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NRF2 and Keap1
Genetic Polymorphisms in Breast Cancer

Diana Raquel Careano da Costa

Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
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Orientadora: Prof.ª Doutora Luiza Augusta Tereza Gil Breitenfeld Granadeiro
Co-orientadora: Prof.ª Doutora Ana Cristina Ramalhinho Tavares Patricio
Co-orientadora: Mestre Micaela Carina Pereira Almeida

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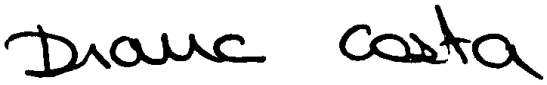

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A ti, pai.

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

Marie Curie

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Resumo

O cancro é uma das doenças com maior taxa de mortalidade, sendo o cancro da mama o mais frequente e com maior taxa de mortalidade no sexo feminino.

A exposição do ser humano ao stress oxidativo pode estar correlacionada com o desenvolvimento de diversas patologias, entre elas o cancro. As vias envolvidas no stress oxidativo representam um fator de risco para o desenvolvimento do cancro da mama. Um dos fatores mais importantes na regulação do stress oxidativo é o complexo *NF-E2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1* (Keap1) (Nrf2-Keap1).

A regulação deste complexo pode estar comprometida devido a mutações somáticas que influenciem a perda da regulação negativa ou à presença de *Single Nucleotide Polymorphisms* na região promotora do gene NRF2, que podem estar relacionados com alterações na expressão deste fator de transcrição e respetivas consequências biológicas.

Os polimorfismos genéticos tornaram-se potenciais alvos de investigação no cancro da mama devido à influência que os mesmos podem representar na carcinogénese e a sua possível associação torna-se um elemento-chave para investigação futura.

O objetivo principal deste trabalho consistiu na identificação de *Single Nucleotide Polymorphisms* não descritos na literatura nas regiões polimórficas dos genes NRF2 e KEAP1 em 40 doentes com cancro da mama confirmado histologicamente como hormono-dependente e estabelecer uma possível associação com o grau histológico do tumor. Para a identificação dos polimorfismos em estudo usou-se PCR e sequenciação de Sanger. De forma a poder relacionar e comparar os resultados obtidos no sangue com o tecido tumoral, foi otimizada a técnica de extração de gDNA de tecido parafinado de rato, usando para tal 9 amostras de tecido mamário de rato.

Os polimorfismos rs6721961, rs6706649, rs35652124 do NRF2 e rs1048290 da KEAP1 foram identificados em todas as amostras, não tendo sido identificadas mutações *de novo*. Estes resultados enfatizam a necessidade de estudar o tecido tumoral e verificar se este apresenta alterações quer a nível da expressão de genes como a nível do aparecimento de novas mutações relacionadas com o tumor.

A extração de gDNA de amostras de tecido parafinado de cortes mamários de rato foi bem-sucedida pelo que o protocolo foi devidamente otimizado, permitindo que o mesmo seja replicado, no futuro, em amostras humanas.

Palavras-chave

Cancro da mama; Polimorfismos genéticos; SNPs; NRF2; KEAP1

Resumo alargado

De acordo com os dados estatísticos da Agência Internacional de Pesquisa sobre o Cancro (IARC), o cancro da mama apresenta a maior taxa de incidência e mortalidade em mulheres.

Vários fatores de risco, como envelhecimento, menarca precoce, menopausa tardia, terapia de reposição hormonal, uso de contracetivos orais, obesidade, histórico familiar, lesões benignas, radioterapia, nutrição e outros hábitos de vida, estão envolvidos no desenvolvimento do cancro da mama.

A exposição a estrogénios é um fator de risco para o desenvolvimento e progressão do cancro da mama. O metabolismo do estrogénio desempenha um papel importante na iniciação e desenvolvimento do tumor. A exposição a altos níveis de estrogénio ao longo da vida e a desregulação de enzimas envolvidas na biossíntese e na via metabólica do estrogénio são consideradas fatores de risco para o cancro da mama.

O desequilíbrio metabólico desempenha um papel vital na etiologia do cancro da mama. A progressão da doença, a terapêutica contínua e a exposição a oxidantes levam ao aumento de espécies reativas de oxigénio e de azoto, promovendo instabilidade e resistência, resultando na progressão da doença e num pior prognóstico. As quantidades elevadas de espécies reativas nas células tumorais podem ser responsáveis por desencadear stress oxidativo que, consequentemente, pode causar danos no DNA, alterações nos genes supressores tumorais e, eventualmente, desenvolvimento e progressão do cancro. Um dos principais complexos que protege as células contra o stress oxidativo é o complexo *NF-E2-related factor 2 - Kelch-like ECH-associated protein 1* (Nrf2-Keap1).

O Nrf2 é um fator de transcrição, de zíper de leucina da região básica (bZIP) e um membro da família *cap "n" collar* (CnC) localizado, principalmente, no citoplasma das células. A Keap1, principal inibidora do Nrf2, é uma proteína adaptadora que apresenta na sua constituição um domínio que permite a sua associação a proteínas adaptadoras substrato-específicas relacionadas com a ubiquitina ligase (Cul3, Rbx, E2). O Nrf2 encontra-se no citoplasma acoplado à proteína Keap1. Quando as células são sujeitas a stress oxidativo, a Keap1 liberta o Nrf2, promovendo a sua deslocação para o núcleo, o que permite a ativação da transcrição de genes citoprotetores que codificam enzimas antioxidantes de fase II e proteínas responsáveis pelo transporte de xenobióticos. Desta forma, o Nrf2 pode proteger células e tecidos contra múltiplas fontes que provocam danos, incluindo stress eletrofílico, xenobiótico, metabólico e oxidativo.

No entanto, a ativação ou acúmulo aberrante do Nrf2, um evento comum em muitos tumores, confere uma vantagem seletiva às células tumorais e está associada à progressão maligna, resistência à terapia e um pior prognóstico dos pacientes. Isto sugere que é necessário alcançar um equilíbrio ideal entre as funções paradoxais do NRF2 na malignidade, o que pode resultar numa melhoria seletiva para identificar estratégias terapêuticas no tratamento do cancro.

A pesquisa de marcadores genéticos de suscetibilidade ao cancro da mama tem levado a um número crescente de estudos epidemiológicos de polimorfismos genéticos, mais propriamente *Single Nucleotide Polymorphisms*, que apresentam um papel importante no metabolismo dos estrogénios e/ou na ativação e detoxificação de carcinogénicos.

O objetivo principal deste trabalho consistiu na identificação de *Single Nucleotide Polymorphisms* não descritos na literatura nas regiões polimórficas dos genes NRF2 e KEAP1 em mulheres diagnosticadas com cancro da mama. Pretende-se analisar a hipótese de estes polimorfismos estarem associados a um aumento do risco e consequente aumento do grau histológico do tumor, de forma a estabelecer o complexo Nrf2-Keap1 como biomarcador do prognóstico tumoral.

Numa primeira parte deste trabalho, foram sequenciadas 40 amostras de sangue de doentes recrutadas no Centro Hospital Universitário Cova da Beira diagnosticadas com cancro da mama confirmado histologicamente como hormono-dependente de forma a identificar e genotipar *Single Nucleotide Polymorphisms* do NRF2 e da KEAP1. Verificou-se a presença dos polimorfismos rs35652124, rs6706649, rs6721961 e rs104829 em todas as amostras. No entanto, nenhuma outra mutação foi identificada. Este resultado reforça a necessidade de estudar o tecido tumoral das pacientes e comparar os resultados obtidos com os resultados previamente analisados do sangue.

Para avançar para estudos no tecido tumoral, foi necessária uma otimização da técnica de extração de gDNA de tecido parafinado. A parafina funciona como uma barreira física que protege o tecido, mas, no entanto, pode interferir com a extração eficiente de DNA e, por isso, a sua remoção requer especial atenção. De forma a evitar o desperdício de amostras biológicas humanas, foram usados 9 cortes de tecido mamário de rato para esta otimização. De referir que nenhum animal foi eutanasiado apenas para este trabalho. Após extração de gDNA do tecido com o uso do protocolo otimizado, procedeu-se à quantificação da concentração de DNA das amostras onde se obteve uma concentração de DNA de 20,5 ng/µL e A260/A280 = 1,864. Para complementar, foi realizado PCR com o uso de dois primers de rato de dois genes *housekeeping*, Gliceraldeído-3-fosfato desidrogenase e Ciclofilina A, e uma eletroforese em gel que permitiu visualizar uma

correta amplificação das sequências de interesse, o que evidencia uma extração de DNA bem-sucedida. Esta otimização é de extrema importância uma vez que permitirá avançar para estudos em tecido tumoral e possivelmente identificar mutações *de novo* associados ao tumor.

Abstract

Cancer is one of the diseases with the highest mortality rate, with breast cancer being the most frequent and with the highest mortality rate in females.

Human exposure to oxidative stress may be correlated with the development of several pathologies, including cancer. The pathways involved in oxidative stress represent a risk factor for the development of breast cancer. One of the most important factors in oxidative stress regulation is the NF-E2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) (Nrf2-Keap1) complex.

The regulation of this complex may be compromised, due to somatic mutations that influence the loss of negative regulation or the presence of Single Nucleotide Polymorphisms in the promoter region of the NRF2 gene, which may be related to changes in the expression of this transcription factor and its biological consequences.

Genetic polymorphisms have become potential research targets in breast cancer due to their influence on carcinogenesis and their possible association becomes a key element for future research.

The main objective of this work was to identify Single Nucleotide Polymorphisms not described in the literature in the polymorphic regions of the NRF2 and KEAP1 genes in 40 patients with histologically confirmed hormone-dependent breast cancer and to establish a possible association with the histological grade of the tumour. To identify the polymorphisms under study, PCR and Sanger sequencing were used. In order to be able to relate and compare the results obtained in the blood with the tumour tissue, the technique of extracting gDNA from mouse paraffin-embedded tissue was optimized, using 9 samples of mouse mammary tissue.

Polymorphisms rs6721961, rs6706649, rs35652124 from NRF2 and rs1048290 from KEAP1 were identified in all samples, and *de novo* mutations were not identified. These results emphasize the need to study the tumour tissue and verify if it presents alterations in gene expression and appearance of new tumour-related mutations.

The gDNA extraction from paraffin-embedded tissue samples from mouse mammary sections was successful, so the protocol was properly optimized, allowing it to be replicated, in future, in human samples.

Keywords

Breast cancer; Genetic polymorphisms; SNPs; NRF2; KEAP1

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List of Abbreviations

A	
A	Adenine
ARE	Elements Responsible for the Antioxidant Response
B	
BTB	Broad-complex, Tramtrack and Bric-à-brac
bZIP	Basic Leucine Zipper
C	
C	Cytosine
CnC	Cap'n'Collar
Cul-3	Cullin 3
CypA	Cyclophilin A
D	
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
F	
FFPE	Formalin-Fixed Paraffin-Embedded
G	
G ; g	Guanine ; grams
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic DNA
GSH-Px	Glutathione Peroxidase
GSTM1	Glutathione S-Transferase M1
GSTT1	Glutathione S-Transferase T1
GSTP1	Glutathione S-Transferase P1
H	
H2O2	Hydrogen Peroxide
HO·	Hydroxyl Radicals
HO-1	Heme Oxygenase-1
I	
IARC	International Agency for Research on Cancer
IVR	Intervening Region
K	
KEAP1	Kelch-like ECH-associated Protein 1

M	
Maf	Masculoaponeurotic Fibrosarcoma Proteins
mL	Millilitre
mRNA	Messenger RNA

N	
Neh	Nrf2-ECH Homology
nM	Nanometre
NO	Nitric Oxide
NQO1	NAD(P)H:quinone Oxidoreductase 1
NRF2	Nuclear Factor Erythroid 2–related Factor 2

O	
O2 ·-	Superoxide Anion Radicals
ONOO-	Peroxynitrite
OS	Oxidative Stress

P	
PCR	Polymerase Chain Reaction
PK	Proteinase K

R	
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Revolutions per Minute
RT	Room Temperature
RXRα	Retinoid X receptor alpha

S	
sMaf	Small Maf protein
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase

T	
T	Thymine

V	
V	Volt

Others	
3'-UTR	3'-Untranslated Region
µl	Microliter

Chapter 1

Introduction

1. Introduction

1.1. Breast Cancer

Worldwide, cancer is a major public health problem (1), being breast cancer the most prevalent among women, representing 30% of female cancers, besides its prevalence is also a type of cancer with high morbidity and mortality, with an incidence of 15% (figure 1) (2).

