Relation to Breast Cancer
rs1062577	Chen et al. [33]	2016	Chinese	Increased risk of breast cancer
	Dehghan et al. [44]	2017	Iranian	
rs2046210	Wang et al. [39]	2014	Chinese	Increased risk of breast cancer
	Thanh et al. [46]	2018	Vietnamese	
	Mizoo et al. [47]	2013	Japanese	
	Campa et al. [48]	2011	European	
rs2077647	Son et al. [34]	2015	Korean	Increased risk of breast cancer
rs2234693 (PvuII)	Wang et al. [39]	2014	Chinese	Increased risk of breast cancer
	Carrillo-Moreno et al. [38]	2018	Mexican	Increased risk of breast cancer
	Gonzalez-Mancha et al. [37]	2008	Spanish	Moderately correlated with breast cancer risk
	Ramalhinho et al. [2]	2013	Portuguese	No association with breast cancer risk
rs2228480 (T594T)	Wang et al. [39]	2014	Chinese	Increased risk of breast cancer
	Kallel et al. [42]	2009	Tunisian	
	Curran et al. [43]	2001	Australian	
rs2273206	Son et al. [34]	2015	Korean	Increased risk of breast cancer
rs2881766	Son et al. [34]	2015	Korean	Increased risk of breast cancer
	Chen et al. [33]	2016	Chinese	Increased risk of breast cancer
	Dai et al. [60]	2019	Chinese	Associated with lymph node metastasis and ER expression and decreased breast cancer risk
rs926778	Son et al. [34]	2015	Korean	Increased risk of breast cancer
rs9340799 (XbaI)	Wang et al. [39]	2014	Chinese	Increased risk of breast cancer
	Ladd et al. [40]	2008	Dutch	No correlation with breast cancer risk
	Shen et al. [41]	2006	Chinese	No correlation with breast cancer risk
	Ramalhinho et al. [2]	2013	Portuguese	Reduced risk of breast cancer for xx (homozygous mutant) carriers
rs9479118	Chen et al. [33]	2016	Chinese	Increased risk of breast cancer

Several mutations in *ESR1* have been described, among them, single nucleotide polymorphisms (SNPs). The most studied SNPs in *ESR1* are rs2234693 (PvuII), rs9340799 (XbaI) and rs2228480 (T594T) (Table 1) [2, 31, 32].

Other SNPs studied in *ESR1* are rs1062577, rs2046210, rs2077647, rs2273206, rs2881766, rs926778 and rs9479118 (Table 1) [31, 33, 34].

On what concerns to the SNPs rs2234693 (PvuII), rs9340799 (XbaI)), both polymorphisms are located at intron 1 and are thought to be functional polymorphisms [2, 31, 32, 35]. The possible mechanism of action is a change in *ESR1* transcription activity, contributing to carcinogenesis [2, 31, 32, 35].

The PvuII [c.453-397T > C] polymorphism has been suggested to increase ERα transcription or produce ERα isoforms [36]. Several studies have been performed in order to understand the correlation of PvuII and breast cancer risk, however there are conflicting results. Gonzalez-Mancha et al. (2008) performed a study on which 1148 samples of Spanish women were genotyped and PvuII T allele (altered allele) was moderately correlated with the increased risk of developing breast [37]. Ramalhinho et al. (2013) performed a similar study in 228 Portuguese women from Central Eastern Portugal and PvuII genotypes were not associated with breast cancer variation risk, the same research team found similar results during a research performed in 2019 [2]. Carrillo-Moreno et al. (2019) also studied PvuII polymorphism in Mexican population, correlating positively breast cancer risk with PvuII and similar results were found by Wang et al. (2014) [38, 39].

The polymorphism XbaI [c.454-351A>G], was studied by Ladd et al. (2008) in Dutch post-menopausal women and no correlation with breast cancer risk was found, similar results were found by Shen et al. (2006) in a Chinese case-control study [40, 41]. However, Wang et al. (2014) associated XbaI polymorphism with increased risk of breast cancer. In the opposite, Ramalhinho et al. (2013) associated the homozygous mutant genotype with a reduced risk of breast cancer [2, 39].

