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Influence of low penetrance genes in susceptibility for breast cancer in the Portuguese population of Beira Interior

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Aos meus Pais,

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Thesis Outline

This thesis is organized in 11 chapters. Chapters 1 to 4 explore the essential theoretical background that was behind the formulation of the hypothesis of the research. Chapter 5 establishes general and specific aims of this work. In Chapter 6 to 10 are presented the results of the investigation in the form of accepted or in preparation papers.

Chapter 6 explores the association of null genotype in GSTM1, GSTT1 and the polymorphism in GSTP1 (A/G) with susceptibility to breast cancer. This work was published in 2011 in Molecular and Cellular Biochemistry (Ramalhinho AC et al. Mol Cell Biochem 2011; 355: 265-271, DOI 10.1007/s11010-011-0863-9).

Chapter 7 presents the results of a study conducted in order to evaluate the potential role of polymorphic genes encoding enzymes involved in estrogen biosynthesis (CYP19A1) and metabolism (GSTM1, GSTT1 and GSTP1), and their action in modulating individual susceptibility to breast cancer. The paper was published in DNA and Cell Biology in 2012 (Ramalhinho AC et al. DNA Cell Biol 2012; 31(6): 1100-1106, DOI: 10.1089/dna.2011.1538).

In Chapter 8 is presented the association between -397 PvuII (T>C) and -351 XbaI (A>G) restriction fragment length polymorphisms (RFLPs) in intron 1 of ERα gene and susceptibility of breast cancer, by undertaking a case-control study in BRCA1 185delAG and 5382insC / BRCA2 6174delT negative Portuguese women. The results of this work were published in Molecular Biology Reports in 2013 (Ramalhinho AC et al. Mol Biol Rep 2013; 40(8): 5093-5103, DOI: 10.1007/s11033-013-2611-6).

Chapter 9 brings forward the work presented as oral communication at the 17th World Congress on Controversies in Obstetrics, Gynecology and Infertility (COGI) and published in the Proceedings of the mentioned congress. The work explores the association between polymorphisms in low penetrating genes of estrogen biosynthetic pathway (CYP17A1 T27C and CYP19A1 codon 39 Trp/Arg), estrogen metabolic pathway (CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GSTM1/GSTT1 deletion genotypes, GSTP1 Ile105Val, MTHFR C677T), DNA damage signaling and repair pathway (TP53 Arg72Pro), and estrogenic response (ER alpha XbaI and PvuII), with breast cancer susceptibility in a Portuguese population (Ramalhinho AC et al. Proceedings of the 17th World COGI 2013; 217-221, ISBN 978 88 6521 063 5).

Chapter 10 presents a paper submitted for publication. This work aimed to evaluate the association between polymorphisms in the same low penetrating genes studied in Chapter 9 with breast cancer risk predicted by Gail model.

Chapter 11 explores the use of dermal fibroblasts cultures as models of study of response to estrogens. Cultures of dermal fibroblasts were established and characterized by fluorescence microscopy. Cellular toxicity, flow cytometry and real time-PCR assays were performed in MCF-7 cells and normal human dermal fibroblasts (NHDF) to evaluate cell response to 17β-estradiol. This work is presented as a paper submitted for publication.

Finally, the most important conclusions from the conducted studies are brought up in terms of actual knowledge and future perspectives.

Resumo Alargado

Em Portugal, o cancro da mama apresenta as maiores taxas de incidência e de mortalidade de entre as doenças femininas. Foram já identificados vários fatores de risco genéticos e não genéticos envolvidos no desenvolvimento do cancro da mama, contudo os mecanismos moleculares de carcinogénese da mama permanecem pouco claros. Menos de 5% de todos os casos de cancro de mama são devidos a genes de suscetibilidade de alta penetrância para o cancro da mama, por isso polimorfismos em genes de baixa penetrância podem ser responsáveis por um aumento relativamente pequeno, mas mais frequente, no risco de cancro. Os polimorfismos em genes de baixa penetrância relacionados com o cancro da mama podem ser encontrados em vários processos biológicos, nomeadamente nas vias de síntese e metabolismo dos estrogénios, de resposta aos estrogénios, ou de regulação do ciclo celular. Os estrogénios foram claramente identificados como agentes carcinogénicos, e a maioria dos fatores de risco para o desenvolvimento de cancro da mama estão relacionados com exposição aumentada ou prolongada a estrogénios. Diferenças inter-individuais nas vias de síntese e metabolismo de estrogénios podem definir uma subpopulação de mulheres com perfil "sintetizadora rápida/metabolizadora rápida" que estão mais expostas à formação de aductos no ADN e/ou a mutações devido ao crescimento celular dependente de estrogénios, ou a danos celulares provocados pela exposição aos estrogénios e aos seus metabolitos durante a sua vida. As variações genéticas em genes que codificam os recetores de estrogénios podem também modular uma fração de suscetibilidade associada à exposição aos estrogénios. As enzimas de reparação do ADN podem ainda exercer uma função de proteção do genoma contra potenciais agentes carcinogénicos endógenos ou exógenos. O comprometimento desta função protetora dos genes de reparação do ADN causada, por exemplo, por mutações que determinam polimorfismos, está associada a um aumento da sensibilidade a agentes promotores de lesão no ADN e da predisposição para o cancro. Apesar da associação entre polimorfismos em genes de baixa penetrância e o desenvolvimento de cancro da mama, e em particular os polimorfismos que foram estudados nesta tese, ter já sido estudada noutras populações com resultados discrepantes e não definitivos, na população Portuguesa a informação é escassa e, particularmente na população da Beira Interior, não há qualquer trabalho descrito.

O principal objetivo desta tese é avaliar a influência de polimorfismos em genes de baixa penetrância para a suscetibilidade para o cancro da mama numa população Portuguesa específica, a população da Beira Interior, na qual este efeito genético nunca foi estudado. De modo a atingir este objetivo, foi analisada nesta população a influência de genes polimórficos de baixa penetrância das vias de síntese (CYP17A1 e CYP19A1) e de metabolização de estrogénios (CYP1B1, CYP1A1, COMT, GTSM1, GSTT1, GSTP1 e MTHFR), de reparação do ADN (TP53), e de resposta aos estrogénios (ERα), na suscetibilidade ao cancro da mama. Os nossos

resultados indicam que a deleção total de apenas um ou, de ambos os genes GSTM1 e GSTT1, está associada com um aumento do risco de desenvolver cancro da mama. Também a presença em homozigotia ou heterozigotia do alelo variante do codão 39 do gene CYP19A1 está associada com um aumento significativo do risco de vir a desenvolver cancro da mama. Contudo, o efeito deste polimorfismo da via de biossíntese dos estrogénios parece ser modulado pelo polimorfismo de deleção da GSTM1 e da GSTT1 na via de metabolização dos estrogénios, dado que a suscetibilidade para o desenvolvimento de cancro da mama se verificou ser menor em portadores dos genes GSTM1 e GSTT1, independentemente do genótipo da CYP19A1. Portadores dos genótipos xx no gene ERα, ou portadores simultâneos dos genótipos xx e pp, também parecem ter um risco reduzido de desenvolver cancro da mama, quando comparado com portadores de outras combinações destes genótipos. A presença do alelo variante no gene TP53 em homozigotia ou heterozigotia também parece estar associado com um aumento significativo do risco de desenvolver cancro da mama.

Foi também avaliado no âmbito desta tese se a influência dos polimorfismos de baixa penetrância se reflete nos métodos de avaliação de risco já estandardizados, usados na prática clínica, e baseados em parâmetros não genéticos. Para atingir este objetivo foi avaliada a prevalência dos polimorfismos nos genes de baixa penetrância estudados em grupos de mulheres às quais foram calculados valores de risco relativo para desenvolver cancro da mama em cinco anos pelo Modelo de Gail Modificado. Quando comparámos o risco de vir a desenvolver cancro da mama conferido pelos genótipos considerados "de risco", e o risco de vir a desenvolver cancro da mama em 5 anos calculado pelo Modelo de Gail, constatou-se que os dois são independentes entre si. No entanto, verificou-se que a presença simultânea de três, quatro ou cinco genótipos de risco era mais frequente nas mulheres afetadas por cancro da mama do que nas mulheres não afetadas pela doença. Não foi encontrada relação estatisticamente significativa entre os genótipos de risco com os scores de "alto risco" e de "baixo risco" calculados pelo Modelo de Gail pelo que demonstramos que, pelo menos nesta população, a combinação de dois ou mais polimorfismos nos genes de baixa penetrância estudados deveriam ser considerados como fatores de risco relevantes no desenvolvimento de cancro da mama, de modo independente do cálculo pelo Modelo de Gail.

De modo a colmatar a necessidade de aumentar o conhecimento acerca dos mecanismos subjacentes ao cancro da mama e à carcinogenicidade dos estrogénios, é necessário desenvolver novos modelos de estudo. Os fibroblastos são facilmente obtidos e cultivados a partir de uma biópsia de pele, dado que conseguem proliferar rapidamente na presença de soro. Esta capacidade, e o facto de os fibroblastos expressarem recetores de estrogénios do tipo alfa e beta, aumentou o interesse nestas células como modelos de estudos de resposta aos estrogénios. Assim, esta tese tem ainda como objetivo apresentar resultados preliminares relativos à utilização de culturas de fibroblastos da derme como modelos de estudo da resposta aos estrogénios. Foram estabelecidas condições ótimas de cultura destas células para serem usadas em ensaios *in vitro* com 17β-estradiol, e confirmou-se que os fibroblastos da derme humana são responsivos aos estrogénios *in vitro*. Foi ainda desenvolvido um método de

isolamento de fibroblastos da derme humana, o que deixa uma porta aberta para estudos futuros com este modelo celular.

Em suma, esta tese fornece novos dados acerca do perfil genético da população feminina da Beira Interior no que concerne ao risco de cancro da mama, e explora um novo modelo celular para ser aplicado na investigação no campo desta doença.

Palavras-chave

Cancro da mama, genes de baixa penetrância, polimorfismos, estrogénios, fibroblastos

Abstract

In Portugal, breast cancer presents the highest incidence and mortality rates among women diseases. Several genetic and non-genetic risk factors for the development of breast cancer have been identified, however, the molecular mechanisms related to breast carcinogenesis remain unclear. Less than 5% of all breast cancer cases are accounted for high-penetrance cancer susceptibility genes, so polymorphisms in low penetrance genes may be responsible for a relatively small, but frequent increase of cancer risk. The main objective of this thesis is to assess the influence of polymorphisms in low penetrating genes in breast cancer susceptibility, in a specific Portuguese population, the population of Beira Interior, in which this genetic effect was never accessed. The influence of polymorphic low penetrating genes of the estrogen biosynthetic pathway (CYP17A1 and CYP19A1), the estrogen metabolic pathway (CYP1B1, CYP1A1, COMT, GTSM1, GSTT1, GSTP1 and MTHFR), DNA damage signaling and repair pathway (TP53), and estrogenic response (ERα), in breast cancer susceptibility was analyzed. We found that deletion of GSTM1 and GSTT1, alone or in association, is associated with increased risk of breast cancer. Also, the homozygous or heterozygous presence of the variant allele in CYP19A1 codon 39 is significantly associated with an increased risk of breast cancer. The effects of this polymorphism in estradiol biosynthesis appear to be modulated by GSTM1 and GSTT1 deletion polymorphism in estrogen metabolic pathway, as breast cancer susceptibility was found to be lower in carriers of GSTM1 and GSTT1, independently of CYP19A1 genotype. Carriers of xx genotype in ERα gene, or simultaneous carriers of xx and pp genotypes, seemed to have a significant reduced risk of breast cancer. It was also found that TP53 variant allele in homozygosity or heterozygosity was associated with a significant increased risk for breast cancer. We also studied the prevalence of these polymorphisms in groups of women with different 5-year relative risk for developing breast cancer scores, calculated by the Modified Gail Model. We found that breast cancer risk score calculated by Gail Model was independent of the prevalence of risk genotypes, but the simultaneous presence of three, four and five risk genotypes was revealed to be more prevalent in breast cancer affected women than in unaffected women. As it was not found statistical association of risk genotypes with “high risk” or “low risk” scores calculated by Gail Model, it is demonstrated that, in this population, combinations of two or more polymorphisms in the low penetrating genes studied should be considered important risk factors for breast cancer development in an independent way of Gail Model. It is also aim of this thesis to present preliminary results on the use of dermal fibroblasts cultures as models of study of response to estrogens. Optimal conditions for culture of cells for *in vitro* assays with 17β-estradiol were established and fibroblasts responsiveness to estrogens was confirmed. Also, a method for isolation of human dermal fibroblasts was developed, what leaves open doors for future studies in this cell model. In summary, this thesis provides new data about the genetic profile

of the female population of Beira Interior concerning breast cancer risk, and explores a new cell model to be used in molecular research in the field of the disease.

Keywords

Breast cancer, low penetrance genes, polymorphisms, estrogens, fibroblasts

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List of Acronyms

•OH	Hydroxyl radical
17β-HSD	17β-hydroxysteroid dehydrogenase
2- OHE	2-hydroxyestradiol
2-MeOE_2	2-methoxyestradiol
3β-HSD	3β-hydroxysteroid dehydrogenase
4-OHE	4-hydroxyestradiol
5-HTR	Serotonin receptors (also known as 5-hydroxytryptamine or 5-HT receptors)
A	Adenine
AI	Aromatase inhibitor
ADP	Adenosine diphosphate
Akt	Protein Kinase B (also known as PKB)
Ala	Alanine
AR	Androgen receptor
Arg	Arginine
Asn	Asparagine
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia Rad3-related
BM	Basement membrane
bp	Base pair
BPA	Bisphenol A
BSA	Bovine serum albumin
C	Cytosine
CAFs	Cancer-associated fibroblasts
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CE	Cathecol estrogens
CE-Q	Catechol estrogen quinones
CE-SQ	Catechol estrogen semiquinones
-CH_3	Methyl group
CHCB	Centro Hospitalar Cova da Beira (Hospital Centre of Cova da Beira)
COMT	Catechol-O-methyltransferase
CRH-1R	Type 1 corticotropin-releasing hormone receptor
c-Src	Tyrosine-protein kinase (also known as CSK)
Cu^{2+}	Copper ion
Cys	Cysteine
BCDDP	Breast Cancer Detection and Demonstration Project
DCIS	Ductal carcinoma in situ
DDE	Dichlorodiphenyldichloroethylene
DDR	DNA damage response
DDT	Dichlorodiphenyltrichloroethane
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified eagle medium
DNA	Desoxyribonucleic acid

DNMT	DNA methyltransferase
DNTPs	Deoxynucleotide Triphosphates
DSB	Double-strand break
E_1	Estrone
E_2	Estradiol, 17ß-estradiol
ECM	Extra-cellular matrix
EDC	Endocrine disrupting chemical
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase (also known as NOS3)
ER	Estrogen receptor
ER-	Estrogen receptor negative
ER+	Estrogen receptor positive
ERE	Estrogen responsive elements
ER-X	Novel estrogen receptor
ERα	Estrogen receptor alfa
ERß	Estrogen receptor beta
ESR1	Estrogen receptor 1 (the same as ERα)
ESR2	Estrogen receptor 2 (the same as ERß)
FBS	Fetal bovine serum
Fe^{2+}	Iron ion
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
G	Guanine
GF	Growth factor
GHR	Growth hormone receptor
Glu	Glutamine
Gly	Glycine
GPR30	G protein-coupled receptor
GSH	Glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HAA	Heterocyclic aromatic amine
HBOC	Hereditary breast and ovarian cancer
HBS	Hormone-binding site
HER2/neu	Human Epidermal growth factor Receptor 2 (also known as ErbB2)
HR	Homologous recombination
HRT	Hormone replacement therapy
Hsp	Heat shock protein
htSNPs	Haplotype-tagging single nucleotide polymorphisms
Hz	Hertz
IDC	Infiltrating ductal carcinoma
kb	Kilobase
LCIS	Lobular carcinoma in situ
Leu	Leucine
LOH	Loss of heterozigozity
MAPK	Mitogen-activated protein kinase
MB-COMT	Membrane-bound Catechol-O-methyltransferase

MC1R	Melanocortin 1 receptor
Melatonin-1R	Melatonin 1 receptor
mER	Membrane estrogen receptor
Met	Methionine
$MgCl_2$	Magnesium chloride
miRNA	MicroRNA
MMP	Metalloproteinases
MnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
MTHFR	5,10-methylenetetrahydrofolate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
myb	Myeloblastosis
NADPH	Nicotamide adenine dinucleotide phosphate
NAFs	Normal epithelial (or breast) associated fibroblast
NHDF	Normal human dermal fibroblasts
NHEJ	Non-homologous end-joining
NHS	Nurses' Health Study
NO	Nitric oxide
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered solution
PCBs	Polychlorinated biphenyls
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PI_3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PLP	Pyridoxal
PR	Progesterone receptor
Pro	Proline
PTHR/PTHrPR	Parathyroid hormone receptor/ peptide-related with parathyroid receptor
RBI	Retinoblastoma tumor supressor
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXRα	Retinoid X receptor type alpha
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
S-COMT	Soluble Catechol-O-methyltransferase
Ser	Serine
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphim
T	Thymine
TF	Transcription factor
TGF	Transforming growth factor
TNFα	Tumor necrosis factor alpha
Trp	Tryptophan
U.S.	United States (the same as USA)
Val	Valine

VEGF	Vascular endothelial growth factor
WHI	Women's Health Initiative

Chapter 1

Breast Cancer

Breast cancer is one of the most frequently diagnosed cancers in the Western world, with an estimated 1.38 million new cancer cases diagnosed in 2008 (23% of all cancers) and a significant cause of mortality worldwide. Breast cancer is the fifth most common cause of cancer death overall and is now the most frequent cancer among women both in developed and developing regions with around 690 000 new cases estimated in each region (population ratio 1:4). The lowest incidence rate is seen in Eastern Africa (19.3 per 100 000 women), and is greater than 80.0 per 100 000 women in developed regions of the world (except Japan). In Europe, the incidence rate of breast cancer is 89.7 per 100 000 women. The range of mortality rates is much less, 6 to 19 per 100 000 women, because of the more favorable survival of breast cancer developed regions (Ferlay et al., 2010). In Europe, breast cancer is the most common cancer-affecting woman, representing the leading cause of cancer death between 35 to 55 years old (Jemal et al., 2004). In Portugal, breast cancer is the most incident and mortal women disease (Pinheiro et al., 2003).

Breast cancer is a multifactorial disease caused by complex inherited and environmental factors and although several risk factors for the development of breast cancer have been identified, the molecular mechanisms related to breast carcinogenesis remain unclear. However, it is assumed that initiation of breast cancer, like other cancers, is a consequence of cumulative genetic and epigenetic damages, resulting in activation of proto-oncogenes and inactivation of tumor suppressor genes. These in turn are followed by uncontrolled cellular proliferation and/or aberrant programmed cell death, or apoptosis (Badve et al., 2011, Su et al., 2011). Reactive oxygen species (ROS) and estrogens are two cellular environment factors related to the etiology of cancer. ROS are known to be mitogenic to a variety of cells, and therefore capable of tumor promotion (Mitrunen and Hirvonen, 2003). Estrogens have been clearly identified has carcinogens, and most of the risk factors for breast cancer are related to the increased or prolonged exposure to estrogen. Stimulation of breast-cell proliferation has been proposed as the main effect of estrogens in breast carcinogenesis. Besides breast-cell proliferation, estrogen also induces aneuploidy and structural chromosomal changes (Zhu et al., 1998a). Furthermore, metabolic by-products of estrogens are responsible for free-radical-mediated DNA damage, single-strand breaks, estrogen–DNA adducts formation, protein oxidation and lipid peroxidation, what triggers genetic instability and cellular damage (Mueck, 2007). These issues will be further discussed in Chapters 2 and 3.

In the present chapter it will be discussed the distribution of breast cancer in Portugal, specifically in the Central Eastern region of the country. It will also be addressed the influence of genetic variation in cancer rates among different populations, the role of estrogens in mammary development, breast cancer pathology, and mathematical models of breast cancer risk assessment.

1.1. Breast cancer in Portugal and in the Central Eastern region of the country (Beira Interior)

The distribution of breast cancer in Portugal presents a well-defined geographic gradient, northwest-southwest, and the areas with higher mortality are the metropolitan areas of Lisbon and Oporto. In Portugal, the mortality rate, in 2005, was 27.2 per 100 000 women. In the Central Eastern region of Portugal, where this study was conducted, the mortality rate was 26.2 per 100 000 women (Direcção Geral de Saúde, 2008). Concerning incidence, the National Publication of Oncologic Registry in 2010 refers 91.68 per 100 000 women in Portugal, and 90.98 per 100 000 women in the Central Region of the country (IPO, 2010).

The Central Eastern region of Portugal (Beira Interior), comprises three cities (Covilhã, Fundão and Belmonte), with a total of 87 869 habitants and 1375 km^2. Hospital Centre of Cova da Beira (Centro Hospitalar Cova da Beira) is the main healthcare institution in the region. Seven-hundred years ago, Covilhã and Belmonte were two of the major seven Jewish centers of Portugal. Since the end of the Roman Empire existed a Jewish minority in the territory that later became Portugal and, at the founding of the nation, in 1143, this minority was already widespread in some important locations. The Jewish Community of Belmonte is the only legitimate peninsular community heir of the ancient historical presence of the Sephardic Jews, and the most active Jewish Community in this region. Throughout and after the time of Inquisition were preserved many of the customs and social relations, and many of the New Christians continued to marry only among themselves for centuries (Martins, 2006).

The Gynecologic Oncology medical consultation begun in Hospital Centre of Cova da Beira in 2005, and since then an increasing higher number of breast cancer cases have been reported. In 2008 and 2009, the period of time in which samples were collected for this study, 63 new breast cancer cases were diagnosed in this medical consultation; from 2005 to 2012, 207 new breast cancer cases were diagnosed in the same hospital.

1.2. Populational differences in breast cancer rates resulting from genetic variation

Ethnic differences in cancer incidence and mortality exist and are probably the result of genetic and epidemiological risk factors. Hypotheses regarding lifestyle, reproductive, and

screening factors explain some of the differences in breast cancer incidence. However, the processes by which different risk factors specifically act and interact to promote cancer are largely unknown. An endogenous factor that must be considered is the role of inherited, or germline, mutations in ethnic differences in cancer risk. Genes for more than 20 cancer syndromes have been identified. Genetic differences among ethnic groups for cancer risks in some of these genes have been recognized and are caused by a common germline mutation within an ethnic group (Neuhausen, 1999).

Ethnic differences may also arise from founder effects. Examples of populations in which founder effects are well documented include Afrikaners of South Africa (Diamond and Rotter, 1987), Finns (de la Chapelle, 1993), Ashkenazic Jews (Motulsky, 1995) and French Canadians (Labuda et al., 1997). An example is the founder BRCA1 and BRCA2 mutations in Jewish. In general, mutations in both BRCA1 and BRCA2 in one individual are rare, but the three common mutations identified in Ashkenazi Jewish, the BRCA1 185delAG and BRCA2 6174delT have a combined population prevalence of 2 to 2.5%, which is approximately 10 to 50 times higher than the allele frequency in the general population (Roa et al., 1996; Oddoux et al., 1996, Ramus et al., 1997). It was suggested an increased risk of breast cancer in Jewish women with a family history, which could reflect the frequency of the founder BRCA1 and BRCA2 mutations. Despite, even though mutations in BRCA1 and BRCA2 genes are more common in Ashkenazic Jewish women, there was little to no overall increased risk of breast or ovarian cancers among these women compared with non-Jewish Caucasians (Egan et al., 1996). It is important to note that mutations in BRCA1 and BRCA2 are not peculiar, or exclusive, to the Jewish people, as BRCA1 and BRCA2 mutations have been identified in individuals of all racial and ethnic groups. The fact is that relatively homogenous populations have founder mutations in which small genetic alterations that cause disease are easy to find. These groups (e.g., Ashkenazic Jews, French Canadians, Finns, and Afrikaners) are then the first to be studied because information obtained from studying the effect of a mutation in a well-defined population may be beneficial for determining effects in larger, more heterogeneous populations. Knowledge of which factors - genetic, environmental, or both - affect cancer development is essential for designing effective screening methods, providing information on ways to reduce cancer risk, and developing effective treatments once cancer develops. By studying the effect of a single, frequent mutation, or founder mutation, in a well-defined population, knowledge is gained that can be applied to larger, more heterogeneous populations. The data that are generated as a result of these studies are likely to provide information that will aid in the development of strategies for more successful prevention and treatment of breast cancer (Neuhausen, 1999).

1.3. Human breast normal and malignant development: the role of estrogens

The female mammary gland undergoes most of its development after birth, achieving a fully differentiated state only late in pregnancy. Hormones, growth factors, and stromal factors regulate this dynamic process. Female human mammary gland development (mammogenesis) begins with budding and branching between 6 and 20 weeks of gestation, yielding at birth a primitive gland composed of ducts ending in ductules. From birth to the onset of puberty, mammary growth is minimal and proportional to that of the body representing isometric growth. This period is regarded as a quiescent phase in the evolution of the gland. At puberty, mammary growth is activated and accelerates dramatically. This phase is characterized by a rapid extension and branching of the duct system. Under the influence of systemic hormones the ducts begin to expand into the surrounding stroma. Additional mammary gland proliferation and regression events occur with each luteal phase of the ovulatory cycle, and the complexity of this ductal system increases through addition of branching. At mid/late pregnancy there is significant differentiation of the terminal structures with lobular–alveolar development, and alveoli form all over the ductal system. Thus, the critical events in mammary gland development include mammary development in the fetus, exponential epithelial outgrowth during puberty, and the rapid transition to lactational competency that occurs during late pregnancy. This dynamic process is under strict hormonal control (Lamote et al., 2004; Rudel et al., 2011).

The mammary gland is composed of two major cellular compartments: a highly dynamic epithelium that undergoes cycles of proliferation, differentiation and apoptosis in response to local and endocrine signals, and an underlying stroma containing fibroblasts, endothelial cells and adipocytes, which collectively form the mammary fat pad. The epithelium of the mammary gland is composed of luminal and basal/myoepithelial cell lineages. Luminal cells line the ductal lumen and secrete milk upon terminal differentiation into lobulo-alveolar cells. Basal/myoepithelial cells reside between these luminal cells and the basement membrane to assure ductal contractility (Pelekanou and Leclercq, 2011). The development of the mammary ductal tree depends on stromal–epithelial interactions, and these interactions are important in embryonic and postnatal evolvement. The stroma not only modulates the normal development of the mammary gland but also actively participates in malignant transformation of the tissue. Mammary ducts consist of luminal cells associated with myoepithelial cells surrounded by the basement membrane (BM) that separates the epithelium from the stroma. The stromal compartment is composed of mesenchymal cells (fibroblasts, blood cells and leukocytes) and extra-cellular matrix (ECM) (laminin, fibronectin, collagen, proteoglycans, etc.), which influence mammary development (Kass et al., 2007).

Estrogen-mediated proliferation is fundamental to mammary gland development. The fact is that estrogen-mediated proliferation is also fundamental to breast tumorigenesis. At the onset of puberty, circulating ovarian steroid hormones act as pivotal mediators of ductal

morphogenesis (LaMarca and Rosen, 2007). Current understanding of the central role of hormones in the genesis of breast cancer is based on over 100 years of studying breast cancer. Not only estrogen, but also the other ovarian hormone, progesterone, plays a pivotal role in normal and neoplastic development of the mammary gland. These hormones have a paradoxical role, as long action of estrogen and progesterone is associated with increased breast cancer risk, while short duration of pregnancy level doses are associated with a reduced breast cancer risk (Medina, 2005).

1.4. Estrogens biosynthesis, metabolism and action

Estrogens are steroid hormones produced mainly by ovaries and placenta but also in lower quantities by testis, supra-kidney, brain, adipose tissue, breast, skin, blood vessels, bone and cartilage (Czajka-Oraniec and Simpson, 2010). In humans there are three main estrogenic compounds: estradiol, estrone and estriol. Estradiol is the main and more powerful estrogen produced by the ovary (Mitrunen and Hirvonen, 2003). Estrogens affect growth and differentiation of breast, as explored previously, but also prostate, brain, bone, cardiovascular system and skin (Prins and Korach, 2008; Pike et al., 2009; Bhupathy et al., 2010; Imai et al., 2010; Kok and Linn, 2010).

The mature follicles in the ovaries of pre-menopausal non-pregnant women synthesize estrogens. Also, estrogen biosynthesis can be performed in other tissues, like adipose tissue, that is the major source of estrogen production after menopause. Biosynthesis of estrogens involves series of enzymatic steps from cholesterol to C-19 androgens and C-18 estrogens. In the biosynthesis of estrogen CYP11A, CYP17A1, and CYP19A1 are particularly important (Figure 1.1). In addition to CYPs, hydroxysteroid dehydrogenases, 3β-HSD and 17β-HSD, are involved in the estrogen biosynthesis (Mitrunen and Hirvonen, 2003).

Estrogens production sites outside ovaries can convert C-19 steroids in C-18 steroids but cannot synthesize precursors of C-19 steroids, so they depend on serum availability of C19 steroids. Also, steroids that are synthesized in these locations, mainly in bone, breast and brain, are only biologically active in the local of production. Despite the small quantities of estrogens that are produced in these locals, the local concentrations are high (Imai et al., 2010).

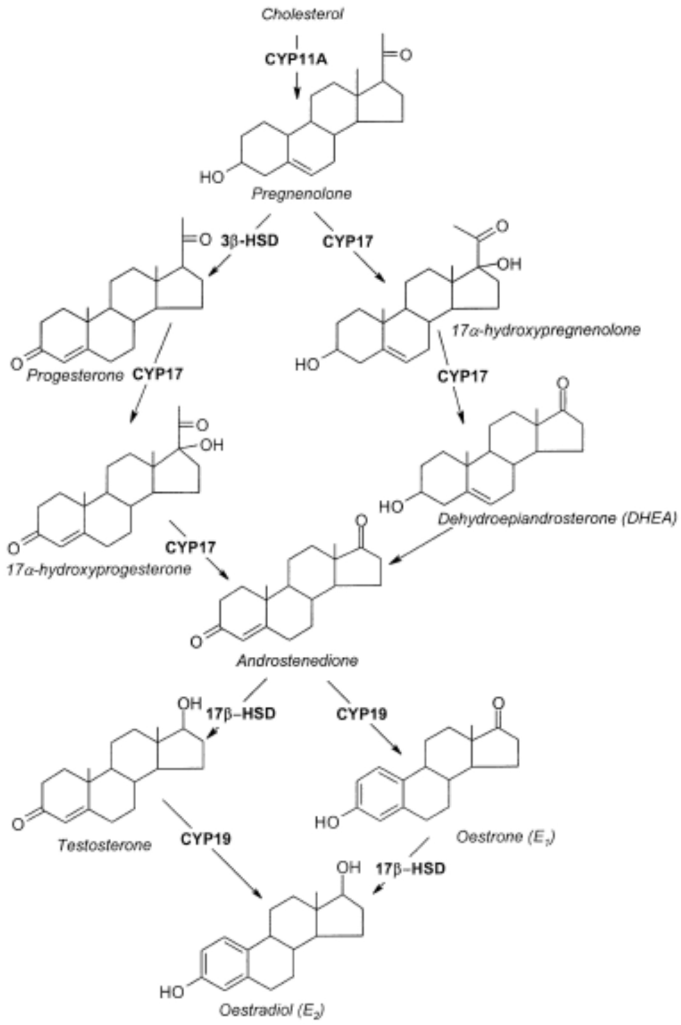

Figure 1.1. Biosynthesis of estradiol from cholesterol (Mitrunen and Hirvonen, 2003).

Estrogens undergo extensive oxidative metabolism via the action of several other CYPs. The generation of hydroxyl groups at different sites of the molecule affects the biological property of the respective estrogen metabolites yielding estrogenic, non-estrogenic or even carcinogenic metabolites (Figure 1.2). Catechol estrogens are the major metabolites of estrogen. Hydroxylation of estradiol and estrone leads to formation of catechol oestrogens (CE), namely 2- or 4 hydroxyestrons or -estradiols, and also 16ß-hydroxyestrone. The major metabolic pathway for estrogen in liver is 2-hydroxylation that is mainly catalyzed by CYP1A2, CYP3A3 and CYP3A4, while CYP1A1 is responsible for extrahepatic 2-hydroxylation. In contrast, CYP1B1 appears to be the main CYP responsible for the 4-hydroxylation, which dominates in many extrahepatic tissues such as human breast and uterus (Mitrunen and Hirvonen, 2003).

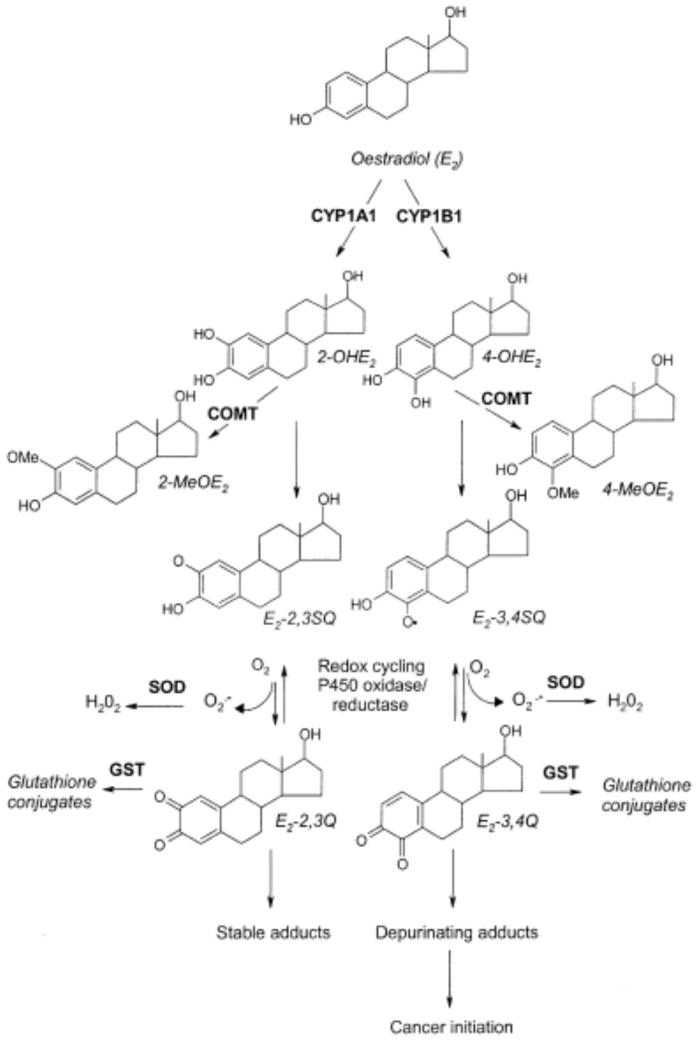

Figure 1.2. Metabolism of estradiol (Mitrunen and Hirvonen, 2003).

A greater role in carcinogenesis has been suggested for 4-OHE_2 than for 2-OHE_2. In fact, the 2-hydroxylation pathway is thought to be anti-carcinogenic. The 2- and 4-hydroxylated estrogens can be inactivated by O-methylation catalyzed by COMT. The observed lack of carcinogenic activity of the 2-hydroxyoestrogens may be due to faster rate of O-methylation. Quantitatively, the most active conjugation pathway for catechol estrogens is methylation, but they can also be conjugated by glucoronidation or sulfation. Further metabolism of the catechol metabolites of estrogen leads to formation of semiquinones (CE-SQ) and quinones (CE-Q). CE-2,3Q can bind stably to DNA, while CE-3,4Q forms depurinating adducts, which are lost from DNA by cleavage of the glucosidic bond leaving apurinic sites, which is hypothesized to cause tumor-initiation similarly to that seen with the depurinating polycyclic aromatic hydrocarbon (PAH)–DNA adducts. In addition, redox cycling between CE-SQs and CE-Qs produces ROS, which can cause oxidative damage to lipids and DNA. Quinones may be conjugated with glutathione (GSH) catalyzed by glutathione S-transferases (GSTs) or reduced to catechol estrogens by quinone reductase. The semiquinones may react with molecular

oxygen (H_2O_2) to form superoxide radicals, which are reduced to hydrogen peroxide either spontaneously or catalyzed by superoxide dismutases such as MnSOD. H_2O_2 is neutral and rather nonreactive except in the presence of reduced transition metal ions (i.e. Fe^{2+}), which evoke the formation of the most powerful oxidant, the hydroxyl radical (•OH). H_2O_2 can also readily cross the cellular and nuclear membranes and reach the DNA in neighboring cells (Mitrunen and Hirvonen, 2003).

Estrogens act especially over the reproductive organs, but they also act on other organ systems such as cardiovascular, skeletal, immune, gastrointestinal, and neural sites. Their major actions are genomic, mediated by nuclear estrogen receptors, but they also have non-genomic actions (Imai et al., 2010).

Estrogen signaling is primarily mediated through ERs. ERs include classic receptors as ERα and ERβ, members of the nuclear receptor superfamily acting as transcription factors and modulate the transcription of target genes, and membrane receptors such as GPR30 (an orphan G-protein coupled receptor) and ER-X. ERα is primarily expressed in the gonadal organs (uterus, ovary, prostate, testes, and breast), but is also present at lower levels in other tissues such as bone, liver, kidney, adipose tissue, and brain. ERβ is primarily expressed in non-gonadal tissues – colon, bone marrow, vascular endothelium, lung, bladder, and brain. ERα and ERβ co-localize in many cell types, including the endothelium, epithelium, muscle, bone, cartilage, hematopoietic cells, neurons, and glia, although each receptor exhibits a distinct pattern of tissue-specific distribution throughout the body. Although it is unclear how these patterns are dictated, it is known that separate genes, ESR1 and ESR2, encode ERα and ERβ located on different chromosomes (Cui et al., 2013).

The GPR30 receptor is an orphan G-protein-coupled receptor that has recently been shown to bind estrogen and cause estrogen-mediated adenylyl cyclase stimulation. It is a multi-pass membrane protein that localizes to the endoplasmic reticulum. GPR30 is widely distributed in neural, breast cancer, placental, heart, ovarian, prostate, hepatic, vascular, epithelial and lymphoid tissue. The protein binds estrogen, resulting in intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus. This protein therefore plays a role in the rapid non-genomic signaling events widely observed following stimulation of cells and tissues with estrogen. The receptor acts independently of ERα and ERβ and triggers ER-dependent EGFR action (Revankar et al., 2007).