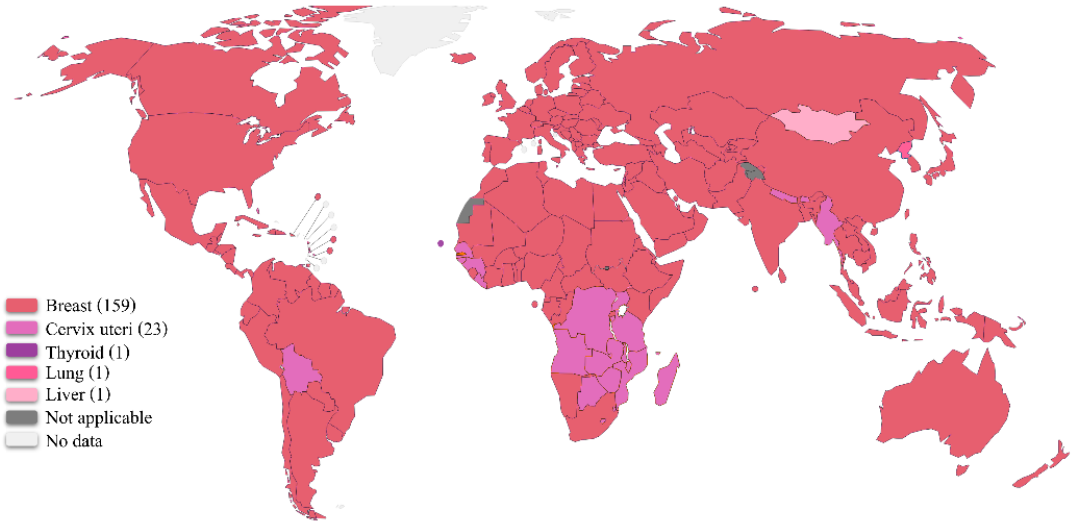
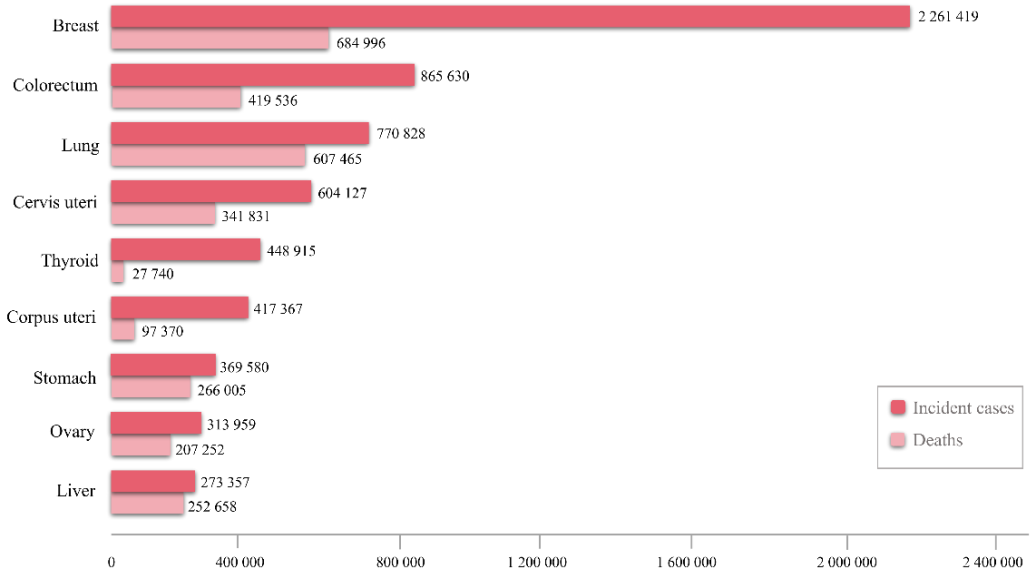


Figure 1: Top cancer per country, estimated number of new cases in 2020, females, all ages. The pink colour represents breast cancer (Adapted from (3)).

In 2020, breast cancer was diagnosed in 2.26 million women and led to 684,996 deaths (graph 1) (4). At the end of 2020, in a 5 years follow-up, there were 7.8 million women alive who were diagnosed with breast cancer, which makes this disease the most prevalent cancer in the world (5). In Portugal, in 2020, were diagnosed 7 041 new cases in women and 1 864 deaths (3).



Graph 1: Estimated number of incident cases and deaths worldwide, females, all ages (Adapted from (3)).

1.2. Breast Cancer Risk Factors

The development of the mammary gland is a complex process, whose phases and functions are driven by a vast number of hormones and growth factors, with estrogen and progesterone responsible for most of the physiological changes (6). As in other tissues, breast tissue homeostasis is maintained through a balance between proliferation, differentiation, and apoptosis (7, 8).

There are several factors identified as breast cancer risk factors, among them is exposure to estrogens, hormone therapy, lifestyle factors such as diet, alcohol intake and smoking (9). Other known risk factors include a history of ductal carcinoma *in situ*, high body mass index, first birth at age greater than 30 years or nulliparity, early menarche (before age 13 years), family history of breast or ovarian cancer, late menopause, and postmenopausal hormone therapy (10).

About 10% of all cases of breast cancer are related to genetic predisposition or family history (11).

Breast cancer risk is correlated with lifetime exposure to higher levels of both endogenous and exogenous estrogens and these have been identified has carcinogens (12). Most breast cancers are estrogen receptor-positive (ER+) (13). However, the carcinogenic effects of estrogens can be mediated by both estrogen receptor-dependent and estrogen receptor-independent pathway (14). Also, estrogen metabolism plays an important role in tumour initiation and development, the deregulation of estrogen biosynthetic and metabolic pathway enzymes are an important factor which contributes to an increased risk of developing breast cancer (15). The production of reactive estrogen quinones and the subsequent synthesis of oxygen free radicals play a crucial role in the beginning of estradiol-induced breast cancer (16). These genotoxic metabolites can interact with DNA to create unstable DNA adducts that cause mutations and promote breast cancer development (17).

The permanent modification of the genetic material of cells, which results from oxidative damage, is one of the main causes of mutagenesis that could lead to the further development of a carcinogenic process (18).

Oxidative stress (OS) has been studied for many years, with the first studies reported since the 1970s (19). The excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as nitric oxide (NO), hydrogen peroxide (H2O2), peroxynitrite (ONOO-), hydroxyl radicals (HO·) and superoxide

anion radicals (O2 ·-) can promote damage to DNA, cell membranes, and proteins, leading to pathological processes (20).

High cellular ROS levels result from an imbalance between ROS production and the antioxidant system. To combat oxidative damage, several defense mechanisms allow the maintenance of the physiological conditions. The first line of cellular defense is ensured by antioxidant substances, such as vitamins C and E, glutathione and β–carotene. The molecules involved in the second-phase response are essentially protein nature (21).

There are several studies that have presented evidence of the role of OS in the etiology of breast cancer (22, 23).

Ramalhinho A *et al.* (2011) reported that Glutathione S-transferase M1 (GSTM1) and Glutathione S-transferase T1 (GSTT1) null polymorphisms, that imply the total absence of GSTM1 and GSTT1 enzymes, are related, alone or in association, with increased risk of breast cancer (24).

Also, Almeida M. *et al.* (2019) referred that the cumulative presence of polymorphisms in phase II enzymes and in its regulators Nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-Like ECH-Associated Protein 1 (KEAP1) might result in metabolic imbalance, which can compromise antioxidant response (25).

1.3. Oxidative Nrf2-Keap1 Pathway

Nrf2 is a transcription factor and a master regulator of multiple antioxidant responses playing an essential role in cytoprotection (25, 26).

Nrf2 is a soluble protein, consisting of 589 amino acids, and belongs to the cap'n'collar (CnC) subfamily of proteins that have a "basic leucine zipper" (bZIP) in their constitution (27). This set of proteins has structural characteristics that are homologous to each other, such as the presence of a CnC domain in the N-terminal region adjacent to the DNA binding site and a bZIP domain in the C-terminal region, characterized by the periodic repetition of leucine residues (28, 29). The Nrf2 protein contains seven domains, Neh1-Neh7, each with an important role for its correct performance (figure 2a). The Neh1 domain is a basic region leucine zipper motif that confers stability to the protein, DNA binding and helps in dimerization with Maf proteins. The Neh2 domain contains two high and low affinity binding motifs, ETGE and DLG respectively, responsible for the interaction between Nrf2 and Keap1. It is the interactions between these two binding sites and the DGR domain of Keap1 that are responsible for mediating Nrf2 activity. Since Keap1 functions as a substrate for the Cullin 3 (Cul3)-dependent E3 ubiquitin ligase complex, this protein is able to repress the activity of Nrf2 (30). Transactivation activity is mediated by the Neh3, Neh4 and Neh5 domains. The Neh6 domain, a serine-rich region, is important for stability. Finally, the Neh7 domain interacts with a nuclear receptor, more specifically Retinoid X Receptor Alpha (RXRα), that inhibits the Nrf2-ARE signalling pathway (31, 32).

Keap1 is the master negative regulator of Nrf2 (33). Keap1 protein contains three major domains: BTB (broad-complex, tramtrack and bric-à-brac) domain mediates Keap1 homodimerization and associates with Cul3; IVR (intervening region) domain contains critical cysteine residues and connects the BTB domain with the C terminus Kelch/DGR domain; and Kelch/DGR domain mediates binding with the Neh2 domain of Nrf2 (figure 2b) (34, 35).

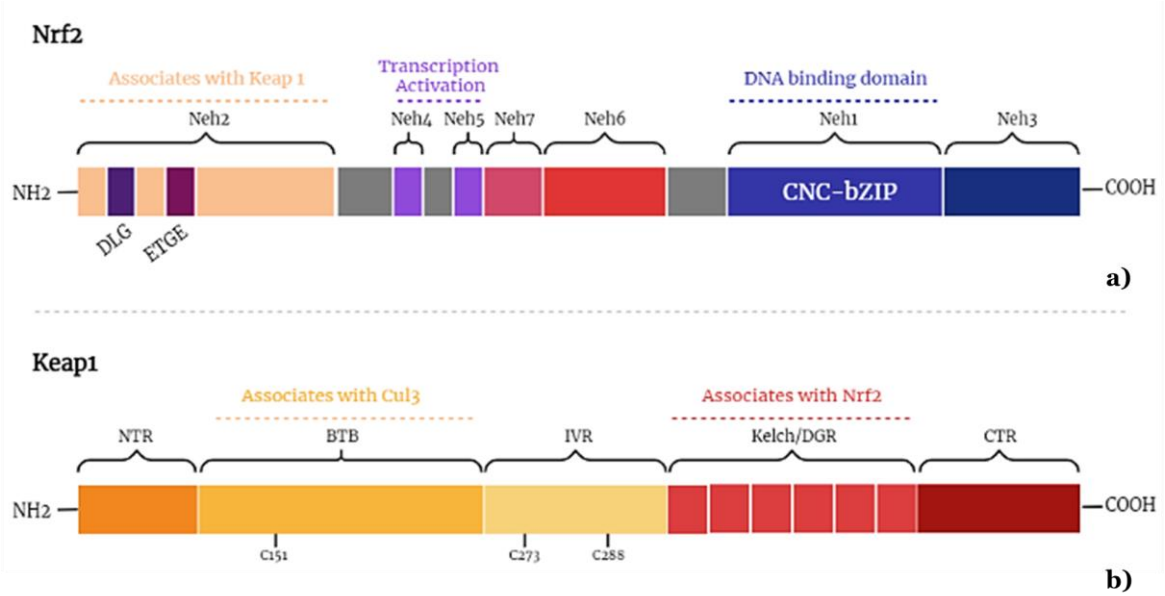


Figure 2: Domain structure of Nrf2 and Keap1. **a)** Functional Nrf2-ECH homology (Neh) domains. **b)** Functional Keap1 domains (NTR, BTB, IVR, Kelch/DGR and CTR).

The binding model, called "hinge-and-latch", proposes that when cells are subjected to OS, Keap1 undergoes a conformational change that prevents it from binding to the DLG domain, and Nrf2 ubiquitination does not occur (36).

In figure 3, it can be seen that under basal circumstances, Nrf2 is found in the cytoplasm and forms a complex with Keap1. When exposed to OS, Nrf2 is released by Keap1, allowing it to move to the nucleus where it stimulates the activity of components of antioxidant defense, more specifically, inducing the expression of cytoprotective genes like phase-II detoxification enzymes such as glutathione S-transferase M1 (GSTM1), glutathione S-transferase T1 (GSTT1), glutathione S-transferase T1 (GSTP1), superoxide dismutase (SOD), glutathione reductase, ferritin, thioredoxin reductase, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (37, 38).