As previously referred, others found out that polymorphism in *ESR1* is the polymorphism T594T [c.1782G>A], located at exon 8, although the

exact functionality is unknown, probably has a role on co-regulators recruitment [31]. The T594T polymorphism was correlated with breast cancer risk in Tunisian, Chinese and Australian women [39, 42, 43].

Other SNPs have been studied in breast cancer, among them, the rs1062577 located in the 3'-UTR of *ESR1* is thought to promote higher ERα expression, leading to higher proliferation rate in breast cancer cells [44, 45]. This polymorphism was studied in Chinese and Iranian population by Chen et al. (2016) and Dehghan et al. (2017), respectively, being correlated with the increased risk of breast cancer in both populations [33, 44]. The rs2046210, located upstream *ESR1* was studied in the Chinese, Vietnamese, Japanese population and European population, all studies correlated rs2046210 with an increased risk of breast cancer [39, 46-48].

Son et al. 2015 also studied SNPs in *ESR1* and their correlation with breast cancer risk, and the research group found a positive correlation between rs2881766, rs2077647, rs926778, and rs2273206, and breast cancer risk in Korean population [34]. The polymorphism rs2881766 was also studied by Chen et al. (2016) and was found positively correlated with breast cancer risk in Chinese population, this research group also found an association between breast cancer risk and the polymorphism rs9479118 [33].

All these results emphasize the need to better understand the risk potentiated by *ESR1* SNPs, the impact on ERα, and consequently in breast cancer and therapeutic outcome.

Along with SNPs, other *ESR1* mutations have been studied, mainly posttranslational modifications (PTM) that affect ER signalling, creating an imbalance between ER degradation and stability, that in turn might have impact in therapeutic resistance and clinical outcome [23, 25, 49, 50].

The most common point mutations in *ESR1* are Y537N, Y537S, D538G and K303R, these mutations are related with constant ER activity [23, 49, 51, 52].

The Y537S mutation was first described by Weis et al. (1996) and has been shown to confer hormone independent activation [25, 53, 54]. The Y537N mutation, first described by Fuqua lab in 1997, has found to promote a constitutive transactivation function to ER [25, 53, 55].

Merenbakh-Lamin et al. (2013) first described the mutation D538G that occurs in LBD and results in higher levels of ERα, which is independent of the ligand [49, 56]. Toy et al. (2017) found Y537S, Y537N and D538G in patient samples with metastatic and endocrine-refractory breast cancer, however these mutations were not found in the primary samples, these mutations probably promote hormone-independent growth and activity, and are acquired after endocrine treatment [49].

On what concerns to K303K mutation, it was first described by Fuqua lab in 1997 [55]. The mutation leads to polyubiquitination of ERα in response to antiestrogens, and to an altered recruitment of co-regulators, which is associated with lymph node metastasis and enlarged tumor size, and consequently lead to poor prognosis [23, 25, 57].

It seems that all these point mutations on ERα affects its functional role and lead to endocrine resistance [25]. Moreover, these mutations are possibly more frequently acquired after treatment, when they were not identified in the corresponding sample of the primary tumor but were present in metastatic ER^+ tumors after treatment [49, 58]. Besides the biochemical and clinical implications of these point mutations, there is another challenge, Ross et al. (2019) refer that ER immunohistochemistry is not predictive of *ESR1* mutation status, in this regard, not only the *ESR1* mutations should be analysed in clinical practice, but therapeutics should also be more personalized regarding the result of the SNPs and point mutations [59].

THERAPEUTICS IN BREAST CANCER

As previously discussed, the majority of breast cancer expresses estrogen receptors. Breast cancer molecular subtypes can be evaluated through immunohistochemistry, and they can be classified in four major subtypes, Luminal A, Luminal B, HER-2 enriched and Triple negative (Table 2), according to the hormone receptors stage and proliferation rate [26, 29, 51, 61]. The molecular subtype Luminal A is $ER^+/PR^+/HER\text{-}2^-$, the Luminal B $ER^+/PR^+/HER\text{-}2^+$, the HER-2 enriched is $ER^-/PR^-/HER\text{-}2^+$, and

in the Triple negative, there is no expression of hormone receptors ($ER^-/PR^-/HER2^-$) [26, 51, 61].