ER-X was designated as a novel, plasma membrane-associated, putative estrogen receptor, in 2002. ER-X expression is highly regulated during development, and has been shown to be enriched in the fetal baboon brain and in the neo-cortex, uterus, and lungs of postnatal rodents. The expression of ER-X peaks 7–10 days after birth and declines over the first postnatal month. In adults, ER-X expression is almost undetectable, but re-emerges after ischemic injury or in animal models of Alzheimer Disease (Toran-Allerand et al., 2002).

Although both types of ER transduce estrogen signals into a large variety of physiological responses in various organs, the nuclear ERs initiate the biological events in a slow motion

such as in hours or even days, while the cell membrane ERs triggers an intracellular signaling cascade response in a much fast manner such as in seconds (Cui et al., 2013).

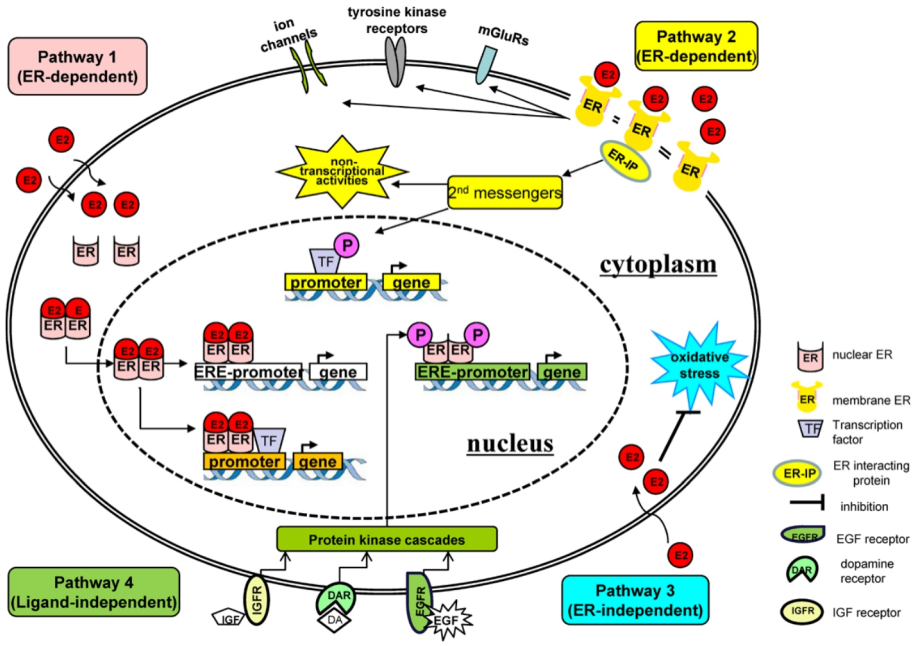

Figure 1.3. Signaling pathway mediated by E_2 and ERs. Pathway 1: The nuclear initiated estrogen signaling mediated through classical ERs leads to the transcriptional changes in estrogen-responsive genes with or without EREs. Pathway 2: The membrane initiated estrogen signaling leads to diverse cytoplasmic effects, including the regulation of membrane-based ion channels, regulation of second messenger systems, and modification of transcription factors or other membrane receptors. Pathway 3: Estrogen can also exert anti-oxidant effects in an ER-independent manner. Pathway 4: Ligand-independent genomic actions. Growth factors (GF) activate protein-kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs (Cui et al., 2013).

Although most estrogen-mediated signaling pathways are ER dependent, ER-independent signaling mechanisms exist (Figure 1.3). There are two different, but inter-related, ER-dependent mechanisms, which are classified as either “genomic” or “non-genomic”, based on whether the end result of ER signaling is transcription regulation. Despite these traditional classifications, recent studies have implicated some “non-genomic” pathways in gene transcription. In addition to being either genomic or non-genomic, ER-dependent pathways can initiate either in the nucleus or at the plasma membrane. Unlike ER-dependent pathways, instead of binding to estrogen receptors, estrogen initiates the ER-independent signaling pathways through regulating enzymatic activities or interacting with non-sex steroid-hormone-nuclear-receptors in certain cells (Cui et al., 2013).

Both receptors ERα and ERβ act as cytoplasmic transcription factors activated by ligand. In the cytoplasm they stay inactivated and associated to heat shock proteins (Hsp), but after they bond to the hormone, Hsp are dissociated and receptors are transferred to the nucleus, where gene transcription occurs. Separation from the Hsp leads to dimerization of the receptor with formation of a stable ER homo or heterodimer (ERα-ERα; ERβ-ERα; ERβ-ERβ) (Powell et al., 2010). The second step of this pathway involves the interaction with co-

regulator (co-activators or co-repressors) molecules (Powell et al., 2010; Zárate and Seilicovich, 2010). The activated ER dimmer interacts with estrogen responsive elements (ERE) (Powell et al., 2010). ERE are small DNA sequences located in a gene promoter that are capable to bond to the ER dimmer and regulate transcription. In the last step, ER-ERE complex promotes the formation of a transcription pre-initiation complex and facilitates chromatin disruption in ERE. Finally, RNA polymerase II and the remaining transcription machinery bonds to the promoter and gene are transcribed. Depending on the cell and on the co-regulator, the receptor that is connected to the DNA can have a positive or negative effect in the expression of the target gene (Barone et al., 2010).

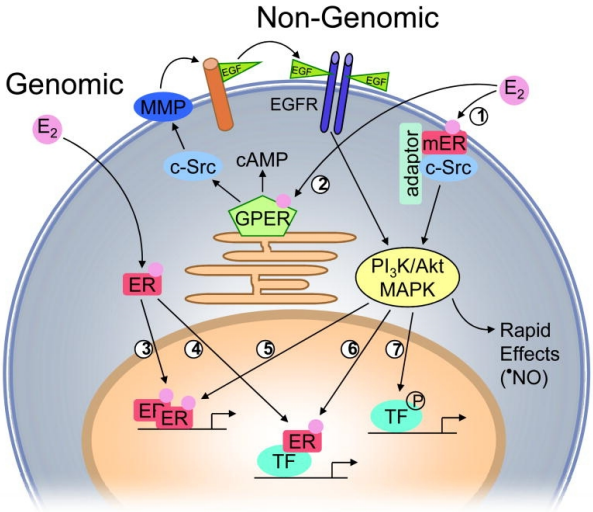

Figure 1.4. Genomic and non-genomic signaling mediated by estrogen receptor (Meyer et al., 2009).

Estrogen can also bond to G protein-coupled receptor (GPR30), which is located in the endoplasmic reticulum (Figure 1.4). GPR30 activates adenylate cyclase, what results in the production of cAMP and c-Src, that activates the metalloproteinases (MMP) matrix and leads to the activation of MAPK and PI_3K. Once activated, MAPK and PI_3K/Akt can induce rapid non-genomic effects, by the release of NO, or also influence gene transcription (Meyer et al., 2009). Rapid non genomic effects caused by estrogens, lasting from few seconds to some minutes, are transmitted by enzymatic pathway or by ionic channels through the activation of membrane estrogen receptors (mER) (Figure 1.5).

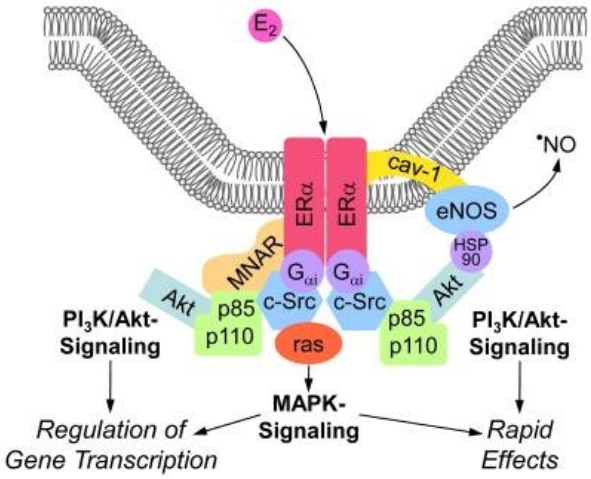

Figure 1.5. Non-genomic action of estrogen, mediated by membrane estrogen receptor alpha (Meyer et al., 2009).

Furthermore, activated ER modulates the function of other classes of transcription factors (TF) throughout protein-protein interaction. Transcriptional activity can be augmented by phosphorylation, or by other transcription factors that can be activated or interact directly with ER, or independently bond to ER in the promoter region of the target gene (Meyer et al., 2009).

Table 1.1. Differences between genomic and non-genomic estrogen action (adapted from Krolic and Milnerowicz, 2012).

Genomic action mechanism	Non-genomic action mechanism
Binding with ERα and ERβ	Activation/repression of other pathways than receptor ERα or ERβ
Late response after exposure to the hormone	Fast response after exposure to the hormone
Gene expression, mRNA and new proteins synthesis	Rapid adaptation to changes in cell external environment

Epidemiological studies in humans and biological studies in animals have shown estradiol as a carcinogen. Small elevations of circulating estrogen levels caused by increased endogenous production or by taking them as drugs may lead to breast or uterine cancer (Roy et al., 2007; Krolic and Milnerowicz, 2012). Estrogens can promote carcinogenesis by different ways (Figure 1.6). Combining the steroid receptor complex with DNA causes one of the direct influences of estrogens on target cells. In that way, it can regulate the expression of genes and transform proto-oncogenes into oncogenes. Also, ER-mediated proliferation of cells has been associated with carcinogenic events, as continuous stimulation increases the chances of occurrence of spontaneous mutations (Krolic and Milnerowicz, 2012).

One of the theses of tumorigenesis in the non-genomic pathway is that estrogen stimulates the induction of several histone modifications in ERα target gene promoters like phosphorylation, acetylation and methylation (Mann et al., 2011). Also, the involvement of estrogens in several signaling pathways like G protein coupled receptors, tyrosine kinases and mitogen-activated protein kinases (MAPKs), cell membrane ion channels, activation of adenylate cyclase production or phospholipase C (PLC) activation may regulate many processes such as gene expression or proliferation of cells in tissues where there are no traditional targets of these hormones, e.g. in the vascular wall or the central nervous system (Krolic and Milnerowicz, 2012).

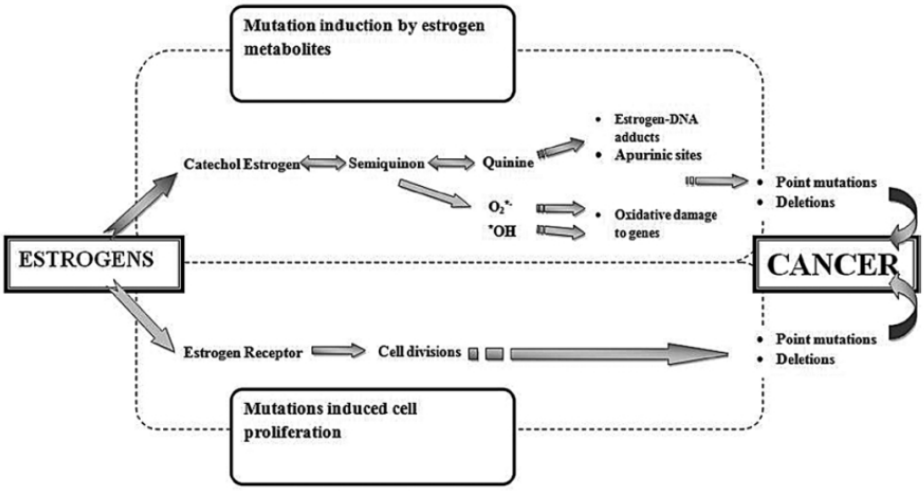

Figure 1.6. Different pathways of estrogen promoted carcinogenesis (Krolic and Milnerowicz, 2012).

Another pathway involved in the carcinogenic process is insufficient conjugation of estrogens and their metabolites. Estrone and estradiol are not capable of forming adducts with DNA, while the oxidation products of catechol estrogens are able to bind covalently to bases of DNA and to the nucleophilic sites of the proteins. 4-hydroxy CE-Qs create depurinating adducts, which can lead to apurinic DNA sites and permanent mutations. This consequently leads to tumorgenesis. This adducts were found in various target tissues of cancers (Cavalieri and Rogan, 2010).

Oxidative damage to genes is another example of the negative effect of estrogens on the cells. Catechol-estrogens and 17α-ethinylestradiol can be oxidated by Cu^{2+} and the production of hydroxyl radicals was shown (Seacat et al. in 1997). Alternatively, semiquinones may react with molecular oxygen and form superoxide radial and quinone. This free radical damage induced by estrogens may lead to 8-hydroxylation of the purine bases of DNA, strand breakage and lipid hydroperoxide-mediated DNA modification (Roy et al., 2007; Cavalieri and Rogan, 2010).

1.5. Breast cancer pathology: subtypes of breast cancers

Breast cancer is genetically and clinically a heterogeneous disease. Management of breast cancer relies on the availability of robust clinical and pathological prognostic and predictive factors to guide patient decision-making and the selection of treatment options. In order to organize this heterogeneity and standardize the language, breast cancer classification systems have been developed. These classification schemes have evolved over many decades into a tool that is used to aid in treatment and prognosis (Malhotra et al., 2010). The treatment approach of breast cancer is dependent of three main prognostic determinants used in routine practice: tumor size, lymph node status, presence of metastasis (TNM classification) (Rakha et al., 2010).

In-situ carcinoma is considered the pre-invasive state of breast cancer. The behavior of in-situ breast cancer is still unknown and there are no predictive bio-molecular markers for invasive progression. Breast carcinoma in situ is a histologic diagnosis, sub-classified as either ductal or lobular. Ductal carcinoma *in situ* (DCIS) is considerably more common than lobular carcinoma *in situ* (LCIS) counterpart and encompasses a heterogeneous group of tumors. Traditionally, DCIS has been classified taking in account their architectural features in five well recognized subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid (Malhotra et al., 2010). The ductal and lobular subtypes constitute the majority of all breast cancers worldwide, with the ductal subtype accounting for 40–75% of all diagnosed pre-invasive and invasive breast cancers cases (Bombonati and Sgroi, 2011).

Similar to *in situ* carcinomas, invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. The major invasive tumor types include infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas. Infiltrating ductal carcinoma (IDC) is, by far, the most common subtypes accounting for 70–80% of all invasive lesions. With prognostic significance, IDC is classified according their histologic differentiation, as either well differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3), based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Malhotra et al., 2010).

Clinical practice has shown that TNM staging is insufficient to define prognosis and to schedule appropriate therapy for breast cancer. New prognostic markers have been investigated. Molecular markers as ER, PR, ErbB2 (Her2/neu) and p53 have been widespread used, however the larger medical community has not accepted the routine use of these markers for DCIS. In contrast to DCIS, where the use of molecular markers is still debated, the utility of ER, PR and HER2/neu is well accepted for IDC and it is recommended that their status be determined on all invasive carcinomas. Already, the status of these markers helps determine which patients are likely to respond to targeted therapies (i.e., tamoxifen or aromatase inhibitors for ER+/PR+ patients and trastuzumab or lapatinib for HER2/neu patients). The molecular subtypes display highly significant differences in prediction of overall survival, as well as disease-free survival with the basal-like/triple-negative (ER-/PR-/ErbB2-) subtype having the shortest survival. Recently, microarray-based gene expression

analysis is also being used to define molecular subtypes of breast cancer, however, routine use of microarray analysis or genome sequencing is still cost prohibitive (Malhotra et al., 2010).

1.6. Mathematical models of breast cancer risk assessment

Cancer risk prediction models provide an important approach to assess risk and susceptibility by identifying individuals at high risk, facilitating the design and planning of clinical chemoprevention trials, and allowing the evaluation of interventions. Conventional breast cancer risk model includes the cumulative estrogen exposure data such as age, age at menarche and menopause, age at first live birth, and use of HRT in risk calculation, since estrogens are the main risk factor for mammary carcinogenesis (Chang, 2011).

The most widely known and commonly used model for breast cancer risk assessment is the Gail model (Gail et al., 1989). This model was initially designed in 1989 using data that were collected as part of the Breast Cancer Detection and Demonstration Project (BCDDP), a nested case–control study of almost 300 000 women, was validated in the Nurses' Health Study (NHS) (Spiegelman et al., 1994), and was subsequently modified to project the absolute risk of developing breast cancer in 1999 (Costantino et al., 1999). Gail Model is a risk-assessment model that focuses primarily on non-genetic risk factors, with limited information on family history (Evans and Howell, 2007). Both the original and the modified versions of the Gail model use six-breast cancer risk factors, namely age, hormonal or reproductive history (age at menarche and age at first live birth), previous history of breast disease (number of breast biopsies and history of atypical hyperplasia), and family history (number of first-degree relatives with breast cancer). The Gail model is the only model that has been validated in three large population-based databases (Cummings et al., 2009). The major limitation of the Gail model is the inclusion of only first-degree relatives, which results in underestimating risk in the 50% of families with cancer in the paternal lineage and also takes no account of the age of onset of breast cancer (Evans and Howell, 2007).

Several authors have tried to improve breast cancer risk models by adding single-nucleotide polymorphisms to original breast cancer risk models (Gail, 2008; Gail, 2009; Mealiffe et al., 2010; Dai et al., 2012; Darabi et al., 2012). Although some models have attempted to include the enzyme kinetics of phases I and II such as CYP1A1, CYP1B1, COMT, or GSTP1 in a qualitative manner, estrogen metabolic pathways are interconnected and complex, and each of the metabolizing enzymes have genetic polymorphisms that could result in alteration of catalytic activity of the enzyme. Obviously, the fact that genetic variation does not quantify the functional consequences of the enzyme activity makes it more complicated to develop a quantitative model. Although, the models could reflect the factors associated with estrogen metabolism into risk calculations only in a qualitative manner. It is, therefore, necessary to develop a refined model that utilizes a pathway-based functional and quantitative approach.

The most recent proposed risk prediction model is being developed to incorporate estrogen exposure parameters, individual phenotypic factors such as body mass index or family history, and the functional effects of genetic variants of CYP1A1, CYP1B1, and COMT (Chang, 2011).

Genetic risk prediction models use pedigree analysis methods, which are based on information about the exact relationships among individuals within a family. These models include the most widely used and validated model, BRCAPRO, as well as the Yale University model, the International Breast Cancer Intervention Study (IBIS) model (also known as the Tyrer–Cuzick model), and the Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm (BOADICEA). The algorithm is based in assumptions about the number of susceptibility genes involved, the allele frequencies in the general population, and the cancer risks that are conferred by these alleles. The main advantage of these models is that they can, in principle, compute cancer risks and mutation carrier probabilities regardless of the family structure and disease pattern. However, their accuracy depends on their underlying assumptions. At best, the current genetic risk prediction models give approximate risk estimates because not all breast cancer susceptibility genes have been identified (Amir et al., 2010).

Chapter 2

Non-genetic Factors Associated with Breast Cancer

Several non-genetic risk factors are involved in the etiology of breast cancer. Being a woman is the main risk factor for developing breast cancer; the disease is about 100 times more common among women than men. Also, risk of developing breast cancer increases with aging. About 1 of 8 invasive breast cancers are found in women younger than 45, while about 2 of 3 invasive breast cancers are found in women age 55 or older (Mavaddat et al., 2010). The majority of non-genetic risk factors associated with breast cancer is related to reproductive and hormonal factors and is widely thought to reflect longer lifetime exposures to endogenous steroid hormones. The genetic component of the disease is reflected on a tendency to cluster in families, although this could also reflect shared life-style and environment (Mavaddat et al., 2010). Thus, although the conventional risk factors of breast cancer are related to reproductive factors, lifetime exposure to endogenous sex hormones and inheritance, other life-style derived and environmental risk factors like the use of exogenous estrogens, radiation exposure and alcohol consumption, and other endocrine disruptors constitute risk factors for the disease (Coyle, 2004).

2.1. Menarche and Menopause

Menarche and menopause are markers of onset and cessation, respectively, of ovarian and related endocrine activity associated with reproduction. During women's reproductive years (broadly the time between menarche and menopause) the ovary produces steroid hormones that directly affect the development and function of the breast. Early menarche and late menopause are known to increase women's risk of developing breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2012).

The increased breast cancer risk associated with early age at menarche (<12 years), probably results from prolonged exposure of breast epithelium to estrogens, earlier onset of regular menstrual cycles, and higher estrogen levels for several years after menarche in women with early age at menarche (Mitrunen and Hirvonen, 2003). It has been hypothesized that early menarche induces an early proliferation of mammary gland cells through early exposure to high hormonal levels (Harrison et al., 1999). Regarding this risk factor, contradictory findings have arisen. Modest elevation in breast cancer risk was found to be associated with early age of menarche in a study (Russo and Russo, 2006) but there was no association between age at

menarche and breast cancer risk in the Japan Collaborative Cohort Study (Tamakoshi et al., 2004). Regular ovulatory menstrual cycles seem to increase women risk of breast cancer: breast cancer risk can be more than two times greater among women whose menstrual cycles become regular within one year of their first menstrual period than among women with a five years or longer delay in the onset of regular cycle (De Stavola et al., 2004).

According to epidemiological studies, women who reach menopause at a late age are more likely to have a higher risk of breast cancer, although no consistent trend is observed (Goldman and Hatch, 2000; Oran et al., 2004; Zografos et al., 2004). Later age at menopause maximizes the number of ovulatory cycles, and may therefore lead to increased breast cancer risk. The higher breast cancer risk in women with a late menopause can be explained by both the longer duration and higher level of exposure to estrogen and progesterone. They also may experience a larger number of anovulatory cycles resulting in a lack of cyclic progesterone. Artificial menopause by bilateral oophorectomy also markedly reduces breast cancer risk and the effect is greater than natural menopause. Differences between effect of natural and artificial menopause on risk of breast cancer can be explained by the fact that ovarian function does not stop at the time of menopause among women with intact ovaries, but declines over period a of a few months or year (Goldman and Hatch, 2000; Zografos et al., 2004).

2.2. Parity, age at first full term pregnancy, lactation and abortion

Higher parity and early age at first full term pregnancy have both been associated with decreased lifetime incidence of breast cancer. The mammary gland epithelium could reach full differentiation at the first full-term pregnancy and differentiated cells do not divide or proliferate under normal conditions and are less susceptible to the effects of carcinogens (Parsa and Parsa, 2009).

Women having their first child before the age of 20 have about half of the risk compared to those having their first child after the age of 30. The mechanism of the protective effect of parity is not completely understood, but probably reflects early full differentiation of mammary gland cells, rendering them less susceptible to carcinogenic transformations (Russo et al., 2000; Parsa and Parsa, 2009). The protective effect of pregnancy on breast cancer may also be due to two beneficial consequences of completed pregnancy. Firstly, prolactin levels are substantially lowered in multiparous women, when compared to nulliparous, and several epidemiological studies indicated that prolactin may function as a progression factor for human breast cancer. Secondly, multiparous women have lower levels of circulating estradiol and higher level of bioavailable or free estradiol (Lee et al., 2004).

Also, prolonged lactation has been associated with decreased risk (Lipworth et al., 2000; Lee et al., 2004). An explanation for the protection afforded by lactation is that the cumulative number of ovulatory menstrual cycles a woman experiences will be lower among women

substantial lactation experience because breast-feeding delays ovulation following a completed pregnancy. Breast-feeding is a potentially modifiable behavior, thus its impact on reducing risk of breast cancer is extremely important particularly among premenopausal women (Goldman and Hatch, 2000; Parker et al., 2001; Lee et al., 2004; Zografos et al., 2004).

A number of studies have examined the risk of breast cancer associated with spontaneous and induced abortion and there has been some controversy about this association. A collaborative reanalysis of data from 53 epidemiological studies, that included 83,000 women with breast cancer from 16 countries described the inconsistent finding across studies and difficulties in evaluation of that relationships. The conclusion was that breast cancer risk did not appear to be associated with an increased number of either spontaneous or induced abortions (Collaborative Group on Hormonal Factors in Breast Cancer, 2012), despite some studies have suggested that abortion may moderately increase the risk of breast cancer (Brind et al.,1996; Wingo et al. 1997; Zografos et al., 2004).

2.3. Body mass index

Weight gain and obesity is a potential risk factor that may influence the incidence of breast cancer. Numerous observational studies have investigated the correlation between obesity and breast cancer, with inconsistent results, thus there is no universal consensus on the relationship between BMI and breast cancer (Cheraghi et al., 2012).

Obesity has been related to higher endogenous estrogen levels and to increased risk of breast cancer in postmenopausal women who have most of their circulating estrogen derived from conversion of androgen to estrogen in adipose tissue (Hunter and Willett, 1993). Some studies suggest that body mass index greater than 30 may increase the risk of breast cancer both in pre and postmenopausal periods (Johnson-Thompson and Guthrie, 2000), whereas others claim that obesity may reduce the risk of breast cancer during premenopausal period, but increase the risk during postmenopausal period (Jefcoate et al., 2000). It was shown that *in situ* aromatization catalyzed by cytochrome P450 19 (CYP19A1) can effectively increase tissue estradiol levels more than production by the uptake of estrogen from circulation (Yue et al., 1999).

To date, few meta-analyses have been conducted to estimate a summary measure of the effect of overweight and obesity on breast cancer. A recent meta-analysis showed that body mass index has no significant effect on the incidence of breast cancer during premenopausal period. On the other hand, overweight and obesity may have a minimal effect on breast cancer risk in this period of women's life (Cheraghi et al., 2012).

Physical activity has also been associated with decreased breast cancer risk. Suggested mechanisms include reduction of regular ovulatory cycles and increase of the amount of methylated catechol estrogens (Mitrunen and Hirvonen, 2003).

2.4. Endogenous steroid hormone concentration

Sex steroid hormones have long been hypothesized to increase breast cancer risk, and epidemiologic evidence provides strong support for the etiologic role of endogenous hormones (Endogenous Hormones and Breast Cancer Collaborative Group, 2002; Missmer et al., 2004; Zeleniuch-Jacquotte et al., 2004). Prospective studies consistently report that higher levels of endogenous estrogens and androgens and lower levels of sex hormone binding globulin (SHBG) are associated with increased risk of postmenopausal breast cancer (Baglietto et al., 2010). It has long been considered that established risk factors for breast cancer, such as early menarche, late menopause, postmenopause obesity, can be thought of as measures of the "cumulative dose of endogenous estrogen that breast epithelium is exposed to over time" (Henderson and Feigelson, 2000).

There is evidence to support an important role for sex hormones on breast cancer risk through the accumulation of genetic damage that these hormones cause to DNA in breast cells. Experimental and clinical evidence support a role for estrogens in breast carcinogenesis (Russo and Russo, 2006), but the effect of androgens on the mammary gland is more complex, as androgens may increase breast cancer risk directly, by stimulating cell proliferation, or indirectly, by providing the substrate to aromatase for the synthesis of estrogens in peripheral or mammary adipose tissue (Hankinson and Eliassen, 2007). However, there is also evidence that androgens may act as anti-estrogens and exert an anti-proliferative and apoptotic effect in the breast under certain circumstances (von Schoultz, 2007).

2.5. Exogenous estrogens

Since breast cancer is the most important malignancy affecting women, the assessment of its possible association with widely used hormonal steroids is of great importance, whether the estrogens and/or progestogens are used as contraceptives or for menopause hormone treatment. Studies of this association are part of a large group of long-term follow-up studies, some of which have demonstrated a well-established protective effect of such hormonal steroids against endometrial, ovarian and colorectal cancer, but even a small association between use of hormonal contraception and breast cancer would be clinically relevant because hormonal contraceptives are among the most frequently prescribed drugs in the world (ESHRE Capri Workshop Group, 2004).

The role of exogenous hormones in the form of hormone replacement therapy (HRT) on breast cancer incidence has become a field of study since the publication of the results from the randomized Women's Health Initiative (WHI) trial in the United States in 2002. Before it, HRT was widely used by postmenopausal women for a variety of reasons, including menopausal symptoms and prevention of chronic diseases such as cardiovascular disease and osteoporosis. After the publication of the WHI, multiple professional societies worldwide changed their HRT prescribing guidelines and recommended only short-term use, if at all (Chen, 2008). In the

WHI study, women who took a combination of estrogen and progesterone had a greater chance of being diagnosed with a node-positive or more advanced stage breast cancer than those on placebo. This finding has not been consistently seen in the observational studies, and the clinical significance of these findings is not clear (Chlebowski et al., 2003).

2.6. Cigarette smoking

The potential role of smoking in breast cancer risk has been subject of several scientific publications. Tobacco exposure is a well-established cause of lung cancer, and is thought to account for nearly one third of all cancer deaths (Reynolds, 2012). The studies on smoking and breast cancer susceptibility have given controversial results. The risk of developing breast cancer has been only weakly associated with cigarette smoking. However, statistically significant effects have been seen for early age at starting, and for heavy, current, and passive smoking (Wartenberg et al., 2000). Tobacco smoke contains thousands of chemical compounds, including 30 carcinogens, and many of which are known to be mammary carcinogens. Some agents in tobacco smoke may also have anti estrogenic effects, and nicotine has been shown to inhibit the CYP19A1 enzyme. Accordingly, smokers are known to have lower age at menopause than non-smokers (Mitrunen and Hirvonen, 2003).

The most important carcinogens in tobacco smoke are polycyclic aromatic hydrocarbons (PAHs), aryl amines, heterocyclic aromatic amines (HAAs), and N-nitrosamines. The ingested or inhaled PAHs are converted to water-soluble derivatives mainly via oxidative activation by cytochrome P4501A1 (CYP1A1) followed by detoxification by phase II enzymes such as glutathione S-transferases (GSTs). PAHs have been shown to be mutagenic to breast cell lines, and, as lipophilic compounds, they are stored in adipose tissues, including breast. Accordingly, PAH–DNA adducts have been identified in normal breast tissue from women with and without breast cancer (Rundle et al., 2000). Cigarette smoke is also a rich source of oxygen radicals, and some antioxidant enzymes, like manganese superoxide dismutase (MnSOD), that seems to be induced by tobacco smoke. The cigarette tarsemiquinone radical can reduce oxygen to produce superoxides, and hence stimulate production of hydrogen peroxide and the hydroxyl radical (•OH). The latter is an extremely reactive radical and therefore a prime candidate for producing endogenous oxidation of DNA (Marnett, 2000).

2.7. Diet

Both diet and nutrition have been studied in relation with breast cancer risk, suggested in part by the great variation seen among different countries in breast cancer incidence, which could be possibly explained through the antioxidant properties of selected nutrients. Is known that human diet contains a great variety of natural anti-carcinogens, but also carcinogenic compounds. Many of these substances may act by generating oxygen radicals, which in turn

may lead to DNA damage, and influence, among others, inflammatory and immune responses, the progression of cells through cell cycle and DNA repair, DNA mutations, DNA adducts, metabolic detoxification, the stimulation of growth factors, and also by the potential anti-estrogenic influence of some nutrients (Michels et al., 2007).

A high intake of fat, especially of polyunsaturated fatty acids, has been suggested to increase breast cancer risk (Bartsch et al., 1999), but not all reports are in agreement with these findings (Byrne et al., 2002). Intake of fruits and vegetables, sources of natural antioxidants, has been shown to decrease breast cancer risk, while the consumption of meat has been associated with increased breast cancer risk (Aune et al., 2012). Dietary fat has long been suspected to be the reason for this association but recent studies support the role of heterocyclic aromatic amines (HAAs) found in well-done meat, that require metabolic activation by N-acetyltransferases (NATs) to be able to exert their harmful effects (Hem, 2000).

Also, some foods and nutrients have been suggested to increase the risk for breast cancer through an increase in circulating levels of endogenous estrogen, insulin like growth factor 1 or other growth factors. Soy, more specifically genistein, with a chemical structure similar to steroidal estrogens, has been shown to have both anti-carcinogenic and cancer promoting effects (Nagata, 2010).

2.8. Alcohol

Alcohol has been described as being one of the most constant enhancer of breast cancer risk. Data from a variety of epidemiologic studies suggest that chronic alcohol consumption even in moderate amounts increases women risk for breast cancer (Rohan et al., 2000). The vast majority of the case-control studies (84% of 69) and cohort studies (76% of 21) published to-date have show a positive association between alcohol consumption and breast cancer. Nevertheless, the magnitude of the association is modest (Poschl et al., 2004). The mechanism underlying the carcinogenic effect associated with alcohol is incompletely understood. Several mechanisms have been hypothesized, including effects on estrogen levels and estrogen receptors, release of carcinogenic metabolites of alcohol, such as acetaldehyde or reactive oxygen species, and decreased absorption of essential nutrients like folate, a micronutrient known to be important in DNA synthesis and repair. It has been observed that the magnitude of the alcohol–breast cancer association differs by levels of folate consumption (Baglietto et al., 2005). Another proposed mechanism underlying the relationship between alcohol consumption and breast cancer is carcinogenesis resulting from alcohol metabolism. The metabolism of alcohol occurs through a two-stage process. During the first stage, acetaldehyde is a primary product. This and other products of alcohol metabolism are known to induce DNA modifications, by causing strand deletions, chromosomal aberrations, or generating protein adducts. Once DNA modifications occur, acetaldehyde may promote breast tumorigenesis by interfering with DNA repair mechanisms (Dumitrescu and Corarla, 2005).

Other proposed mechanisms include alcohol-induced production of ROS (Wright et al., 1999), and increased adduct formation, possibly due to decreased protein expression of detoxification enzymes (Barnes et al., 2000).

Several epidemiologic studies have shown an association between alcohol and breast cancer exclusively for estrogen receptor-positive (ER+) tumors. Also, women consuming alcohol have been hypothesized to exhibit elevated estrogen levels. Alcohol may affect breast cancer risk through the ER signaling pathways as the elevated levels of intracellular estrogens resulting from alcohol intake may act through the estrogen receptor to promote breast tumor growth (Dorgan et al., 2001). In human breast cancer cells, ethanol stimulates cell proliferation and enhances ER-alpha and aromatase expression, supporting a role for ER signaling in the proliferation of breast cancer cells. In addition to increasing the transcriptional activity of ER alpha, ethanol may also affect breast cancer risk by down-regulating the expression of the tumor suppressor gene BRCA1 (Fan et al., 2000; Dumitrescu and Corarla, 2005).

2.9. Epigenetic factors

Epigenetics was defined as a discipline more than 50 years ago, and originally described changes in the development of organisms that could not be explained by changes in DNA. Nowadays, the concept of epigenetics describes mitotically stable states and changes of gene activity that do not involve alterations of the primary DNA sequence, thus provide a second layer of information above the pure genomic print (Veek and Esteller, 2010). The term is generally considered to encompass changes in DNA methylation, histone modifications, microRNA (miRNA) expression, and nucleosome positioning and higher order chromatin as epigenetic changes affecting gene regulation (Huang et al., 2011).

Epigenetic mechanisms coordinate crucial biological processes, like X-chromosome inactivation, genomic imprinting, position effect variegation, reprogramming of genomes during differentiation and development, or RNA interference leading to post-transcriptional gene silencing. Subsequently it became clear that epigenetic modifications play important roles in diseases, including breast cancer (Esteller, 2008).

In recent years, two epigenetic mechanisms have emerged as the most critical players of transcriptional regulation: methylation of DNA and chemical histone tail modifications. It is generally believed that DNA methylation is the initiating event that marks certain genomic sites for the establishment of a transcriptionally inactive chromatin state (Veek and Esteller, 2010). DNA methylation and histone modifications interact with each other in the regulation of gene expression.

DNA methylation refers to the covalent post-replicative addition of a methylgroup (-CH3) onto the 5-carbon of the cytosine ring within CpG dinucleotides. This enzymatic reaction is conferred by DNA methyltransferases (DNMT), which catalyze the transfer from the methyl group donor S-adenosyl methionine. Typically, gene promoters or first exons are enriched in such CpG dinucleotides, where they cluster to form a so-called CpG island. Approximately 60%

of protein-coding mammalian genes harbor CpG islands in their promoter region. These are normally unmethylated in transcriptionally active genes like housekeeping genes, whereas developmental and tissue-specific genes mostly appear to be methylated and silenced in differentiated tissues. In cancer, however, numerous genes, which are unmethylated in the non-malignant tissue, become aberrantly methylated in the tumor. Since the first discovery of a hypermethylated gene in cancer, the retinoblastoma tumor suppressor (RB1), many tumor suppressor genes have been identified being hypermethylated in tumorous tissues as compared to their normal counterparts, for example, BRCA1 (Veek and Esteller, 2010).

Besides DNA methylation, the second key player in chromatin conformation and transcriptional regulation are histone modifications. Histone proteins constitute the nucleosome around which DNA is tightly packaged. Their N-terminal tails reach out of the nucleosomal core and harbor numerous spots for protein modifications, such as acetylation, methylation, phosphorylation, sumoylation, ubiquitination or ADP ribosylation (Bhaumik et al., 2007). Both the type of modification and the affected amino acid residue determine the tightness of the DNA-histone interaction, leading to either an open chromatin state allowing active transcription (e.g. acetylation of lysine) or to a compact chromatin state associated with transcriptional repression (e.g. deacetylation of lysine). Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. In general, methylation of histone H3 lysine 4 (H3K4me), H3K36, or H3K79 is associated with active transcription, whereas methylation of H3K9, H3K27, or H4K20 is associated with gene silencing. Histone methylation is regulated in breast cancer in an even more complicated manner than histone acetylation via a large number of chromosomal remodeling regulatory complexes (Huang et al., 2011).