These enzymes are essential for preventing the mutagenesis that underlies OS-associated carcinogenesis. When basal state is recovered, Keap1 moves to the nucleus, rebinding to Nrf2 and bringing it back to the cytoplasm to suffer proteasomal degradation (39, 40).

By preventing possible DNA damage and mutations, NRF2 is considered a cancer inhibitor (25). Several studies have been carried out that reflect the importance that Nrf2 assumes in protecting cells from damage promoted by internal and/or external factors such as OS and xenobiotics, respectively (30, 41). However, Nrf2-Keap1 complex might be deregulated contributing to chemoresistance (25).

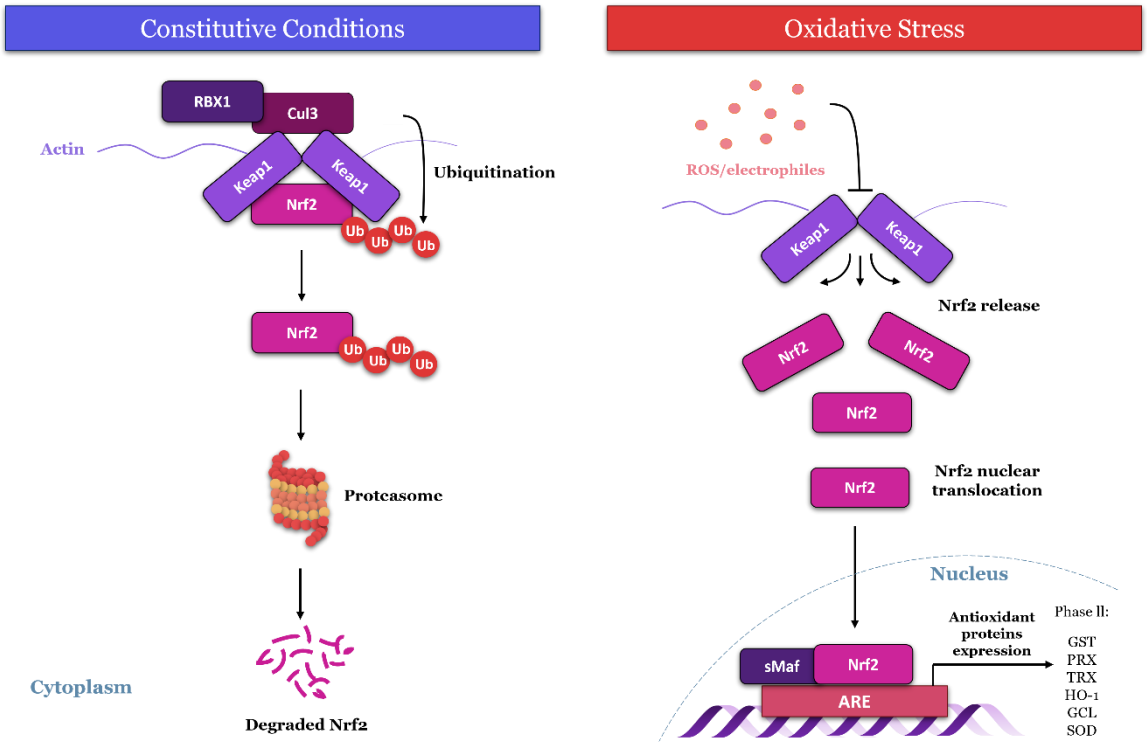


Figure 3: Nrf2-Keap1 pathway. Under constitutive conditions, Keap1 targets Nrf2 for proteasomal ubiquitination. However, when subjected to OS, conformational changes are triggered in Keap1 that allow Nrf2 to move to the nucleus, where it associates with small Maf (sMaf) proteins from the masculoaponeurotic fibrosarcoma (Maf) family, forming a dimer that facilitates its binding to Elements Responsible for the Antioxidant Response (ARE) present in the promoter regions of several cytoprotective genes. Consequently, the formation of this complex promotes the transcription of phase II enzymes.

1.4. The Dual Role of NRF2-KEAP1 Complex: Tumour Suppressor vs Oncogene

NRF2 is responsible for the transcription of cytoprotective genes, the activation of this transcription factor preserves cells from carcinogenic stimuli (42). This fact is supported by studies carried out *in vivo* in mice, which, after silencing the NRF2 gene, showed a reduction in the expression of phase II enzymes, which increased the sensitivity to exposure to carcinogenic elements and prevented the Nrf2-inducing compounds from performing their function (43, 44).

NRF2 downstream genes can be grouped into different categories that play important roles in metabolism, apoptosis, intracellular redox-balancing, and autophagy (figure 4) (45-48). This demonstrates that Nrf2 activation is pivotal in protecting against various stresses resulting from toxic exposure, which makes it one of the major cellular defense mechanisms (49).

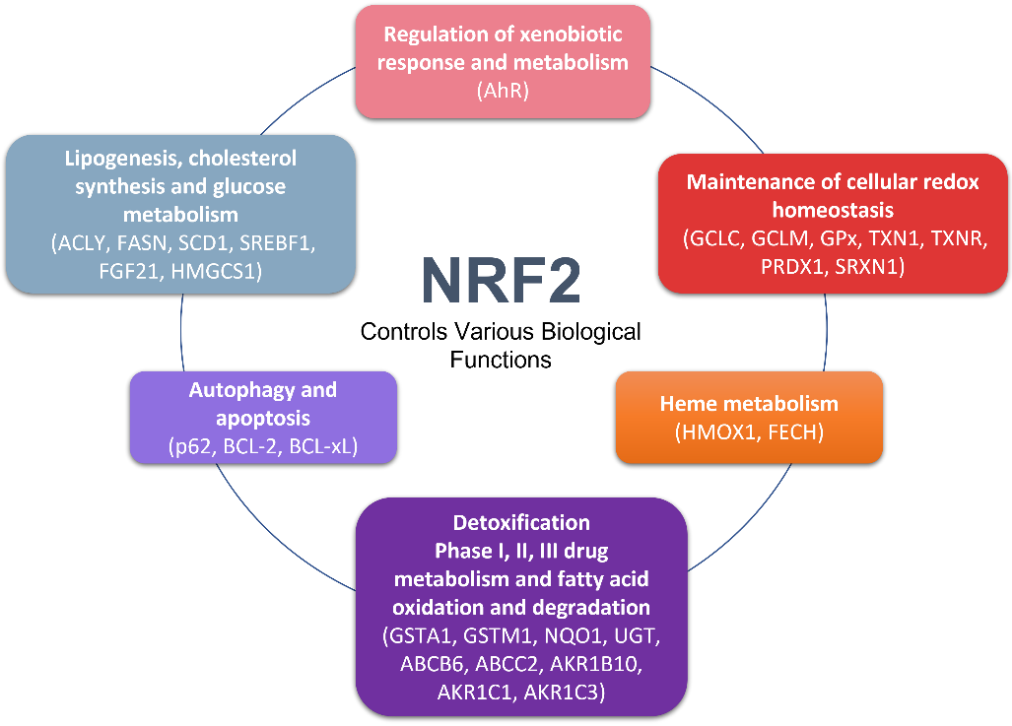


Figure 4: NRF2 downstream genes and the role they play in different mechanisms.

Despite evidence that Nrf2 under normal conditions has a positive influence as a protector of cellular OS, new researchs suggested that Nrf2 deregulation due to NRF2-KEAP1 polymorphisms might compromise detoxification and even lead to chemoresistance (50). It was found that, despite protecting normal cells, Nrf2 also promotes the survival of cancer cells, acting as an oncogene which raised doubts about the "dark side of Nrf2" (51-53).

Nrf2 overexpression is found in several types of cancers such as lung, pancreas, endometrium, bladder, ovaries, and breast (54-57).

Since OS is associated with the initiation of cancer, several hypotheses have emerged about the role of Nrf2: an anticancer role and chemoprevention or hyperactivation of Nrf2 in tumours that create a favourable environment for the survival of cancer cells by protecting them from excessive OS, chemotherapeutic agents or radiotherapy (figure 5) (53, 58).

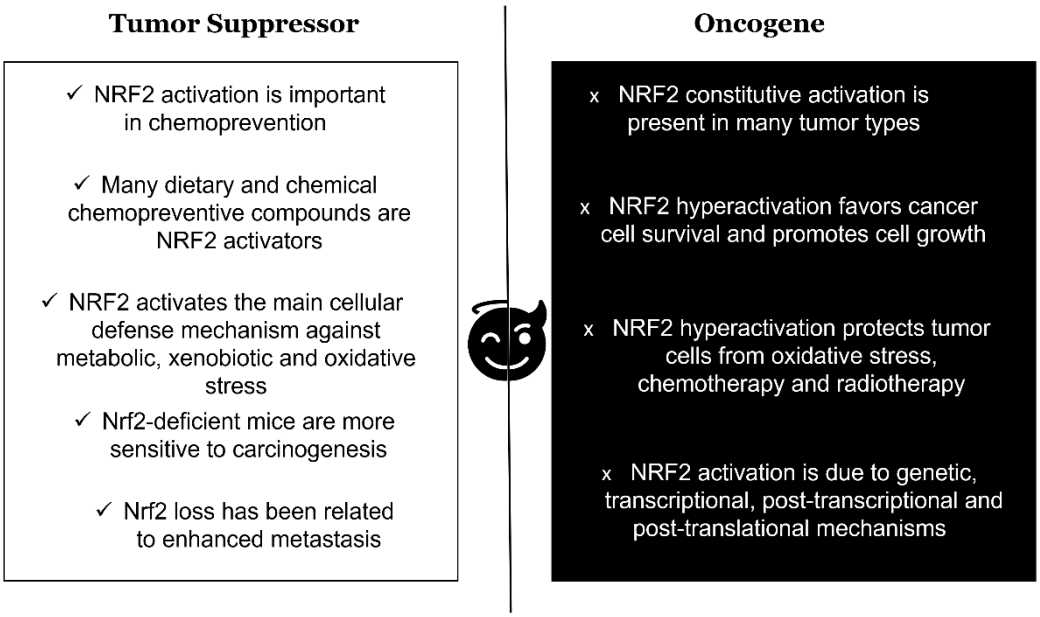


Figure 5: Dual roles of NRF2. Evidence has highlighted that the complex that involves NRF2 appears to be dysregulated in some types of cancer and may promote chemoresistance (Adapted from (53)).

The dysregulation of the Nrf2 signalling pathway, specifically its aberrant activation, has been associated with the harmful effect of tumour cells since the Nrf2 target genes play important roles in cell resistance, survival and proliferation, and may promote carcinogenesis in several cancer types (59, 60).

Overexpression or constitutive activation of the Nrf2-Keap1 pathway that lead to an increase in the antioxidant response in tumour cells that, consequently, allows their survival, overexpression is mainly due to single nucleotide polymorphisms (SNPs) in NRF2-KEAP1 complex (61, 62).

1.5. NRF2-KEAP1 Genetic Polymorphisms

When alternative forms of a gene or intergenic sequence have a frequency of 1% or more in a population, they are called DNA polymorphisms (63). Polymorphisms are considered normal variations in a population, so none of the forms significantly affects the carrier and does not directly cause a disease, but rather, it may serve as a predisposing factor (64). They may be associated with the alteration of a single nucleotide in genomic DNA (gDNA) - Single Nucleotide Polymorphism (SNP) - or with the variation of a repetitive number of DNA sequences - microsatellites or minisatellites (65).

Both transcription and translation can be affected by mutations located at specific important gene sites such as the coding sequence, intron-exon boundaries, and regulatory elements (66). Studies from the sequencing of cancer genomes have shown that most mutations occur in genes in which the products impair signalling pathways that are involved in important cellular functions (67, 68).

SNPs are the most abundant genetic variations, occurring in 100-300 base pairs in the human genome and represent 90% of human sequence variations (69-71). SNPs can be functionally neutral or non-functional, as most are, or have functional effects. These last are related to population diversity, susceptibility to diseases and the way each individual responds to therapy (72, 73). Some SNPs are associated with breast cancer risk, however, their association with prognosis is not yet fully understood (figure 6) (74).