Table 2. Molecular subtypes of breast cancer

Molecular Subtype	Immunohistochemistry phenotype
Luminal A	$ER^+/PR^+/HER\text{-}2^-$
Luminal B	$ER^+/PR^+/HER\text{-}2^+$
HER2 enriched	$ER^-/PR^-/HER\text{-}2^+$
Triple negative	$ER^-/PR^-/HER2^-$

We will focus on the therapeutic approach of Luminal breast cancer which expressed ERs on targeted therapy. There are two "gold standard" approaches of endocrine therapy to $ER\alpha^+$ breast cancers, by blocking the activation of ERα, through selective estrogen receptor modulators (SERMs) or selective estrogen receptor degraders (SERDs), or by decreasing estrogen levels, through aromatase inhibition, using aromatase inhibitors (AIs) [49, 51, 62, 63].

Selective Estrogen Receptor Modulators (SERMs)

The selective estrogen receptor modulators are a category of therapeutic agents, acting as antagonists of ERα in the breast, that are used for the prevention and treatment of diseases such as breast cancer [12, 63]. The mechanism of action of SERMs is through estrogen competition. The active metabolite has an high affinity to the LBD, inhibiting the recruitment of co-regulators by AF-2 of ERα, displacing the estrogen and blocking the recruitment of co-regulators, inhibiting the proliferative effect of estrogen [12, 30, 62-64].

The first approved drug by Food and Drug Administration (FDA), in 1997, was Tamoxifen, which a has a long biological half-life [65]. The IBIS-I trial, performed between 1992 and 2001, enrolled women with 37 to 70 years old, from Unite Kingdom, Australia, New Zealand, Finland, Spain, Switzerland, Belgium, and Ireland [66]. The main findings of the trial IBIS-

I is that Tamoxifen offers a long period of protection after treatment cessation [66]. Due to the side effects of Tamoxifen, like endometrial cancer, other drugs have been developed, among them are Raloxifene [67].

Raloxifene is a second generation SERM, a drug with short half-life, approved for breast cancer treatment by FDA in 2007 [68]. The clinical trial MORE, performed between 1994 and 1998, in twenty five countries, verified that it reduces the high risk of breast cancer in postmenopausal women with osteoporosis [69]. Other SERMs such as Bazedoxifene, Lasofoxifene, Toremifene and Ospemifene are also used in breast cancer treatment, however Tamoxifen is the only approved drug for $ER\alpha^+$ breast cancers in premenopausal women [62, 63, 70, 71]. Beside the good results on breast cancer therapeutics, *de novo* or acquired treatment resistance has been observed, and remains a challenge for breast cancer treatment [51, 63, 64, 72].

Selective Estrogen Receptor Degraders (SERDs)

The selective estrogen receptor degraders, like SERMs, act through antagonizing ERα, but also through its degradation, disrupting the dimerization and nuclear localization of ER and reducing the cellular levels of ERα [62, 63]. Fulvestrant was approved by FDA in 2002, being the first authorized SERD for breast cancer treatment, specifically for hormone receptor-positive metastatic breast cancer in postmenopausal women [73]. Fulvestrant administration consists in an intramuscular injection, however there are bioavailability limitations, what has lead to several researches, mostly on the development of orally bioavailable formulations [74]. Among the new molecules in clinical trials, there is AZD9496, which was in a phase I clinical trial, between September 2014 and June 2019 [75, 76]. Another molecule under trial is RAD1901, two phase I clinical trials have been performed, in ER–positive, HER2–negative, postmenopausal advanced breast cancer patients [77-80]. Other two molecules were also under phase I clinical trials, namely GDC-0927 and LSZ102, which occurred in December of 2014-November 2019 and April 2016-October 2019, respectively [81,

82]. Probably due to these clinical trials in the next few years, new therapeutic based on SERDs might be approved.