2.10. Other risk factors

Rudel et al. (2007) identified 216 chemicals as mammary gland carcinogens in experimental animals, many of which have been listed as potential endocrine disrupting chemicals (EDCs). EDCs are termed because of their capacity to perturb normal hormonal actions, and include industrial chemicals, chlorinated solvents, products of combustion, pesticides, dyes, radiation, drinking water disinfection byproducts, pharmaceuticals and hormones, natural products and research chemicals. Twenty-nine are produced in the U.S at more than 1 million pounds/year; 35 are air pollutants, 25 have involved occupational exposures to more than 5000 women, and 73 have been present in consumer products or as contaminants of food. Thus, exposure is widespread. Nearly all of the chemicals were mutagenic and most caused tumors in multiple organs and species, and these characteristics are generally believed to indicate likely carcinogenicity in humans. Despite this evidence there are few works that aim to characterize possible links between breast cancer risk and occupation, particularly in farming and manufacturing. A recent work approached different jobs in sectors with

potentially high exposures to carcinogens and endocrine disruptors and found an elevated risk in women whose job was in agriculture, bars-gambling, automotive plastics manufacturing, food canning, and metalworking (Brophy et al., 2012).

Bisphenol A (BPA) is a xenoestrogen that has been widely used since the 1950s as a monomer that is polymerized to manufacture polycarbonate plastic and epoxy resins and found as environmental contaminant. Human exposure occurs when BPA leaches into food from common items such as plastic-lined food and beverage cans. Leaching increases when polycarbonate is scratched and discolored, or when items are exposed to low pH and high temperatures. Evidence of the estrogenic effects of BPA has been reported in several studies showing that it activates estrogen receptors alpha and beta and stimulates MCF-7 breast cancer cell growth. Although BPA mimics 17ß-estradiol by competitively binding and activating endogenous ERs, its affinity is at least 10,000-fold less than E_2 for both ERα and ERß, suggesting that other mechanisms could be responsible for its biological effect (Fernandez and Russo, 2010).

Phytoestrogens (flavonoids, isoflavonoids, coumestanes and lignans) are non-steroidal ligands to ERs. Research revealed a higher binding affinity for ERß, which is the preponderant isoform in normal mammary epithelium, associated with antitumor properties.

Estrogenicity/antiestrogenicity of active phytoestrogens results from some structural analogy with both natural and synthetic estrogens: at their extremities they possess phenolic hydroxyls oriented in such way that they may mimic the role of hydroxyls of strong natural estrogens. As steroidal estrogens, phytoestrogens not solely regulate gene transcription; they may also induce rapid effects via extranuclear signaling cascades. As polyphenols, phytoestrogens may exhibit a wide range of biologic activities not necessarily linked to ERs. Known inhibitory effects of polyphenols in tumorigenesis and tumor growth are attributed to two main actions: modification of the redox status and interference with basic cellular functions (apoptosis, cell cycle, angiogenesis, invasion and metastasis) (Pelekanou and Leclercq, 2011).

Organochlorines, classic examples of persistent organic pollutants, have been of worldwide concern owing to their persistence, bio-accumulative ability, and potential negative impacts on humans and animals. Organochlorine pesticides and polychlorinated biphenyls (PCBs) are ubiquitous contaminants that can be detected quite far from the pollutant source. Food is the major source of organochlorines in humans. Organochlorine pesticides, including dichlorodiphenyltrichloroethane (DDT) and PCBs, were widely used for insect control in forestry, agriculture, and building protection (Wolff et al., 2000). Organochlorines are strongly lipophilic and resistant to biotransformation. Some of more persistent organochlorines have half-lives of up to several decades in human tissue. DDT and PCBs residues are found in adipose tissue, breast adipose tissue, blood, and milk (Rusiecki et al., 2005). The data of epidemiological surveys assessing relationship between organochlorines and breast cancer risk are not consistent. Some epidemiological studies identified a positive association between breast cancer risk and adipose or blood levels of the organochlorine

pesticide DDT, PCBs, and/or their metabolite, dichlorodiphenyldichloroethylene (DDE) (Zhang et al., 2004).

Ionizing radiation is a well-established mammary carcinogen (Ronckers et al., 2005). Ionizing radiation has been suggested to increase breast cancer risk among professional occupations, namely flight attendants, nurses, chemists and insulators (Mitrunen and Hirvonen, 2003). Increased breast cancer risk has been shown following acute radiation exposure from the atomic bombings in Japan (Preston et al., 2007) and following high cumulative doses associated with the treatment of some diseases and multiple diagnostic radiographic examinations (Ronckers et al., 2008). Radiotherapy for breast cancer contributed to the development of contralateral breast cancer among women who underwent irradiation at a relatively young age (<35 years) and especially among those with a family history of breast cancer (Hooning et al., 2008).

Although electromagnetic fields have also been hypothesized to affect breast cancer risk by suppressing melatonin production, the data does not provide strong evidence to support this theory. The hypothesis that long-term exposure to relatively weak electromagnetic fields in the power frequency range of 50–60 Hz could increase the risk of breast cancer is based on the assumption that magnetic field exposure suppresses nocturnal melatonin. Experimental studies support the relationship between melatonin and breast cancer. Studies on human breast cancer cell lines have shown that melatonin modulates several estrogen dependent regulatory proteins, suppresses the activity of the estrogen receptor gene, and arrests the metastatic capacity of cells (Srinivasan et al., 2008).

Divalent metals – cadmium, copper, cobalt, nickel, lead, mercury, tin, and chromium – have showed the ability to activate ERα and stimulate cell proliferation in experimental studies with the human breast cancer cell line MCF-7. Some amino acids have been identified as potential interaction sites, suggesting that divalent metals and metal anions activate the receptor through formation of a complex within the hormone-binding domain of the receptor (Martin et al., 2003). Specifically cadmium has potent estrogen-like activity in vivo. It is able to interact with estrogen receptor, thereby preventing 17β-estradiol from binding the receptor. It has been found that cadmium functionally acts like steroidal estrogen in breast cancer cells because of its ability to form a high-affinity complex with the hormone-binding domain of ERα (Johnson et al., 2003).

The breast cancer mortality and incidence rates have been found to be inversely associated with the increasing levels of total average sunlight (Boscoe and Schymura, 2006). It has been suggested that the relationship between breast cancer and sunlight could be partially explained by vitamin D, that is hypothesized to lower the risk of breast cancer by inhibiting cell proliferation via the nuclear vitamin D receptor (McKay et al., 2009). However, data on the risk of breast cancer in relation to sunlight are not consistent (Kuper et al., 2009).

Chapter 3

Genetic Factors Associated with Breast Cancer

Heritable factors are observed in one-fourth of breast cancer cases (Lichtenstein et al., 2000), and 10 to 15% of breast cancer cases have family history of the disease (Dunning et al., 1999). However, only a small proportion of all breast cancer cases, less than 5%, are accounted for high-penetrance cancer susceptibility genes such as BRCA1 and BRCA2, due to the low frequencies of risk alleles that are found in the general population (Eccles and Tapper, 2010). While rare alterations in tumor suppressor genes dramatically raise cancer risk, far more common and less penetrant differences in low penetrance genes may be responsible for a relatively small, but rather frequent increase of cancer risk among individuals. A higher number of studies suggest that the risk associated with each low penetrating variant may be small, but it can be potentiated by association with other genetic or environmental factors. Low penetrance genes can be found in several pathways like detoxifications of environmental carcinogens, steroid hormone metabolism and DNA damage repair pathways.

The genetic variants associated with breast cancer risk can be classified as high-penetrance mutations, that are rare in the population but associated with very high risk (relative risk of carriers versus non-carriers of 5 to >20); moderate penetrance variants associated with moderate increases in risk; and low-penetrance polymorphisms which are common and associated with small increases in risk (relative risk <1.5) (Rebbeck, 1999). Polymorphisms are classified as commonly occurring genetic variations in the general population (>1%). Compared to rare mutations, polymorphisms have been considered as functionally insignificant, however, current evidence emphasizes that a considerable fraction affects the intrinsic properties and proteins function to a variable degree (Ayoub et al., 2011). Although each variant may be associated with a small increased risk for breast cancer in an individual, the attributable risk in the population as a whole and the genetic effect of combinations of relevant polymorphisms may additively or synergistically be higher than for rare, high-penetrance susceptibility genes. Also, the effects of these genetic variants might be modulated by different genes and by behavioral, environmental and other external risk factors. Breast cancer susceptibility related polymorphisms can be found in several biologic pathways, like estrogen and carcinogen detoxification metabolic pathways, cell cycle, apoptosis, cell signaling, growth factors and receptors molecules, cell adhesion, angiogenesis, DNA damage signaling and DNA repair (Eccles and Tapper, 2010).

To date, genetic studies have identified and confirmed BRCA1, BRCA2, TP53, PTEN, STK11 and CDH1 as rare high penetrance genes, CHEK2, ATM, BRIP1 and PALB2 as rare moderate-penetrance genes, and approximately 20 common low-penetrance variants in 19 genes or loci identified by genome-wide association studies that contribute to a woman's risk of breast cancer (Easton et al., 2007; Mavaddat et al., 2010; Zhang et al., 2011).
This chapter will specifically review the known genetic risk factors of breast cancer that were the main object of our research, and discuss their potential role in the modulation of individual susceptibility to the disease.

3.1. High Penetrance Mutations

3.1.1. BRCA1 and BRCA2

The greatest genetic risk factor for breast and ovarian cancer is inheritance of a mutation in one of the breast cancer susceptibility genes, BRCA1 or BRCA2. They are tumor suppressor genes that are estimated to be involved in about half of the familial clustering of early onset breast cancer (Easton et al., 1993), but only in about 5–10% of all breast cancers (Cui and Hoper, 2000). They tend to predispose both to earlier onset of the disease and to multifocal tumors. The coding regions of BRCA1 and BRCA2 show no homology to previously described proteins or to each other. If one copy of either gene is mutated in the germline, the result is hereditary breast and ovarian cancer (HBOC) syndrome, which is inherited in an autosomal-dominant manner. This syndrome is associated with not only early-onset breast cancer but also with an increased risk of ovarian, pancreatic, stomach, laryngeal, fallopian tube and prostate cancer. HBOC syndrome accounts for 5 to 7% of all cases of breast cancer, and individuals with HBOC syndrome have a lifetime risk of developing breast cancer of 50 to 80%, and 30 to 50% of developing ovarian cancer (Roy et al., 2011). Germline mutations in BRCA1 that truncate or inactivate the protein lead to a cumulative risk of developing breast cancer by age of 70 years of up to 80%, whereas the risk of ovarian cancer is of 30 to 40%. For germline BRCA2 mutations, the breast cancer cumulative risk approaches 50%, whereas for ovarian cancers, it is between 10 and 15% (Antoniou et al., 2003). BRCA2 families also show a marked increase in breast and ovarian cancer. However, unlike BRCA1 families, they exhibit an increased risk of male breast, pancreas and prostate cancers (The Breast Cancer Linkage Consortium, 1999; Thompson and Easton, 2002). In the absence of known germline predisposition for breast cancer, mutations in BRCA1 and BRCA2 are uncommon in sporadic breast cancer.
BRCA1 is located in chromosome 17q21.3 and spans approximately 100 kb of genomic DNA. The gene has 22 exons and encodes a protein with 1863 aminoacids. BRCA2 is located in chromosome 13q12, has 27 exons, spans around 70 kb and encodes a protein of 2418 aminoacids. Both are ubiquitously expressed in humans, with the highest levels in the testis, ovaries, and thymus (Thompson and Easton, 2004). The genomic regions of both BRCA1 and

BRCA2 contain very high densities of repetitive DNA elements that may contribute to genetic instability. In both cases, 47% of the genomic regions consist of repetitive sequences. Genes that contain such a high density of repetitive DNA are rare. Alu-dense regions of the genome, as the ones found in 42% and 20% of the genomic regions of BRCA1 and BRCA2, respectively, are associated with high density of genes and localize predominantly to R bands of metaphase chromosomes, which are involved in homologous and non-homologous chromosomal exchange. (Welcsh and King, 2001). BRCA1 protein contains an amino-terminal RING domain that has E3 ubiquitin ligase activity (which catalyzes protein ubiquitylation) and a BRCT domain that facilitates phospho-protein binding. Many inherited cancer-associated BRCA1 mutations have been found within the RING and BRCT domains, indicating that both domains are involved in suppressing breast and ovarian cancer (Roy et al., 2011).

Biochemical, genetic and cytological studies have revealed multiple functions for BRCA1 and BRCA2. Both BRCA1 and BRCA2 proteins are involved in maintaining genome integrity by participating in DNA repair, cell cycle checkpoint control and the regulation of key mitotic and cell division steps, particularly controlling homologous recombination and double-strand break repair in response to DNA damage. Thus, the complete loss of function of either protein leads to a dramatic increase in genomic instability (O'Donovan and Livingston, 2012). One of the most toxic DNA lesions to a cell is the DNA double-strand break (DSB). DSBs are considered to be the most threatening form of DNA damage, as the integrity of both strands of the DNA duplex is compromised simultaneously, thus no intact complimentary strand is available as a template for repair (Khanna et al., 2001). Double strand breaks normally occur during DNA replication, during the generation of antibody diversity and during meiosis. DSBs can be induced exogenously by agents such as ionizing and ultraviolet radiation. Failure to repair a DSB can result in apoptosis, and misrepair may lead to mutations or chromosomal rearrangements such as translocations and deletions. The repetition of these alterations over time can promote carcinogenesis (Jackson et al., 2009; Ohnishi et al., 2009). Maintenance of genomic integrity, in order to prevent such pathological outcomes, is mediated by a cellular network of signaling events, the DNA damage response (DDR), which is triggered in response to genotoxic stress. The DNA damage response to double-strand breaks involves sensor proteins that can detect broken ends, effector proteins that execute the repair, and mediator proteins that facilitate interactions between sensors and effectors. The DDR also includes the activation of checkpoints that delay the cell cycle before or during replication (G1/S or intra-S-phase checkpoints) or before cell division (G2/M checkpoint) to ensure that genetic errors are not transmitted to subsequent generations by allowing time for DNA repair (Roy et al., 2011). In order to repair DSBs, mammalian cells have evolved two major pathways: non-homologous end-joining (NHEJ), more susceptible to errors, and homologous recombination (HR), which is mostly error free (Hartlerode et al., 2009). NHEJ is highly efficient and is the primary DSB repair mechanism used in the G0-, G1- and early S-phases of the cell cycle. It involves the direct re-ligation of the ends of a DSB. NHEJ can be error-free or error-prone depending on the nature of the sequence at a DSB, because the termini at some but not all

DSBs are processed before ligation, and removal of bases can result in loss of DNA sequence at the break (O'Donovan and Livingston, 2010). The second major pathway for DSB repair is HR, generally regarded as being error-free. HR relies on the presence of an intact sister chromatid to act as template for correct repair of the break without loss of sequence information. As such, HR can only take place in the S- and G2-phases of the cell cycle. The protection of the genome by HR involves damage recognition by the kinases ATM and ataxia telangiectasia and Rad3-related (ATR), signal mediation by CHK2 and BRCA1, and initiation of repair by the effectors BRCA2 and RAD51. There are also several facilitators of the HR pathway, such as PALB2 and BRIP1, and each of these facilitators is a predisposing factor for HBOC syndrome when mutated, which suggests that it is the BRCA1–BRCA2–HR pathway that suppresses tumorigenesis (O'Donovan and Livingston, 2010; Roy et al., 2011).

Common genetic alterations are associated with heterozygous BRCA1 or BRCA2 mutations, and these include loss of the wild-type BRCA1 or BRCA2 allele (LOH). These additional alterations may allow cells to bypass checkpoint controls and evade apoptosis, and thereby initiate tumorigenesis. The fact that both BRCA1 and BRCA2 mutation carriers display these similar somatic alterations further confirms that their role in HR-mediated repair is important for tumor suppression (Roy et al., 2011).

Given that BRCA1 and BRCA2 protect the genome from errors that arise during DNA replication, it is logical that cells driven to replicate would develop potentially oncogenic genetic alterations in the absence of BRCA1 or BRCA2 function. However, most cancers are driven to grow and divide, so this feature alone does not determine why there is a major predisposition to breast and ovarian cancer in individuals who lack functional BRCA1 or BRCA2. One common feature is that breast and ovarian epithelial cells are subject to strong growth signals by hormonal stimulation during the normal menstrual cycle. So it seems that cells hormonally induced to growth are more vulnerable to genetic instability in the context of BRCA1 or BRCA2 deficiency. Despite a number of theories have surfaced to explain the tissue specificity of HBOC syndrome, none is considered definitive. An additional unsolved mystery is why BRCA1 mutation carriers develop predominantly, but not exclusively, ER-negative tumors, whereas BRCA2 mutation does not favour the development of any particular subtype of breast cancer. However, the breast cancers that arose in Ashkenazi Jewish women with founder mutations in BRCA1 or BRCA2 were mostly triple-negative (ER-negative, progesterone receptor (PR)-negative and ErbB2-non-amplified), so the association with ER expression and BRCA genes is not rigid. These observations pose the question of why there should be a difference in the biological subtypes of breast cancer when the two proteins appear to be working in a common pathway of DNA repair (Roy et al., 2011).

3.2. Low Penetrance Polymorphisms

3.2.1. Polymorphisms in genes involved in estrogen biosynthetic and metabolic pathways

There is substantial evidence that estrogens play an important role in the etiology of breast cancer. In fact there is an established strong positive association between circulating levels of estrogens and the risk of breast cancer, and several mechanisms have been postulated to be responsible for the carcinogenic effect of estrogens (Dumas and Diorio, 2011). As previously approached, in premenopausal non-pregnant women, nearly all estrogens are synthetized in the ovaries; after menopause, most estrogens are formed by aromatization of androstenedione to estrone in peripheral adipose tissue. Several enzymatic steps lead to the conversion of cholesterol into C-19 androgens and C-18 estrogens, involving several enzymes (Figure 3.1) (Mitrunen and Hirvonen, 2003).

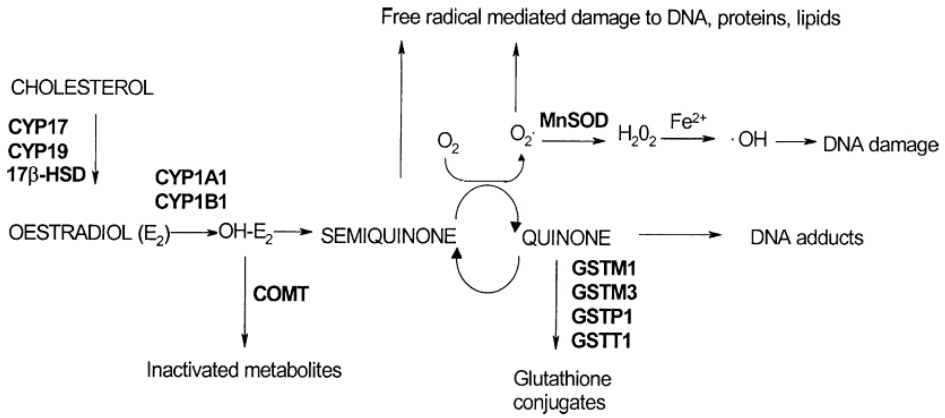

Figure 3.1. A simplified scheme showing the role of the polymorphic enzymes in estrogen synthesis and metabolism (Mitrunen and Hirvonen, 2003).

Once formed, estrogens are extensively metabolized by a number of oxidative and conjugative reactions that can lead to their deactivation and subsequent elimination, or to the generation of metabolites that have distinct biologic activities, including altered hormonal properties, genotoxicity, through the formation of reactive species that modify cellular DNA and protein, and/or chemotherapeutic properties, by forming derivatives that are antagonistic at the estrogen receptor (ER) or potentially antiangiogenic (Zhu and Conney, 1998b).

Many of the enzymes involved in estrogen metabolism and biosynthesis have a polymorphic distribution within the human population and, for some, there are known gene variants that may account for a proportion of enzymatic variability (Thompson and Ambrosone, 2000).

3.2.1.1. CYP17A1

The CYP17A1 gene is located on chromosome 10q24.3 and encodes for the cytochrome P450C17α enzyme, which is primarily expressed in women in ovary and adrenal cortex (Mitrunen and Hirvonen, 2003). The enzyme plays a key role in sex steroid synthesis and has a bi-functional catalytic activity: it mediates both steroid 17-hydroxylase and 17,20-lyase activities, and catalyzes a rate limiting step in sex steroids synthesis, the conversion of 17 α-hydroxypregnenolone and progesterone to dehydroepiandrosterone (DHEA) and to androstenedione, respectively, that are precursors of testosterone and estrogens (Weston et al., 1998). Genetic variation in CYP17A1 gene has been studied extensively in relation to gonadal development and the synthesis of androgens and estrogens. Mutations in CYP17A1 gene cause 17-hydroxylase deficiency, a rare form of congenital adrenal hyperplasia, and sexual infantilism (Costa-Santos et al., 2004). Approximately 40 different mutations in CYP17 have been reported to cause 17-hydroxylase deficiency (Martin et al., 2003). The absence of 17,20-lyase activity results in impaired production of gonadal sexual steroids and high levels of basal progesterone (which is a substrate of 17a-hydroxylase). These severe clinical phenotypes have led researchers to hypothesize that CYP17 may also play a role in polycystic ovarian syndrome, a hormone dependent disorder characterized by hyperandrogenism and chronic annovulation (Carey et al., 1994). Genetic variation in CYP17 has also been extensively studied in relation to breast and prostate cancer. One of the most focused polymorphism in CYP17A1 gene (denoted T27C or A1/A2 in the literature) is a single T to C base substitution at position 1931 in the 5' untranslated region of the gene, which creates a MspA1 restriction enzyme recognition site. The A2 allele corresponds to the creation of an additional Sp-1-type (CCACC box) promoter site 34 bp upstream from the site of initiation of translation in the variant allele, in the 5' untranslated region of CYP17A1. Since it was thought that the number of 5' promoter elements correlates with promoter activity, it might be expected that an additional CCACC site might influence promoter activity, thereby up-regulating transcription. Thus, the additional promoter site was thought to influence gene expression resulting in increased enzyme activity leading to an increased amount of bioavailable estrogen, and it was originally associated with polycystic ovary syndrome risk, and later with breast cancer risk (Carey at al., 1994). A later study however, showed that the 5-Sp-1-type site does not actually bind the transcription factor (Nedelcheva et al., 1999; Setiawan et al., 2007) but there is still some evidence indicating that this polymorphism may influence endogenous steroid hormone levels (Feigelson et al., 1998; Hainma et al., 1999; Jernstromet al., 2001). Serum estrogen and progesterone levels have been found to be higher in premenopausal women with the CYP17A1 A2 allele (Feigelson et al., 1998) whereas postmenopausal women with the A2/A2 genotype had elevated levels of estrone and dehydroepiandrosterone (Hainma et al., 1999). Furthermore, the urinary ratio of 2-hydroxyestrone to more potent 16α-hydroxyestrone was shown to be lowest in women with the CYP17A1 A2/A2 genotype (Jernstromet al., 2001). The studies completed so far on CYP17A1 and breast cancer risk have given controversial results. The A2 allele has been found

to be significantly associated with advanced breast cancer, male breast cancer, and both postmenopausal and early-onset breast cancer. The latter association has been found especially in women with a family history of the disease. The most consistent finding, however, is that the reduced risk of breast cancer, usually associated with a later age at menarche, is limited to women with the CYP17A1 A1/A1. Also parity was associated with decreased breast cancer risk only among premenopausal women with the A1/A1 genotype. The other studies have not shown any significant association between the CYP17A1 genotypes and breast cancer risk (Mitrunen and Hirvonen, 2003).

3.2.1.2. CYP19A1

CYP19A1 gene, located on chromosome 15q21.1, encodes CYP19A1 or aromatase, also called estrogen synthetase, the enzyme that catalyzes three consecutive hydroxylation reactions converting C19 androgens, such as testosterone, to aromatic C18 estrogenic steroids. Aromatase is present in many tissues including skin, liver, muscle, fat, breast, ovary, placenta and nerve. This widespread may contribute to sex-specific differences and suggests that estrogen produced by this enzyme has physiologic functions not only as a sex steroid hormone but also in growth or differentiation (Toda et al., 1990; Harada et al., 1992). The human aromatase gene CYP19A1 comprises a 93 kb 5'-regulatory region and a 30 kb 3'-coding region (Figure 3.2.). The regulatory region contains 10 tissue-specific promoters for local estrogen biosynthesis under normal physiological or pathological conditions such as breast cancer and endometriosis. Activation of each promoter gives rise to alternatively spliced forms of mature mRNA with the first exon a tissue-specific, untranslated region (5'-UTR) upstream of the coding region. The coding region spans nine exons (exon II-X), which are identical in all mRNA species and encode the same protein and 3'-UTR of the mRNA regardless of the tissue or the promoter used (Cui et al., 2013).

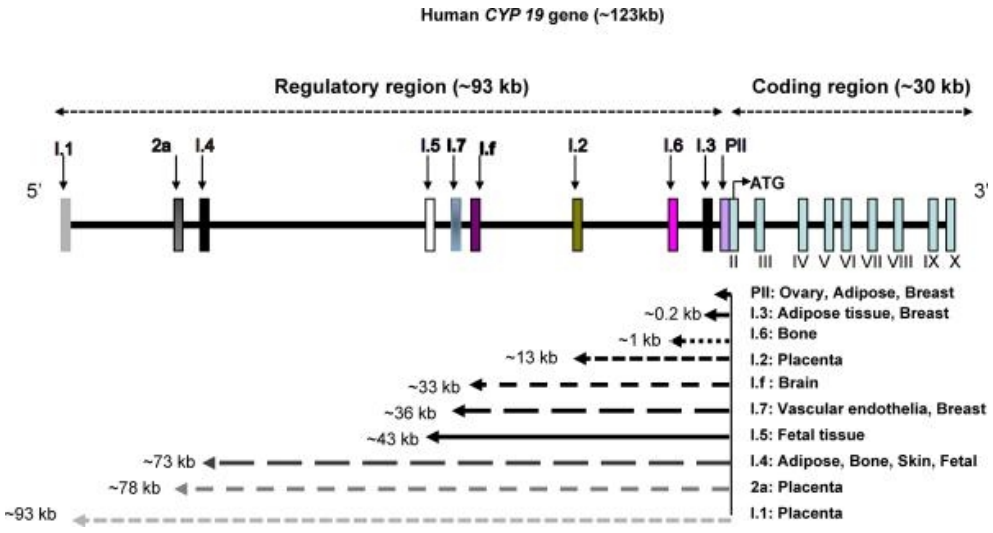

Figure 3.2. Schematic representation of the human CYP19A1 gene showing alternative splicing and tissue specific promoters (Cui et al., 2013).

Distinct aromatase promoters are specifically regulated in different tissues by distinct sets of hormones or cytokines and second messenger signaling pathways, among other factors. For example, aromatase gene expression in the ovaries is regulated by follicle-stimulating hormone (FSH), which acts through cAMP via the proximal promoter II. In the placenta, aromatase gene expression is regulated via the distal promoter I.1, responsible for increasing circulating estrogen levels in pregnant women. Aromatase gene expression in adipose and bone is driven by the distal promoter I.4 in response to glucocorticoids, class 1 cytokines, and tumor necrosis factor alpha (TNFα), whereas in breast cancer patients, the increase in aromatase expression in breast adipose is partly due to a switch in promoter usage from the distal promoter I.4 to the cAMP responsive promoter II (Cui et al., 2013).

In women of reproductive age, the ovaries express high levels of aromatase and they are the main source of estrogens. After menopause peripheral tissues become sites of estrogen synthesis of great importance. In postmenopausal women, estrogen is predominantly circulating as estrone, derived from the peripheral aromatization of androstenedione (Mitrunen and Hirvonen, 2003). Estrogens synthesized locally in extragonadal sites mostly do not enter the circulation but exert intracrine, autocrine, paracrine, and juxtacrine effects, acting directly in the cells of synthesis or on the neighbouring cells (Harada et al., 1999). These interactions at the tissue level are very difficult to measure in the clinical setting, and they remain mostly unrecognized. However, it is believed that in the post-menopausal breast, it is local estrogen synthesis that determines their tissue level and affects breast cancer risk (Yue at al., 1998).

The aromatase activity has been found to be more strongly expressed in adipose stromal cells surrounding mammary carcinoma cells than in stromal cells distal to the tumor, indicating that enzymatic aromatization could be an important source of estrogen for mammary tumors (Mitrunen and Hirvonen, 2003). Genetic variations at this locus may alter aromatase activity and thereby affect hormone levels. The most focused polymorphism In literature is a tetranucleotide repeat $[TTTA]_n$ in intron 4, that may have a functional effect in the translated enzyme due to an alteration on the splice site. This polymorphism has been reported to be associated with breast cancer risk among Caucasian women (Sourdaine et al., 1994; Kristensen et al., 1998; Hairman et al., 2000) although negative results also exist (Healey et al., 2000; Miyoshi et al., 2000; Baxter et al., 2001). Recently, a case control study on the association of another polymorphism of CYP19A1, CYP19A1 Val(80), along with $[TTTA]_n$ polymorphism, and the risk of breast cancer in BRCA carriers and non-carriers suggested that the CYP19A1 Val(80) polymorphism is associated with increased breast cancer risk in young women from a population of Ashkenazi Jews with BRCA1 mutations (Raskin et al., 2009). Also, a C826T variation in exon 7 which gives rise to the non-conservative amino acid substitution Arg264Cys, has been used in breast cancer studies despite not having an apparent effect on aromatase activity (Watanabe et al., 1997; Siegelmann-Danieli and Buetow, 1999; Long et al., 2006). Miyoshi et al (2000) identified codon 39 Trp/Arg polymorphism in CYP19A1 gene and showed that it was significantly associated with breast cancer risk among Japanese women

but there are no results available for other populations. This polymorphism locates on exon 2, close to several tissue-specific promoters, including adipose and breast cancer tissue promoters, which are located between promoter I.1 and exon 2. Few studies have evaluated the association of genetic polymorphisms in this region with breast cancer risk, and results on this association are conflicting (Haiman et al., 2000; Miyoshi et al., 2000; Cai et al., 2008).
In the therapeutic field of breast cancer, aromatase has been extensively studied due to the beneficial effects of the inhibition of its activity. Aromatase inhibitors (AIs) are a group of drugs that have the ability to cease the production of oestrogens by inhibiting their conversion from androgens. AIs are used in the endocrine therapy of breast cancer that express estrogen receptors because local estrogens produced in the tumor and surrounding cells are major stimulants for the cancer growth in these patients. However, treatment with aromatase inhibitors result of global inhibition of the catalytic activity of aromatase in all tissues. The perfect approach would be the blockage of estrogen production only in the breast, so the development of tissue-selective inhibitors, based on the tissue-specific regulation of CYP19A1 expression is an extensively researched subject (Sainsbury, 2013).

3.2.1.3. COMT

Catechol-O-methyltransferase (COMT) is a significant phase II enzyme involved in the conjugation and inactivation of catechol estrogens, catalyzing the transfer of a methyl group from the methyl donor SAM (S-adenosyl methionine) to one hydroxyl moiety of the catechol ring of a substrate (Guldberg and Marsden, 1975). In humans, COMT protein exists as two isoforms: a soluble form in the cytoplasm (S-COMT), and as a membrane-bound form (MB-COMT) (Jeffery and Roth, 1984; Malherbe et al., 1992; Ulmanen et al., 1997). Both S-COMT and MB-COMT proteins are encoded by a single gene localized in chromosome 22q11.2 containing six exons, of which exons 1 and 2 are noncoding (Grossman et al., 1992). The amino acid sequence of S-COMT and MB-COMT is identical, except for an NH_2-terminal extension of 50 hydrophobic amino acids in MB-COMT that serves as an anchor to the membrane (Bertocci et al., 1991; Ulmanen and Lundstrom, 1991; Dawling et al., 2001a). A proximal promoter gives rise to the 1.3-kb S-COMT mRNA, whereas a distal promoter gives rise to the 1.5-kb MB-COMT mRNA (Tenhunen et al., 1994). The MB-COMT and S-COMT proteins occur constitutively but differ in tissue expression. S-COMT is the predominant form in virtually all tissues, whereas MB-COMT generally accounts for approximately 10% of total enzyme activity (Dawling et al., 2001a). For example, S-COMT constituted less than 90% of total COMT activity in normal breast tissue as well as in MCF-7 breast cancer cells (Tenhunen et al., 1994). COMT is found in various mammalian tissues including liver, kidney, red blood cells and breast (Assicot et al., 1977; Merriam et al., 1980), and the level of the protein has been shown to be higher in breast tumor tissue compared to normal mammary tissue (Tenhunen et al., 1999). A single G to A transition in the COMT gene results in valine to methionine amino acid change in codon 108/158 in the cytosolic/membrane-bound form of

the protein. This amino acid change is believed to result in a 3 to 4-fold decrease in enzymatic activity (Dawling et al., 2001b; Goodman et al., 2002). Thus, the COMT*1 (Val) allele encodes for the high-activity enzyme, and the COMT*2 (Met) allele encodes for the thermolabile low-activity enzyme. Since the variant form (Met) has been associated with decreased activity of the enzyme, when compared to the wildtype (Val), these two forms are frequently represented as COMT-L allele and COMT-H allele, respectively. It has been hypothesized that the individuals who inherit the low activity COMT-L gene may be at increased risk for breast cancer because of an increased accumulation of the catechol estrogen intermediates (Lavigne et al., 1997; Thompson et al., 1998; Mitrunen et al., 2001). To study these differences in COMT activity according to the genotype, COMT activity was determined in red blood cells from individuals with different genotypes using 3,4-dihydroxybenzoic acid as substrate. It was found that activity of the protein in homozygous Met/Met individuals was approximately 60% lower than that of homozygous Val/Val individuals. Heterozygotes showed intermediate activity (Syvanenet al.,1997). Physiological substrates of COMT for inactivation and detoxification include catecholamine neurotransmitters and the catechol estrogens, produced by cytochrome P-450-mediated hydroxylation of estradiol and estrone, which can cause oxidative damage, and are known to have a role in estrogen-linked carcinogenesis (Mitrunen et al., 2001). COMT methylates catechol estrogens to less polar monomethyl ethers, which can then be excreted. If production of these conjugates is incomplete, catechol estrogens may be oxidated to reactive quinone/semiquinone intermediates capable of free radical formation, or direct formation of DNA adducts (Cavalieri et al., 1997; Mitrunen et al., 2001). It is likely that the Val/Met polymorphism also affects catechol estrogen metabolism, specifically the four main catechol estrogens, 2-OHE_2, 2-OHE_1, 4-OHE_2, and 4-OHE_1. Despite conflicting results, several molecular epidemiological studies indicate that the Val/Val, Val/Met, and Met/Met genotypes may be associated with differences in risk of developing breast cancer. The inactivation of catechol estrogens by COMT reduces the level of 2-OH and 4-OH estrogen metabolites, thereby lowering the potential for mutagenic damage through DNA adduct formation or through superoxide and hydroxy radicals produced during catechol estrogen quinone-semiquinone redox cycling (Mitrunen et al., 2001). Also, most studies have shown that methylated catechol estrogens have little or no binding affinities for the classical estrogen receptor (Mitrunen and Hirvonen, 2003). The product of COMT, 2-methoxyestradiol (2-$MeOE_2$), which is produced by COMT from 2-OHE_2, may also have a protective role against estrogen-induced carcinogenesis by inhibiting angiogenesis and cell proliferation both *in vitro* and *in vivo* (Michnovicz et al., 1986; Lottering et al., 1992; Mitrunen et al., 2001). This inhibitory effect of 2-$MeOE_2$ appears to be attributable to several biological mechanisms: 2-$MeOE_2$ inhibits tubulin polymerization, promoting the disruption of microtubule function; it also induces tumor cell apoptosis by super induction of wild-type p53 protein; 2-$MeOE_2$ suppresses tumor growth and inhibits angiogenesis (D'Amato et al., 1994; Klauber et al., 1997; Mukhopadhyay and Roth, 1998; Huang et al., 2000; Mitrunen et al., 2001). Finally, 2-$MeOE_2$, but not 4-MeO_2, was suggested to

be an endogenous estrogen metabolite that inhibits mammary carcinogenesis (Zhu and Conney, 1998). COMT allele frequencies have been shown to vary significantly between different ethnic groups. Nearly equal frequency of the two alleles exists in European populations, while the H allele is much more common than the L allele in populations in all other parts of the world. COMT genotypes also seem to be related to progression of breast cancer to the metastatic state (Matsui et al., 1999). Most studies reported so far on COMT genotypes and breast cancer risk have given discrepant results (Mitrunen and Hirvonen, 2003).

3.2.1.4. CYP1A1

Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), encoded by CYP1A1 gene is one of the most important phase I enzymes expressed in breast tissue (Li et al., 2004). It is involved in the conversion of estrone and estradiol to 2-hydroxyestradiol (2-OHE_1) in several extra-hepatic tissues including breast (Cavalieri et al., 2001). The CYP1A1 gene, located at 15q22-q24, comprises seven exons and six introns and spans 5,810 base pairs (Masson et al., 2005). In humans, CYP1A1 is under the regulatory control of the aryl hydrocarbon receptor, a transcription factor that regulates gene expression (Masson et al., 2005). CYP1A1 also has aryl hydrocarbon hydroxylase activity which is responsible for metabolizing polycyclic aromatic hydrocarbons (PAH) to aryl epoxides, that is the first step in the metabolism of PAHs (Law, 1990). Due to its aryl hydrocarbon hydroxylase activity, CYP1A1 has been mostly studied as the principal metabolizer enzyme of cigarette smoke constituents and other environmental pollutants such as PAHs leading to electrophilic, carcinogenic molecules (Bartsch et al., 2000). The activity of aryl hydrocarbon hydroxylase encoded by the CYP1A1 gene has been observed in both normal and neoplastic human breast epithelium. Some studies suggest that heterocyclic amines are activated by CYP1A1 via N-hydroxylation in breast tissue. CYP1A1 gene polymorhisms have been extensively studied, especially in relation to cancer susceptibility (Mahmoud et al., 2010). Four common polymorphisms of the CYP1A1 gene have been identified to date. A T to C substitution at nucleotide 3801, that originates a MspI restriction site in the 3'-noncoding region (Kawajiri et al., 1990); a A to G substitution at nucleotide 2455, that results in an amino acid change at codon 462 of isoleucine to valine within the heme-binding domain of exon 7 (Hayashi et al., 1991); a nucleotide 3205 T to C substitution, that creates a MspI restriction fragment length polymorphism (RFLP) in the 3'-noncoding region (Crofts et al. 1993); and the nucleotide 2453 C to A substitution, that results in an amino acid substitution at codon 461 of threonine to asparagine (Cascorbi et al., 1996). The first described mutation, located downstream from the polyadenylation site (in CYP1A1 ∗2A allele), and the mutation in the heme-binding region of the gene, leading to an amino acid change from isoleucine to valine at codon 462 (in CYP1A1 ∗2C allele), are strictly linked in Caucasians (Hayashi et al., 1991). The frequencies of the CYP1A1 ∗2A and CYP1A1 ∗2C alleles vary considerably by race, being considerably less prevalent in Caucasians than in Asians. These mutations have been suggested to lead to higher enzyme activity and therefore

higher rates of carcinogen activation. Studies of CYP1A1 in cultured human lymphocytes showed significantly elevated levels of inducible enzyme activity among CYP1A1 *2C genotypes in exon 7 compared with the wild-type genotype (Landi et al., 1994). Also, the Valine allele in exon 7 was also reported to be more readily inducible than the CYP1A1 Isoleucine wild-type allele (Kawajiri et al., 1990). The third described mutation, found in the 3'noncoding region upstream from the polyadenylation site (in CYP1A1∗3 allele), is an African-American specific mutation; also, these mutated alleles appeared to be associated with CYP1A1 inducibility at the level of transcription followed by threefold elevation in aryl hydrocarbon hydroxylase enzyme activity (Crofts et al. 1993). Finally, the C to A substitution in exon 7 that leads to a threonine to aspartic acid change at codon 461 (in CYP1A1 ∗4 allele) has been reported to cause lower catalytic activity against progesterone (Schwarz et al., 2000). Despite these findings, the functional significance of variant CYP1A1 genotypes is unclear (Chen et al., 2007).