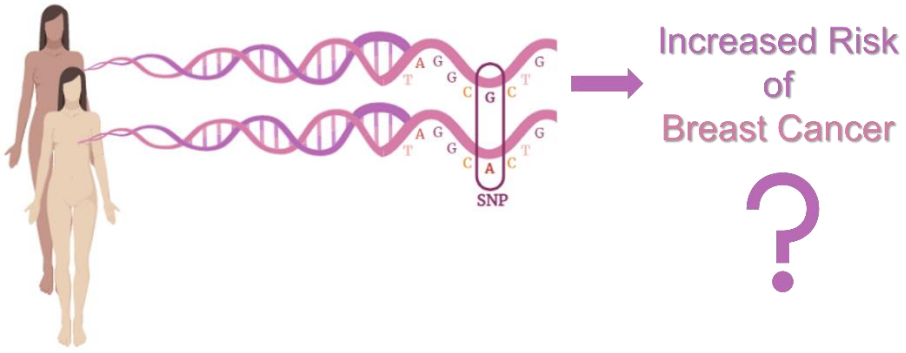


Figure 6: Schematic representation of a point mutation, Single Nucleotide Polymorphism (SNP). Several of these mutations are associated with breast density parameters and risk of breast cancer and are increasingly studied as possible tumour prognostic markers.

NRF2 gene is located in the cytogenetic band 2q31.2 of chromosome 2, has 5 exons and the transcript encodes a Nrf2 transcription factor containing 605 amino acid residues (figure 7) (75).

Studies have shown that it is a highly polymorphic gene (41, 76). The pioneer study of the structure of NRF2 was carried out by Yamamoto *et al.*, in which three SNPs, rs6721961 (-617, C>A ; location on chromosome 2:177265309), rs6706649 (-651, G>A and location on chromosome 2:177265343) and rs35652124 (-653, A>G and location on chromosome 2:177265345) were discovered in the regulatory region of the gene (figure 7) (77). Later, Marzec *et al.* studied the effect of these SNPs on the regulation of gene expression (78). The influence of these in the transcriptional activity of NRF2 was evaluated, verifying the reduction of the NRF2 expression and the consequent reduction of the basal levels of the Nrf2 protein and the transcription of the genes dependent on it (79, 80). These genetic polymorphisms have been reported as possible tumour prognostic markers (81).

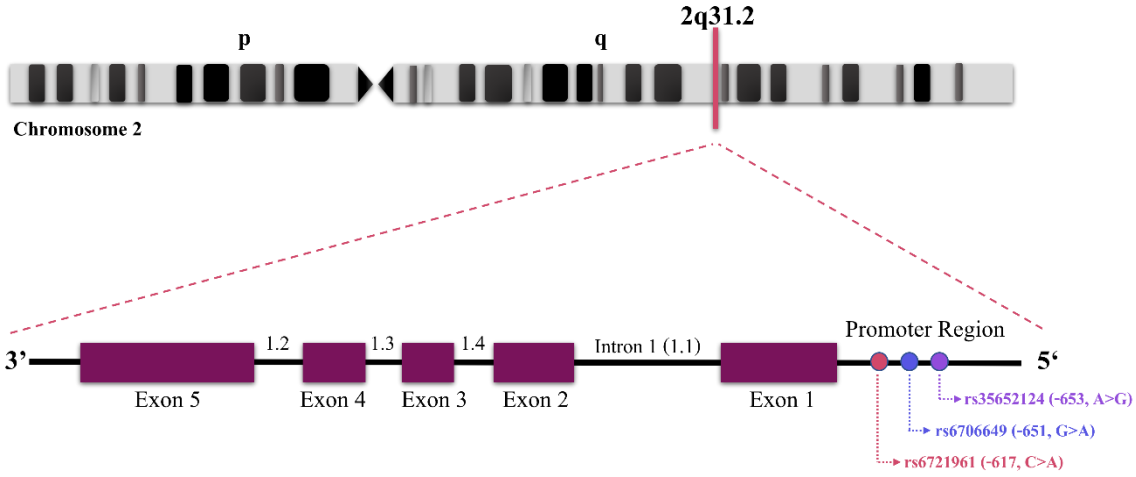


Figure 7: Single Nucleotide Polymorphisms rs6721961, rs6706649 and rs35652124 represented in the promoter region of NRF2 gene located on chromosome 2. (Adapted from (75)).

The effect of NRF2 gene polymorphisms has been related to the development of various pathological conditions (81-85).

A study by Shimoyama Y. *et al.* (2014) demonstrated an association of the rs35652124 SNP with cardiovascular disease, it was observed that diabetics with rs35652124 T promoter allele had an increased risk of having cardiovascular disease (82).

Another study by Okano Y. *et al.* (2013) was carried out with non-smoking women in Japan, where the SNP rs6721961 T allele was related to susceptibility to lung adenocarcinoma (81).

Shaheen S. *et al.* (2010) associated an early gestational acetaminophen exposure with an increased risk of asthma in children aged 7 years. They found that when copies of the SNP rs6706649 T allele was present, this association increased significantly (83).

A study by Hartikainen J. *et al.* (2012) demonstrated that there are SNPs in the NRF2 pathway that affect both susceptibility for breast cancer and the outcome of the breast cancer patients. Since the T allele was related to decreased Nrf2 protein expression in breast cancer tissue, the NRF2 promoter polymorphism rs6721961 was associated with a higher risk of breast cancer (41).

The meta-analysis by Almeida M. *et al.* (2019) concludes that high levels of Nrf2 expression is associated with a poor outcome in breast cancer patients. This high expression can promote the cytoprotection of tumour cells from OS leading to therapeutic resistance and, consequently, tumour progression (49).

Besides NRF2 SNPs, also polymorphisms in KEAP1 might lead do Nrf2-Keap1 complex deregulation. KEAP1 gene is located on chromosome 19p13.2 (figure 8). Keap1 has been found to have an abnormal function in cancer cells, leading to an increase in unbound Nrf2 that accumulates in the nucleus and promotes carcinogenesis and tumour resistance to adjuvant therapies (25, 86).

Hartikainen J. *et al.* (2015) were the first to associate polymorphisms in the KEAP1 gene with breast cancer risk. They identified the rs1048290 SNP located in the exon 4, genomic region encoding the DGR domain of Keap1, which interacts directly with Nrf2 (87). This SNP characterized by the substitution of a cytosine (C) for a guanine (G) is associated with increased Keap1 protein expression and, consequently, may interfere with Nrf2 protein levels and with the Nrf2-Keap1 signalling pathway itself (86).

Figure 8: Schematic representation of chromosome 19, where the KEAP1 gene is located at 19p13.2.

The influence of these polymorphisms in KEAP1 and NRF2 are of main importance in order to understand its role in breast cancer development and prognosis. Thus, is of main importance to identify new SNPs in the germline of the complex NRF2-KEAP1 besides the already described by our research team. Also, the identification of SNPs in tumour tissue will allow to confirm if tumour development is due to acquisition of new mutations.

Chapter 2

Aims

2. Aims

The main objective of this work is the identification of undescribed mutations in the polymorphic regions of NRF2 and KEAP1 genes and to establish an association with the histological grade of the tumour. More specifically, it is intended to:

i. Identify new SNPs in NRF2 and KEAP1 genes in women diagnosed with breast cancer and establish a relationship with tumour histological grade;

ii. Following the previous objective, we aimed to optimize the technique of gDNA extraction from paraffin-embedded tissue, that will be used, in future, to identify *de novo* SNPs in tumour tissue.

Chapter 3

Materials and Methods

3. Materials and Methods

3.1. Sequencing of SNPs from Blood Tissue

3.1.1. Study Population

Breast cancer patients were recruited at Child and Women Health Department, Gynaecologic Oncology Division of Hospital Centre of Cova da Beira (Covilhã), accounting with a total of 40 breast cancer patients. Informed consent was obtained from all participants included in the study and all patients have histologically confirmed hormone-dependent breast cancer.

3.1.2. Equipment and Consumables

- Wizard® Genomic DNA Purification Kit (Promega)
- Cell Lysis Solution (provided by kit)
- Nuclei Lysis Solution (provided by kit)
- Protein Precipitation Solution (provided by kit)
- Isopropanol
- Ethanol
- Micropipettes and Tips
- Eppendorfs
- Centrifuge Tubes
- Agarose
- 1x TAE Buffer
- GreenSafe DNA Gel Stain
- Thermocyclers
- Water bath
- Microcentrifuge
- Vortexer

3.1.3. 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 which are summarized in figure 9.

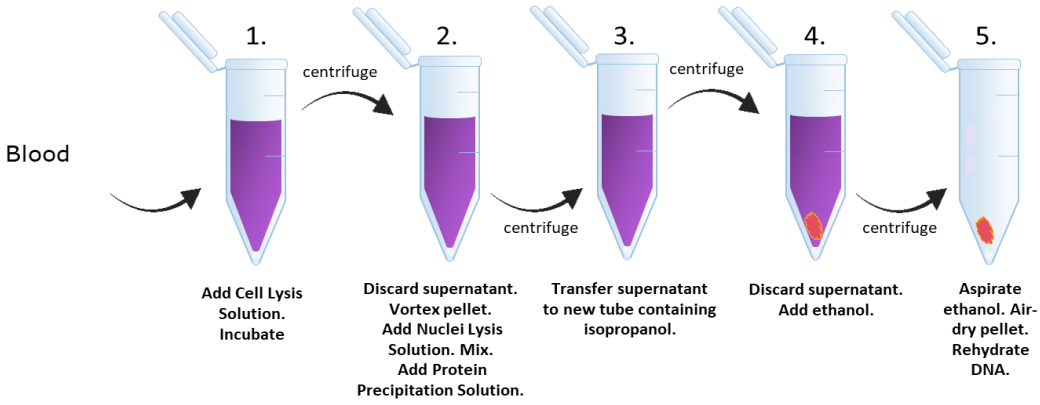


Figure 9: Main steps of DNA extraction from blood according to the kit.

3.1.4. SNPs amplification

Amplification of the NRF2 and KEAP1 fragments was performed through PCR reaction. The mixture was carried out in a total volume of 25 µL and contained 10 pmol of each primer, 1.5 mM of MgCl2, 100 nM of each deoxynucleotide triphosphate, 1 unit of DreamTaq DNA polymerase and 100 ng of genomic DNA. The amplification was performed on the Bio-Rad MyCycler Thermal Cycler® equipment.

The region containing the SNPs rs35652124, rs6721961 and rs6706649 of NRF2 was amplified with the primers: forward primer 5'-CCC TGA TTT GGA GGT GCA GAACC-3' and reverse primer 5'-GGG GTT CCC GTT TTT CTC CC-3' with the following conditions: 95°C for 10 min for initial denature followed by 35 cycles at 95°C for 1 min, at 60°C for 1 min, at 72°C for 1 min and additionally 72°C for 5 min.

For KEAP1 rs1048290 it was used the follow sequence of primers: forward primer 5'-TGT CCC CAT TTT TCT TAC GC-3' and reverse primer 5'- AGC AAA AGC AAA AGC AGT CC-3'. PCR conditions were 95°C for 5 min for initial denature followed by 35 cycles at 95°C for 30 sec, at 53°C for 30 sec, at 72°C for 30 sec and additionally 72°C for 10 min.

PCR product amplification was confirmed in a 2% agarose gel electrophoresis. To prepare the gel, 2 g of agarose were diluted in 100 mL of 1X TAE buffer by heating the solution to which, after cooling, GreenSafe® was added at a rate of 1 µL/mL. After polymerization, the gel was loaded with 7 µL of final product and 2 µL of 100 base pair (bp) molecular weight marker. The gel was subjected to a voltage of 120 V for 30 min. The electrophoresis results were observed by exposing the gel to UV light with the aid of the Uvitec Cambridge® equipment.

3.1.5. Sequencing

After confirming the amplification, the samples were purified using a mix of 1:2 of with Fastap and EXOI, respectively, during 20 min at 37ºC, 20 min at 80ºC and 1 min at 12°C.

The KEAP1 and NRF2 PCR amplified fragments were sequenced through Sanger sequencing (performed by STABVIDA®). For this, 5 µL of H2O plus 3µL of Primer (Forward or Reverse) plus 5 µL of purified PCR product are placed on the plate or in the 1.5 µL eppendorf. An online request is made on the STABVIDA ® website, where it is necessary to fill in the table with the name to be given to each sample, activating the code on the labels according to the information in the table. Note that when filling out the request, it must include in the notes: “Use a protocol for detection of heterozygotes”. Label the plate or each eppendorf (as applicable). The samples are then placed in an envelope in the cold until collection is made.

3.2. Optimization of DNA Extraction from paraffin-embedded Tissue

3.2.1. Mouse Breast Tissue

Since the protocol used required prior optimization due to the critical step for paraffin removal, so that it would not interfere with DNA extraction, mouse breast sections were used to avoid wasting human breast tissue until that step has been succeed.