Aromatase Inhibitors (AIs)

The biosynthesis of estrogen, through androgens conversion, is catalysed in local tissue, like adipose breast tissue, by enzymes such as aromatase [62, 71]. Therefore, supressing estrogens production through aromatase inhibition is a therapeutic approach in order to diminish estrogen action and thus tumor growth and proliferation, on these regard, therapeutics with Aromatase Inhibitors (AIs) arises. The therapeutics with AIs are of main importance in postmenopausal women, once estrogen production by ovary is reduced, and the main source of estrogen is breast adipose tissue. This therapeutic approach has two main advantages, it is not associated with endometrial cancer risk and thromboembolic events, unlike SERMs [67]. Although, long-term therapeutic is related with osteoporosis and insomnia [63, 83].

Aromatase Inhibitors might be classified into two major categories, steroidal aromatase inhibitors (SAIs) and nonsteroidal aromatase inhibitors (NSAIs). The Exemestane is the steroidal AI approved in 1999 by FDA, for adjuvant breast cancer treatment in postmenopausal women with ERα$^+$ breast cancer with prior treatment with Tamoxifen during 2 to 3 years [84]. The effects of Exemestane are irreversible, preventing androgens to bind to the catalytic site of aromatase [85]. Goss et al. (2011) performed a randomized placebo-controlled trial in 4560 healthy postmenopausal women with 5-year Gail risk score greater than 1.66% and found that Exemestane reduced the risk of invasive breast cancer, and in a 3-years follow-up Exemestane was not associated with serious toxic effects [86].

On what concerns to NSAIs, they interact with aromatase through noncovalent interactions, competing with endogenous ligands [87, 88]. There are two approved drugs for breast cancer adjuvant treatment, Anastrozole and Letrozole, approved by FDA in 2001 and in 1998, respectively [88, 89].

The clinical trial IBIS-II, an international double-blind, randomised placebo-controlled trial, evaluated Anastrozole in postmenopausal women with increased risk of breast cancer, between 2003 and 2012 [90]. Through IBIS-II trial it was verified that Anastrozole reduces the risk of ERα$^+$ breast cancer [90]. Another trial. ATAC, a double-blinded randomised trial, compared treatment with Tamoxifen and Anastrozole, alone or in association, during five years in postmenopausal ERα$^+$ breast cancer patients and verified that women treated with Anastrozole experienced less side effects and less recurrence when compared to Tamoxifen, concluding that Anastrozole should be considered as the preferred initial adjuvant treatment [91].

Relatively to Letrozole, a phase III, double-blinded trial (BIG 1-98) compared during five years the therapy with Tamoxifen and Letrozole alone, in postmenopausal breast cancer women [92]. It was verified that women undertaking Tamoxifen experienced more thromboembolic events and endometrial pathology, while women treated with Letrozole experienced more fractures and cardiovascular events, but the main conclusion is that women undertaking Letrozole have higher disease-free survival [92].

Thus, NSAIs can be more tolerable than SAIs and clinical data indicate that they are more effective in postmenopausal women with ERα$^+$ breast cancer. Beside these positive advances in breast cancer treatment, resistance to endocrine therapy remains a challenge in ERα$^+$ breast cancer.

Therapeutic Resistance

Estrogens and estrogen receptors play a key role in breast cancer development, differentiation and progression. During the time, several efforts have been made in order to counteract the effects of estrogen and estrogen receptors, however side effects and resistance to endocrine therapy lead to a decreased overall survival, and about 40 to 50% of ERα$^+$ breast cancer cases have lower disease-free survival, being a matter of concern [93]. Resistance to endocrine therapy might be due to long periods of exposure, the estrogen deprivation might lead to activation of growth factor

pathways inducing cancer progression [94, 95]. Moreover, acquired mutations during treatment might promote resistance to treatment [49, 94].