The CYP1A1 Ile462Val polymorphism in exon 7 and the T6235C polymorphism on the 3' non-coding region (MspI) have been intensively studied in relation to breast cancer risk. Several reports have shown that there is a significant association between CYP1A1*2 polymorphism and breast cancer risk (Chacko et al., 2005; Singh et al., 2007; Surekha et al., 2009). Similarly other studies from USA (Zhang et al., 2004; Taioli et al., 1999), China (Huang et al., 1999) also found that presence of CYP1A1*2 polymorphism had increased risk for breast cancer. The frequency of presence of Val allele was found to be slightly increased in breast cancer patients and was dependent on the stage of the disease (Surekha et al., 2009). Despite this findings, in several other reports from Korea (Shin et al., 2007), African-American white women (Li et al., 2004), UK (Basham et al., 2001), Brazil (da Fonte de Amorim et al., 2002), Caucasian and African Americans (Bailey et al., 1998a) and North Indians (Singh et al., 2007), CYP1A1*2 polymorphism was not observed to be a risk factor to breast cancer. Overall, the CYP1A1* 2 polymorphism was found to show a null or weak association with breast cancer risk (Rozati et al., 2008).

3.2.1.5. CYP1B1

Cytochrome P450, family 1, subfamily B, polypeptide 1 is a key member of the cytochrome P450 super-family of enzymes (Tang et al., 1996). The encoded estrogen-metabolizing protein appears to be the main CYP450 enzyme responsible for the 4-hydroxylation of estradiol (Hayes et al., 1996; Stoilov et al., 1998). Its 4-hydroxylase action generates 4-OHE_2, which is thought to provide excessive mitogenic stimulation of breast cells because its binding to ER is of longer duration than that of E_2 (Dumas and Diorio, 2011). Moreover, since it is co-localized with CYP19A1, the enzyme responsible for estrogen biosynthesis, this could lead to high local production of potentially carcinogenic estrogen metabolites (Jefcoate et al., 2000). As 4-hydroxylated metabolites represent only a minor portion of total urine estrogens, this was considered as a minor route for metabolism. However, CYP1B1 is present virtually in all adult

and fetal tissues, with high levels found in breast. CYP1B1 also activates many PAHs and arylamines (Mitrunen and Hirvonen, 2003). The activity of this enzyme is known to be up-regulated by estrogen (Tsuchiya at al., 2004) and over-expression of CYP1B1 may cause accumulation of estrogen metabolites that directly or indirectly damage DNA (Yager, 2000).
The gene coding for the CYP1B1 enzyme is located on chromosome 2 locus p21 (Stoilov et al., 1998). Several polymorphisms have been described in the CYP1B1 gene. The most studied SNP in relation to breast cancer risk is Val432Leu, in exon 3 of the CYP1B1 gene. This polymorphism has been proposed to have the most profound impact on the catalytic properties of CYP1B1, since the Val432 allele displays three-fold higher 4-hydroxilase activity compared to Leu432 allele, while other mutations seem not to have any effect on the enzyme activity (Li et al., 2000). However, polymorphisms given by a C to G transition (Ala48Gly) and a G to T transition (Ala119Ser) in exon 2 may alter the enzyme activity, and a A to G transition in exon 3 (Asn543Ser) was also proposed to be associated with increased enzymatic activity for carriers of Ser453 (Mitrunen and Hirvonen, 2003). Although the 4-hydroxylase activity of the Val432 allele is higher compared to Leu432 allele, the expected effect of the mutated Leu variant on breast cancer risk is unclear since it has been associated with both increased and reduced breast cell proliferation. Research on the possible influence of this SNP on breast cancer risk showed inconclusive results, whether assessed among the overall population, by race, or within levels based on estrogen-related factors (Dumas and Diorio, 2011). The CYP1B1 Val/Val genotype has been associated with estrogen and progesterone receptor positive breast cancer in Caucasian women (Bailey et al., 1998b), while Chinese women carrying the Leu/Leu genotype were at increased risk for postmenopausal breast cancer (Zheng et al., 2000). In contrast, in Japanese women this polymorphism had no effect, whereas the Ala119Ser change was associated with increased breast cancer risk (Watanabe et al., 2000). These discrepancies are likely to be at least partly due to ethnic differences. A recent meta-analysis found no association between the CYP1B1 Leu432Val polymorphism and risk of breast cancer among Caucasian women (Paracchini et al., 2007). One analysis in Polish women demonstrated no significant haplotype effects among eight CYP1B1 tagging SNPs (Gaudet et al., 2006). A recent population-based case-control study examined several CYP1B1 haplotype-tagging single nucleotide polymorphisms (htSNPs) in relation to invasive breast cancer risk and found no evidence that Caucasian women with any common haplotype in CYP1B1 had a substantially altered risk of developing invasive breast cancer (Huang et al., 2009). In summary, results suggest that genetic variation in CYP1B1 has, at most, a minor influence on breast cancer susceptibility among Caucasian women.

3.2.1.6. Glutathione S-transferases (GSTs)

Glutathione *S*-transferases (GSTs) are a family of Phase II detoxification enzymes. They detoxify environmental chemicals and are involved in oxidative stress pathways. GSTs catalyze the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous

electrophilic compounds. Several allelic variants of polymorphic GSTs, mainly deletion polymorphisms, show impaired enzyme activity and are suspected to increase the susceptibility to various diseases (Ramalhinho et al., 2013). The involvement of GSTs polymorphisms in breast cancer has been approached in the form of a book chapter, published as "Glutathione and Glutathione S-Transferases: Risk Factors in Multifactorial Diseases", by Nova Science Publishers in 2013 (please see Appendix).

3.2.1.7. MTHFR

5,10-methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism pathway that regulates the intracellular folate pool for synthesis and methylation of DNA (Rosenberg et al., 2002). It catalyzes 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the donor for the remethylation of homocysteine to methionine, the precursor for the universal methyl donor *S*-adenosylmethionine (SAM) (Matthews et al., 1998; Fodinger et al., 2000). Also, MTHFR is involved in conjugation and inactivation of catechol estrogens mediated by COMT, as COMT catalyzes the transfer of a methyl group from SAM to the catecholic ring moiety of a substrate, as previously explored (Figure 3.3).

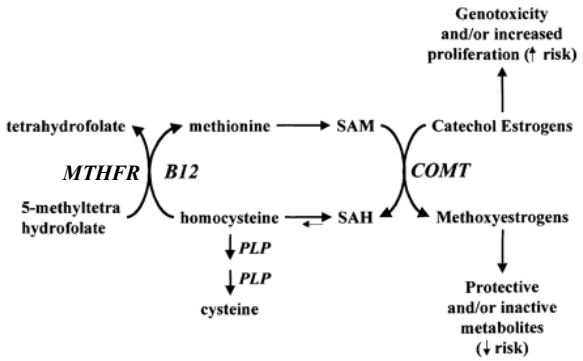

Figure 3.3. Role of the folate metabolic pathway and COMT in breast cancer risk (B12, vitamin 12; COMT, catechol-*O*-methyltransferase; PLP, pyridoxal 5′-phosphate; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine) (Addapted from Goodman et al., 2001).

Folate is a water-soluble vitamin naturally found in green leafy vegetables, cereals, legumes and fruits (Aune et al., 2011). Folate is necessary for synthesis of thymine, which is important in DNA synthesis, integrity and stability (Kim, 2004). Deficiency of folate may cause defective DNA repair and chromosomal fragile site expression, leading to chromosomal breaks and micronucleus formation (Aune et al., 2010). Furthermore, folate is the primary methyl group donor which has a central role in DNA methylation (Kim, 2004, Lu et al., 2011). So folate is involved in DNA methylation, synthesis, and repair. More and more evidence has indicated the association of folate, as well as the aberrant DNA methylation, with the risk of human cancers (Lu et al., 2011). However, the role of dietary folate in cancers is still controversial. Only few studies reported that dietary folate was negatively associated with the risk of endometrial

cancer (Xu et al., 2007), breast cancer (Shrubsole et al., 2001) and rectal cancer (Jiang et al., 2005).

The MTHFR gene, located at chromosome 1p36.3, codifies the MTHFR protein (Rosenberg at al., 2002). Two common allele variants of the MTHFR gene have been described. They lead to amino acid substitutions, C677T (Ala222Val) and A1298C (Glu429Ala), and to decreased enzyme activity (Frosst et al., 1995; Kim, 2004). It was shown that homozygous individuals for the 1298C allele have approximately the same enzyme activity as those heterozygous for the 677T allele (Hosseini et al., 2011). The C677T polymorphism has been examined in relation to several cancers (Mason and Choi, 2000) and specifically to breast cancer risk (Gershoni-Baruch et al., 2000; McGlynn et al., 2000; Sharp et al., 2002). In the first study in Jewish women, there was no significant difference of MTHFR C677T genotype between sporadic cases and controls (Gershoni-Baruch et al., 2000). In Caucasian women it was reported that the MTHFR 677T allele was more prevalent in cases than controls (McGlynn et al., 2000), while in other studies the reported risk for breast cancer was associated with both the C677T and A1298C polymorphisms (Sharp et al., 2002). A recent meta-analysis of 51 studies that analyzed the association of C677T (Ala222Val) with breast cancer risk provided the evidence that MTHFR Ala222Val gene polymorphisms contributed to the breast cancer development (Yu and Chen, 2012).

3.2.2. Polymorphisms in estrogenic response genes

The most important determinants of risk for breast cancer are related to endogenous hormone levels and major reproductive events, thus suggesting that genes in the estrogen pathway, and namely the genes that encode proteins involved in the estrogenic response, may influence breast cancer risk (González-Zuloeta Ladd et al., 2007). In this context, ERα has been described as the most relevant.

3.2.2.1. Estrogen Receptor Alpha (ERα)

ERs are nuclear receptor proteins that have an estrogen binding domain and a DNA binding domain. There are two types of ERs, ERα and ERß. The ERα gene (ESR1) is localized on chromosome 6q25.1, and the ERß gene (ESR2) is localized on chromosome 14q22-24. ERα is of special interest because it mediates the biologic effects of estrogen in estrogen-sensitive tissues like breast, and its protein levels are elevated in pre-malignant and malignant breast cells. ERα has been demonstrated to be a significant prognostic factor for breast cancer. Consequently, inhibition of ERα has become one of the major strategies for the prevention and treatment of breast cancer (Cai et al., 2003).

ERα comprises eight exons separated by seven intronic regions, spans more than 140 kilobases, and is genetically polymorphic (Gosden et al., 1986; Green et al., 1986; Greene et al., 1986). The most studied polymorphisms of ERα are the PvuII (T>C) and XbaI (A>G), both in intron 1, 397 and 351 bp upstream of exon 2 respectively (Figure 3.4.). In practical terms,

genotypes are distinguished by the absence or presence of PvuII or XbaI restriction sites: PP (TT) and XX (AA) indicate the absence of restriction sites; pp (CC) and xx (GG) correspond to presence of PvuII or XbaI restriction sites, respectively, on both alleles, and Pp (TC) and Xx (AG) represent heterozygotes. As they are separated by approximately only 50 bp, these polymorphisms are in strong linkage disequilibrium. It might also be possible that these SNPs are in linkage disequilibrium with another SNP in ERα gene that affects the expression of ERα (González-Zuloeta Ladd et al., 2008).

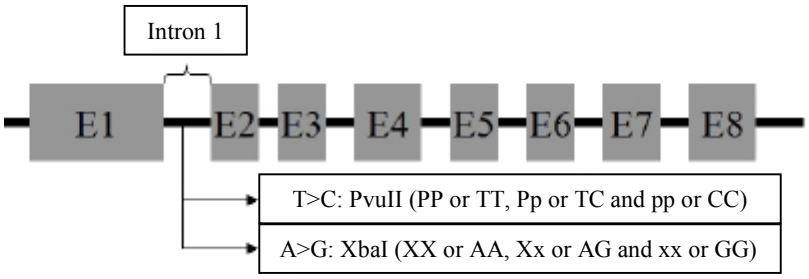

Figure 3.4. Schematic representation of ERα, with the localization of PvuII and XbaI polymorphisms. Grey boxes represent exons (adapted from Araújo et al., 2011).

These polymorphisms have been associated with several gynecological diseases like breast cancer (Dunning et al., 1999), endometrial cancer (Weiderpass et al., 2000), endometriosis, adenomyosis and leiomyoma (Kitawaki et al., 2006), with various estrogen-dependent characteristics such as the onset of menopause (Weel et al., 1999), lumbar spine bone mineral density, vertebral bone area and vertebral fracture risk in post-menopausal women (van Meurs et al., 2003), waist circumference (Fox et al., 2005), blood pressure (Peter et al., 2005), coronary reactivity (Lehtimaki et al., 2002) and lipid profile (Molvarec et al., 2007a), and other pathological conditions, including cardiovascular disorders (Lawlor et al., 2006), venous thromboembolism (Straczek et al., 2005), miscarriage (Silva et al., 2010), severe pre-eclampsia (Molvarec et al., 2007b) or Alzheimer's Disease (Brandi et al., 1999).

For breast cancer, contradictory findings have arisen: while some studies have found an increased risk of developing the disease for carriers of both A and T alleles of the XbaI and PvuII polymorphisms, others have found an increased risk only for the X (A) allele of XbaI (González-Zuloeta Ladd et al., 2007).

Molecular mechanisms by which these polymorphisms influence receptor activity are still unclear. Initial reports had suggested an alteration in protein expression secondary to changes in mRNA splicing of PvuII gene (Hill et al., 1989). As these are present in the non-coding region (activation domain) of gene, the structure of receptor protein should not be altered. Instead there is growing evidence that these SNPs affect the binding of transcription factors like myb to ERα gene leading to decreased expression of ERα receptor. However, whether these SNPs affect the quality or quantity of estrogen receptor alpha gene mRNA transcripts or protein expression remains to be established (Herrington et al., 2002). Also, these SNPs (p and x alleles) seem to be associated with lower serum estradiol levels in postmenopausal

women, by affecting the expression of one of the subtypes of 17ß-hydroxysteroid dehydrogenase enzyme that catalyzes the transformation of estrone into estradiol (Schuit et al., 2005).

3.2.3. Polymorphisms in cell cycle regulation genes

Carcinogenesis in humans is believed to result from uncontrolled cell proliferation. Cell-cycle checkpoints are one of the primary defense mechanisms against mutagenic exposure. These checkpoints are regulatory pathways that control the order and timing of cell-cycle transitions to ensure DNA replication and chromosome segregation. The key point in cell-cycle regulation is the transition of the restriction point late in the G1 phase, which is crucial in determining the cell's destiny: division, differentiation, senescence, or apoptosis. It is believed that once the restriction point has been overcome, cell-cycle progression occurs almost automatically. Cells may be arrested at the restriction point, temporarily halting the cell cycle and allowing DNA repair to be completed. The loss of this checkpoint and the perturbation of cell-cycle control may not only disrupt the balance between normal growth and terminal differentiation but may also be accompanied by genomic instability, which may facilitate the development of cancer, as evidenced by the frequent inactivation of cell-cycle control genes, including those for p53, p16, p27, and retinoblastoma protein, in various cancers (Cai et al., 2011).

3.2.3.1. TP53

The TP53 tumor suppressor gene is located on chromosome 17p13 (Hollstein et al., 1991). It is known to be crucial for maintaining genomic integrity and preventing cells from undergoing oncogenic transformation and is one of the most commonly mutated genes in all types of human cancer (Hollstein et al., 1991; Levine, 1997; Vogelstein et al. 2000). TP53, like BRCA1, BRCA2 and ATM, are estimated to be involved in around half of the familial clustering of early onset breast cancer, but in only about 5 to 10% of all breast cancer cases (Easton et al., 1993; Oesterreich and Fuqua, 1999; Cui and Hopper, 2000; Mitrunen and Hirvonen, 2003). TP53 encodes tumor protein p53 protein, a transcription factor which is involved in the regulation of several target genes that induce cell cycle arrest, DNA repair, senescence and apoptosis in response to cellular stresses such as DNA damage (Vogelstein et al., 2000; Prives et al., 1999; Vogelstein et al., 2004). Mutations in TP53 may result in loss of p53 normal functions, leading to unrestrained cell proliferation, and possible tumorigenesis (Petitjean et al., 2007). Thus, p53 is believed to contribute to cellular transformation and malignancy, and in fact p53 protein is expressed at low level in normal cells and at a high level in a variety of transformed cell lines (Hollstein et al., 1991; Petitjean et al., 2007). Also, p53 is mutated in 30 to 50% of breast cancer cases and its mutations occur in tumors with BRCA1 or BRCA2

mutations with greater frequency than in sporadic tumors with wild-type BRCA1 and BRCA2, and are often associated with poor prognosis, what makes TP53 the most frequently altered gene in human cancers, including breast carcinoma (Zhang et al., 2010; Roy et al., 2012). Multiple studies suggest that the loss of p53 cooperates with the loss of BRCA1 or BRCA2 in tumorigenesis. A susceptibility to develop breast carcinomas is also a clinical feature of Li-Fraumeni syndrome, which is caused by germline mutation of TP53 and is associated with predisposition to develop several types of cancer (Roy et al., 2012).

Several SNPs have also been revealed to modulate p53 function and are therefore thought to be risk factors for numerous types of cancer, including mammary carcinomas (Al-Qasem et al., 2011). Among the polymorphisms reported in the TP53 coding region, only two alter the amino acid sequence: SNPs in codon 72 and in codon 47 (Pietsch et al., 2006; Whibley et al., 2009). The most common and widely studied SNP in TP53 gene is the polymorphism in codon 72 of exon 4, that encodes an arginine-proline substitution (C>G). This polymorphism occurs in the non-conserved proline-rich region of exon 4 and encodes either arginine (CGC) or proline (CCC) leading to three variant forms: Arg/Arg, Arg/Pro and Pro/Pro (Wei et al., 2005; Hartmann et al., 2006; Toyama et al., 2007). The two polymorphic variants of the codified protein seem to present structural differences and also different biological properties (Harris et al., 1986; Dumont et al., 2003). The Pro72Arg polymorphism occurs in the proline-rich domain of p53, which is necessary for the protein to fully induce apoptosis. It was noticed that p53(Pro) was a stronger inducer of transcription than p53(Arg), whereas p53(Arg) induced apoptosis faster and was a more potent suppressor of transformation than p53(Pro) (Dumont et al., 2003). The distribution of frequencies of the two alleles among healthy individuals and among ethnic groups is significantly different. The frequency of the Pro allele is 17% in Sweden, and 63% in Nigeria (Hartmann et al., 2006); the Pro allele frequency is approximately 60% in African Americans, but 30-35% in Caucasian Americans (Murphy, 2006). Previous studies have shown that this common polymorphism is involved in modulating p53 apoptotic function, and as such it may affect the response to chemotherapy (Wei et al., 2005).

Chapter 4

Dermal fibroblasts as a model of study of estrogenic response

Fibroblasts are cells with origin in mesodermal layer and one of their main functions is to produce collagen fibers (Doljanski, 2004). In classical cell biology, fibroblasts were considered as a population of cells relatively inert and uninteresting. This vision was dramatically changed, and currently they are recognized as a central component of tissue biology (Sorrell and Caplan, 2009), that have important roles in structural and physiological regulation of tissues (Mine et al, 2008).

4.1. Why dermal fibroblasts can be used as models of study of estrogenic action?

Fibroblasts are a heterogeneous and dynamic population of cells that synthetize and release a collection of precursors of the extracellular matrix (ECM), particularly fundamental substance, collagen, reticulin and elastin, that contribute for structural support of tissues (Kanitakis, 2002; Sorrell, 2009). Fibroblasts also participate in paracrine and autocrine interactions in skin, that are particularly important in different steps of wound healing (Darby and Hewitson, 2007; Werner et al., 2007), in inflammatory response by secretion of cytokines (Sorrel, 2009), in angiogenesis by production of angiogenic growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF) (Newman et al., 2011). Although the first cell cultures were established for the first time near 100 years ago, nowadays fibroblasts are a frequently used tool in several fields of research. Fibroblast cultures have raised interest because they are easy to obtain from biopsy of the skin, which constitutes the main source of fibroblasts, and to the fact that after isolation and establishment of cultures, fibroblasts present rapid and continuous proliferation in presence of serum, when compared to other cell types that require additional growth factors. *In vitro* cultures of fibroblasts present heterogenic genetic expression profiles, depending on their provenience from distinct anatomic locals, and based in the metabolic differences between fibroblasts from different tissues. These topographic differences are maintained throughout cellular passages, what means that fibroblasts present positional memory (Chang et al., 2002). Besides the heterogeneity attributable to anatomic positioning, fibroblasts of a same tissue do not

establish a homogeneous population, so it becomes important that fibroblasts with origin in different parts of the body, and in different layers of the same tissue, are faced as distinct cellular types (Sorrel, 2009; Chang et al., 2002). Concerning dermal fibroblasts, they can be classified in two subpopulations, according to their positioning on the papilar dermis or the reticular dermis. These two populations present phenotypic differences that are revealed in the production and organization of ECM, the production of growth factors and cytocines, and in the participation of inflammatory responses (Sorrel, 2009). The main attraction of using dermal fibroblasts as an *in vitro* model is associated with the expression of several specific receptors for hormones and neurotransmitters. Dermal fibroblasts have been found to express several hormone receptors, including ERα and ERβ, parathyroid hormone receptor/ peptide-related with parathyroid receptor (PTHR/PTHrPR), thyroid-stimulating hormone receptor (TSHR), *type 1 corticotropin-releasing hormone receptor* (CRH-1R), *melanocortin 1 receptor* (MC1R), melatonin 1 receptor (melatonin-1R), *serotonin receptors*, also known as 5-hydroxytryptamine receptors or 5-HT receptors (5-HTR), growth hormone receptor (GHR), androgen receptor (AR), retinoid X receptor type alpha (RXRα) (Zouboulis, 2004; Tsui et al., 2011).

Sex steroid hormones are involved in regulation of skin development and functions as well as in some skin pathological events, and several studies have shown that estrogens have many important beneficial and protective roles in skin physiology. They have been shown to accelerate cutaneous wound healing, to improve inflammatory skin disorders such as psoriasis during pregnancy, to protect against skin photoaging, and epidemiological studies indicate that the mortality rates from both non-melanoma skin cancers and melanoma are significantly lower in women (Stevenson and Thornton, 2007).

As demonstrated by the changes seen in the skin of post-menopausal women, compared with pre-menopausal women, estrogens play an important role in skin homeostasis: menopause causes hypoestrogenism, accelerating age-related deterioration, which results in thinner skin, increased number and depth of wrinkles, increased skin dryness, and decreased skin firmness and elasticity (Brincat, 2000; Stevenson and Thornton, 2007) and estrogen receptor expression has been shown to be reduced after menopause (Punnonen et al., 1980; Nelson and Bulun, 2001). Hormone replacement therapy (HRT) has been shown to increase epidermal hydration, skin elasticity, skin thickness, and also reduces skin wrinkles. Furthermore, the content and quality of collagen and the level of vascularization is enhanced (Stevenson and Thornton, 2007). There is also a variation in skin thickness during the menstrual cycle, with skin thickness lowest at the start of the menstrual cycle, when estrogen and progesterone levels are low, which then increases with the rising levels of estrogen (Eisenbeiss, 1998).

Skin has the capacity to synthesize estrogens from androgens, to use it in paracrine or intracrine fashions, and may contribute to the circulating pool of estrogens. Aromatase, which is found in skin fibroblasts, is responsible for the local conversion of C19 steroids to estrogens (Sasano and Harada, 1998; Nelson and Bulun, 2001), and dermal fibroblasts have been found to express ERα and ERβ (Tsui et al., 2011). Thus, taking advantage of the explored

characteristics, the position of human skin and the easy with which it can be obtained, dermal fibroblasts can be used as models of study of estrogenic action.

4.2. Tumor micro-environment in breast cancer and involvement of stromal fibroblasts

Breast cancer is composed of both parenchymal or carcinoma cells and stromal cells. Tumor stroma consists of fibroblasts, adipocytes, inflammatory cells such as lymphocytes and macrophages, and lymphatic and blood capillaries including pericytes and endothelial cells. Despite these complicated architecture of breast cancer tissue environment, most of the research has been directed toward that in carcinoma or parenchymal cells. However, tumor microenvironment has been recently identified as a major factor influencing treatment resistance of cancer to radiotherapy and chemotherapy (Miki et al., 2012). It is also well known that tumor microenvironment plays a pivotal role in neoplastic cell initiation or tumorigenesis, progression/ development, and metastatic spread of tumor cells (Carmeliet and Jain, 2000; Bhowmick and Neilson, 2004; Siemann, 2010).

As previously emphasized in Chapter 1, stromal-epithelial interactions have a fundamental role in normal mammary development such as determining normal duct formation and the initiation and maintenance of estrogen and/or progesterone responsiveness in mammary epithelial cells. Furthermore, normal stroma controls epithelial cell polarity, loss of which leads to an increase in cell proliferation and tumorigenesis (Polyak and Kalluri, 2010). Mammary gland fibroblasts are the predominant cells of the mammary stroma, responsible for the production of most of the components of connective tissue and ECM molecules, which influence cell adhesion and proliferation. Thereby, modifications in stromal fibroblasts can play a significant role in overall cancer development. Indeed, several recent publications have reported genetic and epigenetic changes in stromal fibroblasts that modulate the expression of many genes encoding growth factors and cytokines (Hu and Polyak, 2008; Lin et al., 2009). These soluble factors affect the microenvironment, and promote the growth of the tumor. To progress and spread, tumor cells keep interacting with their stromal fibroblasts through secreted molecules. This reciprocal heterotypic signaling plays a major role in the various steps of tumorigenesis (Aboussekhra, 2011).

Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells. In healthy organs, fibroblasts have a low proliferation index and minimum metabolic capacity. By contrast, during wound healing and in cancers, fibroblasts become activated, start to proliferate, secrete higher amounts of ECM components, and acquire contractile features. In tumors, these fibroblasts are known as reactive fibroblasts, peri-tumoral fibroblasts, myofibroblasts, tumor-associated or cancer-associated fibroblasts (CAFs) (Polyak and Kalluri, 2010). Both normal epithelial associated fibroblast (NAFs) and CAFs can inhibit the growth or cell proliferation of pre-cancerous breast epithelial cells. Normal breast-associated fibroblast

demonstrated greater inhibitory capacity than CAFs, which suggest that the ability of fibroblasts to inhibit epithelial cell proliferation detected in normal human breast tissue is lost during the process of breast carcinogenesis. CAFs also have key roles in transformation, proliferation, and invasion of several type of carcinoma including breast carcinoma (Aboussekhra, 2011).

While most CAFs are active fibroblasts, a small proportion remains inactive. Indeed, in breast tumors 80% of fibroblasts are in active form. Active fibroblasts play similar roles in wound healing and in cancer, which is considered as a wound that does not heal (Dvorak, 1986). However, while in normal wounds active fibroblasts are transients, CAFs are persistent in tumors. Active fibroblasts secrete high levels of various growth factors, cytokines, chemokines, and ECM degrading proteases such as the MMPs (Bhowmick et al., 2004). These factors are used by activated fibroblasts to communicate with cancer cells as well as with other stromal cells. CAFs exhibit some cancer-specific changes, including defective p53/p21-dependent signaling pathway in response to g-rays, high level of survivin, increased resistance to cisplatin and UV light and strong expression of the proliferation markers Ki-67 and PCNA (Hawsawi et al., 2008). However, these cells are non-neoplastic because they senesce in culture and do not grow *in vivo* in tumor xenografts (Kojima et al., 2010).

There is substantial evidence that CAFs actively contribute to the growth, expansion and dissemination of neoplastic epithelial cells. Thereby, stromal fibroblasts have been considered as the “contracted farmers” that prepare a terrain for the tumor’s ease and fertile growth (Cheng and Weiner, 2003). This effect was revealed *in vivo* by showing that human breast cell line MCF-7 cells are hardly tumorigenic in SCID mice, but their tumorigenicity was dramatically increased when inoculated with fibroblasts (Trimis et al., 2008).

Chapter 5

Aims

The main objective of this thesis is to highlight the influence of polymorphisms in low penetrating genes in breast cancer susceptibility, specifically in the population of Beira Interior, in which this genetic effect was never accessed.

To achieve our main objective, we accomplished three specific goals:

1. Analyze the influence of polymorphic low penetrating genes of the estrogen biosynthetic pathway, the estrogen metabolic pathway, DNA damage signaling and repair pathway, and estrogenic response, in breast cancer susceptibility in a Portuguese population, hosted in the Central Eastern part of the country (Beira Interior).
2. Study the prevalence of these polymorphisms in groups of women with different 5-year relative risk for developing breast cancer scores, stratified by the Modified Gail Model.
3. Explore the use of dermal fibroblasts cultures as models of study of responses to estrogens.

Chapter 6

Glutathione S-Transferase M1, T1 and P1 Genotypes and Breast Cancer Risk: a Study in a Portuguese Population

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Glutathione *S*-transferase M1, T1, and P1 genotypes and breast cancer risk: a study in a Portuguese population

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Abstract Glutathione *S*-transferases are a superfamily of multifunctional enzymes that play a key role in Phase II metabolism, detoxifying therapeutic drugs, and various carcinogens by conjugation with glutathione. We undertook a case–control study in Central-Eastern Portuguese population to evaluate the association of null genotype in GSTM1 and GSTT1 along with the polymorphism in GSTP1 (A/G) and susceptibility to breast cancer. The population sample consisted of 85 patients with histological diagnosis of breast cancer and 102 healthy women. Genomic DNA was extracted from blood samples, and genotyping analyses were performed by PCR-based methods. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated by unconditional logistic regression. We found a increased breast cancer risk associated with GSTM1 null genotype (OR = 3.597; 95% CI = 1.849–6.999; $P = 0.0001$) and GSTT1 (OR = 2.592; 95% CI = 1.432–4.690; $P = 0.002$), but the presence of valine alleles compared to isoleucine alleles in codon 105 in GSTP1 did not increase the risk of breast cancer development. The two-way combination of GSTM1 and GTTT1 null genotypes resulted in 8-fold increase for breast cancer risk (OR = 8.287; 95% CI = 3.124–21.980; $P = 0.0001$) and the three-way combination of GSTP1 105AA/AG and null genotypes for both GSTM1 and GSTT1 resulted in 5-fold increase for breast cancer risk (OR = 5.040; 95% CI = 1.392–18.248; $P = 0.016$). Our results suggest that GSTM1 and GSTT1 null genotype alone, both combined or combined with GSTP1 valine alleles, are associated with higher susceptibility to breast cancer development.

Keywords GSTM1 · GSTT1 · GSTP1 · Polymorphisms · Breast cancer

Introduction

Breast cancer is the most common cause of cancer-related deaths among women in Western world [1]. Mutations in high penetrance genes account for only a small percentage of cases of familial and sporadic breast cancer, because the low frequencies of risk alleles in the general population. So it seems interesting to analyze the role of other genetic factors such as low penetrance candidate genes that may alter breast cancer predisposition. Low penetrance genes can participate on detoxification of environmental carcinogens, steroid hormone metabolism, and DNA damage repair pathways [2]. Glutathione *S*-transferases (GSTs) are a superfamily of ubiquitous, dimeric, and multifunctional enzymes that play a key role in Phase II metabolism, which generally detoxifies carcinogens, by conjugation with glutathione (GSH) [3]. A multiplicity of exogenous and endogenous compounds with electrophilic functional groups can be degraded by glutathione (GSH) conjugation by neutralizing their electrophilic sites, increasing their water solubility and excretability, and also protecting the cells from DNA damage and adducts formation [4]. In estrogen metabolism, GSTs play a role in the catalysis of GSH conjugation of catechol estrogen quinones, the

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reactive intermediates of estrogen metabolism capable to bind to DNA [2]. In humans, GSTs can be differentiated into at least three major families of proteins: cytosolic or soluble, mitochondrial and peroxisomal, and microsomal (currently named membrane-associated proteins involved in eicosanoid and glutathione metabolism, MAPEG) GSTs [5]. Seven classes of cytosolic GSTs were created based on amino acid sequence similarities, physical structure of the genes, and immunological crossreactivity, and are termed alpha (α), mu (μ), kappa (κ), pi (π), theta (θ), omega (Ω), and zeta (ζ) [5–7]. The active site of a GST protein is formed by the glutathione-binding site, which is preserved in the different classes, and the hydrophobic substrate-binding site, that has variations in the forming residues, leading to wide substrate specificity [2]. GSTM1, GSTT1, and GSTP1 isoenzymes are present in both normal and breast tumor tissues and belong to the best-characterized classes, along with class alpha and omega. GSTM1 is codified by a gene located on chromosome 1p13.3 [8, 9]. The homozygous deletion (null genotype) of the GSTM1 gene results in the total absence of a functional gene product, and then a total absence of the respective enzyme activity [8]. Like GSTM1, GSTT1 gene, located at 22q11.2, has a genetic variant that consists of a complete deletion of the whole gene, resulting in the lack of the codified enzyme [10]. GSTP1 is a polymorphic gene located on chromosome 11q13. One of the functional genetic variants identified is the point mutation at nucleotide 313 that results in a single amino acid change from isoleucine (Ile) to valine (Val), at codon 105 [11]. As this residue lies in close proximity to the hydrophobic-binding site for electrophilic substrates, the variant allele seems to have altered specific activity and affinity depending on the substrate [12, 13]. GSTM1 and GSTP1 isoenzymes catalyze the nucleophilic conjugation of glutathione to several exogenous and endogenous chemicals molecules with electrophilic functional groups, including polyaromatic compounds, responsible for the formation of lipophilic polyaromatic adducts that can be stored in breast tissue [14]. GSTT1 is better known by its role in the detoxification of smaller reactive hydrocarbons like activated metabolites of the heterocyclic amines formed by cooking meat at high temperatures [15, 16]. Carcinogen metabolizing enzymes like GSTs are involved in the activation and deactivation of diverse chemical carcinogens, including xenobiotics and sex hormones. Individuals carrying a variant in one of these low penetrating genes are estimated to have low risk to develop breast cancer when compared to carriers of mutations in high penetrating genes like BRCA1 and BRCA2, but the high frequency in the population of some of the variants makes the population to be interpreted as being of high risk. This study was designed to investigate the oncologic risk of genotypic variations of GSTM1, GSTT1, and GSTP1 low penetrance genes involved in xenobiotic metabolic pathways in breast cancer.

Materials and methods

Study population

Case subjects, 85 women with histologically confirmed breast cancer, diagnosed at Child and Women Health Department, Gynecologic Oncology Division, Hospital Centre of Cova da Beira, Covilhã—Portugal, were enrolled between May 2008 and March 2009. 102 female controls with no previous history of any type of cancer and without family histories of cancer, from the same geographic region, were also studied. All subjects were Caucasian. Both cases and controls completed a questionnaire that assessed parameters such as age, family history of breast cancer, parity, ethnicity, reproductive and menstrual history, and history of breast diseases and procedures. An informed consent was obtained before entering in the study. The study was approved by the Institutional Review Board of Hospital Centre Cova da Beira, Covilhã—Portugal.

DNA extraction and genotyping

Blood was collected by venous puncture in EDTA-containing tubes, and genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol, and stored at 4°C. Genotyping for GSMT1, GSTT1, and GSTP1 was performed using PCR-based methods slightly modified from procedures earlier described [17, 18]. For GSTM1 and GSTT1, the presence of the wild-type and/or the null alleles was analyzed by multiplex PCR together with co-amplification of a fragment of beta-globulin gene as a positive control. Regarding to GSTP1, the variant alleles containing a base substitution at the nucleotide 313, resulting in Ile105Val amino acid change, were differentiated from the wild-type allele (Ile/Ile) by BsmA1 restriction enzyme digestion (New England Biolabs) subsequent to PCR amplification. In brief, each PCR reaction mixture was carried out in a total volume of 25 µl and contained 10 pmol of each primer, 1.5 mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Promega, USA), and 100 ng of genomic DNA, using MyCycler Thermal Cycler (Bio-Rad, Munich, Germany). Reaction mixtures were pre-incubated for 10 min at 95°C, and the amplified or digested DNA was electrophoresed through agarose gels stained with ethidium bromide. The genotyping assays used for GSTM1 and GSTT1 do not identify homozygous wild-type and

heterozygous individuals but classifies as "present" individuals with one or two copies of the relevant gene and individuals with homozygous deletions as "null." The assay for GSTP1 classifies individuals as homozygous Ile/Ile, homozygous Val/Val, or heterozygous Ile/Val depending on the band pattern created by the digestion. Results were confirmed by re-genotyping 10% of randomly selected samples, and all the results were in agreement with the ones obtained previously.