The mouse mammary tissue samples were acquired at the Health Sciences Research Centre of the University of Beira Interior (CICS-UBI, Covilhã). Therefore, 9 samples of mouse mammary tissue were used to optimize the protocol. From those 9 samples, 3 were slices of 5 μm and 6 were 10 μm slices. We emphasize that no animals were euthanized specifically for the present work, they were previously sacrificed for other researchers, and we removed breast tissue.

3.2.2. Equipment and Consumables

- QIAamp DNA FFPE Tissue Kit (QIAGEN®)
- Xylene
- Ethanol (96–100%)
- 1.5 ml or 2 ml Tubes
- Micropipettes and Tips
- Eppendorfs
- Tubes
- Heat Block
- Microcentrifuge
- Vortexer
- Nanophotometer
- Agarose
- 1x TAE Buffer
- GreenSafe DNA Gel Stain
- Thermocyclers
- Mouse primers (table 1)

Table 1: Mouse primers used for amplification of housekeeping genes GAPDH and CypA.

Gene	Primer	Sequence	Reference
rGAPDH	FW	5' AGA CAG CCG CAT CTT CTT GT 3'	(88)
	RV	5' TGA TGG CAA CAA TGT CCA CT 3'	
rCypA	FW	5' CAA GAC TGA GTG GCT GGA TGG 3'	(89)
	RV	5' GCC CGC AAG TCA AAG AAA TTA GAG 3'	

Chapter 4

Results and Discussion

4. Results and Discussion

In the present study we intended to identify new polymorphisms in the polymorphic regions of NRF2 and KEAP1 genes, in peripheral blood of 40 patients with histological confirmed hormone-dependent breast cancer. PCR was performed followed by electrophoresis to confirm the amplification of the target genes. In figure 10 is shown the amplification of NRF2 and KEAP1 PCR products of 8 participants of the study. The samples were purified to eliminate possible contaminating waste using Fastap and EXOI enzymes. The resulting products were sent for sequencing analysis, which was performed by STABVIDA®.

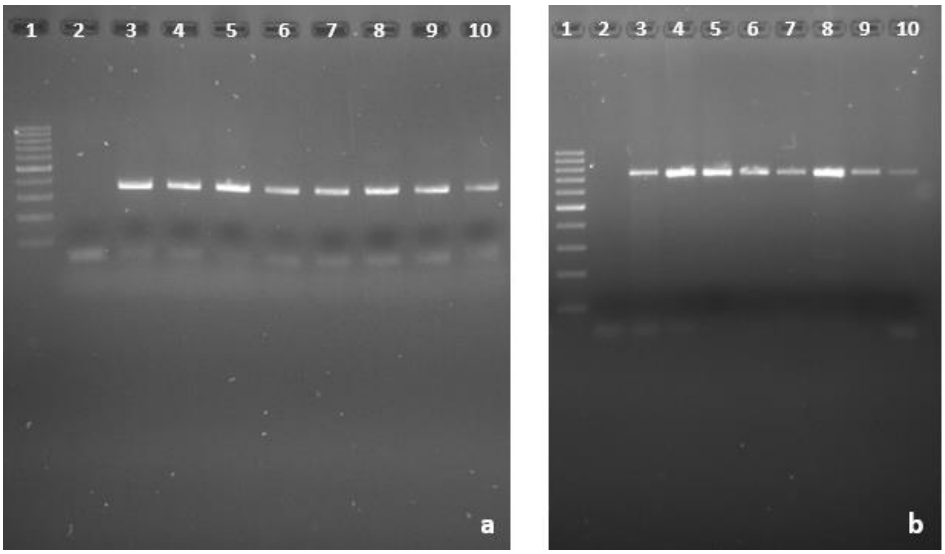


Figure 10: PCR product electrophoresis results of 8 patients. **a)** Verified amplification of the NRF2 gene in all samples. **b)** Verified amplification of the KEAP1 gene in all samples. **Well 1** - molecular weight marker; **Well 2 -** negative control; **Well 3-10 -** samples. The primer sequences used were: NRF2 - FW: 5' CCCTGATTTGGAGGTGCAGAACC 3'; RV: 5' GGGGTTCCCGTTTTTCTCCC 3' and for KEAP1 - FW: 5' TGTCCCCATTTTTCTTACGC 3'; RV: 5' AGCAAAAGCAAAAGCAGTCC 3'.

The analysis of the sequencing results allowed us to identify the presence of single nucleotide polymorphisms described in literature, rs35652124, rs6706649 and rs6721961 from NRF2 and rs1048290 from KEAP1, in all samples (figure 11).

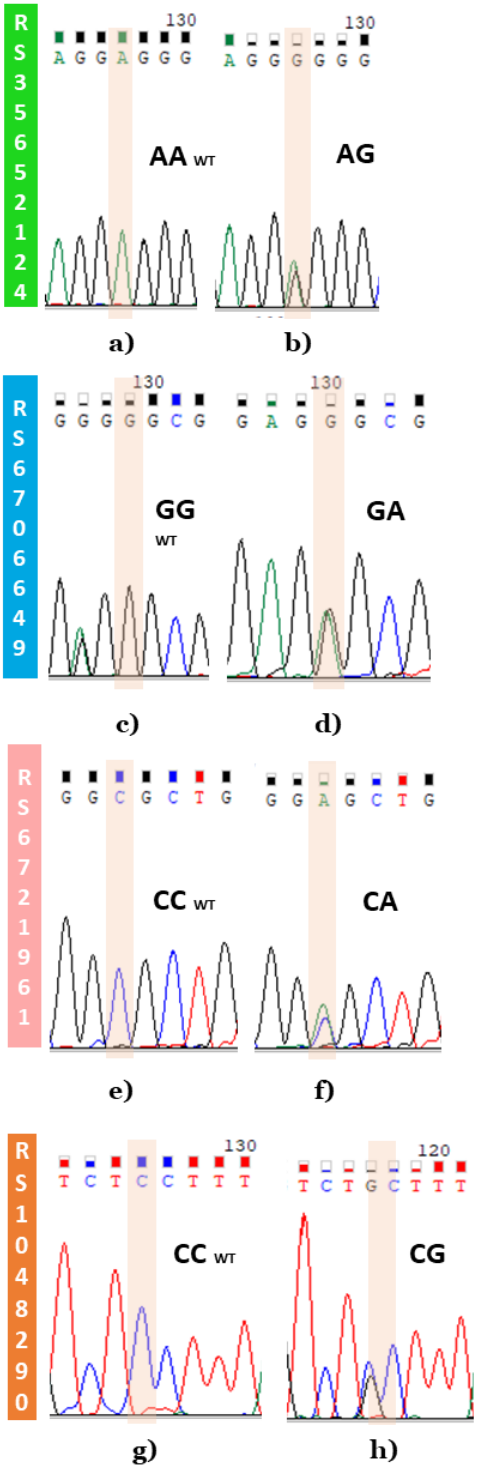


Figure 11: Sanger sequencing results for the SNPs found for KEAP1 and NRF2. **a)** NRF2 rs35652124 AA wild type (wt); **b)** NRF2 rs35652124 AG genotype; **c)** NRF2 rs6706649 GG wt; **d)** NRF2 rs6706649 GA genotype; **e)** NRF2 rs6721961 CC wt; **f)** NRF2 rs6721961 CA genotype; **g**) KEAP1 rs1048290 CC wt and **h**) KEAP1 rs1048290 CG genotype.

However, *de novo* mutations were not identified (table 2).

Table 2: Summary of the polymorphisms identified for the 40 patients in study.

Patient	*KEAP1*: rs1048290 *NRF2*: rs35652124; rs6706649; rs6721961	*de novo* SNPs in KEAP1 / NRF2
1	identified	not identified
2	identified	not identified
3	identified	not identified
4	identified	not identified
5	identified	not identified
6	identified	not identified
7	identified	not identified
8	identified	not identified
9	identified	not identified
10	identified	not identified
11	identified	not identified
12	identified	not identified
13	identified	not identified
14	identified	not identified
15	identified	not identified
16	identified	not identified
17	identified	not identified
18	identified	not identified
19	identified	not identified
20	identified	not identified
21	identified	not identified
22	identified	not identified
23	identified	not identified
24	identified	not identified
25	identified	not identified
26	identified	not identified
27	identified	not identified
28	identified	not identified
29	identified	not identified
30	identified	not identified
31	identified	not identified
32	identified	not identified
33	identified	not identified
34	identified	not identified
35	identified	not identified
36	identified	not identified
37	identified	not identified
38	identified	not identified
39	identified	not identified
40	identified	not identified

In addition to the identification of SNPs rs35652124, rs6706649 and rs6721961 from NRF2 and rs1048290 from KEAP1 genotypes (table 3), a statistical analysis using the SPSS® Statistics program for Windows (version 25) allowed to correlate the individual genotypes with the tumour histological grade. Histological grade 1 was considered as low grade and histological grades of 2 and 3 were considered as high grade (table 4).

Table 3: Number of cases identified for each genotype of NRF2: a) rs35652124, b) rs6706649, c) rs6721961 and for KEAP1 d) rs1048290.

a)	
NRF2 rs35652124	***n (%)***
AA (WT)	21 (52.5)
AG	16 (40)
GG	3 (7.5)

b)	
NRF2 rs6706649	***n (%)***
GG (WT)	30 (75)
GA	9 (22.5)
AA	1 (2.5)

c)	
NRF2 rs6721961	***n (%)***
CC (WT)	32 (80)
CA	7 (17.5)
AA	1 (2.5)

d)	
KEAP1 rs1048290	***n (%)***
CC (WT)	13 (32.5)
CG	21 (52.5)
GG	6 (15)

Table 4: Description of the histological grade of breast cancer of the patients in study. Low histological grade was identified in 10 (25%) of the cases and high histological grade was identified in 30 (75%) of the cases.

Histological Grade	***n (%)***
Low (1)	10 (25)
High (2 or 3)	30 (75)

Taking into account the previous data, a correlation analysis of NRF2 and KEAP1 polymorphisms with histological grade was performed, using Chi square test. Also, odds ratio (OR) and 95% confidence intervals (95% CI) were calculated.

For NRF2 rs35652124, rs6706649 and rs6721961 (tables 5, 6 and 7) no correlation between the genotypes of these SNPs and histological grade was found (*p*-value = 0.361, OR 0.510 95% CI 0.119 – 2.188; *p*-value = 0.206, OR 3.857 95% CI 0.424 – 35.112 and *p*-value = 0.068, OR 0.231 95% CI 0.045 – 1.197 for rs35652124, rs6706649 and rs6721961, respectively).

Table 5: Association between rs35652124 NRF2 polymorphism and breast cancer.

NRF2 rs35652124	**Histological Grade, *n (%)***		***OR (95% CI)***[a]	***p*-value**
	Low (1)	**High (2 or 3)**		
AA (WT)	4 (10)	17 (42.5)		1
AG/GG	6 (15)	13 (32.5)	0.510 (0.119 – 2.188)	0.361

[a] *OR:* odds ratio; *CI:* confidence interval

Table 6: Association between rs6706649 NRF2 polymorphism and breast cancer.

NRF2 rs6706649	**Histological Grade, *n (%)***		***OR (95% CI)***[a]	***p*-value**
	Low (1)	**High (2 or 3)**		
GG (WT)	9 (22.5)	21 (52.5)		1
GA/AA	1 (2.5)	9 (22.5)	3.857 (0.424 – 35.112)	0.206

[a] *OR* odds ratio; *CI* confidence interval

Table 7: Association between rs6721961 NRF2 polymorphism and breast cancer.

NRF2 rs6721961	Histological Grade, *n (%)*		*OR (95% CI)*[a]	*p*-value
	Low (1)	**High (2 or 3)**		
CC (WT)	6 (15)	26 (65)		1
CA/AA	4 (10)	4 (10)	0.231 (0.045 – 1.197)	0.068

[a] *OR* odds ratio; *CI* confidence interval

About KEAP1 rs1048290 we found that although there is no statistical significance (*p*-value = 0.079), there is a tendency for the carriers of the G allele to have tumours with high histological grade. In fact, the majority of the cases with high histological grade (45%) are carriers of the G allele, when compared to the wild type (CC) genotype (table 8).

Table 8: Association between rs1048290 KEAP1 polymorphism and breast cancer.