Several mechanisms have been found related to therapy resistance, among them, point mutations in *ESR1* gene like Y537S and D538G [56]. Merenbakh-Lamin et al. (2013), identified D538G mutation, that leads to a conformational change in LBD, thus resistance to Tamoxifen and Fulvestrant might result from this conformational change in helix 12, which interferes with the binding to the receptor, leading to acquired endocrine resistance [56]. Also the mutation K303R was related to resistance to Anastrozole, this point mutation results in an increased binding between ERα-mutated and PI3K, leading to activation of Akt survival pathways, thus not only endocrine therapy with AIs become more ineffective, as also, PI3K/Akt pathway activation leads to tumor progression [96]. Another study from Fribbens et al. (2016), verified that patients with *ESR1* mutations has worse progression-free survival with Fulvestrant when compared to wild-type, thus, *ESR1* mutations analysis prior to treatment, may lead to the choice of a more effective treatment in order to reduce therapeutic resistance [97]. Guerrero-Zotano et al. (2018), performed a study in order to evaluate the resistance of $ER\alpha^+$ breast cancers in prolonged exposure to Letrozole, and verified that probably treatment with Letrozole remodeled the transcriptional landscape, once an higher number of mutations were found in patients with prolonged treatment with Letrozole [98].

In 2019, Xie et al. performed a retrospective study in order to evaluate the differences in treatment with Fulvestrant and Exemestane, and verified that a better efficacy was fund for Fulvestrant when compared to Exemestane after treatment failure with NSAIs [99].

Pan et al. (2017) performed a meta-analysis from 88 trails in women with ER^+ breast cancer that were submitted to endocrine therapy and were disease-free during the next 5 years [100]. The study evaluated the outcomes during the 5 to 20 years post treatment, and verified that twenty years after diagnosis, women still have a persistent risk of recurrence and death [100].

These studies emphasise the need to develop new SERMs/SERDs and AIs with better pharmacological properties, which should also take into account *ESR1* mutations.

Moreover and as previously referred, SNPs are often related to breast cancer risk, but the impact of SNPs in the tumor profile and in breast cancer treatment is not stablished. Several polymorphisms might promote altered transcriptional activity of ERα, contributing to carcinogenesis [2, 31, 32, 35, 44, 45]. Once there are genetic variants that are considered risk factors and that may influence malignant transformation [101], further studies should be performed in order to understand the pathway involved in this process and consequently genetic heterogeneity should be taken into account when choosing the treatment, in order to promote a more individualized and successful treatment.

Menopausal Hormone Therapy in Breast Cancer Patients

Women naturally enter in menopause, or due to treatment, experience a premature ovarian insufficiency, thus the main source of estrogen is adipose breast tissue, where estrogen is catalysed by aromatase [62, 71, 102].

Menopause hormone therapy (MHT) consists in the therapeutic replacement with exogenous estrogen and/or progesterone in order to avoid side effects of menopause [103]. However, there is a potential that MHT induces tumors in women with no history of breast cancer, also, women with previous history of breast cancer, mainly $ER\alpha^+$ breast cancer, might have a recurrence [102]. These two major considerations are not straightforward, as previously referred, there are several signalling pathways for ERα, each women has an unique genetic profile, resulting in an individual response to MHT, thus generalizing if breast cancer survivors should enter in MHT, or not, is dependent of individual characteristics. In this regard, women individual estrogens characterization should be take into account in the evaluation of the best MHT.

CONCLUSION

In this chapter, we provide an overview and the challenges of ERα in breast cancer carcinogenesis. The ERα signalling pathway along SNPs and point mutations are critical to the response of breast cancer to treatment, which they are intimately related to ERα stability. The ERα status is considered as the main factor to predict the response to endocrine therapy. However, further studies should be performed in order to better understand how individual heterogeneity affects the incidence, development and response of breast cancer to endocrine therapy. Although many clinical trials are carried out with new molecules, and that breast cancer patients may benefit from a more effective treatment, prevention through genetic profiles evaluation should also be concerned.

Along with prevention, there is also a need to perform further studies in breast cancer survivals, due to menopause and respective MHT. Overall, much has been done in the past decades on what was concerned about ERα positive breast cancers, but the progress in genomic and biological technologies will complement the knowledge that we know about ERα, making it possible to prevent and treat women, taking into account its individual characteristics. This is the main challenge for breast cancer treatment and will be the turning point to decrease relapse and increase overall survival.

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