Statistical analysis

Logistic regression method was used to access odds ratio (ORs) and 95% confidence intervals (95% CI) as estimates of relative risk. The frequencies of the GSTP1 Val/Val genotype were too low, thus the genotype data were divided into two groups, GSTP1 Ile/Ile and GSTP1 Ile/Val + Val/Val to increase the statistical power. Joint effects were estimated using GST putative low risk genotypes combination (GSTM1 present, GSTT1 present, and GSTP1 Ile/Ile) as referent group. P values less than 0.05 were considered statistically significant. Calculations were done using computer software SPSS for Windows (version 16.0).

Results

The mean age was 55.5 years (SD, 20.3 years; range, 17–84 years) for the controls and 62.8 years (SD, 12.9 years; range, 34–85 years) for the cases. The association between presumed risk factors and the development of breast cancer is shown in Table 1. The majority of study participants were postmenopausal at the time of blood donation (64.5% controls and 72.9% cases). The risk of breast cancer was lower for women who had at least one full term pregnancy (OR = 0.248; 95% CI = 0.089–0.694) and in general, other factors like menopausal status, history of first degree family history of breast cancer, history of benign breast disease, or BMI have not increased or decreased breast cancer risk significantly. Genotypes distribution of GSTM1, GSTT1, and GSTP1 in cases and controls is summarized in Table 2. GSTM1 deletion occurred in 59.8% of the controls. GSTM1 null genotype was significantly more common among breast cancer cases compared to controls (OR 2.592; 95% CI 1.432–4.690; $P = 0.002$). GSTT1 null genotype was also significantly more frequent in cases than in controls (OR 3.597; 95% CI 1.849–6.999; $P = 0.0001$). We did not find any significant increase of breast cancer risk associated with GSTP1 genotypes (OR 1.103; 95% CI 0.611–1.989; $P = 0.765$) when compared Val allele carriers in homozygosity or heterozygosity with Ile/Ile carriers. To further investigate if GSTs genotypes are related with breast cancer risk, we analyzed their association in two and three-way combinations to evaluate the impact of gene–gene interaction. Presence of GSTM1, GSTT1 alleles along with Ile/Ile genotype in GSTP1 were considered as low risk genotypes, and null genotype in both GSTM1 and GSTT1 along with Val allele in homozygosity or heterozygosity for GSTP1 was referred as the presumed high-risk genotype combination. Table 3 exhibits the risk of breast cancer with the two-way combination of GSTM1 and GSTT1. More than observing an increased risk for breast cancer associated with GSTM1 and GSTT1 null genotypes, we found a 8-fold increased breast cancer risk in women who carry null genotype both in GSTM1 and GSTT1 (OR 8.287; 95% CI 3.124–21.980; $P = 0.0001$). It was not observed any association of breast cancer risk with GSTP1 polymorphisms in combination with GSTM1 (Table 4). In relation to GSTT1 combined with GSTP1, it was found a significant elevation of breast cancer risk associated with GSTP1 Ile/Ile genotypes when associated to GSTT1 null genotype (OR 3.627; 95% CI

Table 1 Selected characteristics of the population studied

	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)
Total	102 (100)	85 (100)	
Age at menarche			
≤12 years	51 (50.0)	58 (68.2)	1.0
13–14 years	35 (34.3)	19 (22.3)	2.095 (1.068–4.108)
≥15 years	16 (15.7)	8 (9.4)	2.275 (0.899–5.755)
Age at first full term pregnancy			
≤25 years	56 (54.9)	39 (45.9)	1.0
26–30 years	12 (11.8)	18 (21.2)	0.464 (0.201–1.072)
≥31 years	13 (12.7)	16 (18.8)	0.566 (0.245–1.308)
Number of full-term pregnancies			
Nulliparous	21 (20.6)	12 (14.1)	1.0
1	10 (9.8)	23 (27.1)	0.248 (0.089–0.694)
2	36 (35.3)	27 (31.8)	0.762 (0.320–1.814)
3+	35 (34.3)	23 (27.1)	0.870 (0.360–2.103)
Menopausal status			
Premenopausal	36 (35.3)	23 (27.01)	1.0
Postmenopausal	66 (64.7)	62 (72.9)	0.680 (0.363–1.274)
First degree family history of breast cancer			
No	91 (89.2)	70 (82.3)	1.0
Yes	11 (10.8)	15 (17.6)	0.564 (0.244–1.304)
History of benign breast disease			
No	55 (53.9)	37 (43.5)	1.0
Yes	47 (46.1)	48 (56.5)	0.659 (0.369–1.176)
BMI			
≤25	56 (54.9)	36 (42.3)	1.0
>25	46 (45.1)	49 (57.6)	0.603 (0.338–1.079)

Table 2 Association between GSTs genotypes and breast cancer

	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)[a]	*P* value
GSTM1				
Present	61 (59.8)	31 (36.5)	1.0	
Null	41 (40.2)	54 (63.5)	2.592 (1.432–4.690)	0.002
GSTT1				
Present	84 (82.4)	48 (56.5)	1.0	
Null	18 (17.6)	37 (43.5)	3.597 (1.849–6.999)	0.0001
GSTP1				
Ile/Ile	48 (47.1)	39 (45.9)	1.0	
Ile/Val + Val/Val	54 (52.9)	46 (54.1)	1.103 (0.611–1.989)	0.765

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

Table 3 Association between GSTM1 and GSTT1 genotype combinations and breast cancer

GSTM1	*GSTT1*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)[a]	*P* value
+	+	49 (48.0)	17 (20.0)	1.0	
+	−	12 (11.8)	14 (16.5)	3.363 (1.303–8.679)	0.014
−	+	33 (32.3)	31 (36.5)	2.708 (1.294–5.664)	0.011
−	−	8 (7.8)	23 (27.0)	8.287 (3.124–21.980)	0.0001

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

Table 4 Association between GSTM1 and GSTP1 genotype combinations and breast cancer

GSTM1	*GSTP1*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)[a]	*P* value
+	Ile/Ile	29 (28.4)	19 (22.3)	1.0	
+	Ile/Val + Val/Val	32 (31.4)	12 (14.1)	0.572 (0.237–1.380)	0.271
−	Ile/Ile	19 (18.6)	24 (28.2)	1.928 (0.837–4.443)	0.144
−	Ile/Val + Val/Val	22 (21.6)	30 (35.3)	2.081 (0.937–4.624)	0.076

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

Table 5 Association between GSTT1 and GSTP1 genotype combinations and breast cancer

GSTT1	*GSTP1*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)[a]	*P* value
+	Ile/Ile	38 (37.2)	22 (25.9)	1.0	
+	Ile/Val + Val/Val	46 (45.1)	26 (30.6)	0.976 (0.479–1.990)	1.000
−	Ile/Ile	10 (9.8)	21 (24.7)	3.627 (1.448–9.084)	0.008
−	Ile/Val + Val/Val	8 (7.8)	16 (18.8)	3.455 (1.273–9.372)	0.016

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

1.448–9.084; $P = 0.008$) and less with Ile/Val + Val/Val genotypes (OR 3.455; 95% CI 1.273–9.372; $P = 0.016$), as shown in Table 5. After analyzing the three-way combination of GSTM1, GSTT1, and GSTP1 polymorphisms (Table 6), we found that Ile/Ile genotype seems to be strongly associated with risk of breast cancer when combined with both GSTM1 and GSTT1 null genotypes (OR 12.600; 95% CI 2.358–67.315; $P = 0.001$). Also, the combination of GSTM1 and GSTT1 null genotypes with presence of the Val allele in GSTP1 was associated with a 5-fold increase in breast cancer risk when compared with the combination of GSTM1 present, GSTT1 present, GSTP1 Ile/Ile (OR 5.040; 95% CI 1.392–18.248; $P = 0.016$), so it appears that the increase of breast risk is mainly given by GSTM1 together with GSTT1 deletion, rather than by any genotype of GSTP1.

Table 6 Association between GSTM1, GSTT1, and GSTP1 genotype combinations and breast cancer

GSTM1	*GSTT1*	*GSTP1*	Controls, *n* (%)	Cases, *n* (%)	OR (95% CI)[a]	*P* value
+	+	Ile/Ile	21 (20.5)	10 (11.8)	1.0	
−	+	Ile/Ile	17 (16.6)	12 (14.1)	1.482 (0.516–4.258)	0.593
+	−	Ile/Ile	8 (7.8)	9 (10.6)	2.362 (0.702–7.955)	0.221
+	+	Ile/Val and Val/Val	29 (28.4)	8 (9.4)	0.579 (0.196–1.716)	0.411
−	−	Ile/Ile	2 (2.0)	12 (14.1)	12.600 (2.358–67.315)	0.001
−	+	Ile/Val and Val/Val	17 (16.7)	18 (21.1)	2.224 (0.815–6.064)	0.140
+	−	Ile/Val and Val/Val	3 (2.9)	4 (4.7)	2.800 (0.524–14.959)	0.387
−	−	Ile/Val and Val/Val	5 (4.9)	12 (14.1)	5.040 (1.392–18.248)	0.016

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

Discussion

We examined the association of three common GSTM1, GSTT1, and GSTP1 polymorphisms, both alone and in combination, with breast cancer risk in a population-based, case–control study of Caucasian women in a region of Portugal. Genotypes distribution in control subjects was in agreement with previous reports for Caucasian populations. GSTM1 deletion in 59.8% of the controls agrees with a meta-analysis of 30 studies [19] involving over 10,000 individuals, which identified the GSTM1 null genotype in 53% Caucasians (with a range of 42–62%). About 20% of Caucasians are homozygous for a GSTT null allele [20], and the occurrence of the deletion in our control group was somewhat lower, 17.6%. Concerning GSTP1, the frequency of GSTP1 Val allele containing genotypes in our population study (52.9%) was similar to that reported for Caucasians (45–60%) [21]. It was not found any significant breast cancer risk associated with GSTs genotypes and menopausal status, parity, or BMI, however, we observed an increased risk for breast cancer related with GSTM1 and GSTT1 null genotype, both alone or in a two-way combination, confirming previous reported data [22–25]. In the used assay, the absence of a PCR product indicates the GSTM1 or GSTT1 null/null genotype, and study participants were categorized as either "present" (wild-type) or "null" genotypes. This analytical approach does not positively identify the null allele and, therefore, cannot distinguish homozygous wild type from heterozygous present/null individuals, but it conclusively identifies the null/null genotypes. GSTM1 and GSTT1 null genotypes have been the focus of several studies because of their special condition: the majority of polymorphisms affecting genes involved in carcinogen metabolism are single nucleotide polymorphisms, deletions are less common, and the complete absence of a gene in form of a null allele is rare. GSTs are phase II detoxifying enzymes, and the polymorphisms in the codifying genes may be associated with the increased susceptibility to breast cancer, as normal or increased GSTs activity may facilitate detoxification of electrophilic carcinogens, protecting susceptible tissues from somatic DNA mutations [20, 22]. Thus, population with reduced GST enzyme activity, as result of homozygous deletions of GSTM1 or GSTT1, may be at greater risk for developing cancers due to their impaired ability to metabolically eliminate carcinogenic compounds and reduced detoxification efficiency. We found no significant elevation of breast cancer risk associated with GSTP1 genotypes alone but significantly higher breast cancer risk when all genotypes were analyzed together. Women who carry null genotype both in GSTM1 and GSTT1 and wild-type genotype in GSTP1 have shown 12-fold increased risk for breast cancer. This means that Ile/Ile genotype seems to increase risk for breast cancer only when associated to GSTT1 null genotype or both GSTM1 and GSTT1 null genotypes, and that Val allele tends to act as a protective factor rather than as a risk factor, in agreement with the findings of other research groups [22, 24]. It has also been reported that GSTP1 Ile allele acted in the opposite way, protecting against the risk of breast cancer, and Val allele was associated with the risk of developing the disease [23, 26]. Another study that comprised 500 breast cancer cases and 395 controls did not found association of breast cancer risk with any combination of GSTM1, GSTT1, and GSTP1 genotypes [21]. The inconsistent results between studies might be due to the fact that the enzyme encoded by GSTP1 Val allele exhibits different activity, affinity, and thermostability according to substrates [26]. GSTP1 variants encode the change at position 105 from Ile to Val. It was hypothesised that this amino acid substitution may limit free access of the substrate to the H-site required for conjugating with glutathione as well as its thermostability [27, 28]; however, the exact biological mechanism and effect of the polymorphism is not completely understood [26, 29]. Furthermore, GSTs are known to have overlapping substrate specificities, and the

absence of GST isoenzymes may be compensated by other isoforms. Different population studied with different risk agent exposures may explain the differences in the outcomes of the reported studies conducted on this topic. GSTM1 and GSTP1 isoenzymes catalyze the detoxification of epoxides formed by polycyclic hydrocarbons in cigarette smoke and other polyaromatic compounds; in breast tissue, tobacco smoke is one of the major sources for these deposits, but smoking effects on breast cancer are still controversial because it seems to have putative anti-estrogenic effects [30–32]. We did not find association between GSTM1 and GSTP1 alone with breast cancer risk; however, our population was not stratified by smoking status. The same occurs with alcohol consumption, use of oral contraceptives, and hormone replacement therapy, so we decided to not consider these data on our analysis. Another conflicting point in literature is the inverse relationship between intake of dietary isothiocyanates derived from cruciferous vegetables like broccoli and the risk of cancers, particularly lung, colon, and breast. Their protective effects appear to be influenced by GST genotype: individuals with GSTT1 and GSTM1 null are better protected than those with GSTT1 and GSTM1 positive [33]. The three genes analyzed in this study are considered as low penetrating genes, and alone or in combination, they may identify subjects as poor or fast metabolizers. Poor metabolizers are more exposed to the formation of carcinogen–DNA adducts and/or mutations, which confers them higher susceptibility to complex genetic disorders such as cancer. Common differences in genes encoding for metabolism enzymes like GSTM1, GSTT1, and GSTP1 may be responsible for a relatively small but rather frequent increase of cancer risk among individuals, than those so called high-penetrating genes like tumor suppressor genes. A higher number of studies suggest that the risk associated with each low penetrating variant may be small, but it can be potentiated by association with other genetic or environmental factors. Our results indicate that GSTM1 and GSTT1 null genotypes, alone or in association, are related with increased susceptibility to breast cancer development. All the individuals included in the analysis share a common characteristic, their geographic area of residence at Central-Eastern region of Portugal, thus they are probably subjected to similar environmental factors. This fact could have an important contribution to the reported association. Further studies need to be conducted to evaluate the value of these genes in association with other known low penetrance genes in defining breast cancer risk, in the Portuguese population.

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Chapter 7

Positive Association of Polymorphisms in Estrogen Biosynthesis Gene, CYP19A1, and Metabolism, GST, in Breast Cancer Susceptibility

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Positive Association of Polymorphisms in Estrogen Biosynthesis Gene, CYP19A1, and Metabolism, GST, in Breast Cancer Susceptibility

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Purpose: This case–control study was conducted in order to evaluate the potential role of polymorphic genes encoding enzymes involved in estrogen biosynthesis (*CYP19A1*) and metabolism (*GSTM1*, *GSTT1*, and *GSTP1*), and their action in modulating individual susceptibility to breast cancer. ***Methods:*** Genomic DNA was extracted from blood samples of 101 patients with histological diagnosis of breast cancer and 121 healthy women. Genotyping analyses of *CYP19A1* codon 39 Trp/Arg (*T/C*), *GSTM1* and *GSTT1* homozygous deletions, and *GSTP1* codon 105 *Ile/Val* (*A/G*) were performed by polymerase chain reaction–based methods. ***Results:*** Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated by unconditional logistic regression. Significant statistical association of the *TC/CC* genotypes combined with breast cancer risk was found, with reference to *TT* genotype (OR = 1.770; 95% CI = 1.036–3.024; $p = 0.036$). Also, *CYP19A1* arginine allele in homozygosity or heterozygosity (*TC/CC*) was associated with a significant increased risk for breast cancer when associated to *GSTM1* null genotype (OR = 6.158; 95% CI = 2.676–14.171; $p < 0.001$) and *GSTT1* null genotype (OR = 4.870; 95% CI = 2.216–10.700; $p < 0.001$). The three-way combination of *CYP19A1 TC/CC*, *GSTM1* null, and *GSTT1* null polymorphism was related with significant increased risk for breast cancer (OR = 11.429; 95% CI = 3.590–36.385; $p < 0.001$). Valine alleles compared with isoleucine alleles in codon 105 in *GSTP1*, in combination with *CYP19A1* genotypes, were not associated with an increase of breast cancer development. ***Conclusions:*** Our results suggest that the effect of *CYP19A1 T/C* polymorphism in susceptibility to breast cancer development can be modulated by the presence of *GSTM1* and *GSTT1*, but not *GSTP1*.

Introduction

In Europe, breast cancer is the most common cancer affecting women, representing the leading cause of cancer death between 35 and 55 years old (Jemal *et al.*, 2004). Breast cancer is the leading cause of death among women in developing countries. In Portugal, it presents the highest incidence and mortality rates among women diseases (Pinheiro *et al.*, 2003). Although many risk factors for the development of breast cancer have been identified, the molecular mechanisms related to breast carcinogenesis remain unclear. Estrogens have been clearly identified as carcinogens, by inducing aneuploidy and structural chromosomal changes, and stimulation of breast cell proliferation has been proposed as the main effect of estrogens in breast carcinogenesis—as more rapidly cells proliferate, greater the chance of acquiring a potentially cancer-causing mutation (Zhu and Conney, 1998). Also, metabolic by-products of estrogens are responsible for free radical–mediated DNA damage, single-strand breaks, estrogen–DNA adducts formation, protein oxidation, and lipid peroxidation, which triggers genetic instability and cellular damage (Mueck and Seeger, 2007). Germline mutations in the so-called high penetrance genes to breast cancer susceptibility, *BRCA1* and *BRCA2*, appear to account for the majority of hereditary breast cancer, but they represent only 5% to 10% of all breast cancer cases (Yang and Lippman, 1999). Thus, low penetrance genes, acting together with endogenous or life-style risk factors, can be linked with a significant percentage of breast cancer cases (Mitrunen and Hirvonen, 2003). Low penetrance genes can be found in several pathways like detoxification of environmental carcinogens, steroid hormone metabolism, and DNA damage repair pathways. Interindividual variability has been observed in estrogen biosynthesis and metabolic pathways, namely in genes encoding for proteins involved in estrogen biosynthesis, like *CYP19A1*, and in genes encoding for xenobiotic metabolizing enzymes, like Glutathione *S*-transferases (*GSTs*) (Thompson and Ambrosone, 2000). *CYP19A1* gene,

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CYP19A1 AND GST GENOTYPES AND BREAST CANCER RISK

located on chromosome 15q21.1, encodes *CYP19A1* or aromatase (EC 1.14.14.1), the enzyme that catalyzes three consecutive hydroxylation reactions converting C19 androgens, such as testosterone, to aromatic C18 estrogenic steroids (Bulun *et al.*, 2004). Genetic variations at this locus may alter aromatase activity and thereby affect hormone levels. The most focused is a tetranucleotide repeat $[TTTA]_n$ in intron 4 that may have a functional effect in the translated enzyme due to an alteration on the splice site; it has been reported to be associated with breast cancer risk among Caucasian women (Sourdaine *et al.*, 1994; Kristensen *et al.*, 1998; Hairman *et al.*, 2000), although negative results also exist (Healey *et al.*, 2000; Miyoshi *et al.*, 2000; Baxter *et al.*, 2001). Recently, a case–control study on the association of *CYP19 Val*(80) and $[TTTA]_n$ polymorphisms and the risk of breast cancer in *BRCA* carriers and noncarriers suggests that the *CYP19 Val*(80) polymorphism is associated with increased breast cancer risk in young women from a population of Ashkenazi Jews with *BRCA1* mutations (Raskin *et al.*, 2009). Also, a *C826T* variation in exon 7 that gives rise to the nonconservative amino acid substitution *Arg*264*Cys* has been used in breast cancer studies despite not having an apparent effect on aromatase activity (Watanabe *et al.*, 1997; Siegelmann-Danieli and Buetow, 1999; Long *et al.*, 2006). Miyoshi *et al.* (2000) identified codon 39 *Trp/Arg* polymorphism in *CYP19* gene and showed that it was significantly associated with breast cancer risk among Japanese women but there are no results available for other populations. Glutathione S-transferases (EC 2.5.1.18) constitute a superfamily of ubiquitous, multifunctional enzymes that play a key role in cellular detoxification (Ketterer, 1998). *GSTs* catalyze the conjugation of glutathione (*GSH*) to a wide variety of exogenous and endogenous chemicals with electrophilic functional groups like environmental carcinogens, reactive oxygen species (ROS), and chemotherapeutic agents. Conjugation with *GSH* renders metabolic products with higher hydrophilicity, which are easier to excrete (Hayes and Pulford, 1995). Human cytosolic *GST* family is the most complex and relevant to disease investigation. Cytosolic GSTs are subdivided into seven distinct classes that are designated as α (A), μ (M), π (P), σ (S), θ (T), ω (O), and ζ (Z). This classification is based on similarities in amino acid sequence, gene structures, and immunological crossreactivity (Hayes *et al.*, 2005; Di Pietro *et al.*, 2010). Polymorphic variants of three members of the *GST* family, *GSTM1*, *GSTT1*, and *GSTP1*, have been studied due to their potential importance in carcinogenesis. The human μ (M) class is encoded by a 100-kb gene cluster located on chromosome 1p13.3 (Landi, 2000) and the θ (T) class of *GSTs* consists of two genes located at 22q11.2 (Meyer *et al.*, 1991). Homozygous gene deletion (null genotype) has been identified at both *GSTM1* and *GSTT1* (Xu *et al.*, 1998; Alexandrie *et al.*, 2002), and these deletion variants lead to the total absence of the codified protein, thus total absence of enzyme activity (Bruhn *et al.*, 1998). The π (P) class of human *GSTs* is encoded by a gene located on chromosome 11q13.2 (Ali-Osman *et al.*, 1997). The polymorphism in exon 5 *A*313*G* (*Ile*105*Val*) of the *GSTP1* gene is in close proximity to the substrate binding site of *GSTP1*, and the Valine variant has been demonstrated to have either lower or higher specific activity and affinity than that of Ile variant, depending on the substrate (Ali-Osman *et al.*, 1997; Sundberg *et al.*, 1998). Associations of *GSTM1* and *GSTT1* null genotypes, and *GSTP1 Ile*105*Val* polymorphism with breast cancer have been reported with inconsistent results (Helzlsouer *et al.*, 1998; Millikan *et al.*, 2000; Gudmundsdottir *et al.*, 2001; Mitrunen *et al.*, 2001). Our group has previously shown, in a Portuguese population, that *GSTM1* and *GSTT1* null genotypes alone, both combined or combined with *GSTP1* Valine alleles, are associated with higher susceptibility to breast cancer development (Ramalhinho *et al.*, 2011). In light of this background, our aim is to analyze the influence of polymorphic genes encoding for enzymes involved in estrogen biosynthesis (*CYP19A1*) and in the conversion of estrogen metabolites and their by-products (*GSTM1*, *GSTT1*, and *GSTP1*) in breast cancer susceptibility.

Materials and Methods

Study population

A study group consisting of 101 women with histologically confirmed breast cancer, diagnosed at Child and Women Health Department, Gynecologic Oncology Division, Hospital Centre of Cova da Beira, Covilhã, Portugal, was enrolled between May 2008 and March 2009. A nonmalignant control group, 121 healthy female blood donors with no previous history of cancer and without family histories of cancers, was also studied. All subjects were Caucasian and had the same geographic area of residence. Both cases and controls completed a questionnaire that assessed parameters such as age, family history of breast cancer, parity, ethnicity, reproductive and menstrual history, and history of breast diseases and procedures. Informed consent was obtained from both patients and controls before entering in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã, Portugal.

DNA extraction

Genomic DNA of all cases and controls was isolated from either frozen or fresh blood samples using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instruction, and stored at 4°C.

Genotyping

CYP19A1 codon 39 *Trp/Arg* (*T/C*) polymorphism, *GSTM1* and *GSTT1* homozygous deletions, and *GSTP1* codon 105 *Ile/Val* (*A/G*) polymorphism analyses were performed by polymerase chain reaction (PCR)–based methods. *CYP19A1* genotyping was performed using PCR with confronting two-pair primers, modified from the procedure described by Hirose *et al.* (2004). Briefly, each PCR mixture was carried out in a total volume of 25 μL that contained 10 pmol of each primer, 1.5 mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Promega), and 100 ng of genomic DNA, using MyCycler Thermal Cycler (Bio-Rad). Reaction mixtures were preincubated for 10 min at 95°C. PCR conditions were 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, for 30 cycles. The final extension was at 72°C for 5 min. The amplified DNA was electrophoresed through 2% agarose gels stained with ethidium bromide. Genotypes were distinguished by the presence of a 200-bp band for T allele, a 264-bp band for C allele, and a 427-bp common band. *GSTM1*, *GSTT1*, and *GSTP1* genotyping was performed as previously published by our group (Ramalhinho

Table 1. Selected Characteristics of the Population Studied

	Controls, n *(%)*	*Cases*, n *(%)*	*OR (95% CI)*
Total	121 (100)	101 (100)	
Age at menarche			
≤12 years	57 (47.1)	63 (62.4)	1.0
13–14 years	41 (33.9)	25 (24.7)	0.552 (0.299–1.018)
≥15 years	23 (19.0)	13 (12.9)	0.511 (0.237–1.103)
Age at first full term pregnancy			
≤25 years	51 (53.1)	40 (50.6)	1.0
26–30 years	19 (19.8)	21 (26.6)	0.710 (0.337–1.496)
≥31 years	26 (27.0)	18 (22.8)	1.133 (0.546–2.350)
Number of full term pregnancies			
Nulliparous	25 (20.7)	22 (21.8)	1.0
1	19 (15.7)	26 (25.7)	1.555 (0.682–3.543)
2	45 (37.2)	29 (28.7)	0.732 (0.350–1.533)
3+	32 (26.4)	24 (23.8)	0.852 (0.391–1.859)
Menopausal status			
Premenopausal	46 (38.0)	31 (30.6)	1.0
Postmenopausal	75 (62.0)	70 (69.3)	1.385 (0.791–2.424)
First degree family history of breast cancer			
No	121 (100)	79 (78.2)	1.0
Yes	0 (0)	22 (21.8)	2.532 (2.133–3.005)
History of benign breast disease			
No	74 (61.2)	45 (44.5)	1.0
Yes	47 (38.8)	56 (55.4)	1.959 (1.146–3.350)
BMI			
≤25	65 (53.7)	40 (39.6)	1.0
>25	56 (46.3)	61 (60.4)	1.770 (1.036–3.024)

OR, odds ratio; CI, confidence interval; BMI, body mass index.

et al., 2011). Results were confirmed by re-genotyping 10% of randomly selected samples, and all the results were in agreement with the ones obtained previously.

Statistical analysis

To examine the association between genotypes and the development of breast cancer, we calculated odds ratios (ORs) and 95% confidence intervals (95% CIs) as estimates of relative risk, using logistic regression analysis with computer software SPSS for Windows (version 16.0). Chi-square test was applied to compare the allelic frequencies between normal controls and breast cancer patients. *p*-Values <0.05 were considered statistically significant.

Results

The mean age was 55.5 years (SD, 20.3 years; range, 17–84 years) for the controls and 62.8 years (SD, 12.9 years; range, 34–85 years) for the cases. Demographic data from cases and controls are shown in Table 1. The majority of study participants were postmenopausal (62.0% controls and 69.3% cases). The risk of breast cancer was higher for women with first degree family history of breast cancer (OR = 2.532; 95% CI = 2.133–3.005) and for women with history of benign breast disease (OR = 1.959; 95% CI = 1.146–3.350). Also, women with body mass index (BMI) superior to 25 seemed to have increased risk for breast cancer (OR = 1.770; 95% CI = 1.036–3.024). *CYP19A1* codon 39 genotype results in cases and controls are presented in Table 2. Significant statistical association of the *TC/CC* genotypes combined with breast cancer risk, with reference to *TT* genotype, was documented (OR = 1.770; 95% CI = 1.036–3.024; $p = 0.036$). Additionally, we analyzed the allele frequency in cases and controls (Table 3) and a positive correlation between *C* allele carriers (homozygous and heterozygous) and breast cancer risk was found (OR = 1.540; 95% CI = 1.013–2.341; $p = 0.043$). Results regarding the genotype distribution of *GSTM1*, *GSTT1*, and *GSTP1* in cases and controls in this population were previously published by our group, as well as the results of the association in two- and three-way combinations to evaluate the impact of gene–gene interaction (Ramalhinho *et al.*, 2011). To investigate whether profiles of *GSTs* and *CYP19A1* genotypes might be associated with the risk of breast cancer, we examined combinations of genotypes. The

Table 2. Distribution of *CYP*19 Codon 39 Polymorphisms in Patients and Controls

	Controls, n *(%)*	*Cases*, n *(%)*	*OR (95% CI)*	p-*Value*
TT	65 (53.7)	40 (39.6)	1.0	
TC/CC	56 (46.3)	61 (60.4)	1.770 (1.036–3.024)	0.036

Table 3. CYP19 Codon 39 Allele Frequency and Genotype, in Cases and Controls

	Controls, n *(%)*	*Cases*, n *(%)*	*OR (95% CI)*	p-*Value*
T, *n* (%)	185 (76.4)	137 (67.8)	1.0	
C, *n* (%)	57 (23.5)	65 (32.2)	1.540 (1.013–2.341)	0.043

CYP19A1 AND GST GENOTYPES AND BREAST CANCER RISK

Table 4. Association Between *GSTM*1 and *CYP*19 Genotype Combinations and Breast Cancer

GSTM1	CYP19	*Controls*, n *(%)*	*Cases*, n *(%)*	*OR (95% CI)*	p-*Value*
+	TT	39 (32.2)	13 (12.9)	1.0	
+	TC/CC	37 (30.6)	22 (21.8)	1.784 (0.786–4.050)	0.164
–	TT	26 (21.5)	27 (26.7)	3.115 (1.362–7.124)	0.006
–	TC/CC	19 (15.7)	39 (38.6)	6.287 (2.676–14.171)	<0.001

reference group consisted of individuals with all putative low-risk genotypes, that is, the presence of *GSTM1* and *GSTT1*, the homozygous *Ile/Ile* genotype for *GSTP1*, and the homozygous *TT* genotype for *CYP19A1*. Heterozygous and homozygous individuals for the *Val* allele and also heterozygous and homozygous individuals for *C* allele were grouped for this analysis, due to the low number of individuals with homozygous *Val* allele and homozygous *C* allele, and so to increase the statistical power. We analyzed the two-way combination of *GSTM1* and *CYP19A1* genotypes (Table 4) and found that when *GSTM1* is present, there is no significant association with breast cancer risk (OR = 1.784; 95% CI = 0.786–4.050; $p = 0.164$) but we found a significant increase of breast cancer risk for carriers of *GSTM1* null genotype along with *CYP19A1* TT wild-type genotype (OR = 3.115; 95% CI = 1.362–7.124; $p = 0.006$), and this risk is higher for carriers of both variants, *GSTM1* null and *CYP19A1* C allele, simultaneously (OR = 6.158; 95% CI = 2.676–14.171; $p < 0.001$). We could find the same relation in the two-way analysis of *GSTT1* and *CYP19A1* genotypes (Table 5). There is no significant increase in breast cancer risk for carriers of *GSTT1* present along with *CYP19A1 C* allele (OR = 1.841; 95% CI = 0.939–3.609; $p = 0.074$), but for carriers of *GSTT1* null genotype the risk is significantly higher in individuals with simultaneous *CYP19A1 TT* genotype (OR = 4.599; 95% CI = 1.792–11.804; $p = 0.001$) or *CYP19A1 TC/CC* genotype (OR = 4.870; 95% CI = 2.216–10.700; $p < 0.001$). Regarding the two-way analysis of *CYP19A1* and *GSTP1* genotypes (Table 6), we found no association of any genotype combination and risk of breast cancer. By analyzing the three-way combination of *CYP19A1*, *GSTM1*, and *GSTT1* polymorphisms (Table 7), we found a positive association with breast cancer risk for women who carry C allele in *CYP19A1* and null genotypes in *GSTM1* (OR = 5.000; 95% CI = 1.491–16.771; $p = 0.007$) or *GSTT1* (OR = 6.333; 95% CI = 2.195–18.271; $p = 0.001$). We also found that when both *GSTs* are deleted, the risk of breast cancer is higher, independently of *CYP19A1* genotype, and the combination of all putative high-risk genotypes, *CYP19A1 TC/CC*, *GSTM1* null, and *GSTT1* null, carries a strong association with breast cancer risk (OR = 11.429; 95% CI = 3.590–36.385; $p < 0.001$).

Discussion

High estradiol levels have been consistently associated with an increased breast cancer risk (Dorgan *et al.*, 1996; Key *et al.*, 2002; Missmer *et al.*, 2004; Zeleniuch-Jacquotte *et al.*, 2004), as estrogen promotes cellular growth and contributes to tumor growth by promoting the proliferation of cells with existing mutations or perhaps by increasing the opportunity for mutations (Zhu and Conney, 1998). Also, ROS have been related to the etiology of cancer, as they are known to be mitogenic to a variety of cells, and therefore capable of tumor promotion (Mitrunen and Hirvonen, 2003). Interindividual differences in estrogen biosynthetic and metabolic pathways may define subpopulations of women with higher lifetime exposure to hormone-dependent growth promotion or to cellular damage from estrogens or their metabolites. Such variation could explain a portion of the cancer susceptibility associated with reproductive events and hormone exposure. In most studies, polymorphisms in estrogen biosynthesis and metabolism genes are considered separately (Helzlsouer *et al.*, 1998; Kristensen *et al.*, 1998; Haiman *et al.*, 2000; Healey *et al.*, 2000; Millikan *et al.*, 2000; Miyoshi *et al.*, 2000; Mitrunen *et al.*, 2001; Baxter *et al.*, 2001; Gudmundsdottir *et al.*, 2001; Hirose *et al.*, 2004; Raskin *et al.*, 2009; Ramalhinho *et al.*, 2011) but here we hypothesize that *SNPs* in these low penetrance genes can exhibit synergistic effects on modulating individual susceptibility to breast cancer. Furthermore, specific associations of polymorphisms in estrogen biosynthesis and metabolism genes could result in a high-risk profile, by influencing lifetime levels of estrogen that could influence breast cancer risk. The four genes analyzed in this study are considered as low penetrating genes. These genes, alone or in association, may identify subjects as poor or fast synthesizers, like subjects as poor or fast estrogen metabolizers. While fast synthesizers are prolonging their exposure to estrogen, with potential increased carcinogenic activity, poor metabolizers are more exposed to the formation of carcinogen-DNA adducts and/or mutations, which confers them higher susceptibility to complex genetic disorders such as cancer. Thus, women having the "fast synthesizer/poor metabolizer" profile may possess higher risk for

Table 5. Association Between *GSTT*1 and *CYP*19 Genotype Combinations and Breast Cancer

GSTT1	CYP19	*Controls*, n *(%)*	*Cases*, n *(%)*	*OR (95% CI)*	p-*Value*
+	TT	56 (46.3)	23 (22.8)	1.0	
+	TC/CC	41 (33.9)	31 (30.7)	1.841 (0.939–3.609)	0.074
–	TT	9 (7.4)	17 (16.8)	4.599 (1.792–11.804)	0.001
–	TC/CC	15 (12.4)	30 (29.7)	4.870 (2.216–10.700)	<0.001

Table 6. Association Between *GSTP*1 and *CYP*19 Genotype Combinations and Breast Cancer

GSTP1	CYP19	*Controls,* n *(%)*	*Cases,* n *(%)*	*OR (95% CI)*	p-*Value*
Ile/Ile	TT	34 (28.1)	26 (25.7)	1.0	
Ile/Val + Val/Val	TT	31 (25.6)	14 (13.9)	0.591 (0.262–1.130)	0.202
Ile/Ile	TC/CC	23 (19.0)	28 (27.7)	1.592 (0.751–3.376)	0.224
Ile/Val + Val/Val	TC/CC	33 (27.3)	33 (32.7)	1.308 (0.648–2.640)	0.454

developing breast cancer than women with other profiles. In this study we investigated, in a population-based, case–control study of Caucasian women from a Portuguese region, breast cancer risk in association with polymorphism in a gene involved in estrogen biosynthesis (*CYP19A1*) along with polymorphisms in other three genes implicated in estrogen metabolism (*GSTM1*, *GSTT1*, and *GSTP1*). To avoid potential biases, we selected cases and controls from a single institution that only accepts patients from the same geographic area, Central Eastern Portugal. Patients and controls were matched by ages and belonged to the same ethnic group (Caucasian). Regarding *CYP19A1* codon 39 genotype distribution, our results were in agreement with the few previously published reports. Miyoshi *et al.* (2000) identified this polymorphism and performed a case–control study in order to evaluate its association with breast cancer risk in a Japanese population. They concluded that homozygous and heterozygous carriers of the variant *Arg* (*C*) allele at codon 39 had a decreased risk of breast cancer compared with the noncarriers. In our study, breast cancer patients had higher relative frequency of *C* allele and lower prevalence of *Trp* (*T*) allele than controls. Thus, for developing breast cancer, *C* allele carriers apparently have higher risk, while *T* allele carriers have lower risk, consequently *T* allele seems to be protective for carriers. A similar study, also in a Japanese population, was conducted by Hirose *et al.* (2004). They found, in homozygous and heterozygous carriers of the *C* allele, a significantly increased risk of breast cancer among premenopausal women with a late age at first full term pregnancy or a high BMI. In our study, we found positive statistical association of the *TC/CC* genotypes (*C* allele carriers) with breast cancer risk with no association with any particular demographic data. More recently, Tuzuneri *et al.* (2010) also found an association between C allele-bearing and breast cancer risk, and they propose *TT* haplotype as a protective haplotype. No published reports, regarding functional effects of *CYP19* codon 39 *Trp/Arg* amino acid change, are of our knowledge. To explain our data, we can speculate about deleterious effect of *Trp* to *Arg* substitution in codon 39 of *CYP19A1* gene; it seems that the encoded protein *P450* aromatase variant tends to have higher activity, thereby raising estrogens levels, what leads to the association of *C* allele with breast cancer risk. Considering *GSTs* polymorphisms, our group has already demonstrated the significant increase of breast cancer risk associated with *GSTM1* and *GSTT1* null polymorphisms, both alone or in combination, in the same population, while *GSTP1* genotypes seem to have no influence in breast cancer susceptibility (Ramalhinho *et al.*, 2011). In that article we reported that *GSTM1* null genotype was significantly more common among breast cancer cases compared with controls, so as *GSTT1*, and no significant increase of breast cancer risk associated with *GSTP1* genotypes was found. We also observed an eight-fold increased breast cancer risk in women who carry null genotype both in *GSTM1* and *GSTT1*, and we found that *Ile/Ile* genotype, so as presence of *Val* allele, seemed to be associated with risk of breast cancer when combined with both *GSTM1* and *GSTT1* null genotypes; thus, it appears that the increase of breast cancer risk was mainly linked to both *GSTM1* and GSTT1 deletion, rather than to any genotype of *GSTP1*. Grouping *CYP19A1* and *GSTM1* genotypes, breast cancer susceptibility was not altered when *GSTM1* was present. However, breast cancer risk for carriers of *GSTM1* null genotype along with *CYP19A1 TT* wild-type genotype is increased, so as for simultaneous carriers of both variants, *GSTM1* null and *CYP19A1 C* allele. Similar association was observed in the analysis of *CYP19A1* and *GSTT1* genotypes; independently of *CYP19A1* polymorphism, breast cancer risk is lower for carriers of *GSTT1*. Again, no association between both *CYP19A1* and *GSTP1* genotypes taken together and risk of breast cancer was found. To our knowledge, this is the first study to analyze the potential role of *CYP19A1* codon 39 polymorphism in Portuguese women, and its possible combined effect with *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms in the development of breast cancer. We determined that *C* allele, in homozygosity or heterozygosity, is significantly associated with an increased risk of breast cancer in the population studied. Furthermore, it seems that the effects of *CYP19A1 T/C*

Table 7. Association Between *GSTM*1, *GSTT*1, and *CYP*19 Genotype Combinations and Breast Cancer

GSTM1	GSTT1	CYP19	*Controls,* n *(%)*	*Cases,* n *(%)*	*OR (95% CI)*	p-*Value*
+	+	TT	32 (26.4)	8 (7.9)	1.0	
		TC/CC	29 (24.0)	12 (11.9)	1.655 (0.593–4.618)	0.333
+	–	TT	7 (5.8)	5 (4.9)	2.857 (0.715–11.410)	0.128
		TC/CC	8 (6.6)	10 (9.9)	5.000 (1.491–16.771)	0.007
–	+	TT	24 (19.8)	15 (14.8)	2.500 (0.912–6.851)	0.071
		TC/CC	12 (9.9)	19 (18.8)	6.333 (2.195–18.271)	0.001
–	–	TT	2 (1.6)	12 (11.9)	24.000 (4.448–129.490)	0.001
		TC/CC	7 (5.8)	20 (19.8)	11.429 (3.590–36.385)	<0.001

polymorphism in estradiol biosynthesis appear to be modulated by the presence of *GSTM1* and *GSTT1* in estrogen metabolic pathway, because breast cancer susceptibility is lower in carriers of *GSTM1* and *GSTT1*, independently of *CYP19A1* genotype. The results support the accepted hypothesis that estrogen formation and metabolism, which can be dependent of genetic and environmental variation, are determinant in defining breast cancer risk. Further studies are needed to evaluate the influence of the expression of these genes, especially in association with the expression of other known low penetrance genes in defining breast cancer risk.