KEAP1 rs1048290	Histological Grade, *n (%)*		*OR (95% CI)*[a]	*p*-value
	Low (1)	**High (2 or 3)**		
CC (WT)	1 (2.5)	12 (30)		1
CG/GG	9 (22.5)	18 (45)	0.167 (0.019 – 1.491)	0.079

[a] *OR* odds ratio; *CI* confidence interval

These results are in accordance with the literature, once the G allele is related to a higher affinity for the bound of KEAP1 with NRF2, thus NRF2 is less available to move to the nucleus, compromising detoxification.

The identification of individual genotypes is of main importance once it may provide to the physician information on a putative higher risk of receiving a poor prognosis if the mutated allele is present. In the future we aim to correlate germline polymorphisms with new acquired mutation in tumour tissue from breast cancer patients. For that, there is a critical step that needs to be overpast: gDNA extraction from paraffin-embedded tissue.

For this optimization to be successful, the removal of paraffin from the embedded sections is a critical and essential step in order to obtain DNA with integrity and contamination-free. Since this is an optimization, human samples were not handled in order to avoid the waste of biological human samples, so 9 mouse breast sections were used for this purpose.

Several approaches have been taken in an attempt to extract gDNA from paraffin-embedded tissue (table 9). A first approach with the QIAamp DNA Mini Kit and Blood Mini kit (QIAGEN ®) using treatment with xylene did not allow gDNA extraction. In another attempt, QIAGEN's deparaffinization solution was used in combination with the QIAamp DNA FFPE Tissue Kit (QIAGEN®) protocol. For this, 5 µm sections were used. However, once again, it was not possible to extract gDNA. In a third attempt, treatment with xylene was used with the QIAamp DNA FFPE Tissue Kit (QIAGEN®) which also did not allow gDNA extraction.

Table 9: Different protocols used for the optimization of a final protocol for extracting gDNA from paraffin-embedded tissue (Optimized protocol by our research group).

QIAamp DNA Mini and Blood mini kit (QIAGEN®)	**Deparaffinization solution (QIAGEN®)**	**QIAamp DNA FFPE Tissue (QIAGEN®)**	**Protocol from another research group**	**Optimized protocol by our research group**
For each sample, identify a falcon tube (50 mL) and place a 5 µm FFPE section.	For each sample, identify a falcon tube (50 mL) and place a 5 µm FFPE section.	For each sample, identify a falcon tube (50 mL) and place a 5 µm FFPE section.	For each sample, identify a falcon tube (50 mL) and place a 5 µm FFPE section.	For each sample, identify a falcon tube (50 mL) and place a 10 µm FFPE section.
Pipette 1200 µL of xylene and vortex for 10 sec.	Pipette 160 to 360 µL of deparaffinization solution and vortex vigorously for 10 sec. Spin.	Pipette 1000 µL of xylene and vortex for 10 sec.	Pipette 500 µL of xylene and vortex.	Xylene was added until the microscope slide is covered.
			Let stand for 5 min at RT.	Let stand for 7 min at RT.
			Pipette 500 µL of absolute ethanol and vortex.	Absolute ethanol was added until the microscope slide is covered.
			Let stand for 5 min at RT.	Let stand for 5 min at RT.
The tissue was scraped from the microscope slide using a scalpel for	The tissue was scraped from the microscope slide using a scalpel for	The tissue was scraped from the microscope slide using a scalpel for	The tissue was scraped from the microscope slide using a scalpel for	The tissue was scraped from the microscope slide using a scalpel for the eppendorf.
Resuspend the tissue pellet in 180 µL of ATL Buffer.	Resuspend the tissue pellet in 180 µL of ATL Buffer. Centrifuge at 1000 rpm for 1 min.	Resuspend the tissue pellet in 180 µL of ATL Buffer.	Resuspend the tissue pellet in 180 µL of TLB DNA.	180 µL lysis buffer (ATL Buffer)

QIAamp DNA Mini and Blood mini kit (QIAGEN®)	Deparaffinization solution (QIAGEN®)	QIAamp DNA FFPE Tissue (QIAGEN®)	Protocol from another research group	Optimized protocol by our research group
Add 20 µL of proteinase K and vortex.	Add 20 µL of proteinase K and vortex.	Add 20 µL of proteinase K and vortex.	Add 70 µL of proteinase K and vortex.	Add 20 µL of proteinase K and vortex.
			Verify that the tissue is fully immersed in the mixture.	Verify that the tissue is fully immersed in the mixture.
Incubate at 56°C for 1h or until the sample is completely lysed Vortex occasionally during incubation. It can take up to 3 hours to be completely lysed. Lysis can also be done overnight.	Incubate at 56°C for 1h or until the sample is completely lysed	Incubate at 56°C for 1h or until the sample is completely lysed.		Incubate at 56°C for 1h or until the sample is completely lysed.
	Incubate the tubes in a thermal block at 90°C for 60 min.	Incubate the tubes in a thermal block at 90°C for 60 min.	Incubate the tubes in a thermal block at 90°C for 60 min (overnight).	Incubate the tubes in a thermal block at 90°C for 60 min.
Quick spin.	Quick spin.	Quick spin.	Remove the tubes from the thermal block and allow them to cool to RT.	Remove the tubes from the thermal block and allow them to cool to RT. Quick spin.
	Transfer the bottom to a 2 mL tube and continue with the QIAamp DNA FFPE Tissue (QIAGEN) protocol.			
Pipette 200 µL of Buffer AL and mix by pipetting up and down 3x.		Pipette 200 µL of Buffer AL and mix by pipetting up and down 3x.	Pipette 200 µL of DNA PBB and mix by pipetting up and down 3x.	Pipette 200 µL of Buffer AL and mix by pipetting up and down 3x.
Incubate at 70°C for 10 min. Spin.			Incubate at RT for 10 min.	Incubate at RT for 10 min.
Add 200 µL of absolute ethanol. Vigorous vortex and spin.		Add 200 µL of absolute ethanol. Vigorous	Add 100 µL Isopropanol to each tube and mix the	Add 200 µL of absolute ethanol. Vigorous vortex and spin.

QIAamp DNA Mini and Blood mini kit (QIAGEN®)	Deparaffinization solution (QIAGEN®)	QIAamp DNA FFPE Tissue (QIAGEN®)	Protocol from another research group	Optimized protocol by our research group
		vortex and spin.	lysate by pipetting up and down 3x.	
Add the entire mixture, including the precipitate, to the column.		Transfer the entire lysate (≈ 550 µL) to the column in the collection tube.	Transfer the entire lysate (≈ 550 µL) to the column in the collection tube.	Transfer the entire lysate (≈ 550 µL) to the column in the collection tube
Centrifuge at 8.000 g for 1 min.		Centrifuge at 8.000 rpm for 1 min.	Centrifuge at 8.000 rpm for 2 min.	Centrifuge at 8.000 rpm for 2 min.
Place the column in a new collection tube.		Place the column in a new collection tube.	Place the column in a new collection tube.	Place the column in a new collection tube.
Add 500 µL of Buffer AW1 to the column with the filter.		Add 500 µL of Buffer AW1 to the column.	Add 500 µL of WBI to the column.	Add 500 µL of Buffer AW1 to the column.
Centrifuge at 8.000 rpm for 1 min.		Centrifuge at 8.000 rpm for 1 min.	Centrifuge at 8.000 rpm for 2 min.	Centrifuge at 8.000 rpm for 2 min.
Place the column in a new tube.		Place the column in a new collection tube.	Discard the contents of the collection tube. Return the column to the same collection tube.	Discard the contents of the collection tube. Return the column to the same collection tube.
Add 500 ml of AW2 Buffer to the column.		Add 500 µL of AW2 Buffer to the column.	Add 500 µL of WBII to the column.	Add 500 µL of AW2 Buffer to the column.
Centrifuge at 14.000 rpm for 3 min.		Centrifuge at 8.000 rpm for 1 min.	Centrifuge at 8.000 rpm for 2 min.	Centrifuge at 8.000 rpm for 1 min.
Place the column in a new collection tube.		Place the column in a new collection tube.	Place the column in a new collection tube.	Place the column in a new collection tube.
Centrifuge at maximum speed for 1 min to dry the filter membrane.		Centrifuge at maximum speed for 3 min to dry the filter membrane.	Centrifuge at 18.000 rpm for 2 min to dry the filter membrane.	Centrifuge at 14.000 rpm for 3 min to dry the filter membrane.
Place the column in an already		Place the column in an	Place the column in an	Place the column in an already

QIAamp DNA Mini and Blood mini kit (QIAGEN®)	Deparaffinization solution (QIAGEN®)	QIAamp DNA FFPE Tissue (QIAGEN®)	Protocol from another research group	Optimized protocol by our research group
labelled elution tube.		already labelled elution tube.	already labelled elution tube.	labelled elution tube.
Add 200 µl of AE Buffer to the centre of the column, without touching the membrane.		Add 20-100 µl of ATE Buffer in the centre of the column, without touching the membrane.	Add 80 µl of EB DNA to the centre of the column, without touching the membrane.	Add 50 µl of ATE Buffer in the centre of the column, without touching the membrane.
Incubate at RT for 5 min.		Incubate at RT for 5 min.	Incubate at RT for 5 min.	Incubate at RT for 5 min.
Centrifuge at maximum speed for 1 min. Repeat the addition of AE Buffer, incubation and centrifugation.		Centrifuge at maximum speed for 1 min.	Centrifuge at 8.000 rpm for 2 min to collect the eluate from the elution tube. The eluate is the DNA stock. This processed sample can be stored at 2-8°C for a maximum of 2 weeks or -20°C for up to 60 days.	Centrifuge at 14.000 rpm for 1 min. The eluate is the DNA stock. This processed sample can be stored at 2-8°C for a maximum of 2 weeks or -20°C for up to 60 days.

DNA quantification

Vortex the DNA stock for 5 sec.		Vortex the DNA stock for 5 sec.
Measure according to the rules of the spectrophotometer in use.		Measure according to the rules of the spectrophotometer in use.
Note: Each DNA stock must have a minimum concentration of 5 ng/µL. If the concentration of a DNA stock is < 5ng/µl, the DNA Isolation procedure should be repeated for that sample using two 5µm FFPE tissue sections.		Note: Each DNA stock must have a minimum concentration of 5 ng/µL. If the concentration of a DNA stock is < 5ng/µl, the DNA Isolation procedure should be repeated for that sample using two 5µm FFPE tissue sections.

QIAamp DNA Mini and Blood mini kit (QIAGEN®)	Deparaffinization solution (QIAGEN®)	QIAamp DNA FFPE Tissue (QIAGEN®)	Protocol from another research group	Optimized protocol by our research group
If the DNA stock concentration is between 5 ng/µl to 35 ng/µl prepare 35µl of diluted sample: Required DNA stock volume (µl) = (35µl x 5 ng/µl) / DNA stock concentration in ng/µL		If the DNA stock concentration is between 5 ng/µl to 35 ng/µl prepare 35µl of diluted sample: Required DNA stock volume (µl) = (35µl x 5 ng/µl) / DNA stock concentration in ng/µL		

In a new approach, a protocol already established by another research group was used and adapted to the protocol of the QIAamp DNA FFPE Tissue Kit (QIAGEN®). 10 µm sections were used and the volume of xylene and the incubation times with xylene were increased. As a result, the optimized protocol conditions and procedures were as follows:

To remove paraffin, the sample was placed in a 50 mL falcon, xylene was added until the microscope slide is covered (7 min incubation at RT) and then is transferred to a new falcon where it was covered with absolute ethanol (5 min incubation at RT). Subsequently, it was removed from the ethanol and allowed to dry at RT checking that the paraffin has been completely removed.

After deparaffinization, the tissue was scraped from the microscope slide using a scalpel for the eppendorf. 180 µL lysis buffer (ATL Buffer) and 20 µL PK were used to resuspend the pellet (vortexing and incubation at 56ºC, 60 min). After that, it was vortexed and incubated at 90°C for 60 min (or until lysis was complete). It was removed from the block and allowed to cool to RT. A spin was performed to remove excess liquid from the lid. 200 µL of AL buffer was pipetted (mixed and incubated for 10 min at RT). 200 µL of 100% ethanol was then added and vortexed vigorously, and all lysate (approximately 550 µL) was transferred to the column (centrifuged for 2 min at 8.000 rpm). The column was then placed in a new collection tube, where 500 µL of AW1 Buffer was added (centrifugation at 8.000 rpm for 1 min). The contents of the collection tube are discarded, the column is placed back in the same tube and 500 µL of AW2 Buffer is added (centrifugation at 8.000 rpm for 1 min). Next, the column was placed in a new collection tube and centrifuged to dry the filter membrane (14.000 rpm

for 3 min). The column was then placed in an already labelled elution tube and 50 µL of ATE Buffer was added to the centre of the column without touching the membrane (incubation for 5 min at RT, followed by centrifugation at 14.000 rpm for 1 min). Finally, the eppendorf eluate is the DNA "stock", which can be stored at 2-8 °C for 2 weeks or -20 °C for 60 days.