Disclosure Statement

The authors declare that we have no conflict of interest.

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Chapter 8

Genetic Polymorphisms of Estrogen Receptor Alpha -397 PvuII (T>C) and -351 XbaI (A>G) in a Portuguese Population: Prevalence and Relation with Breast Cancer Susceptibility

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Genetic polymorphims of estrogen receptor alpha −397 PvuII (T>C) and −351 XbaI (A>G) in a portuguese population: prevalence and relation with breast cancer susceptibility

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Abstract Estrogen receptor alpha (ERα), that mediates the biologic effects of estrogen in estrogen-sensitive tissues like breast, is genetically polymorphic. To evaluate the association between −397 PvuII (T>C) and −351 XbaI (A>G) restriction fragment length polymorphisms (RFLPs) in intron 1 of ERα gene and susceptibility of breast cancer, we undertook a case–control study in BRCA1 185delAG and 5382insC/BRCA2 6174delT negative Portuguese women. The study population consisted of 107 patients with histological diagnosis of breast cancer and 121 women with no history of breast cancer. Genomic DNA was extracted from blood samples and genotyping analyses were performed by PCR–RFLP. XbaI polymorphism was associated with a significant reduced risk of breast cancer for carriers of the x allele in homozygozity (OR 0.178; 95 % CI 0.070–0.456; $P < 0.001$) or heterozigozity (OR 0.223; 95 % CI 0.089–0.561; $P = 0.001$). The PvuII polymorphism was associated with a non-significantly reduced risk. The combined analysis of PvuII and XbaI polymorphisms revealed none synergistic effect of the two genotypes, except for simultaneous carriers of pp and xx genotypes, that have a reduced risk of breast cancer (OR 0.226; 95 % CI 0.049–1.035; $P = 0.044$). The combination of PvuII and XbaI genotypes into haplotypes showed that carriers of two copies of the px (ppxx) haplotype had a reduced risk of breast cancer (OR 0.405; 95 % CI 0.194–0.843; $P = 0.014$), compared with PX (PPXX + PPXx + PpXX + PpXx) haplotypes. PvuII and XbaI polymorphisms were in linkage disequilibrium both in cases ($D = 0.044$, $r^2 = 0.049$, $X^2 = 5.216$, $P = 0.022$) and controls ($D = 0.090$, $r^2 = 0.139$, $X^2 = 16.819$, $P < 0.001$), but not in the entire sample population analyzed as a whole ($D = 0.087$, $r^2 = 0.0076$, $X^2 = 1.733$, $P = 0.188$). In conclusion, in this case–control study we found that ERα gene XbaI polymorphism may modify individual susceptibility for breast cancer in this population.

Keywords: Estrogen receptor alpha · Polymorphism · Breast cancer · Haplotype · Linkage disequilibrium

Introduction

Breast cancer is one of the major cancers affecting morbidity and mortality of women worldwide [37]. In Europe, breast cancer is the most common cancer affecting women, representing the leading cause of cancer dead between 35 and 55 years-old [26]. In Portugal it is responsible for the highest incidence and mortality rates among women diseases [39]. It is generally believed that initiation of breast cancer is a consequence of cumulative genetic damages leading to genetic alteration that result in activation of proto-oncogenes and inactivation of tumor suppressor genes, inducing uncontrolled cellular proliferation and/or aberrant programmed cell death [32]. Besides the genetic component, several endogenous and lifestyle risk factors have been associated with risk for the development of breast cancer. One of the most important determinants of risk for breast cancer is related to endogenous hormone levels, suggesting that genes in the estrogen pathway may

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influence breast cancer risk [8]. Estrogens have been clearly identified as carcinogens, by inducing aneuploidy and structural chromosomal changes [57] but the main effects of estrogen are thought to be via stimulation of breast-cell proliferation, that increases the chances of proliferation of a potentially mutated cell [25, 32, 35].

Estrogen receptors (ERs) mediate the biologic effects of estrogen in estrogen-sensitive tissues like breast, having an important role in growth and differentiation of normal mammary tissue, and also in the development of breast cancer [18, 45]. There are three types of estrogen receptors, the estrogen receptor alpha (ERα, also named ESR1), the estrogen receptor beta (ERβ, also named ESR2) and the G-protein coupled ER1 (GPER) [31]. The relative distribution of ERα and ERβ differs for different tissues, although there is some overlap. ERα is primarily present in the endometrium, the ovarian stroma, breast cancer cells, and is the most expressed ER in breast epithelium [15], while ERβ is present in the intestine, lung, bone marrow, brain and prostate [16]. The human ERα gene is located on chromosome 6q25.1, comprises eight exons separated by seven intronic regions, and spans more than 140 kilobases [13–15]. Several polymorphisms of ERα gene have been described and the most studied are the PvuII (T>C), rs2234693, and the XbaI (A>G), rs9340799, in intron 1, 397 bp and 351 bp upstream of exon 2, respectively [56, 59]. In practical terms, genotypes are distinguished by the absence or presence of PvuII or XbaI restriction sites: *PP* (*TT*) and *XX* (*AA*) indicate the absence of restriction sites; *pp* (*CC*) and *xx* (*GG*) correspond to presence of PvuII or XbaI restriction sites, respectively, on both alleles, and *Pp* (*TC*) and *Xx* (*AG*) represent heterozygotes. These two polymorphisms are separated by only 50 bp, and are in strong linkage disequilibrium. These polymorphisms have been associated with several gynecological diseases like breast cancer [6], endometrial cancer [55], endometriosis, adenomyosis and leiomyoma [27], with various estrogen-dependent characteristics such as the onset of menopause [54], lumbar spine bone mineral density, vertebral bone area and vertebral fracture risk in post-menopausal women [51], waist circumference [9], blood pressure [38], coronary reactivity [30] and lipid profile [34], and other pathological conditions, including cardiovascular disorders [29], venous thromboembolism [48], miscarriage [46], severe pre-eclampsia [33] or Alzheimer's disease [4]. Association of XbaI and PvuII polymorphisms in ERα gene and breast cancer risk have been described with conflicting results [5, 12, 24, 36, 45, 53]. Molecular mechanisms by which these polymorphisms influence receptor activity are still unclear. Initial reports had suggested an alteration in protein expression secondary to changes in mRNA splicing of PvuII gene [20]. As these are present in the non-coding region (activation domain) of gene, the structure of receptor protein should not be altered. Instead there is growing evidence that these SNPs affect the binding of transcription factors like myb to ERα gene leading to decreased expression of ERα receptor. It was also found that the presence of p allele resulted in >10-fold increase in a downstream reporter activity as compared to only 2.5-fold increase in activity with p allele; both sets of gene stimulated by transcription factor myb. However, whether these SNPs affect the quality or quantity of estrogen receptor alpha gene mRNA transcripts or protein expression remains to be established [19]. These SNPs (p and x alleles) seem to be associated with lower serum estradiol levels in postmenopausal women, by affecting the expression of one of the subtypes of 17β-hydroxysteroid dehydrogenase enzyme that catalyzes the transformation of estrone into estradiol [43]. It might also be possible that these SNPs are in linkage disequilibrium with another SNP in ERα gene that affects the expression of ERα. The aim of this study is to analyze the population distribution of ERα PvuII and XbaI genotypes, the linkage between these SNPs and the potential involvement of these polymorphic genes in breast cancer susceptibility in a population of Portuguese women. Several studies regarding the association of Erα gene polymorphisms and breast cancer are found in literature but this is the first work to report its prevalence and relation with breast cancer susceptibility in a Portuguese population, specifically from the Central Eastern part of Portugal. This population has specific characteristics that lead to its interest: it is more rural and more closed than populations from the littoral part of Portugal, and has Jewish ancestry roots in its foundation. Also, this work is in line with other recent publications from our group, which aim to characterize the genetics of breast cancer in this population.

Materials and methods

Study population

The studied population consisted of 228 Caucasian women from the same geographic area of residence, Central Eastern Portugal. A study group consisting of 107 women with histologically confirmed breast cancer, diagnosed at Child and Women Health Department, Gynecologic Oncology Division, Hospital Centre of Cova da Beira, Covilhã—Portugal, was enrolled between May 2008 and March 2009. A non-malignant control group, 121 healthy female blood donors with no previous history of cancer and without family histories of cancers, was also studied. Most of eligible controls were being treated for gastrointestinal or cardiovascular diseases. Women with amenorrhea, previous history of hysterectomy, oophorectomy or hormone-

related diseases such as thyroid disease, and systematic diseases such as diabetic mellitus or chronic liver disease were excluded from both groups. Were also excluded from both groups women with more than 85 and <18 years. Both cases and controls completed a questionnaire that assessed parameters such as age, family history of breast cancer, parity, ethnicity, reproductive and menstrual history, and history of breast diseases and procedures. Informed consent was obtained from both patients and controls before entering in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã—Portugal.

DNA extraction

Genomic DNA of all cases and controls was isolated from either frozen or fresh blood samples using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction, and stored at 4 °C.

BRCA1 and BRCA2 mutation analysis

BRCA1 185delAG and 5382insC, and BRCA2 6174delT genes mutations were analyzed by PCR–RFLP, using a methodology described elsewhere with slight modifications [1]. The primers used for BRCA1 185delAG analysis were 5′ TGA CTT ACC AGA TGG GAG AC 3′ (forward) and 5′ GAA GTT GTC ATT TTA TAA ACC TTT 3′(reverse). The primers used for BRCA1 5382insC analysis were 5′ GGG AAT CCA AAT TAC ACA GC 3′ (forward) and 5′ CCA AAG CGA GCA AGA GAA TCT C 3′ (reverse). For BRCA2 mutations we used the primers 5′ TGG GAT TTT TAG CAC AGC ACG 3′ (forward) and 5′ CTG GTC TGA ATG TTC GTT AC 3′ (reverse). Each PCR reaction mixture 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 Taq DNA polymerase (Promega, USA) and 200 ng of genomic DNA, using MyCycler Termal Cycler (Bio-Rad, Munich, Germany). Reaction mixtures were pre-incubated for 3 min at 94 °C. PCR conditions were 30 s at 94 °C, 30 s at 55 °C for BRCA1 mutations, or 30 s at 61 °C for BRCA2 mutation, and 30 s at 72 °C, for 30 cycles. The final extension was at 72 °C for 10 min. PCR produced products of 170 bp for 185delAG, 270 bp for 5382insC and 148 bp for 6174delT mutations. PCR products were digested by restriction endonucleases HinfI (185delAG), DdeI (5382insC) and BbrPI (6174delT) (Fermentas, Thermo Fisher Scientific, Canada) for 16 h. Digested products were analyzed on 5 % agarose gels with ethidium bromide. Heterozygous genotype exhibited fragments of 170, 150 and 20 bp for 185delAG, 214, 36 and 20 bp for 5382insC, and 127 and 21 bp for 6174delT mutations. 10 % of randomly selected samples were re-genotyped and all results were confirmed.

ERα polymorphism genotyping

Estrogen receptor alpha gene genotyping was performed using PCR–RFLP method modified from a procedure described earlier [5]. The primers used for analysis were 5′ CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3′(forward) and 5′ TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA 3′ (reverse). Briefly, each PCR reaction mixture was carried out in a total volume of 50 µL and contained 10 pmol of each primer, 3 mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Promega, USA) and 100 ng of genomic DNA, using MyCycler Termal Cycler (Bio-Rad, Munich, Germany). Reaction mixtures were pre-incubated for 3 min at 94 °C. PCR conditions were 45 s at 94 °C, 45 s at 61 °C and 2 min at 72 °C, for 36 cycles. The final extension was at 72 °C for 7 min. The amplified DNA fragment of 1.3 kb contains a part of intron 1 and exon 2 of ER alpha gene. The PCR product was digested by PvuII and XbaI restriction endonucleases for 16 h. Digested fragments were electrophoresed through 1.5 % agarose gels stained with ethidium bromide. Genotypes were distinguished by the absence or presence of PvuII or XbaI restriction sites: *PP* and *XX* indicate the absence of restriction sites; *pp* indicates the presence of PvuII restriction sites on both alleles, identified by two fragments (850 and 450 bp); *xx* indicates the presence of XbaI restriction sites on both alleles, identified by two fragments (900 and 400 bp). *Pp* and *Xx* represent heterozygotes. Results were confirmed by re-genotyping 10 % of randomly selected samples, and all the results were in agreement with the ones obtained previously.

Statistical analysis

Deviations from Hardy–Weinberg equilibrium (HWE) were assessed separately for cases and controls using a goodness-of-fit Chi square test with one degree of freedom. Chi square statistics were used to evaluate the differences in the distribution of allele types and genotypes in normal controls and breast cancer patients. Odds ratio (ORs) and 95 % confidence intervals (95 % CI) were calculated as estimates of relative risk, using logistic regression analysis. *P* values <0.05 were considered statistically significant. For the PvuII and XbaI genotype, women with the PP and XX genotype respectively, served as reference. Many measures of linkage disequilibrium have been proposed but here we used the mathematical model described by (Hill and Robertson [21]) because of its robustness to sample

size of the population. PvuII/XbaI haplotypes were inferred from genotype frequencies. Calculations were based on the maximum likelihood model [7] and the SEM algorithm [49]. The combination of the PvuII and XbaI genotypes into haplotypes was defined by the sum of genotypes frequencies as PX (PPXX + PPXx + PpXX + PpXx), Px (PPxx + Ppxx), pX (ppXX + ppXx) and px (ppxx). Linkage disequilibrium resulting from the non-random association of the genotypes was assessed by Chi square analysis. We estimated linkage disequilibrium coefficients D and r^2. r^2 is assumed to be the most relevant measure for association analysis because there is an inverse relation between this coefficient and the sample size required to detect association between susceptibility loci and SNPs [52]. Statistical analysis was performed with computer software SPSS (version 19.0).

Results

Genotyping was successfully performed in all cases and controls. PP and XX, that indicate the absence of restriction sites, are both identified by presence of one fragment with 1,300 bp; pp indicates the presence of PvuII restriction sites on both alleles, identified by two fragments (850 and 450 bp); xx indicates the presence of XbaI restriction sites on both alleles, identified by two fragments (900 and 400 bp). Pp and Xx heterozygous genotypes are identified by presence of all the fragments (Fig. 1). There were no major differences between cases and controls with regard to the general demographic characteristics or major known risk factors for breast cancer such as age at menarche, age at first full term pregnancy, number of full term pregnancies or menopausal status (Table 1). The majority of the study participants were postmenopausal (69.2 % of cases and 62.0 % of controls). Compared to controls, breast cancer cases had more often a body mass index (BMI) above 25 kg/m^2 (58.9 % of cases and 46.3 % of controls), and increased risk for breast cancer (OR = 1.662; 95 % CI = 0.983–2.810). Also, the risk of breast cancer was higher for women with first degree family history of breast cancer (OR = 2.440; 95 % CI = 2.071–2.876) and for women with history of benign breast disease (OR = 1.864; 95 % CI = 1.100–3.158). The mean age at diagnosis of the cases was 62.2 years (SD = 12.2). Cases and controls included in the study were analyzed for mutations in the BRCA1 (185delAG and 5382insC) and BRCA2 (6174delT) genes and were confirmed not to carry these mutations.

Genotypes distribution of ERα PvuII and XbaI polymorphims in cases and controls is summarized in Table 2. There was no significant difference in the prevalence of p allele among cases (82.2 %) and controls (86.8 %, OR 0.706; CI 0.343–1.454, $P = 0.343$). Approximately 26.2 % of the cases and 37.2 % of the controls were homozygous for this allele, and 56.1 % of the cases and 49.6 % of the

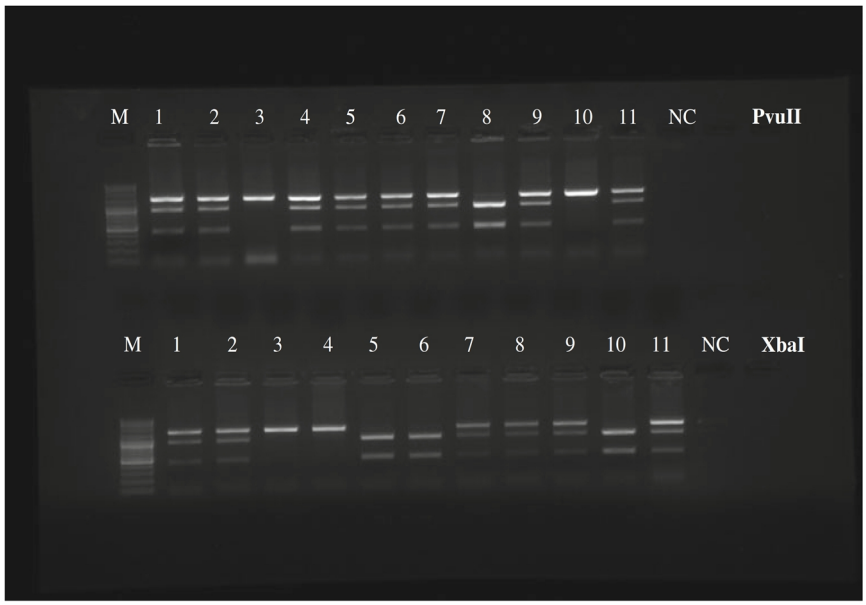

Fig. 1 PCR–RFLP 2 % agarose gel stained with bromidium ethide representing the genotypes observed in this study. Legend: *M* molecular weight marker; PvuII 1, 2, 4, 5, 6, 7, 9 and 11—Pp genotype; PvuII 3 and 10—PP genotype; PvuII 8—pp genotype; XbaI 1, 2, 7, 8, 9 and 11—Xx genotype; XbaI 3 and 4—PP genotype; XbaI 5, 6 and 10—xx genotype; *NC* negative control

Table 1 Selected characteristics of the population studied

	Controls, *n* (%)	Cases, *n* (%)	OR (95 % CI)
Total	121 (100)	107 (100)	
Age at menarche			
≤12 years	57 (47.1)	65 (60.7)	1.0
13–14 years	41 (33.9)	27 (25.2)	0.577 (0.316–1.054)
≥15 years	23 (19.0)	15 (14.0)	0.572 (0.272–1.200)
Age at first full term pregnancy			
≤25 years	51 (53.1)	42 (39.2)	1.0
26–30 years	19 (19.8)	22 (20.6)	1.406 (0.673–2.939)
≥31 years	26 (27.0)	19 (17.8)	0.887 (0.432–1.821)
Number of full term pregnancies			
Nulliparous	25 (20.7)	24 (22.4)	1.0
1	19 (15.7)	28 (26.2)	1.535 (0.684–3.444)
2	45 (37.2)	31 (29.0)	0.718 (0.348–1.479)
3+	32 (26.4)	24 (22.4)	0.781 (0.362–1.688)
Menopausal status			
Premenopausal	46 (38.0)	33 (30.8)	1.0
Postmenopausal	75 (62.0)	74 (69.2)	1.375 (0.793–2.385)
First degree family history of breast cancer			
No	121 (100)	84 (78.5)	1.0
Yes	0 (0)	23 (21.5)	2.440 (2.071–2.876)
History of benign breast disease			
No	74 (61.2)	49 (45.8)	1.0
Yes	47 (38.8)	58 (54.2)	1.864 (1.100–3.158)
BMI			
≤25	65 (53.7)	44 (41.1)	1.0
>25	56 (46.3)	63 (58.9)	1.662 (0.983–2.810)

controls were heterozygous. We did not find any significant variation of breast cancer risk associated with p allele carriers in heterozygosity (OR 0.842; 95 % CI 0.396–1.792; $P = 0.655$) or homozygosity (OR 0.468; 95 % CI 0.205–1.068; $P = 0.069$), with reference to PP genotype. Concerning XbaI polymorphism, x allele was significantly more prevalent among controls (94.2 %) than cases (76.6 %; OR 0.201; 95 % CI 0.083–0.488; $P < 0.001$).

When we stratified the breast cancer cases by some clinical-pathologic tumor characteristics listed in Table 3, we did not find significant differences between genotype frequencies distribution in any group of patients with some specific tumor feature.

Women with the Xx and xx genotype had a reduced risk of breast cancer (OR 0.223; 95 % CI 0.089–0.561; $P = 0.001$ and OR 0.178; 95 % CI 0.070–0.456; $P < 0.001$) compared to women with the XX genotype. The distribution of genotype frequencies was in HWE in the control group, as well as in cases (Table 4).

Table 2 Frequency distribution of ERα polymorphisms PvuII and XbaI in cases and controls

	Cases, *n* (%)	Controls, *n* (%)	OR	95 % CI	*P* value
PP	19 (17.8)	16 (13.2)	1.0		
Pp/pp	88 (82.2)	105 (86.8)	0.706	0.343–1.454	0.343
Pp	60 (56.1)	60 (49.6)	0.842	0.396–1.792	0.655
pp	28 (26.2)	45 (37.2)	0.468	0.205–1.068	0.069
XX	25 (23.4)	7 (5.8)	1.0		
Xx/xx	82 (76.6)	114 (94.2)	0.201	0.083–0.488	<0.001
Xx	47 (43.9)	59 (48.8)	0.223	0.089–0.561	0.001
xx	35 (32.7)	55 (45.4)	0.178	0.070–0.456	<0.001

PvuII and XbaI polymorphisms were in linkage disequilibrium both in cases ($D = 0.044$, $r^2 = 0.049$, $X^2 = 5.216$, $P = 0.022$) and controls ($D = 0.090$, $r^2 = 0.139$, $X^2 = 16.819$, $P < 0.001$). When the entire sample population was analyzed, linkage equilibrium was not rejected ($D = 0.087$, $r^2 = 0.0076$, $X^2 = 1.733$, $P = 0.188$) (Table 5). The combined analysis of PvuII and XbaI polymorphisms (Table 6) revealed a synergistic effect of pp and xx genotypes combination, as simultaneous carriers of these genotypes may have a reduced risk for breast cancer (OR 0.226; 95 % CI 0.049–1.035; $P = 0.044$). The combination of the PvuII and XbaI genotypes into haplotypes (Table 7) showed that PX haplotype seems to be slightly more prevalent in cases (54.2 %) than in controls (43.0 %), while px haplotype was more prevalent in controls (25.6 %) than in cases (13.1 %). Carriers of two copies of the px (ppxx) haplotype had a reduced risk of breast cancer (OR 0.405; 95 % CI 0.194–0.843; $P = 0.014$), compared with PX (PPXX + PPXx + PpXX + PpXx) haplotypes.

Discussion

ERα role as a hormone-dependent transcriptional regulator has attracted attention to the association of ERα genetic polymorphisms and the development of numerous diseases, including breast cancer. Since estrogens actions are mediated by assembly with estrogen receptors, and estrogens are critical in female breast development, interindividual differences in breast cancer susceptibility may be due to ERα genetic variation. Among the several ERα polymorphisms that have been identified, possible associations of PvuII and XbaI polymorphisms with breast cancer risk are the most studied. In this study we conducted a population based case–control study in a subset of Portuguese women in order to analyze the possible association of risk of developing breast cancer and polymorphisms in ERα. We

Table 3 Association of ER alpha gene PvuII and XbaI polymorphisms with tumor characteristics in the studied population

Parameters	Cases, *n* (%)	PvuII polymorphism			XbaI polymorphism		
		PP, *n* (%)	Pp, *n* (%)	pp, *n* (%)	XX, *n* (%)	Xx, *n* (%)	Xx, *n* (%)
Estrogen receptor status							
Negative	16 (14.9)	2 (1.9)	11 (10.3)	3 (2.8)	2 (1.9)	7 (6.5)	7 (6.5)
Positive	91 (85.0)	15 (14.0)	54 (50.5)	22 (20.6)	15 (14.0)	44 (41.1)	32 (29.9)
OR		1.000	0.942	0.997	1.000	0.978	0.930
95 % CI			0.767–1.156	0.796–1.250		0.796–1.201	0.741–1.167
P value			0.604	0.982		0.836	0.562
Progesterone receptor status							
Negative	39 (36.4)	7 (6.5)	24 (22.4)	8 (7.5)	8 (7.5)	17 (15.9)	14 (13.1)
Positive	68 (63.6)	10 (9.3)	41 (38.3)	17 (15.9)	9 (8.4)	34 (31.8)	25 (23.4)
OR		1.000	1.072	1.156	1.000	1.259	1.211
95 % CI			0.691–1.663	0.715–1.868		0.773–2.052	0.730–2.008
P value			0.747	0.542		0.309	0.432
c–erb–B2							
Negative	91 (85.0)	14 (13.1)	56 (52.3)	21 (19.6)	15 (14.0)	46 (43.0)	30 (28.0)
Positive	16 (14.9)	3 (2.8)	9 (8.4)	4 (3.7)	2 (1.9)	5 (4.7)	9 (8.4)
OR		1.000	0.785	0.907	1.000	0.833	1.962
95 % CI			0.238–2.586	0.232–3.548		0.178–3.908	0.473–8.134
P value			0.693	0.888		0.818	0.327
Histological type							
Invasive ductal	94 (87.8)	16 (14.9)	56 (52.3)	22 (20.6)	14 (13.1)	44 (41.1)	36 (33.6)
Invasive lobular	8 (7.5)	0 (0.0)	6 (5.6)	2 (1.9)	1 (0.9)	5 (4.7)	2 (1.9)
Others	5 (4.7)	1 (0.9)	3 (2.8)	1 (0.9)	2 (1.9)	2 (1.9)	1 (0.9)
P value			0.425	0.478		0.454	0.365
Differentiation grade (bloom)							
G1	31 (29.0)	6 (5.6)	17 (15.9)	8 (7.5)	5 (4.7)	14 (13.1)	12 (11.2)
G2	58 (54.2)	9 (8.4)	37 (34.6)	12 (11.2)	8 (7.5)	28 (26.2)	22 (20.6)
G3	18 (16.8)	2 (1.9)	11 (10.3)	5 (4.7)	4 (3.7)	9 (8.4)	5 (4.7)
P value			0.717	0.781		0.820	0.593
Axillary lymph node status							
Negative	96 (89.7)	17 (15.9)	58 (54.2)	21 (19.6)	15 (14.0)	45 (42.1)	36 (33.6)
Positive	11 (10.3)	0 (0.0)	7 (6.5)	4 (3.7)	2 (1.9)	6 (5.6)	3 (2.8)
OR		1.000	0.892	0.840	1.000	1.000	0.654
95 % CI			0.820–0.971	0.708–0.997		0.222–0.4496	0.120–3.565
P value			0.157	0.083		1.000	0.623

selected cases and controls from a single institution that only accepts patients from the same geographic area, to avoid potential biases. Also, patients and controls were Caucasian and matched by ages. Genotypes distribution of ERα PvuII and XbaI polymorphisms in the studied population in this work is comparable to those found in other Caucasian populations [36, 53]. We found a significant reduced risk of breast cancer in women carriers of xx genotype compared to women with XX genotype. No variation of breast cancer risk associated with PvuII genotypes was established, although it has been reported that p allele is associated with increased levels of androstenedione, what suggests that women carrying this allele would have an increased risk for breast cancer [58]. We also investigated the impact of the combination of PvuII and XbaI genotypes in breast cancer development risk. Except for pp/xx carriers, which seem to have a reduced risk, combinations of PvuII and XbaI polymorphisms did not influence the risk of breast cancer. Previously, our group has demonstrated in the same population the increase of breast cancer risk associated with other polymorphisms in low penetrance genes, GSTM1 and GSTT1 null, alone or

Table 4 Hardy–Weinberg equilibrium analysis

	ERα Polymorphism	Genotype	Observed, *n* (%)	Expected, *n* (%)	
Total sample	PvuII	PP	35 (15.4)	$p \times p \times n = 40$ (17.5)	Var allele freq = 0.58
		Pp	120 (52.6)	$2 \times p \times q \times n = 111$ (48.5)	$X^2 = 1.560$
		pp	73 (32.0)	$q \times q \times n = 78$ (34.1)	*P* value = 0.212
	XbaI	XX	32 (14.0)	$p \times p \times n = 32$ (14.0)	Var allele freq = 0.63
		Xx	106 (46.5)	$2 \times p \times q \times n = 107$ (46.7)	$X^2 = 0.008$
		xx	90 (39.5)	$q \times q \times n = 90$ (39.3)	*P* value = 0.930
Cases	PvuII	PP	19 (17.7)	$p \times p \times n = 22$ (20.7)	Var allele freq = 0.54
		Pp	60 (56.1)	$2 \times p \times q \times n = 53$ (50.0)	$X^2 = 1.794$
		pp	28 (26.2)	$q \times q \times n = 31$ (29.2)	*P* value = 0.180
	XbaI	XX	25 (23.4)	$p \times p \times n = 22$ (20.6)	Var allele freq = 0.55
		Xx	47 (43.9)	$2 \times p \times q \times n = 53$ (49.5)	$X^2 = 1.385$
		xx	35 (32.7)	$q \times q \times n = 32$ (29.9)	*P* value = 0.239
Controls	PvuII	PP	16 (13.2)	$p \times p \times n = 18$ (14.7)	Var allele freq = 0.62
		Pp	60 (49.6)	$2 \times p \times q \times n = 57$ (46.7)	$X^2 = 0.329$
		pp	45 (37.2)	$q \times q \times n = 47$ (38.5)	*P* value = 0.566
	XbaI	XX	7 (5.8)	$p \times p \times n = 11$ (9.1)	Var allele freq = 0.70
		Xx	59 (48.8)	$2 \times p \times q \times n = 51$ (42.1)	$X^2 = 2.995$
		xx	55 (45.4)	$q \times q \times n = 59$ (48.8)	*P* value = 0.083

Table 5 Classification of the women in the study according to both PvuII and XbaI

		XX	Xx	xx	Linkage disequilibrium	*P* value
All	PP	9 (3.9 %)	20 (8.8 %)	6 (2.6 %)	D = 0.087	0.188
	Pp	18 (7.9 %)	63 (27.6 %)	39 (17.1 %)	$r^2 = 0.0076$	
	pp	5 (2.2 %)	23 (10.1 %)	45 (19.7 %)	$X^2 = 1.733$	
Cases	PP	6 (5.6 %)	10 (9.3 %)	3 (2.8 %)	D = 0.044	**0.022**
	Pp	15 (14.0 %)	27 (25.2 %)	18 (16.8 %)	$r^2 = 0.049$	
	pp	4 (3.7 %)	10 (9.3 %)	14 (13.1 %)	$X^2 = 5.216$	
Controls	PP	3 (2.5 %)	10 (8.3 %)	3 (2.5 %)	D = 0.090	**<0.001**
	Pp	3 (2.5 %)	36 (29.7 %)	21 (17.3 %)	$r^2 = 0.139$	
	pp	1 (0.8 %)	13 (10.7 %)	31 (25.6 %)	$X^2 = 16.819$	

Bold value indicates statistically significant *P* values

in combination [40], and CYP19 TC/CC, alone or in combination with GSTM1 and GSTT1 null [41]. We did not found, in this study, variation of breast cancer risk associated with any combination of ERα, CYP19 and GSTs polymorphisms (data not shown). ERα gene PvuII and XbaI genetic polymorphisms and their relation with breast cancer risk was investigated in several previous studies, with inconsistent conclusions (Table 8). A recent case–control study in a Pakistani population found that heterozygous genotype of XbaI was 45 % higher than wild type in cancerous cases. Also, the menopausal women carriers of heterozygous or homozygous mutants of PvuII or XbaI genotypes were strongly correlated with breast cancer [24]. In another recent study among Chinese women, association of risk of breast cancer and fibrocystic breast conditions and PvuII and XbaI polymorphisms in ERα gene were examined but was not detected any relation between these genotypes and development of the disease [42]. Shin et al. [45] conducted a hospital-based case–control study to evaluate the association between the XbaI and PvuII genotypes and breast cancer risk in a population of Korean women. In this report, the PvuII genotype distribution did not show any difference between cases and controls, but carriers of XbaI X allele seemed to have a decreased breast cancer risk compared with xx genotype. These findings are not in accordance with ours, as x allele was significantly more prevalent among controls than cases. Thus, in our population, carriers of xx genotype had a reduced risk of breast cancer compared to women with the XX genotype. Similarly, we did not find any significant variation of breast

Table 6 Breast cancer risk in relation to PvuII and XbaI combination genotypes

	Cases, *n* (%)	Controls, *n* (%)	OR	95 % CI	*P* value
PP/XX	6 (5.6)	3 (2.5)	1.0		
PP/Xx	10 (9.3)	10 (8.3)	0.500	0.097–2.577	0.404
PP/xx	3 (2.8)	3 (2.5)	0.500	0.060–4.153	0.519
Pp/XX	15 (14.0)	3 (2.5)	2.500	0.389–16.049	0.326
Pp/Xx	27 (25.2)	36 (29.7)	0.375	0.086–1.636	0.180
Pp/xx	18 (16.8)	21 (17.3)	0.429	0.094–1.964	0.267
pp/XX	4 (3.7)	1 (0.8)	2.000	0.150–26.734	0.597
pp/Xx	10 (9.3)	13 (10.7)	0.385	0.077–1.929	0.238
pp/xx	14 (13.1)	31 (25.6)	0.226	0.049–1.035	**0.044**

Bold value indicates statistically significant *P* values

cancer risk associated with any PvuII genotype. In another study in a Chinese population where PvuII and XbaI polymorphisms were analyzed concerning breast cancer development, Pp and pp genotypes of PvuII polymorphism were associated with an increased risk of breast cancer, comparing to PP genotype, while Xx and xx genotypes were associated with a non-significant elevated risk [5]. Onland-Moret et al. [36] reported a non-significant increased risk of breast cancer in women with PvuII pp genotype compared to women with the PP genotype, while XbaI genotypes were not associated with breast cancer risk. A population-based case–control study was conducted in a subset of Chinese women to examine the association of several genetic polymorphisms and breast cancer risk [44]. In this study, the authors found a non-significant risk of breast cancer associated with PvuII pp and XbaI xx genotype, and the association seemed to be stronger among women with a family history of breast cancer. They also found a putative joint effect on the breast cancer risk between CYP1A1 MspI and ERα XbaI genotypes, between CYP1A1 MspI and ERα PvuII genotypes, and among all the three genotypes together. Hu et al. [22] also found increased risk in premenopausal women carriers of xx or pp genotypes, when they studied 114 cases with early-onset breast cancer or affected relatives and 121 healthy controls. A possible reason for these contradictory findings may remain in the multiple biologic processes where estrogens, and estrogen receptors, are involved. In fact, PvuII and XbaI polymorphisms of ERα have been signaled as possible markers for numerous human diseases, including several types of cancers, neurodegenerative, cardiovascular, immunological, gynecological diseases, and also endocrine disorders. Furthermore, how breast cancer is affected by the intronic PvuII and XbaI polymorphism of the ERα influences the receptor function is still unclear. The position of the polymorphisms in an intron, near the gene promoter, may have an impact on the expression of other genes by influencing mRNA transcription and/or stability of those genes [3, 10]. Also, some introns contain regulatory sequences such as enhancers that affect the levels of expression through transcriptional regulation [2]. Regarding PvuII polymorphism, it was shown that p allele results in a potential Myb transcription factors binding site, what can possibly enhance ERα transcription in carriers of p allele, compared to women with the p allele [19]. However, it is not clear how the higher transcription of ERα gene affects ERα protein because the additional myb binding site can either amplify the transcription of the gene or produce ERα isoforms with different properties [36]. One study has suggested that women who carried PP genotype had earlier onset of menopause than carriers of pp genotype [54]. Concerning XbaI polymorphism, it was suggested that age of menarche was delayed in women with the XX genotype [47]. These findings are consistent with the epidemiologic characteristics of breast cancer, because the age of menarche and the age of menopause are related with cumulative lifetime estrogen exposure, so ERα polymorphisms may influence breast cancer development by regulating ages of menarche and/or menopause. In our study, there were no differences in the distribution of genotypes by age of menarche or menopause. Linkage equilibrium may also explain the observations of associations between these SNPs and breast cancer risk. Intronic polymorphisms may be in linkage disequilibrium with exonic variations, which could affect ER protein function [28]. PvuII and XbaI polymorphisms can also be in linkage disequilibrium with other polymorphisms in the ERα gene, like TA tandem polymorphism in the promoter region [50, 55], or be linked with alterations in another unidentified gene adjacent to ERα gene [28]. Linkage disequilibrium may also partly explain the differential distribution of these SNPs among different populations and discrepancies in results of these polymorphisms on breast cancer risk. Distribution of PvuII and XbaI polymorphisms differs significantly between European, Asian and African populations. Generally, Asian populations show an increased frequency of Px haplotype, and a reduced frequency of PX haplotype when compared to European populations [17, 28], and it was reported that px haplotype is present in lower frequency in African populations [51]. However, divergences in haplotypes distribution are found among several studies. Commonly, pX haplotype is not found or found only in a small percentage of individuals in several populations. Though, in the population studied in this work, pX haplotype was found in 13.1 % of cases and 11.6 % of controls (12.3 % in the whole population). This value, despite higher than the majority of the studies, is similar to what was found in other Caucasian populations of European ancestry [23]. We observed linkage disequilibrium of PvuII and XbaI polymorphisms in both cases

Table 7 Distribution of haplotypes in the studied population

	Cases, *n* (%)	Controls, *n* (%)	Whole population, *n* (%)	OR	95 % CI	*P* value
PX	58 (54.2)	52 (43.0)	110 (48.2)	1.0		
Px	21 (19.6)	24 (19.8)	45 (19.7)	0.784	0.391–1.572	0.493
pX	14 (13.1)	14 (11.6)	28 (12.3)	0.897	0.391–2.056	0.796
px	14 (13.1)	31 (25.6)	45 (19.7)	0.405	0.194–0.843	**0.014**

Bold value indicates statistically significant *P* values

Table 8 Summary of case–control studies performed in XbaI and PvuII polymorphisms and risk of breast cancer

Population	Cases (*n*)	Controls (*n*)	Effect of XbaI polymorphism in breast cancer risk	Effect of PvuII polymorphism in breast cancer risk	Reference
Korean	205	205	XX and Xx: decreased risk	None	[45]
Chinese	1069	1166	Xx and xx: non–significant elevated risk	Pp and pp: increased risk	[5]
Dutch	380	422	None	Pp: increased breast cancer risk	[36]
Chinese	282	298	xx: non–significant increased risk	pp: non–significant increased risk	[44]
Chinese	114	121	xx: non–significant increased risk in premenopausal women	pp: increased risk in premenopausal women	[22]
Pakistani	100	100	Xx and xx: increased risk	Pp and pp: increased risk	[24]
Chinese	614	879	None	None	[42]
Portuguese	107	121	Xx and xx: decreased risk	None	This study

and controls, but not when cases and controls were analyzed as a single population. Incomplete disequilibrium may be due to recombination or multiple mutations which have occurred between, or at, these two polymorphic sites. Multiple demographic and genetic events like natural selection, mutation, random drift, genetic hitchhiking, or gene flow, may contribute to create substantial levels of linkage disequilibrium in any given population. Thus, the inconsistency of haplotypes frequencies throughout different populations may be explained to some extent by the differential patterns of linkage disequilibrium in those populations [11]. In conclusion, in this case–control study we found a significant reduced risk of breast cancer in women carriers of xx genotype; we found no association of breast cancer risk in relation to PvuII genotypes, except for simultaneous carriers of xx and pp genotypes, that were also less susceptible for breast cancer. Thus, the results observed lead us to propose that ERα gene XbaI polymorphism may modify individual susceptibility for breast cancer in this population. Further studies with larger samples sizes are needed to clarify the role of ERα in the etiology of breast cancer.

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Conflict of interest We declare that we have no conflict of interest.

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Chapter 9

A Multigenic Model Based in Low Penetrance Genes to Evaluate Breast Cancer Risk in a Portuguese Population

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A multigenic model based in low penetrance genes to evaluate breast cancer risk in a Portuguese population

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SUMMARY

This study evaluated the association between polymorphisms in low penetrating genes of the estrogen biosynthetic pathway, the estrogen metabolic pathway, DNA damage signaling and repair pathway, and estrogenic response, with breast cancer susceptibility in a Portuguese population. We determined CYP17A1 T27C, CYP19A1 codon 39 Trp/Arg, CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GTSM1/GSTT1 deletion, GSTP1 Ile105Val, MTHFR C677T, TP53 Arg72Pro, ER alpha XbaI and ER alpha PvuII genotypes of 107 patients with histological diagnosis of breast cancer BRCA1/BRCA2 negative, and 121 women with no history of breast cancer. The results obtained show that common polymorphisms in low penetrating genes may modulate and predict breast cancer risk among individuals in this population.

INTRODUCTION

Breast cancer is one of the most frequently diagnosed cancers in the Western world and a significant cause of mortality worldwide. In Europe, breast cancer is the most common cancer affecting women, representing the leading cause of cancer dead between 35 to 55 years-old (Jemal et al., 2004). In Portugal, it presents the highest incidence and mortality rates among women diseases (Pinheiro et al., 2003). Both non-genetic and genetic risk factors are involved in the etiology of breast cancer. While rare alterations in tumor suppressor genes dramatically raise cancer risk, far more common and less intense differences in low penetrance genes may be responsible for a relatively small, but rather frequent increase of cancer risk among individuals. Breast cancer susceptibility related polymorphisms can be found in several biologic pathways, like

estrogen and carcinogen detoxification metabolic pathways, cell cycle, apoptosis, cell signalling, growth factors and receptors molecules, cell adhesion, angiogenesis, DNA damage signalling and DNA repair (Eccles and Tapper, 2010). Inter-individual differences in estrogens biosynthetic and metabolic pathways may define subpopulations of women with higher lifetime exposure to hormone-dependent growth promotion or to cellular damage from estrogens or their metabolites. Fast synthesizers and poor metabolizers are more exposed to the formation of carcinogen-DNA adducts and/or mutations, what confers them higher susceptibility to complex genetic disorders such as cancer. Also, genetic variations in genes that codify ERα may modulate a portion of the cancer susceptibility associated with reproductive events and hormone exposure (Ayoub et al., 2011). Our work aimed to evaluate the association between polymorphisms in low penetrating genes of estrogen biosynthetic pathway (CYP17A1 T27C and CYP19A1 codon 39 Trp/Arg), estrogen metabolic pathway (CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GTSM1/GSTT1 deletion genotypes, GSTP1 Ile105Val, MTHFR C677T), DNA damage signaling and repair pathway (TP53 Arg-72Pro), and estrogenic response (ER alpha XbaI and PvuII), with breast cancer susceptibility in a Portuguese population. These polymorphisms were already shown to modulate breast cancer susceptibility in several populations, despite some conflicting results (Setiawan et al., 2007; Ramalhinho et al., 2011, Ramalhinho et al., 2012, Healey et al., 2000; Economopoulos et al., 2010; Yao et al., 2010; Jakubowska et al., 2012; Onland-Moret et al., 2005; He et al., 2011).

MATERIAL AND METHODS

A study group consisting of 107 women with histologically confirmed breast cancer was diagnosed and enrolled in the study at Child and Women Health Department, Gynecologic Oncology Division, Hospital Centre of Cova da Beira, Covilhã – Portugal. A non-malignant control group, 121 healthy female blood donors with no previous history of cancer and without family histories of cancers, was also studied. Informed consent was obtained from both patients and controls before entering in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã - Portugal. Genomic DNA of all cases and controls was isolated from either frozen or fresh blood samples and genotyping was performed using PCR-based methods.

RESULTS

Besides the results presented in the tables, we also found that the two-way combination of GSTM1 and GSTT1 null genotypes resulted in eightfold increase for breast cancer risk (OR = 8.287; 95% CI = 3.124-21.980; P= 0.0001) and the three-way combination of GSTP1 105AA/AG and null genotypes for both GSTM1 and GSTT1 resulted in fivefold increase for breast cancer risk (OR = 5.040; 95% CI = 1.392–18.248; P = 0.016) (Ramalhinho et al., 2011). Also, CYP19A1 arginine

Gene	Genotype	Cases (n, %)	Controls (n, %)	OR	95% CI	P value
CYP17A1	A1/A1	73 (68.2)	83 (68.6)	1.0		
	A1/A2	30 (28.0)	30 (24.8)	1.137	0.627-2.063	0.673
	A2/A2	4 (3.7)	8 (6.6)	0.568	0.164-1.966	0.367
CYP19A1	TT	40 (39.6)	65 (53.7)	1.0		
	TC/CC	61 (60.4)	56 (46.3)	1.770	1.036 – 3.024	0.036
COMT	Val/Val	17 (15.9)	10 (8.3)	1.0		
	Val/Met	72 (67.3)	98 (81.0)	0.432	0.187-0.999	0.060
	Met/Met	18 (16.8)	13 (10.7)	0.814	0.283-2.346	0.704
CYP1A1	TT	88 (82.2)	98 (81.0)	1.0		
	TC/CC	19 (17.8)	23 (19.0)	0.920	0.470-1.802	0.808
CYP1B1	Val/Val	10 (9.3)	12 (9.9)	1.0		
	Val/Leu	86 (80.4)	104 (85.9)	0.992	0.409-2.408	0.986
	Leu/Leu	11 (10.3)	5 (4.1)	2.640	0.685-10.181	0.154
GSTM1	Present	31 (36.5)	61 (59.8)	1.0		
	Null	54 (63.5)	41 (40.2)	2.592	1.432 – 4.690	0.002
GSTT1	Present	48 (56.5)	84 (82.4)	1.0		
	Null	37 (43.5)	18 (17.6)	3.597	1.849 – 6.999	0.0001
GSTP1	Ile/Ile	39 (45.9)	48 (47.1)	1.0		
	Ile/Val + Val/Val	46 (54.1)	54 (52.9)	1.103	0.611 – 1.989	0.765
MTHFR	Ala/Ala	34 (31.8)	50 (41.3)	1.0		
	Ala/Val	65 (60.7)	64 (52.9)	1.494	0.857-2.604	0.156
	Val/Val	8 (7.5)	7 (5.8)	1.681	0.557-5.069	0.353

Tab. 1 - Association between polymorphisms in genes involved in biosynthesis and metabolism of estrogens and breast cancer.

Gene	Genotype	Cases (n, %)	Controls (n, %)	OR	95% CI	P value
TP53	Arg/Arg	26 (25.7)	54 (44.6)	1.0		
	Arg/Pro	37 (36.7)	38 (31.4)	2.022	1.055-3.878	0.033
	Pro/Pro	38 (37.6)	29 (24.0)	2.721	1.389-5.332	0.003

Tab. 2 - Association between TP53 genotypes and breast cancer.

Gene	Genotype	Cases (n, %)	Controls (n, %)	OR	95% CI	P value
ER alpha	PP	19 (17.8)	16 (13.2)	1.0		
	Pp	60 (56.1)	60 (49.6)	0.842	0.396-1.792	0.655
	pp	28 (26.2)	45 (37.2)	0.468	0.205-1.068	0.069
ER alpha	XX	25 (23.4)	7 (5.8)	1.0		
	Xx	47 (43.9)	59 (48.8)	0.223	0.089-0.561	0.001
	xx	35 (32.7)	55 (45.4)	0.178	0.070-0.456	<0.001

Tab. 3 - Association between ERα genotypes and breast cancer.

allele in homozygosity or heterozygosity (TC/CC) was associated with a significant increased risk for breast cancer when associated to GSTM1 null genotype (OR 6.158; 95% CI=2.676-14.171; P<0.001) and GSTT1 null genotype (OR 4.870; 95% CI=2.216-10.700; P<0.001). The three-way combination of CYP19A1 TC/CC, GSTM1 null and GSTT1 null polymorphism was related with significant increased risk for breast cancer (OR 11.429; 95% CI=3.590-36.385; P<0.001) (Ramalhinho et al., 2012). In addition to homozygous and heterozygous carriers of x allele in ERα gene, simultaneous carriers of pp and xx genotypes had a significant reduced risk of breast cancer (OR 0.223; 95% CI 0.089-0.561; P = 0.001).

	CYP17A1	CYP19A1	GSTM1	GSTT1	GSTP1	COMT	CYP1A1	CYP1B1	MTHFR	ERα PvuII	ERα XbaI	TP53
CYP17A1	No											
CYP19A1	No	**Yes**										
GSTM1	No	**Yes**	**Yes**									
GSTT1	No	**Yes**	**Yes**	**Yes**								
GSTP1	No	No	**Yes**	**Yes**	No							
COMT	No	No	No	No	No	No						
CYP1A1	No	No	No	No	No	No	No					
CYP1B1	No	No	No	No	No	No	No	No				
MTHFR	No	No	No	No	No	No	No	No	No			
ERα PvuII	No	No	No	No	No	No	No	No	No	No		
ERα XbaI	No	No	No	No	No	No	No	No	No	**Yes**	**Yes**	
TP53	No	No	No	No	No	No	No	No	No	No	No	**Yes**

Tab. 4 - Summary of the genetic associations with breast cancer.

CONCLUSIONS

We found an increased breast cancer risk associated with the mutant variants of TP53 and CYP19A1 genes. CYP19A1 arginine allele (TC or CC) was associated with a significant increased risk for breast cancer when associated with GSTM1 null genotype and GSTT1 null genotype, as were GSTM1 and GSTT1 null genotypes, alone, both combined or combined with GSTP1 genotypes. Regarding ERα gene, carriers of x allele, and simultaneous carriers of pp and xx genotypes in ERα gene had a significant reduced risk of breast cancer. In conclusion, common polymorphisms in low penetrating genes may modulate and predict breast cancer risk among individuals in this population.

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Chapter 10

Independence of Low Penetrating Genes in Risk for Breast Cancer Estimated by Modified Gail Model

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INDEPENDENCE OF LOW PENETRATING GENES IN RISK FOR BREAST CANCER ESTIMATED BY MODIFIED GAIL MODEL

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ABSTRACT

Cancer risk prediction models provide an important approach to assess risk and susceptibility by identifying individuals at high risk. Conventional breast cancer risk models were based on cumulative estrogen exposure and mutations in high-penetrance genes. However, mutations in high-penetrance genes have been detected in less than 5% of overall breast cancer patients. Several genome-wide association studies reported many novel breast cancer predisposing single nucleotide polymorphisms in low-penetrance genes. Our study aimed to find out any association between polymorphisms in low penetrating genes of estrogen biosynthetic pathway (CYP17A1 T27C and CYP19A1 codon 39 Trp/Arg), of estrogen metabolic pathway (CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GTSM1/GSTT1 of deletion genotypes, GSTP1 Ile105Val, MTHFR C677T), DNA damage signaling and repair pathway (TP53 Arg72Pro), and of estrogenic response (ER alpha XbaI and PvuII), and breast cancer risk predicted by Gail model. No statistically significant differences in single genotype distribution, or in the distribution of combinations with other risk-associated genotypes was detected, between low or high risk scored women by Gail Model. Although, the simultaneous carriage of two (OR 2.194; 95% CI 1.043-4.612; P=0.037), three (OR 5.370; 95% CI 2.504-11.515; P>0.001), four (OR 13.650; 95% CI 4.432-42.037; P>0.001) or five (OR 5.370; 95% 1.761-149.461; P=0.002) risk genotypes were more prevalent in breast cancer affected women than in unaffected women and was it was found to be significantly associated to breast cancer development. In conclusion, combinations of two or more polymorphisms in low penetrating genes in the estrogen biosynthesis, estrogen metabolism, DNA damage signaling and repair, and estrogenic response pathways are associated to the development of breast cancer, and may be defined as an independent risk factor of risk predicted by Gail model.

KEY-WORDS

Breast cancer, Gail Model, polymorphisms, risk

INTRODUCTION

Both non-genetic and genetic risk factors are involved in the etiology of breast cancer. In the past decades high-penetrance genes (for example, BRCA1, BRCA2, PTEN and TP53) have been identified to be associated with familiar breast cancer (Walsh et al., 2006). However, these genes account for less than 5% of overall breast cancer patients and most of the risk is likely to be attributable to more low-penetrance genetic variants (Antoniou et al., 2002; Antoniou and Easton, 2006). Recently, several genome-wide association studies (GWAS) reported many novel breast cancer predisposing single nucleotide polymorphisms (SNPs) (Easton et al., 2007). Breast cancer susceptibility related polymorphisms can be found in several biologic pathways, like estrogen and carcinogen detoxification metabolic pathways, cell cycle, apoptosis, cell signaling, growth factors and receptors molecules, cell adhesion, angiogenesis, DNA damage signaling and DNA repair (Eccles and Tapper, 2010).

Cancer risk prediction models provide an important approach to assess risk and susceptibility by identifying individuals at high risk. Conventional breast cancer risk models include the cumulative estrogen exposure data such as age, age at menarche and menopause, age at first live birth, and use of HRT for risk calculation, since estrogens are the main risk factor for mammary carcinogenesis (Chang, 2011). The most widely known and commonly used model for breast cancer risk assessment is the Gail model (Gail et al., 1989). This model was initially designed in 1989, and constructed using data that were collected as part of the Breast Cancer Detection and Demonstration Project, a nested case–control study of almost 300 000 women, It was validated by the Nurses' Health Study (Spiegelman et al., 1994) and was subsequently modified in 1999 (Costantino et al., 1993). It is a risk-assessment model that focuses primarily on non-genetic risk factors, with limited information on family history (Evans and Howell, 2007). Both the original and the modified versions of the Gail model use six breast cancer risk factors, namely age, hormonal or reproductive history (age at menarche and age at first live birth), previous history of breast disease (number of breast biopsies and history of atypical hyperplasia), and family history (number of first-degree relatives with breast cancer). The Gail model is the only one model that has been validated by three large population-based databases (Cummings et al., 2009). The major limitation of the Gail model is the inclusion of only first-degree relatives, which results in underestimating risk in the 50% of families with cancer in the paternal lineage and also takes no account of the age of onset of breast cancer (Evans and Howell, 2007).

In the present study we investigated the prevalence of polymorphisms in low penetrating genes of the estrogen biosynthetic pathway (CYP17A1 T27C and CYP19A1 codon 39 Trp/Arg), of the estrogen metabolic pathway (CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GTSM1/GSTT1 deletion, GSTP1 Ile105Val and MTHFR C677T), of the DNA damage signaling and repair pathway (TP53 Arg72Pro), and of the estrogenic response (ER alpha XbaI and ER alpha PvuII genotypes) in 121 women with no history of breast cancer, stratified by 5-year relative risk for developing breast cancer determined by Modified Gail Model score.

MATERIALS AND METHODS

Study Population

The studied population consisted of 121 Caucasian women from the same geographic area of residence, Central Eastern Portugal, enrolled in Hospital Centre of Cova da Beira, Covilhã – Portugal, between May 2008 and March 2009. The healthy female blood donors had no previous history of cancer, no family histories of cancers and were unaffected for BRCA1 185delAG and 5382insC / BRCA2 6174delT mutations. Most of eligible individuals were being treated for gastrointestinal or cardiovascular diseases. Women with amenorrhea, previous history of hysterectomy, oophorectomy or hormone-related diseases such as thyroid disease, and systematic diseases such as diabetic mellitus or chronic liver disease, more than 85, or less than 18 years were excluded. Every individual woman completed a questionnaire that assessed demographic and clinical data such as age, family history of breast cancer, parity, ethnicity, reproductive and menstrual history, and history of breast diseases and procedures. Informed consent was obtained before entering in the study. The study was approved by the Institutional Review Board of Hospital Centre of Cova da Beira, Covilhã - Portugal.

DNA Extraction

Genomic DNA of all cases and controls was isolated from either frozen or fresh blood samples using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction, and stored at 4°C.

Genotyping

Genotyping of all genes was performed using PCR-based methods. The genotyping method used for GSTM1, GSTT1, GSTP1, CYP19A1 and ERα were previously described elsewhere by our group (Ramalhinho et al. 2011; Ramalhinho et al., 2012; Ramalhinho et al., 2013).

COMT gene Val158Met genotyping

COMT gene Val158Met genotyping was performed using PCR-RFLP method with the following primers: 5'-GCCCGCCTGCTGTCACC-3' (forward) and 5'-CTGAGGGGCCTGGTGATAGTG-3' (reverse). Briefly, each PCR reaction mixture was carried out in a total volume of 50 µL and contained 10 pmol of each primer, 1.5mM of $MgCl_2$, 100 nM of each deoxynucleotide triphosphate, 1 unit of Taq DNA polymerase (Promega, USA) and 100 ng of genomic DNA, using MyCycler *Termal Cycler (Bio-Rad*, Munich, Germany). Reaction mixtures were pre-incubated for 2 minutes at 94°C. PCR conditions were 30 seconds at 94°C, 30 seconds at 59°C and 45 seconds at 72°C, for 30 cycles. The final extension was at 72°C for 2 minutes. The amplified

DNA fragment had 210 bp. The PCR product was digested by NlaIII (Fermentas) restriction endonuclease for 16 hours. Digested fragments were electrophoresed through 5% agarose gels stained with 0.5 µg/mL ethidium bromide. Genotypes were distinguished by the pattern of fragments created by the digestion. Homozygous LL (Met/Met) genotype was identified by creation of digestion fragments with 114, 36 and 35 bp; homozygous wild type HH (Val/Val) genotype was identified by fragments of 96, 36, 35 and 18 bp; heterozygous HL (Val/Met) genotype was defined by presence of all fragments.

CYP1B1 gene Val432Leu genotyping

CYP1B1 gene Val432Leu genotyping was performed by PCR-RFLP. The reaction mixtures composition was similar to the described for COMT genotyping. The primers used for analysis were 5'-TCACTTGCTTTTCTCTCTCC-3' (forward) and 5'-AATTTCAGCTTGCCTCTTG-3' (reverse). Reaction mixtures were pre-incubated for 1 minute at 94°C. PCR conditions were 30 seconds at 94°C, 30 seconds at 60°C and 45 seconds at 72°C, for 35 cycles. The final extension was at 72°C for 7 minutes. The amplified DNA fragment had 650bp. The PCR product was digested by Eco571I (Fermentas) restriction endonuclease for 16 hours. Digested fragments were electrophoresed through 3% agarose gels stained with 0.5 µg/mL ethidium bromide. 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.

MTHFR C677T genotyping

MTHFR C677T genotyping was also performed using PCR-RFLP method. Reaction mixtures composition was similar to the described for COMT genotyping. The primers used were 5'-TGAAGGAGAAGGTGTCTGGGGGA-3' AND 5'-AGGACGGTGCGGTGAGAGTG-3'. Reaction mixtures were pre-incubated for 2 minutes at 94°C. PCR conditions were 30 seconds at 94°C, 30 seconds at 61°C and 1 minute at 72°C, for 30 cycles. The final extension was at 72°C for 2 minutes. The amplified DNA fragment had 198 bp. The PCR product was digested by 1 U of HinfI (Fermentas) restriction endonuclease for 16 hours. Digested fragments were electrophoresed through 3% agarose gels stained with 0.5 µg/mL ethidium bromide. 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.

TP53 Arg72Pro genotyping

Arg72Pro SNP of TP53 gene was genotyped by PCR-RFLP using primers previously described (Lin et al. 2008). The primers used were 5'-TTGCCGTCCAAGCAATGGATGA-3' (forward) and 5'-TCTGGGAAGGGACAGAAGATG-3' (reverse). Reaction mixtures composition was similar to the described for COMT genotyping. The reaction was performed by the following conditions: 94°C

for 5 min; 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min; and 72°C for 5 min. The amplified PCR product of 199 bp was digested using 1 unit of restriction enzyme BstUI (New England Biolabs) and incubating at 60°C for 12h. DNA fragments were electrophoresed through a 2.5% agarose gel and stained with ethidium bromide. The Pro72Pro genotype had one fragment with 199 bp, the heterozygous genotypes presents 3 bands with 199, 113 and 86 bp and the Arg72Arg formed 113 and 86 bp.

CYP17A1 T27C genotyping

CYP17A1 genotyping was performed, as described in detail elsewhere (Carey et al., 1994). Briefly, a fragment containing the T27C substitution was amplified with the following primers: 5'-CATTCGCACTCTGGAGTC-3' and 5'-AGGCTCTTGGGGTACTTG-3'. Reaction mixtures composition was similar to the described for COMT genotyping. An initial denaturation step was run at 94°C for 5 min, followed by 30 cycles of amplification with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min. One unit of MspA1 restriction enzyme was used to digest the PCR fragments at 37°C for 2 h. Fragments were separated by gel electrophoresis and stained with ethidium bromide to identify the base pair substitution.

The CYP1A1 gene Ile462Val genotypes were determined by using a polymerase chain reaction–restriction fragment length polymorphism method (PCR–RFLP), as detailed in Ye et al., 2008. The primer sequences used were 5′-GATCTGAGTTCCTACCTGA-3' and 5′-TAGCGTCCAAGAGAAAGACCTCCCAGCGCTCAA-3′. The 25 µl reaction mix for each PCR product comprised 25–50 ng of genomic DNA, 25 mM $MgCl_2$, 10× PCR Buffer, 0.5 pM of each primer, 2.5 mM of each dNTP and 1 U Taq polymerase. An initial denaturation step was run at 94°C for 5 min, followed by 35 cycles of amplification with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min. Digestion of PCR products was accomplished with 2 U of HincII (New England Biolabs) endonuclease for 16 h at 37°C following the supplier's directions for buffer conditions.

All results were confirmed by re-genotyping 10% of the samples.

Breast Cancer Risk Prediction

Five-year relative risk values were obtained for all women included in the control group using Gail Model Risk Assessment Tool computer software from the National Cancer Institute (USA), modified by the National Surgical Adjuvant Breast and Bowel Project (NSABP) for the Breast Cancer Prevention Trial (Wolmark et al., 2001). For comparative analysis, the cut point we established for the Gail scores was at 1.6% risk over 5 years, which is the average 5-year risk of 60-year old women.

Statistical analysis

Logistic regression method was used to access odds ratio (ORs) and 95% confidence intervals (95% CI) as estimates of relative risk conferred by a particular putative risk factor. x^2 test and Fisher's exact test were used when appropriate. Calculations were done using computer software SPSS for Windows (version 16.0). Calculation of Chi-square test for deviation from Hardy-Weinberg equilibrium was performed using STATA (Version 8.0). P values less than 0.05 were considered statistically significant.

RESULTS

The characteristics of the subjects included in this study are presented in Table 1. We scored by Gail Model 35 women as high risk (five year predicted risk ≥ 1.6%) and 86 women as low-risk (five year predicted risk < 1.6%). Menopausal status ($p<0.001$), first degree family history ($p<0.001$) of breast cancer and history of benign breast disease ($p=0.002$) were consistently, differentially distributed between the low risk and high risk scored women. We found no major differences between unaffected and breast cancer affected women with regard to the general demographic characteristics or major known risk factors for breast cancer such as age at menarche, age at first full term pregnancy, number of full term pregnancies or menopausal status, as described early (Ramalhinho et al., 2012). The genotype distribution in the women scored by Gail Model is outlined in Table 2. Affected and unaffected individuals included in the study were analyzed for mutations in the BRCA1 (185delAG and 5382insC) and BRCA2 (6174delT) genes and were confirmed not to carry these mutations. For the studied genoptypes, no statistically significant differences were reported between low or high risk scored women by Gail Model.

In previous reports our group demonstrated that GSTM1 and GSTT1 null genotypes, CYP19A1 arginine allele in homozygosity or heterozygosity (TC/CC) and TP53 proline allele in homozygosity or heterozygosity (Arg/Pro and Pro/Pro) were associated with a significant increased risk for breast cancer. Also, homozygous and heterozygous carriers of x allele in ERα gene, and simultaneous carriers of pp and xx genotypes had a significant reduced risk of breast cancer (Ramalhinho et al., 2011; Ramalhinho et al., 2012; Ramalhinho et al., 2013). In this work we studied the cumulative effects of these six SNPs on breast cancer in the population of affected patients by the disease and in the unaffected group of women (Table 3). We found that women affected by breast cancer had significant prevalence of more than one risk genotype when compared to women not affected by breast cancer. The simultaneous carriage of two (OR 2.194; 95% CI 1.043-4.612; P=0.037) to five (OR 5.370; 95% 1.761-149.461; P=0.002) risk genotypes was found to be significantly associated to breast cancer development. Three (OR 5.370; 95% CI 2.504-11.515; P>0.001), four (OR 13.650; 95% CI 4.432-42.037; P>0.001) and five concomitant risk genotypes were more prevalent in breast cancer affected women than in those unaffected women. We did not found simultaneous carriers of the six risk genotypes in both breast cancer affected and unaffected group of women. In unaffected group of women scored by Gail model, for studied risk genotypes, no statistical association was found between high risk and low risk scores.

Table 1 Selected characteristics of the population studied

	Affected, n (%)	Unaffected, n (%)	Low risk (<1.6), n (%)	High risk (≥1.6), n (%)	OR (95% CI)[a]	P value
Total	107 (100)	121 (100)	86 (71.1)	35 (28.9)		
Age at menarche						
≤ 12 years	65 (60.7)	61 (50.4)	42 (48.8)	19 (54.3)	1.0	
13-14 years	27 (25.2)	40 (33.1)	32 (37.2)	8 (22.9)	0.553 (0.215-1.422)	0.256
≥ 15 years	15 (14.0)	20 (16.5)	12 (13.9)	8 (22.9)	1.474 (0.518-4.194)	0.586
Age at first full term pregnancy						
≤ 25 years	42 (39.2)	73 (60.3)	60 (69.8)	13 (37.1)	1.0	
26-30 years	22 (20.6)	14 (11.6)	8 (9.3)	6 (17.1)	3.462 (1.026-11.684)	0.071
≥ 31 years	19 (17.8)	3 (2.5)	1 (0.8)	2 (5.7)	9.231 (0.778-109.591)	0.098
Number of full term pregnancies						
Nulliparous	24 (22.4)	26 (21.5)	18 (32.1)	8 (22.9)	1.0	
1	28 (26.2)	11 (9.1)	8 (9.3)	3 (8.5)	0.844 (0.176-4.042)	0.832
2	31 (29.0)	40 (33.1)	29 (33.7)	11 (31.4)	0.853 (0.289-2.523)	0.774
3+	24 (22.4)	44 (36.4)	31 (36.0)	13 (37.1)	0.944 (0.329-2.710)	0.914
Menopausal status						
Premenopausal	33 (30.8)	36 (29.8)	34 (39.5)	2 (5.7)	1.0	
Postmenopausal	74 (69.2)	85 (70.2)	52 (60.5)	33 (94.3)	10.788 (2.428-47.932)	<0.001
First degree family history of breast cancer						
No	84 (78.5)	109 (90.1)	86 (100)	23 (65.7)	1.0	
Yes	23 (21.5)	12 (9.9)	0 (0)	12 (34.3)	4.739 (3.296-6.813)	<0.001
History of benign breast disease						
No	49 (45.8)	112 (92.6)	84 (97.7)	28 (80.0)	1.0	
Yes	58 (54.2)	9 (7.4)	2 (2.3)	7 (20.0)	10.500 (2.060-53.517)	0.002
BMI						
≤ 25	44 (41.1)	78 (64.5)	57 (66.3)	21 (60.0)	1.0	
> 25	63 (58.9)	43 (35.5)	29 (33.7)	14 (40.0)	1.310 (0.583-2.947)	0.513

[a] Odds Ratio and 95% Confidence Interval, low risk vs high risk

Table 2 Distribution of the studied polymorphisms in high and low risk women scored by Modified Gail Model

Gene	Genotype	Low risk (<1.6), n (%)	High risk (≥1.6), n (%)	OR[a]	CI[b]	P
CYP17A1	A1/A1	57 (66.3)	26 (74.3)	1.0		
	A1/A2 + A2/A2	29 (32.7)	9 (25.7)	0.680	0.282-1.640	0.390
CYP19A1	TT	45 (52.3)	20 (57.1)	1.0		
	TC + CC	41 (47.7)	15 (42.9)	0.823	0.373-1.817	0.630
CYP1A1	TT	72 (83.7)	26 (74.3)	1.0		
	TC+CC	14 (16.3)	9 (25.7)	1.780	0.689-4.603	0.230
CYP1B1	Val/Val	7 (8.1)	5 (14.3)	1.0		
	Val/Leu + Leu/Leu	79 (91.9)	30 (85.7)	0.532	1.157-1.805	0.325
GSTM1	Present	53 (61.6)	22 (62.9)	1.0		
	Null	33 (38.4)	13 (37.1)	0.949	0.421-2.137	0.899
GSTT1	Present	68 (79.1)	18 (51.4)	1.0.		
	Null	18 (20.9)	6 (17.1)	1.259	0.436-3.635	0.669
GSTP1	Ile/Ile	41 (47.7)	16 (45.7)	1.0		
	Ile/Val + Val/Val	45 (52.3)	19 (54.3)	1.082	0.492-2.380	0.845
TP53	Pro/Pro	21 (24.4)	9 (25.7)	1.0		
	Pro/Arg	23 (26.7)	15 (42.9)	1.522	0.551-4.205	0.417
	Arg/Arg	42 (48.8)	11 (31.4)	0.611	0.219-1.703	0.344
ER alpha	PP	7 (8.1)	3 (8.6)	1.0		
	Pp	50 (58.1)	16 (45.7)	0.747	0.173-3.231	0.695
	pp	29 (33.7)	16 (45.7)	1.287	0.292-5.677	0.738
	XX	4 (4.6)	3 (8.6)	1.0		
	Xx	43 (50.0)	16 (45.7)	0.496	0.100-2.465	0.401
	xx	39 (45.4)	16 (45.7)	0.547	0.110-2.726	0.665

[a] Odds ratio [b] 95% Confidence Interval

Table 3 Cumulative effects of associated SNPs on the risk of breast cancer

	Affected n (%)	Unaffected n (%)	OR[a]	CI[b]	P	Low risk (<1.6), n (%)	High risk (≥1.6), n (%)	OR[a]	CI[b]	P
Counts of risk genotypes*										
0 to 1	16 (14.9)	52 (43.0)	1.0			35 (40.7)	17 (48.6)	1.0		
2	27 (25.2)	40 (33.0)	2.194	1.043-4.612	0.037	30 (34.9)	10 (28.6)	1.457	0.580-3.659	0.493
3	38 (35.5)	23 (19.0)	5.370	2.504-11.515	<0.001	16 (18.6)	7 (20.0)	1.110	0.384-3.206	0.847
4	21 (19.6)	5 (4.1)	13.650	4.432-42.037	<0.001	4 (4.6)	1 (2.8)	1.943	0.201-18.742	0.560
5	5 (4.7)	1 (0.8)	16.250	1.761-149.461	0.002	1 (1.2)	0 (0.0)	1.486	1.229-1.796	0.488
6	0 (0.0)	0 (0.0)	-	-	-	0 (0.0)	0 (0.0)	-	-	-

* CYP19A1, GSTM1, GSTT1, ER alpha PvuII, ER alpha XbaI and TP53

DISCUSSION

We studied the association between polymorphisms and breast cancer risk predicted risk by Gail model, and we found no significant differences in the genotype distribution or in allele frequency in high or low risk women.

Breast cancer risk prediction models are not a diagnostic tool but provide an estimate of likelihood of developing the disease in the future. A well-evaluated risk model, taking genetic and clinical risk factors together, can be used as a screening tool for high risk individuals among the general population. Several authors have tried to improve breast cancer risk models by adding single-nucleotide polymorphisms to original breast cancer risk models (Gail, 2009; Mealiffe et al., 2010; Dai et al., 2012; Darabi et al., 2012). The evidence suggests that SNPs can be used to improve the prediction models. However, there is a number of bias across all studies. For us, the most important limitation is concerned with several genome-wide association studies reporting a lot of different breast cancer predisposing single nucleotide. This number of breast cancer risk-associated SNPs turns difficult to include all in the analyses and to assess all into a putative breast cancer risk model. In addition to the breast cancer risk-associated SNPs, other breast cancer associated risk factors should also be included in the prediction models. In the case of the Modified Gail model, it only accounts with age at menarche, number of previous breast biopsies, presence of atypical hyperplasia on biopsy, age at first childbirth and number of first-degree relatives with a history of breast cancer. As Gail model includes only first-degree relatives, this may underestimate risk associated with cancer in the paternal lineage, so larger family history of breast cancer should be also be considered. The model also takes no account of the age of onset of breast cancer, nor the menopausal status or the body mass index, and these risk factors, associated to SNPs, could provide a more accurate and reliable model to weigh breast cancer risk. The same is true for other environmental risk factors like exogenous hormones use, cigarette and alcohol consumption, physical exercise, nutritive habits, and others, all these data is difficult to evaluate and to assess. In our results, menopausal status, first degree family history of breast cancer and history of benign breast disease were differentially distributed between low risk and high risk scored women by Gail Model, however menopausal status is not a risk factor considered by Gail Model. Recently, a study to examine the associations of endogenous estrogens and testosterone with breast cancer risk in high- and low-risk women, determined by the Gail model, reported that estrone sulfate was statistically associated with breast cancer risk among women with Gail predicted risks <1.66% and ≥ 2.52%. Also, estradiol seemed more strongly associated with breast cancer in women with higher Gail predicted risk compared with women with lower risk, despite the lack of statistical significance. This data suggests, in agreement with several other authors, that higher levels of endogenous estrogens and testosterone are associated with increased breast cancer risk, independently of predicted risk (Eliassen et al., 2006).