PCR was performed to verify the amplification of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and cyclophilin A (CypA) genes. Primers of two mouse housekeeping genes, rGAPDH and rCypA, were used. These genes were specifically chosen to verify if the optimization of the technique was successful.

DNA samples were amplified by the PCR technique. A final reaction volume of 25 µL/sample was obtained, containing 2.5 uL of buffer, 0.5 µL of each necessary primer (table 1), 0.5 µL of dNTPs, 0.75 µL of MgCl2, 0.12 µL of Taq polymerase, 6 µL of DNA and the remaining volume of sterile milliQ water. Amplification was performed on a Bio-Rad MyCycler Thermal Cycler® equipment, with pre-incubation for 5 min at 95°C. After this period, the incubation conditions consisted of performing 35 cycles of PCR for 1 min at 95°C, 1 min at 55°C, 1 min at 72°C followed by 5 min at 72°C.

After the end of the PCR cycles, the samples were loaded with 2 µL of loading dye, to be analysed by agarose gel electrophoresis. Confirmation of the amplification of the PCR products was performed by 2% agarose gel electrophoresis. To prepare the gel, it was necessary to dilute 1 g of agarose in 50 mL of 1X TAE buffer and heating the solution until the agarose is completely dissolved. After cooling, 50 µL of GreenSafe was added. After polymerization, the gel was loaded with 10 µL of final product and 2 µL of molecular weight marker. The gel was subjected to a voltage of 120 V for 45 min. The electrophoresis results were observed by exposing the gel to UV light with the aid of the Uvitec Cambridge® equipment.

With this approach, we accomplished a gDNA extraction concentration of 20.5 ng/ul and A260/A280 = 1.864. Besides quantifications, we performed a PCR with two mouse housekeeping genes (rGAPDH and rCypA) as shown in figure 12, to confirm the integrity of the gDNA.

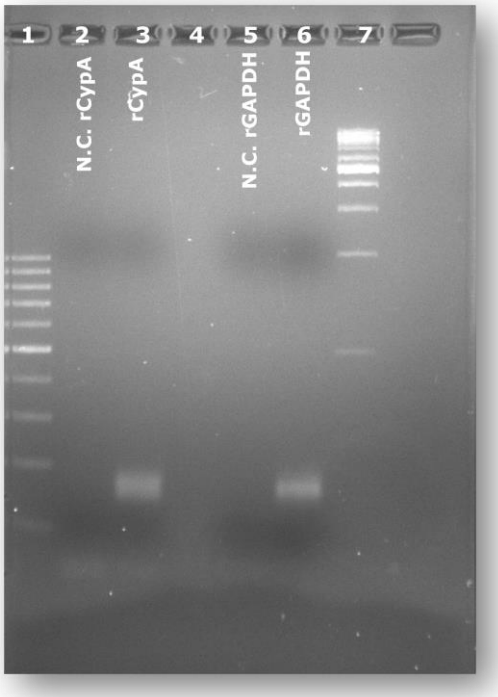


Figure 12: PCR product electrophoresis results. It amplification for housekeeping genes was verified. **Well 1 -** molecular weight marker; **Well 2 -** negative control for rCypA gene; **Well 3 -** amplification of rCypA gene; **Well 4 –** empty; **Well 5 –** negative control for rGAPDH gene; **Well 6 –** amplification of rGAPDH gene; **Well 7 -** molecular weight marker. The mouse primer sequences used were: rCypA - FW: 5' CAA GAC TGA GTG GCT GGA TGG 3'; RV: 5' GCC CGC AAG TCA AAG AAA TTA GAG 3' and for rGAPDH - FW: 5' AGA CAG CCG CAT CTT CTT GT 3'; RV: 5' TGA TGG CAA CAA TGT CCA CT 3'.

Thus, the purity and integrity of gDNA extracted from paraffin-embedded using our optimized protocol was confirmed. These results are of main importance because they prove that the critical step of paraffin removal has been overcome. Besides, no human tissue was wasted for this procedure and no animals were euthanized.

Chapter 5

Conclusion and Future Perspectives

5. Conclusion and Future Perspectives

Breast cancer remains the highest mortality associated disease in women. Understanding the biomolecular mechanisms behind this disease is essential to change the fate of the huge number of women who are unfortunately diagnosed with breast cancer every year.

Oxidative stress continues to be one of the triggers of several diseases, including cancer, and to prevent this, its control is strictly necessary. The Nrf2-Keap1 signalling pathway plays a very important role in the fight against oxidative stress by activating an antioxidant defense mechanism that involves a series of cascading events that maintain the redox balance. However, polymorphisms in this complex seems to promote an imbalance in this pathway, leading to possible consequences that affect its activation. A failure to activate the Nrf2-Keap1 pathway can lead to irreparable DNA damage in normal cells, which can promote OS-associated tumorigenesis. However, studies have also confirmed a negative role played by Nrf2 since tumour cells are also protected upon exposure to OS. Thus, it is clear why in most cases therapies such as chemotherapy and radiotherapy are not effectively enough to combat the disease.

New therapeutic targets that take into account the consequences of SNPs can be used as possible regulators of antioxidant defense and prevent complications associated with oxidative stress. Mutations in Nrf2-Keap1 pathway seem to compromise the normal function of the complex, either by influencing the displacement of Nrf2 to the nucleus or by modulating NRF2 upstream and downstream genes.

This work made possible to identify and genotype the genetic polymorphisms rs35652124, rs6706649 and rs6721961 from NRF2 and rs1048290 from KEAP1, in peripheral blood from breast cancer patients, previously described by our team as being associated with the development and progression of breast cancer. Although *de novo* mutations were not identified, this work highlights the importance of performing studies on tumour tissue in addition to the results already reached in blood by our team.

To achieve the objective of optimizing gDNA extraction from paraffin-embedded tissue, more precisely to optimize the crucial step of paraffin removal, since it can interfere with DNA quality and integrity, different protocols were used. After several approaches, an efficient extraction of gDNA from the embedded-paraffin tissue was achieved. This optimization will allow less waste of human biological samples and

greater rigor in sample handling. It should be noted that no animal suffered or was euthanized just for this work.

By overcoming the challenge of extracting DNA from paraffin-embedded tissue, our team can make a step forward, by studying the NRF2-KEAP1 SNPs in tumour tissue from women diagnosed with breast cancer. This research may lead to new results, as it is expected that new mutations in NRF2 and KEAP1 may be identified in tumour tissue, when compared to peripheral blood. The fact that these mutations are not found in the germline (blood) may be because they were acquired by the tumour genome (somatic mutations). This work has overcome the problematic of gDNA extraction from paraffin-embedded tissue and is a step forward to, in the future, the role of NRF2 and KEAP1 in tumour development and progression can be better understood.

Chapter 6

Bibliographic References

6. Bibliographic References

1. Global Burden of Disease Cancer C, Kocarnik JM, Compton K, Dean FE, Fu W, Gaw BL, et al. Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life Years for 29 Cancer Groups From 2010 to 2019: A Systematic Analysis for the Global Burden of Disease Study 2019. JAMA Oncol. 2022;8(3):420-44.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70(1):7-30.

3. Top cancer per country (incidence) IARC, WHO [Available from: https://gco.iarc.fr/today/online-analysis-map?v=2020&mode=cancer&mode_population=continents&population=900&populations=900&key=total&sex=2&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=10&group_cancer=1&include_nmsc=0&include_nmsc_other=0&projection=natural-earth&color_palette=default&map_scale=quantile&map_nb_colors=5&continent=0&show_ranking=0&rotate=%255B10%252C0%255D (consulted on 3th september, 2022).

4. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Pineros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021.

5. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-49.

6. Brisken C, O'Malley B. Hormone action in the mammary gland. Cold Spring Harb Perspect Biol. 2010;2(12):a003178.

7. Vares G, Ory K, Lectard B, Levalois C, Altmeyer-Morel S, Chevillard S, et al. Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene. 2004;23(26):4603-13.

8. Macias H, Hinck L. Mammary gland development. Wiley Interdiscip Rev Dev Biol. 2012;1(4):533-57.

9. Nur U, El Reda D, Hashim D, Weiderpass E. A prospective investigation of oral contraceptive use and breast cancer mortality: findings from the Swedish women's lifestyle and health cohort. BMC Cancer. 2019;19(1):807.

10. Hou N, Hong S, Wang W, Olopade OI, Dignam JJ, Huo D. Hormone replacement therapy and breast cancer: heterogeneous risks by race, weight, and breast density. J Natl Cancer Inst. 2013;105(18):1365-72.

11. Haber G, Ahmed NU, Pekovic V. Family history of cancer and its association with breast cancer risk perception and repeat mammography. Am J Public Health. 2012;102(12):2322-9.
12. Chen WY. Exogenous and endogenous hormones and breast cancer. Best Pract Res Clin Endocrinol Metab. 2008;22(4):573-85.
13. Elliott MJ, Cescon DW. Development of novel agents for the treatment of early estrogen receptor positive breast cancer. Breast. 2022;62 Suppl 1:S34-S42.
14. Maiti S, Nazmeen A. Impaired redox regulation of estrogen metabolizing proteins is important determinant of human breast cancers. Cancer Cell Int. 2019;19:111.
15. Almeida M, Soares M, Fonseca-Moutinho J, Ramalhinho AC, Breitenfeld L. Influence of Estrogenic Metabolic Pathway Genes Polymorphisms on Postmenopausal Breast Cancer Risk. Pharmaceuticals (Basel). 2021;14(2).
16. Mense SM, Remotti F, Bhan A, Singh B, El-Tamer M, Hei TK, et al. Estrogen-induced breast cancer: alterations in breast morphology and oxidative stress as a function of estrogen exposure. Toxicol Appl Pharmacol. 2008;232(1):78-85.
17. Giudice A, Barbieri A, Bimonte S, Cascella M, Cuomo A, Crispo A, et al. Dissecting the prevention of estrogen-dependent breast carcinogenesis through Nrf2-dependent and independent mechanisms. Onco Targets Ther. 2019;12:4937-53.
18. Cadet J, Davies KJA. Oxidative DNA damage & repair: An introduction. Free Radic Biol Med. 2017;107:2-12.
19. Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and x-irradiation: a mechanism in common. Science. 1954;119(3097):623-6.
20. Jones DP. Redefining oxidative stress. Antioxid Redox Signal. 2006;8(9-10):1865-79.
21. Basak P, Sadhukhan P, Sarkar P, Sil PC. Perspectives of the Nrf-2 signaling pathway in cancer progression and therapy. Toxicol Rep. 2017;4:306-18.
22. Ambrosone CB. Oxidants and antioxidants in breast cancer. Antioxid Redox Signal. 2000;2(4):903-17.
23. Hecht F, Pessoa CF, Gentile LB, Rosenthal D, Carvalho DP, Fortunato RS. The role of oxidative stress on breast cancer development and therapy. Tumour Biol. 2016;37(4):4281-91.
24. Ramalhinho AC, Fonseca-Moutinho JA, Breitenfeld L. Glutathione S-transferase M1, T1, and P1 genotypes and breast cancer risk: a study in a Portuguese population. Mol Cell Biochem. 2011;355(1-2):265-71.

25. Almeida M, Soares M, Ramalhinho AC, Moutinho JF, Breitenfeld L. Prognosis of hormone-dependent breast cancer seems to be influenced by KEAP1, NRF2 and GSTM1 genetic polymorphisms. Mol Biol Rep. 2019;46(3):3213-24.
26. Jiang S, Yang Y, Li T, Ma Z, Hu W, Deng C, et al. An overview of the mechanisms and novel roles of Nrf2 in cardiovascular diseases. Expert Opin Ther Targets. 2016;20(12):1413-24.
27. Shen G, Kong AN. Nrf2 plays an important role in coordinated regulation of Phase II drug metabolism enzymes and Phase III drug transporters. Biopharm Drug Dispos. 2009;30(7):345-55.
28. Sekine H, Motohashi H. Roles of CNC Transcription Factors NRF1 and NRF2 in Cancer. Cancers (Basel). 2021;13(3).
29. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A. 1994;91(21):9926-30.
30. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev. 2013;27(20):2179-91.
31. Namani A, Li Y, Wang XJ, Tang X. Modulation of NRF2 signaling pathway by nuclear receptors: implications for cancer. Biochim Biophys Acta. 2014;1843(9):1875-85.
32. Ooi BK, Chan KG, Goh BH, Yap WH. The Role of Natural Products in Targeting Cardiovascular Diseases via Nrf2 Pathway: Novel Molecular Mechanisms and Therapeutic Approaches. Front Pharmacol. 2018;9:1308.
33. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 1999;13(1):76-86.
34. Canning P, Sorrell FJ, Bullock AN. Structural basis of Keap1 interactions with Nrf2. Free Radic Biol Med. 2015;88(Pt B):101-7.
35. Ogura T, Tong KI, Mio K, Maruyama Y, Kurokawa H, Sato C, et al. Keap1 is a forked-stem dimer structure with two large spheres enclosing the intervening, double glycine repeat, and C-terminal domains. Proc Natl Acad Sci U S A. 2010;107(7):2842-7.
36. Horie Y, Suzuki T, Inoue J, Iso T, Wells G, Moore TW, et al. Molecular basis for the disruption of Keap1-Nrf2 interaction via Hinge & Latch mechanism. Commun Biol. 2021;4(1):576.
37. Sajadimajd S, Khazaei M. Oxidative Stress and Cancer: The Role of Nrf2. Curr Cancer Drug Targets. 2018;18(6):538-57.

38. Pandey P, Singh AK, Singh M, Tewari M, Shukla HS, Gambhir IS. The see-saw of Keap1-Nrf2 pathway in cancer. Crit Rev Oncol Hematol. 2017;116:89-98.
39. Ahmed SM, Luo L, Namani A, Wang XJ, Tang X. Nrf2 signaling pathway: Pivotal roles in inflammation. Biochim Biophys Acta Mol Basis Dis. 2017;1863(2):585-97.
40. Zhang DD. The Nrf2-Keap1-ARE signaling pathway: The regulation and dual function of Nrf2 in cancer. Antioxid Redox Signal. 2010;13(11):1623-6.
41. Hartikainen JM, Tengstrom M, Kosma VM, Kinnula VL, Mannermaa A, Soini Y. Genetic polymorphisms and protein expression of NRF2 and Sulfiredoxin predict survival outcomes in breast cancer. Cancer Res. 2012;72(21):5537-46.
42. Vomund S, Schafer A, Parnham MJ, Brune B, von Knethen A. Nrf2, the Master Regulator of Anti-Oxidative Responses. Int J Mol Sci. 2017;18(12).
43. Duong HQ, You KS, Oh S, Kwak SJ, Seong YS. Silencing of NRF2 Reduces the Expression of ALDH1A1 and ALDH3A1 and Sensitizes to 5-FU in Pancreatic Cancer Cells. Antioxidants (Basel). 2017;6(3).
44. Tonelli C, Chio IIC, Tuveson DA. Transcriptional Regulation by Nrf2. Antioxid Redox Signal. 2018;29(17):1727-45.
45. Xiong L, Xie J, Song C, Liu J, Zheng J, Liu C, et al. The Activation of Nrf2 and Its Downstream Regulated Genes Mediates the Antioxidative Activities of Xueshuan Xinmaining Tablet in Human Umbilical Vein Endothelial Cells. Evid Based Complement Alternat Med. 2015;2015:187265.
46. Niture SK, Jaiswal AK. Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. J Biol Chem. 2012;287(13):9873-86.
47. Hochmuth CE, Biteau B, Bohmann D, Jasper H. Redox regulation by Keap1 and Nrf2 controls intestinal stem cell proliferation in Drosophila. Cell Stem Cell. 2011;8(2):188-99.
48. Dodson M, Redmann M, Rajasekaran NS, Darley-Usmar V, Zhang J. KEAP1-NRF2 signalling and autophagy in protection against oxidative and reductive proteotoxicity. Biochem J. 2015;469(3):347-55.
49. Almeida M, Soares M, Ramalhinho AC, Moutinho JF, Breitenfeld L, Pereira L. The prognostic value of NRF2 in breast cancer patients: a systematic review with meta-analysis. Breast Cancer Res Treat. 2020;179(3):523-32.
50. Gonzalez-Donquiles C, Alonso-Molero J, Fernandez-Villa T, Vilorio-Marques L, Molina AJ, Martin V. The NRF2 transcription factor plays a dual role in colorectal cancer: A systematic review. PLoS One. 2017;12(5):e0177549.

51. Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, et al. Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. Carcinogenesis. 2008;29(6):1235-43.
52. Lau A, Villeneuve NF, Sun Z, Wong PK, Zhang DD. Dual roles of Nrf2 in cancer. Pharmacol Res. 2008;58(5-6):262-70.
53. Menegon S, Columbano A, Giordano S. The Dual Roles of NRF2 in Cancer. Trends Mol Med. 2016;22(7):578-93.
54. Wang R, An J, Ji F, Jiao H, Sun H, Zhou D. Hypermethylation of the Keap1 gene in human lung cancer cell lines and lung cancer tissues. Biochem Biophys Res Commun. 2008;373(1):151-4.
55. Ikeda H, Nishi S, Sakai M. Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis. Biochem J. 2004;380(Pt 2):515-21.
56. Qin JJ, Cheng XD, Zhang J, Zhang WD. Dual roles and therapeutic potential of Keap1-Nrf2 pathway in pancreatic cancer: a systematic review. Cell Commun Signal. 2019;17(1):121.
57. Tossetta G, Fantone S, Montanari E, Marzioni D, Goteri G. Role of NRF2 in Ovarian Cancer. Antioxidants (Basel). 2022;11(4).
58. Hayes JD, McMahon M, Chowdhry S, Dinkova-Kostova AT. Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. Antioxid Redox Signal. 2010;13(11):1713-48.
59. Ganan-Gomez I, Wei Y, Yang H, Boyano-Adanez MC, Garcia-Manero G. Oncogenic functions of the transcription factor Nrf2. Free Radic Biol Med. 2013;65:750-64.
60. Sanghvi VR, Leibold J, Mina M, Mohan P, Berishaj M, Li Z, et al. The Oncogenic Action of NRF2 Depends on De-glycation by Fructosamine-3-Kinase. Cell. 2019;178(4):807-19 e21.
61. Kerins MJ, Ooi A. A catalogue of somatic NRF2 gain-of-function mutations in cancer. Sci Rep. 2018;8(1):12846.
62. Taguchi K, Yamamoto M. The KEAP1-NRF2 System in Cancer. Front Oncol. 2017;7:85.
63. Karki R, Pandya D, Elston RC, Ferlini C. Defining "mutation" and "polymorphism" in the era of personal genomics. BMC Med Genomics. 2015;8:37.
64. Hart JR, Johnson MD, Barton JK. Single-nucleotide polymorphism discovery by targeted DNA photocleavage. Proc Natl Acad Sci U S A. 2004;101(39):14040-4.

65. Weinman LR, Solomon JW, Rubenstein DR. A comparison of single nucleotide polymorphism and microsatellite markers for analysis of parentage and kinship in a cooperatively breeding bird. Mol Ecol Resour. 2015;15(3):502-11.
66. Majewski J, Ott J. Distribution and characterization of regulatory elements in the human genome. Genome Res. 2002;12(12):1827-36.
67. Mardis ER, Wilson RK. Cancer genome sequencing: a review. Hum Mol Genet. 2009;18(R2):R163-8.
68. Nakagawa H, Fujita M. Whole genome sequencing analysis for cancer genomics and precision medicine. Cancer Sci. 2018;109(3):513-22.
69. Nelson MR, Marnellos G, Kammerer S, Hoyal CR, Shi MM, Cantor CR, et al. Large-scale validation of single nucleotide polymorphisms in gene regions. Genome Res. 2004;14(8):1664-8.
70. Kruglyak L, Nickerson DA. Variation is the spice of life. Nat Genet. 2001;27(3):234-6.
71. Chorley BN, Wang X, Campbell MR, Pittman GS, Noureddine MA, Bell DA. Discovery and verification of functional single nucleotide polymorphisms in regulatory genomic regions: current and developing technologies. Mutat Res. 2008;659(1-2):147-57.
72. Wang YM, Zhou P, Wang LY, Li ZH, Zhang YN, Zhang YX. Correlation between DNase I hypersensitive site distribution and gene expression in HeLa S3 cells. PLoS One. 2012;7(8):e42414.
73. Carlton VE, Ireland JS, Useche F, Faham M. Functional single nucleotide polymorphism-based association studies. Hum Genomics. 2006;2(6):391-402.
74. Sartor H, Brandt J, Grassmann F, Eriksson M, Czene K, Melander O, et al. The association of single nucleotide polymorphisms (SNPs) with breast density and breast cancer survival: the Malmo Diet and Cancer Study. Acta Radiol. 2020;61(10):1326-34.
75. Cho HY, Marzec J, Kleeberger SR. Functional polymorphisms in Nrf2: implications for human disease. Free Radic Biol Med. 2015;88(Pt B):362-72.
76. Ishikawa T. Genetic polymorphism in the NRF2 gene as a prognosis marker for cancer chemotherapy. Front Genet. 2014;5:383.
77. Yamamoto T, Yoh K, Kobayashi A, Ishii Y, Kure S, Koyama A, et al. Identification of polymorphisms in the promoter region of the human NRF2 gene. Biochem Biophys Res Commun. 2004;321(1):72-9.
78. Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lanken PN, et al. Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. FASEB J. 2007;21(9):2237-46.

79. Al Azhary NM, Kamel MM, Ismail YM, Mahmoud AA, Radwan EM. The Role of Genetic Polymorphisms in Nrf2 and P73 in Egyptian Women with Breast Cancer. Asian Pac J Cancer Prev. 2016;17(11):4945-9.
80. Matana A, Ziros PG, Chartoumpekis DV, Renaud CO, Polasek O, Hayward C, et al. Rare and common genetic variations in the Keap1/Nrf2 antioxidant response pathway impact thyroglobulin gene expression and circulating levels, respectively. Biochem Pharmacol. 2020;173:113605.
81. Okano Y, Nezu U, Enokida Y, Lee MT, Kinoshita H, Lezhava A, et al. SNP (-617C>A) in ARE-like loci of the NRF2 gene: a new biomarker for prognosis of lung adenocarcinoma in Japanese non-smoking women. PLoS One. 2013;8(9):e73794.
82. Shimoyama Y, Mitsuda Y, Tsuruta Y, Hamajima N, Niwa T. Polymorphism of Nrf2, an antioxidative gene, is associated with blood pressure and cardiovascular mortality in hemodialysis patients. Int J Med Sci. 2014;11(7):726-31.
83. Shaheen SO, Newson RB, Ring SM, Rose-Zerilli MJ, Holloway JW, Henderson AJ. Prenatal and infant acetaminophen exposure, antioxidant gene polymorphisms, and childhood asthma. J Allergy Clin Immunol. 2010;126(6):1141-8 e7.
84. Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, et al. Nrf2 gene promoter polymorphism and gastric carcinogenesis. Hepatogastroenterology. 2008;55(82-83):750-4.
85. von Otter M, Landgren S, Nilsson S, Celojevic D, Bergstrom P, Hakansson A, et al. Association of Nrf2-encoding NFE2L2 haplotypes with Parkinson's disease. BMC Med Genet. 2010;11:36.
86. Muscarella LA, Fazio VM. Keap1/Nrf2 impairing revised: are we missing the single nucleotide polymorphisms? J Thorac Dis. 2016;8(12):E1752-E4.
87. Hartikainen JM, Tengstrom M, Winqvist R, Jukkola-Vuorinen A, Pylkas K, Kosma VM, et al. KEAP1 Genetic Polymorphisms Associate with Breast Cancer Risk and Survival Outcomes. Clin Cancer Res. 2015;21(7):1591-601.
88. He W, Long T, Pan Q, Zhang S, Zhang Y, Zhang D, et al. Microglial NLRP3 inflammasome activation mediates IL-1beta release and contributes to central sensitization in a recurrent nitroglycerin-induced migraine model. J Neuroinflammation. 2019;16(1):78.
89. Furtado A, Mineiro R, Duarte AC, Goncalves I, Santos CR, Quintela T. The Daily Expression of ABCC4 at the BCSFB Affects the Transport of Its Substrate Methotrexate. Int J Mol Sci. 2022;23(5).

Chapter 7

Attachments

7. Attachments

- Poster presented at XVII International CICS-UBI Symposium named ''Optimization of Commercial DNA Extraction Methods for FFPE samples''.