In our study, although no statistically significant differences were reported in the single genotype distribution between low or high risk scored women by Gail Model, we found that

women affected by breast cancer had significant prevalence of more than one risk genotype when compared to women not affected by breast cancer. The simultaneous presence of three, four and five risk genotypes was more prevalent in affected women than in unaffected women by breast cancer, but we could not found significant statistical association between high risk or low risk scores calculated by Gail Model.

In conclusion, our limited series of cases may affect our statistical power and underestimate eventually weak associations with breast cancer risk. However, we surely demonstrated that combinations of two or more polymorphisms of low penetrating genes in the estrogen biosynthesis, estrogen metabolism, DNA damage signaling and repair, and estrogenic response pathways should be considered important factors of risk for breast cancer development in an independent way of Gail Model, at least in this population. Thus, we consider that exists a real need to genotype the women in this population as they may be classified as "high risk" according to the polymorphisms based model presented in this work, while by Gail Model they would be classified as "low risk". So, the combination of these two approaches in assessing breast cancer risk would include women in breast cancer clinical screening programs that could be excluded if only Gail Model was used to access their risk. Similar studies in different populations are needed to access the value of SNPs in defining breast cancer risk.

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Chapter 11

Establishment of Cell Cultures Conditions for in vitro assays with 17Beta-Estradiol: Potential of Dermal Fibroblasts as Models of Study of Responses to Estrogens

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ESTABLISHMENT OF CELL CULTURE CONDITIONS FOR IN VITRO ASSAYS WITH 17BETA-ESTRADIOL: POTENTIAL OF DERMAL FIBROBLASTS AS MODELS OF STUDY OF RESPONSES TO ESTROGENS

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ABSTRACT

Fibroblasts represent a multifaceted, complex group of cells, which have been considered mostly unexciting until the last few years. Dermal fibroblasts have been found to express several hormone receptors, including ERα and ERβ, and it is well known that estrogens play an important role in skin homeostasis, as well in carcinogenesis. Our aim is to propose human dermal fibroblasts as models of study of estrogens action. This work establishes conditions of culture for cells that will be used for in vitro assays with 17beta-estradiol and establishes a protocol of isolation of primary human dermal fibroblasts from skin explants for its putative use as models of study of response to estrogens. NHDF and MCF7 cell lines were stimulated with 17β-estradiol in different conditions and cellular viability was evaluated by MTT assay and flow cytometry was used to determine if cells were viable, apoptotic or necrotic. Furthermore, to evaluate the responsiveness of human dermal fibroblasts to estrogens, GSTM1 expression was analyzed by Real-time PCR. Also, primary cultures of human dermal fibroblasts were established and characterized in terms of estrogen receptors existence and fibroblast specificity by fluorescence microscopy. We found that cells should be maintained in culture medium with constant supplementation of FBS during *in vitro* experiments with 17β-estradiol, in order to guarantee the results reproductability. Exposure to high concentrations of 17β-estradiol led to a decrease in NHDF and MCF7 cell viability but that effect could be reversed after 48 hours of recovery of the cells in culture. Regarding flow cytometry analysis, we found higher rate of cell death by necrosis than early apoptosis for NHDF and MCF7 cells not stimulated with 17β-estradiol, while after stimulation with intermediate concentrations of 17β-estradiol, we found that this tendency was reversed. With real-time PCR experiments we proved that normal human dermal fibroblasts express GSTM1 and that this expression is regulated by 17β-estradiol. Finally, we successfully established a method for isolation of human dermal fibroblasts from an explant of dermal tissue. Our preliminary results indicate that human dermal fibroblasts are responsive to estrogens *in vitro*, and thus they can be used as models of study of estrogenic response.

Key-words: fibroblasts, skin, 17β-estradiol

INTRODUCTION

Sex steroid hormones are involved in regulation of skin development and functions as well as in some skin pathological events. As demonstrated by the changes seen in the skin of post-menopausal women, estrogens play an important role in skin homeostasis (Stevenson and Thornton, 2007). However, despite the knowledge that estrogens have important effects on skin, and estrogen receptors (ERs) have been detected in the skin, the cellular and subcellular sites and mechanisms of estrogen action are still poorly understood (Tsui et al., 2011). Classically, estrogen action is mediated through estrogen receptors (ER), the estrogen receptor alpha (ERα, also named ESR1), the estrogen receptor beta (ERß, also named ESR2) and the G-protein coupled ER1 (GPER) (Maggiolini and Picard, 2010) but estrogenic action is also performed in non-genomic and ER-independent manners, by selective estrogen receptor modulators (SERMs) for example (Tsui et al, 2011). Post-menopausal skin has been shown to have increased dryness (Sator et al., 2004), decreased elasticity and increased wrinkling (Henry et al., 1997; Sumino et al., 2004), and estrogen receptor expression has been shown to be reduced after menopause (Punnonen et al., 1980; Nelson and Bulun, 2001). Breast cancer is a multifactorial disease caused by complex inherited and environmental factors and although several risk factors for the development of breast cancer have been identified, the molecular mechanisms related to breast carcinogenesis remain unclear. However, it is assumed that initiation of breast cancer, as of other cancers, is a consequence of cumulative genetic damages that lead to genetic alterations, resulting in activation of proto-oncogenes and inactivation of tumor suppressor genes (Mitrunen and Hirvonen, 2003). Estrogens have been clearly identified has carcinogens, and most of the risk factors for breast cancer relate to the increased or prolonged exposure to estrogen. Stimulation of breast-cell proliferation has been proposed as the main effect of estrogens in breast carcinogenesis, because as more rapidly cells proliferate, the hypothesis of acquiring a potentially cancer-causing mutation is higher. Besides cell proliferation, estrogen also induces aneuploidy and structural chromosomal changes (Zhu and Conney, 1998). Furthermore, metabolic by-products of estrogens are responsible for free-radical-mediated DNA damage, single-strand breaks, estrogen–DNA adducts formation, protein oxidation and lipid peroxidation, what triggers genetic instability and cellular damage (Mueck and Seeger, 2007). In postmenopausal breast cancer patients, an intra-tumoral production of estrogens occurs as a result of aromatization of androgens into estrogens and is catalyzed by the cytochrome P450 aromatase enzyme (Sasano and Harada, 1998). Breast cancer is composed of both parenchymal or carcinoma cells and stromal cells. Tumor stroma consists of fibroblasts, adipocytes, inflammatory cells such as lymphocytes and macrophage and lymphatic and blood capillaries including pericytes and endothelial cells. Despite these complicated architecture of breast cancer tissue environment, the most of the research has been directed toward that in carcinoma or parenchymal cells. However, tumor microenvironment has been recently identified as a major factor influencing treatment resistance of cancer to radiotherapy and chemotherapy (Miki et

al., 2012). Also, it is also well known that tumor microenvironment plays a pivotal role in neoplastic cell initiation or tumorigenesis, progression/ development, and metastatic spread of tumor cells (Carmeliet and Jain, 2000; Bhowmick et al., 2004; Siemann, 2010). Several studies indicate that stromal fibroblasts surrounding normal and cancerous breast epithelium exert a modulatory effect on the epithelium, the nature of which is dependent upon the state of the fibroblasts and the epithelium. Specifically, stromal fibroblasts in normal breast (NAF – normal breast associated fibroblasts) serve a protective function and exert inhibitory signals on the growth of normal epithelium, while cancer-associated stromal fibroblasts (CAF) act more permissively and allow or promote growth of normal and cancer epithelium, which suggest that the ability of fibroblasts to inhibit epithelial cell proliferation detected in normal human breast tissue is lost during the process of breast carcinogenesis (Sadlonova et al., 2009). Therefore, tumor/cancer stromal cells have been considered not only as a mere physical supporting cell of the parenchymal or carcinoma cells but also functional or regulatory cells in tumor/cancer microenvironment. Consequently, endocrine, autocrine and paracrine interactions between parenchymal and stromal cells are considered pivotal for breast carcinogenesis and malignancy such as metastasis, proliferation, and angiogenesis in breast cancer microenvironment. These findings above all indicated that it is required to establish the *in vitro* system that can examine this important carcinoma/stromal cell interaction in human malignancies (Hawsawi et al., 2008; Sadlonova et al., 2009).

Fibroblasts are cells with origin in mesodermal layer and one of their main functions is to produce collagen fibers (Doljanski, 2004). In classical cell biology, fibroblasts were considered as a population of cells relatively inert and uninteresting. This vision was dramatically changed, and currently they are recognized as a central component of tissue biology (Sorrell and Caplan, 2009), that have important roles in structural and physiological regulation of tissues (Mine et al, 2008). Fibroblasts are a heterogeneous and dynamic population of cells that synthetize and release a collection of precursors of the extracellular matrix (ECM), particularly fundamental substance, collagen, reticulin and elastin, that contribute for structural support of tissues (Kanitakis, 2002; Sorrell and Caplan, 2009). Fibroblasts also participate in paracrine and autocrine interactions in skin, that are particularly important in different steps of wound healing (Darby and Hewitson, 2007; Werner et al., 2007), in inflammatory response by secretion of cytokines (Sorrell and Caplan, 2009), in angiogenesis by production of angiogenic growth factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF) (Newman et al., 2011). Although the first cell cultures were established for the first time near 100 years ago, nowadays they are a tool frequently used in several fields of research. Fibroblast cultures have raised interest due to the ease with which they are obtained by biopsy of the skin, which constitutes the main source of fibroblasts, and to the fact that after isolation and establishment of cultures, fibroblasts present rapid and continuous proliferation in presence of serum, when compared to other cell types that require additional growth factors (Takashima, 2001). *In vitro* cultures of fibroblasts present

heterogenic genetic expression profiles, depending on their provenience from distinct anatomic locals, based in the metabolic differences between fibroblasts from different tissues. These topographic differences are maintained throughout cellular passages, what means that fibroblasts present positional memory (Chang et al, 2002). Besides the heterogeneity attributable to anatomic positioning, fibroblasts of a same tissue do not establish a homogeneous population, so it becomes important that fibroblasts with origin in different parts of the body, and in different layers of the same tissue, are faced as distinct cellular types (Sorrell and Caplan, 2009; Chang et al, 2002). Concerning dermal fibroblasts, they can be classified in two subpopulations, according to their positioning on the papilar dermis or the reticular dermis. These two populations present phenotypic differences that are revealed in the production and organization of ECM, the production of growth factors and cytocines, and in the participation of inflammatory responses (Sorrell and Caplan, 2009). The main attraction of using dermal fibroblasts as an *in vitro* model is associated with the expression of several specific receptors for hormones and neurotransmitters. Dermal fibroblasts have been found to express several hormone receptors, including ERα and ERß, GPER, parathyroid hormone receptor/ peptide-related with parathyroid receptor (PTHR/PTHrPR), thyroid-stimulating hormone receptor (TSHR), type 1 corticotropin-releasing hormone receptor (CRH-1R), melanocortin 1 receptor (MC1R), melatonin 1 receptor (melatonin-1R), serotonin receptors, also known as 5-hydroxytryptamine receptors or 5-HT receptors (5-HTR), growth hormone receptor (GHR), androgen receptor (AR), retinoid X receptor type alpha (RXRα) (Zouboulis, 2004; Tsui et al, 2011).

The main goal of this work is to explore the use of dermal fibroblasts cultures as models of study of responses to estrogens. Cellular viability of NHDF and MCF7 cell lines after exposure to different concentrations of 17-ß-estradiol was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] assay. Propidium iodide (PI) in conjunction to Annexin V staining was used to determine if cells were viable, apoptotic or necrotic through cytometry flow analysis. To evaluate the responsiveness of human dermal fibroblasts to estrogens, GSTM1 expression was analyzed by Real-time PCR. Also, primary cultures of human dermal fibroblasts were established and characterized by fluorescence microscopy.

MATERIAL AND METHODS

Cell lines

MCF-7 breast cancer cell line (ATCC) was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG) and 1% antibiotic/antimycotic (10,000 units/ mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B) (Sigma Aldrich). Normal human dermal fibroblasts (NHDF) cell line (ATCC) was maintained in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% FBS, HEPES (0.01 M) (Sigma Aldrich), L-glutamine (0.02 M) (Sigma Aldrich), sodium pyruvate (0.001 M) (Sigma Aldrich) and 1% antibiotic/antimycotic. Both cell lines were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed with cells between third and ninth passages.

Stimulation with 17ß-estradiol

Approximately 2×10^4 cells were seeded in 24-well plates. Cells were exposed to three different concentrations of 17ß-estradiol (Sigma-Aldrich) 0, 1, 10, 50 and 100 nM in vehicle 0.5% DMSO (Sigma Aldrich), and to four incubation times for each hormone dosage (12, 24, 48 and 96 hours). Vehicle did not induce any alteration to cell culture (data not shown). Three different protocols of FBS supplementation of cell culture medium were performed: without supplementation with 10% FBS during stimulation with E_2, but with supplementation with 10% FBS in the recovery phase (after removal of E_2) (Protocol 1); without supplementation with 10% FBS during stimulation with E_2 and during the recovery phase (Protocol 2); with supplementation with 10% FBS during stimulation with E_2 and during the recovery phase (Protocol 3). After adjusting time exposure to 48 h, and FBS supplementation to Protocol 3, the assay was repeated with and without recovery of cells for 48 hours of culture without 17ß-estradiol. Assays were carried out in triplicate. The cells used in flow cytometry assays and real-time PCR were cultured and stimulated with 17ß-estradiol in 75 cm^3 flasks in order to achieve a sufficient number of cells to successfully perform the experiment.

Cell viability/cytotoxicity assay - MTT test

Cell viability was studied by quantification of the extent of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). At the end of incubation with 17ß-estradiol, medium was removed from wells and replaced by fresh media and 0.5 mg/mL MTT solution, and incubated at 37 °C for 4 hours in absence of light. Thereafter, media

containing MTT was removed and formazan crystals were dissolved with Sorenson's glycine buffer (glycine 0.1M, NaCl 0.1M, pH 10.5) and DMSO (Sigma Aldrich) and absorbance was recorded in Biorad 550 microplate reader at 570 nm. The extent of cell death was expressed as the percentage of cell viability in comparison with control cells.

Flow Cytometry Analysis

Staining with propidium iodide (PI) in conjunction to Annexin V was used to determine the viability status of the cells after stimulation with E_2. NHDF and MCF-7 cells were cultured in 75 cm^3 culture flasks and stimulated with 50 nM of E_2 for 48. After harvesting with trypsin, cells were resuspended in Binding Buffer (0.1M HEPES, pH 7.4, 1.4M NaCl, 25 mM CaCl2). Annexin V and Propidium Iodide (BZ Bioscience) staining was performed according to the protocol provided by the manufacturer. Viability status of the cells was analyzed based in the following: viable cells (PI negative/Annexin V negative); initial apoptosis (PI negative/Annexin V positive); necrotic or death cells (PI positive/Annexin V negative); late apoptosis (PI positive/Annexin V positive).

Total RNA extraction and cDNA synthesis

NHDF and MCF-7 cells were cultured and stimulated with 17β-estradiol in 75 cm^3 flasks. Total RNA from each type of cells was extracted with TRI Reagent (Sigma Aldrich) according to manufacturer instructions. Optical density was determined (Pharmacia Biotech, Ultrospec 3000) and agarose gel electrophoresis analysis were performed to assess the quantity and integrity of total RNA. cDNA was synthesized from 2 µg of total RNA which was denatured for 5 min at 65 ºC together with 500 µM deoxynucleotide triphosphates (Amersham) and 250 ng of random primers (Promega). Reverse transcription was carried out at 37 ºC for 60 min in a 20 µl reaction containing reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM MgCl2, and 0.1 M DTT), 60U RNase inhibitor (Promega), and 200U of M-MLV RT (Promega). Samples were incubated at 25°C, followed by an elongation step at 37°C (ideal temperature for M-MLV RT activity) for 1 hour, and a final step at 70°C for 15 minutes, to inactivate the enzyme. The synthetized cDNA was stored at 4°C for further usage.

Real-time PCR

To evaluate the responsiveness of NHDF cells to estrogens we analyzed by Real-time PCR GSTM1 genes in cells stimulated with 10, 50 and 100 nM of E_2 for 48 hours. Primers of beta-actin (ß-actin) housekeeping gene were used as internal controls to normalize GSTM1 primers.

PCR efficiency was calculated for all primers. Real-time PCR reactions were prepared using 1:1, 1:10, and 1:100 dilutions of NHDF cDNA.

Real time PCR reactions were carried out using 1 µL of cDNA synthesized from E_2 stimulated NHDF cells in a 20 µl reaction containing 10 µl of Maxima Sybr Green/ROX qPCR Master Mix and 300 nM of GSTM1 or 300 nM of ß-actin. The reaction ran in an iQ5 Multicolor Real-Time PCR Detection System Thermocycler (Bio-Rad) according to the following program: a starting DNA denaturation at 95°C for 3 minutes, followed by 60 amplification cycles (consisting in denaturation at 95°C for 10s, annealing at 58°C for 30s and extension at 72°C for 30s). As a quality assay to found if there was primer amplification instead of gene fragment amplification, it was added a melting curve analysis, ranging from 55°C to 95°C, by 0.5°C increments lasting 10s, and fluorescence data was collected at the end of each increment to plot that curve (Figure 12). Data collected was analyzed by iQ5 software v2.0 (Bio-Rad) to plot the amplification graphs for further calculations. The relative expression of target genes was performed recurring to the $\Delta\Delta C_T$ Method, also known as the Double Approximation Method, using the formula $R = \frac{(Etarget)^{\Delta CPtarget(control-sample)}}{(Eref)^{\Delta CPref(control-sample)}}$.

Statistical Analysis

The statistical significance of differences in relative cell viability and recovery rates among experimental groups and conditions of different concentrations of 17ß-estradiol and different cell lines was assessed by Student's *t* test. P values less than 0.05 were considered statically significant. Flow cytometry dot plots were analyzed in FCS Express version 4 Research Edition (De Novo Software). All experimental data are shown as mean ± S.E.M.

Dissociation and culture of dermal fibroblasts

Human dermal fibroblasts were isolated from a specimen originated in a biopsy of skin of consenting random healthy women. Concisely, biopsy specimens of human skin were collected in sterile RPMI 1640 medium (Sigma Aldrich) supplemented with 10% FBS, HEPES (0.01 M) (Sigma Aldrich), L-glutamine (0.02 M) (Sigma Aldrich), sodium pyruvate (0.001 M) (Sigma Aldrich) and 1% antibiotic/antimycotic (a mixture of 10,000 units/ mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B). Skin samples were maintained at 4 ºC until manipulation, performed no longer than 24 hours after collection. In order to establish primary cultures of human dermal fibroblasts, samples were cut into small pieces and digested with 0.025% trypsin/EDTA (Sigma-Aldrich) for 30 minutes to 1 hour, 37 ºC, with continuous magnetic stirring. The digested specimens were placed in 6-well plates, one piece per well, and submersed with RPMI medium supplemented with 10% fetal bovine serum (FBS), HEPES (0.01 M), L-glutamine (0.02 M), sodium pyruvate (0.001 M) and 1%

antibiotic/antimycotic. After dermal fibroblasts start emerging, the skin specimen was discarded, cells were harvested with 0.025% trypsin/EDTA and placed in culture in 25 cm^2 flasks, where were cultured in the same medium until usage in the experiments. Human dermal fibroblasts were routinely maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed with cells between third and ninth passages.

Immunofluorescence microscopy

Human dermal fibroblasts obtained from dermis explant were grown in coverslips coated with collagen. Cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 1% Triton X-100 and blocked with 3% of BSA and 0.2% of Tween 20/PBS. Subsequently, cells were incubated in 3% BSA/PBS for 1 hour with the following primary antibodies: Mouse Monoclonal Human Fibroblast Marker (Sigma-Aldrich) (0.4 µg/ml), mouse monoclonal ICAM 1 (1 µg/ml) (Abcam), mouse monoclonal smooth muscle alpha-actin (5 µg/ml), rabbit polyclonal vWF (0.1 µg/ml), rabbit polyclonal human ERα (2 µg/ml) (Santa Cruz Biotechnology) and rabbit polyclonal human ERß (2 µg/ml) (Santa Cruz Biotechnology, Inc). Secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1 g/mL) or Alexa Fluor 546 goat anti-mouse IgG conjugates (1 g/mL) (Molecular Probes) were used. The expressed proteins were localized using a Zeiss AX10 microscope and the Axio Vision Real 4.6 software. Endothelial cells from human umbilical artery were used as positive controls for vWF and human umbilical artery smooth muscle cells were used as positive control for smooth muscle alpha-actin. Negative control experiments were carried out for all of the antibodies by incubation of cells with primary or secondary antibodies alone.

RESULTS

Hormonal stimulation experiments in human cell lines

We performed several experiments in order to establish the optimal culture conditions of the cells. Dose–response/time-course experiments were carried out using five different concentrations of E2 (0, 1, 10, 50 and 100nM), and four incubation times for each hormone dosage (12, 24, 48 h and 96 hours). After adjusting the incubation time for 48 hours, we performed MTT viability assay in order to assess the relative cellular viability of NHDF cells, and the cytotoxic effects of 17ß-estradiol in this cell line. Three different protocols of FBS supplementation of cell culture medium were performed: without supplementation with FBS during stimulation with E_2, but with supplementation with FBS in the recovery phase (after removal of E_2) (Protocol 1); without supplementation with FBS during stimulation with E_2 and during the recovery phase (Protocol 2); with supplementation with FBS during stimulation with E_2 and during the recovery phase (Protocol 3). These protocols were also evaluated in control NHDF cells, that were cultured in the different conditions established by the three protocols, but stimulation with 17ß-estradiol was not executed (Figure 1).

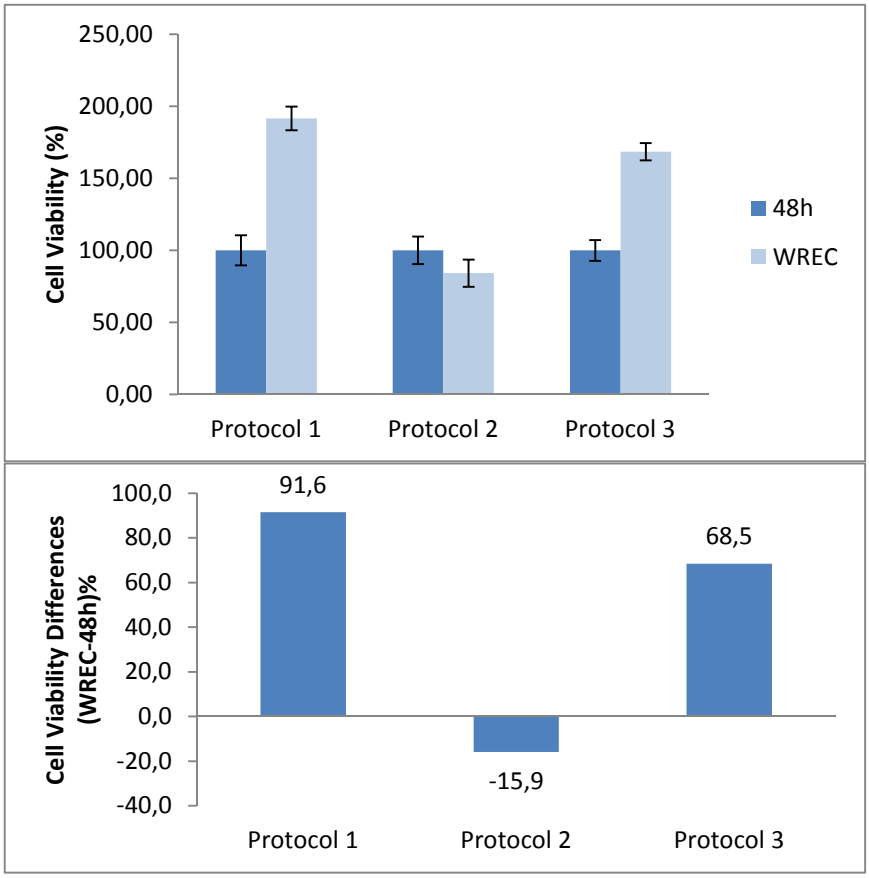

Figure 1 – Upper graph: Evaluation of the effects in NHDF cells of the three tested protocols (48h) and after 48 hours of recovery in controls (without stimulation with 17ß-estradiol). Lower graph: NHDF cell viability differences between 48 hours (48h) and with recovery (WREC) assays. t-student $p<0.005$.

When we used Protocol 1 we found that cell viability was 23,1% higher after recovery when compared with Protocol 3. Privation of FBS during the stimulation phase of Protocol 1 seemed to induce higher susceptibility to FBS steroids, what led to a major recovery and viability rates. When we tested Protocol 2, in which FBS was suppressed from stimulation and recovery phases of the protocol, we found a diminuished cell viability after recovery, what reflects the absence of growth factors and steroids in FBS.

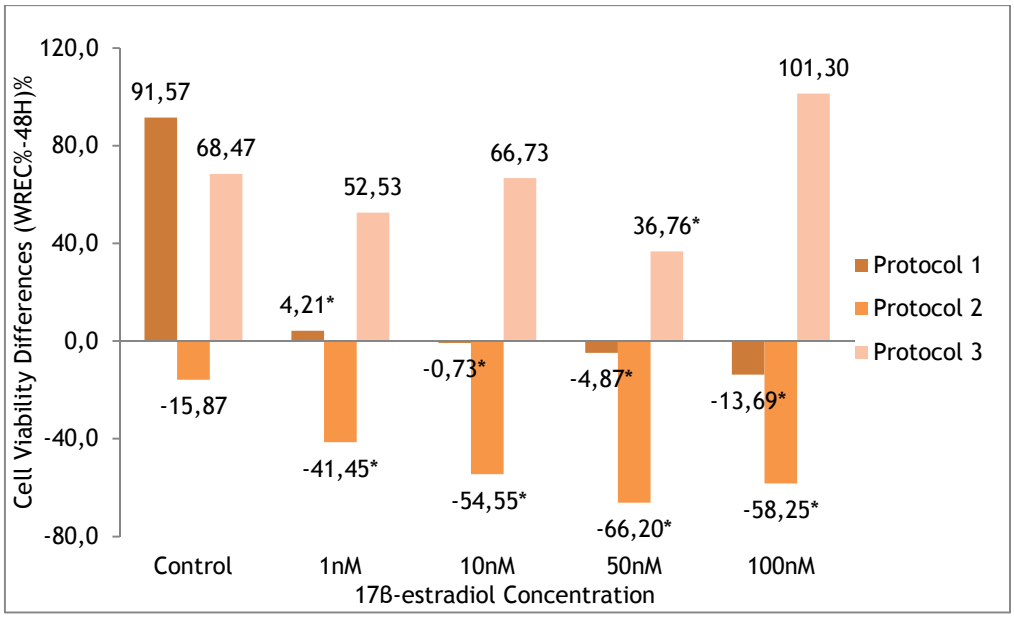

Figure 2 – Graphic representation of the differences of NHDF cell viability after 48 hours of incubation with 17β-estradiol (48H) and after recovery (WREC) with the three tested protocols. * Statistically significant results when compared with controls. t-student $p<0.005$.

When we tested the thre protocols for stimulation with 17β-estradiol (Figure 2) we obtained considerable variability in the results of cell viability when Protocol 1 was used. With Protocol 2, a diminuished cell viability after recovery was found, that can be due to the absence of FBS, and thus, the absence of growth factores and steroids crutials to the good development of cells in culture. Thus, Protocol 3 was considered to be the least sujected to FBS intereferences and was chosen to perform the following assays.

Concentrations of 100 nM lead to a diminuished relative cellular viability, whyle 1 and 50 nM induced an increase of fibroblasts relative viability. For 10 nM we did not found a statitically significant difference (Figure 3).

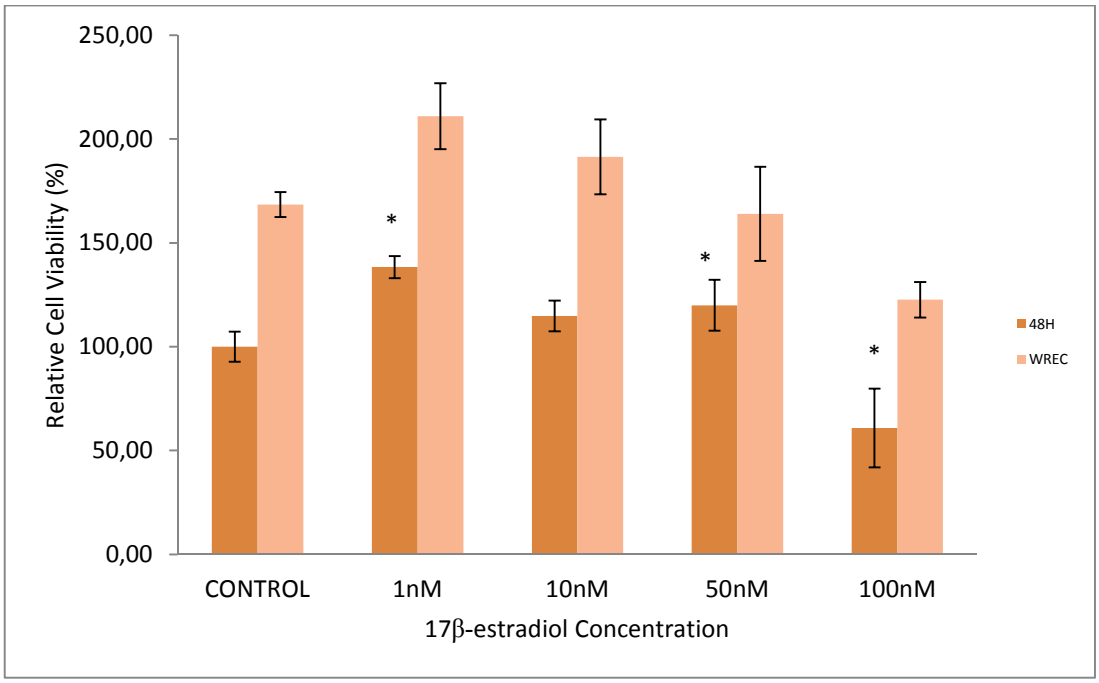

Figure 3 – Effects of 17β-estradiol in NHDF cell line with 48 hours of 17β-estradiol exposure (48H) and after 48 hours of recovery post 17β-estradiol exposure (WREC). t-student $p<0.005$.

We further studied the recovery of fibroblasts after stimulation with 17β-estradiol in the different studied concentrations (Figure 4). We let the cells recover from 17β-estradiol for 48 hours, in normal culture medium. Any cytotoxic effects promoted by 17β-estradiol were reversed after 48 hours of culture in normal "no-stimulation" conditions. For lower 17β-estradiol concentrations (1 and 10 nM), recovery rate was simmilar to the one obtained for controls and recovery rate was significantly lower for 50 nM of 17β-estradiol and significantly higher for the highest concentration, 100 nM.

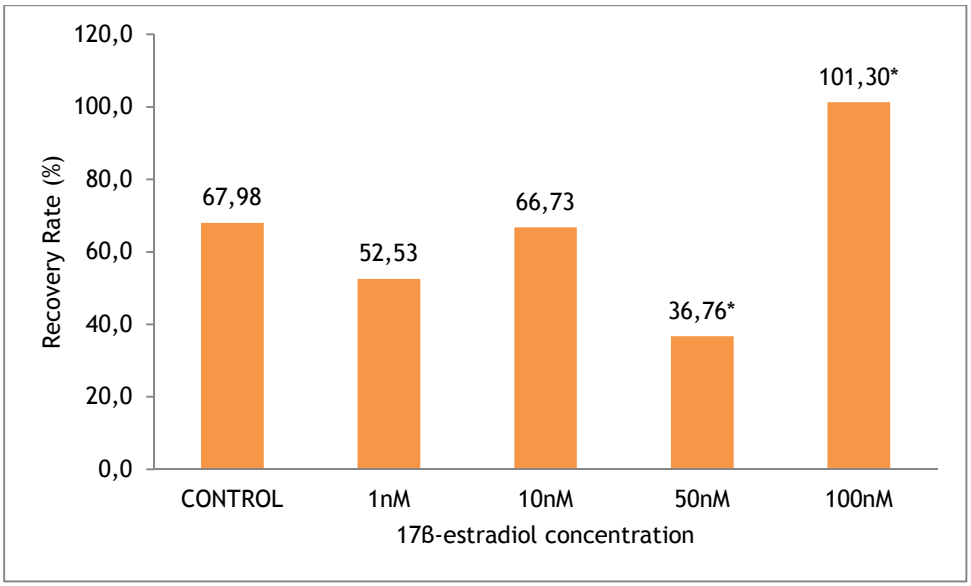

Figure 4 – Recovery rate of NHDF cells after 48 hours of recovery post 17β-estradiol exposure (WREC).

Cell viability assays were also performed in MCF7. A significant reduction of relative viability was found for 50 and 100 nM, the highest studied concentrations of 17β-estradiol (Figure 5).

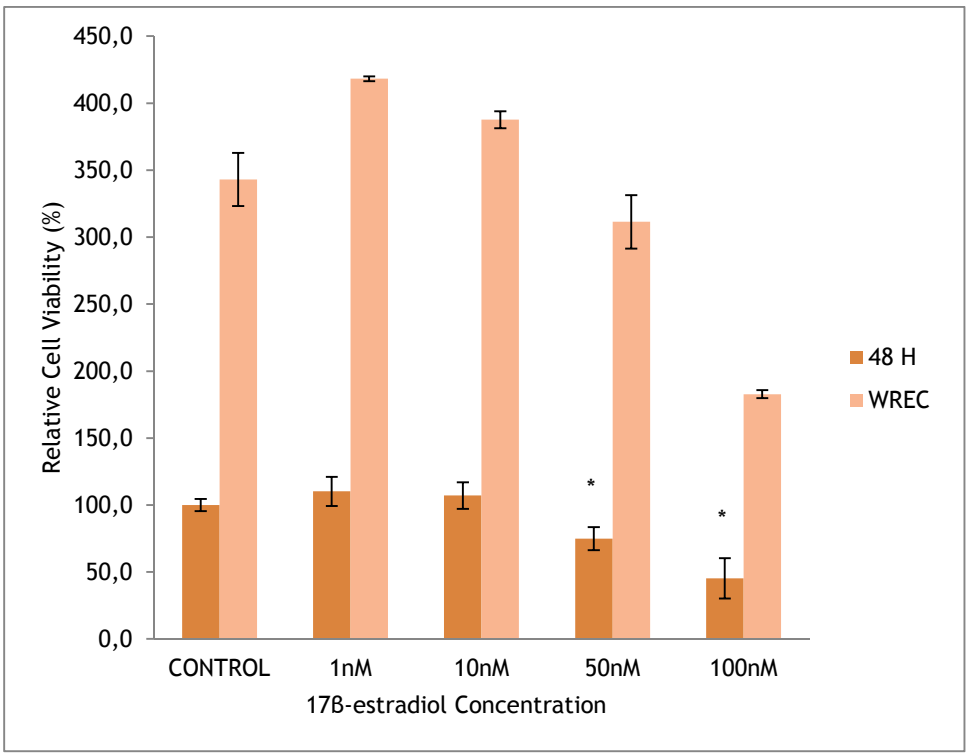

Figure 5 – Effects of 17β-estradiol in MCF-7 cell line with 48 hours of 17β-estradiol exposure (48H) and after 48 hours of recovery post 17β-estradiol exposure (WREC). t-student $p<0.005$.

48 hours after exposure to 17β-estradiol cell recovery rate was also analyzed (Figure 6). MCF7 cells recovered their viability despite the concentration of 17β-estradiol, with recovery rates ranging from 200 to 300%.

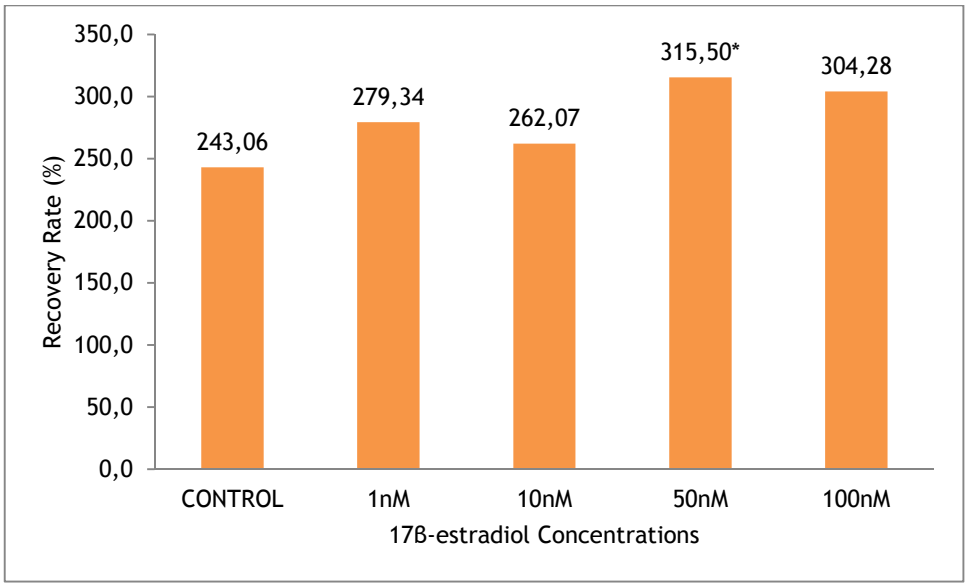

Figure 6 – Recovery rate of MCF-7 cells after 48 hours of recovery post 17β-estradiol exposure (WREC).

Furthermore, we compared cell viability after 48 hours of 17β-estradiol exposure and after recovery for 48 hours post 17β-estradiol exposure (Figure 7 and Figure 8). After 48 hours of 17β-estradiol exposure we did not find statistically significant differences in cell viability between the two cell lines. Regarding recovery rates for 48 hours after 48 hours of 17β-estradiol exposure we found significant different recovery rates of both cell lines, except for the higher concentrations of 17β-estradiol.

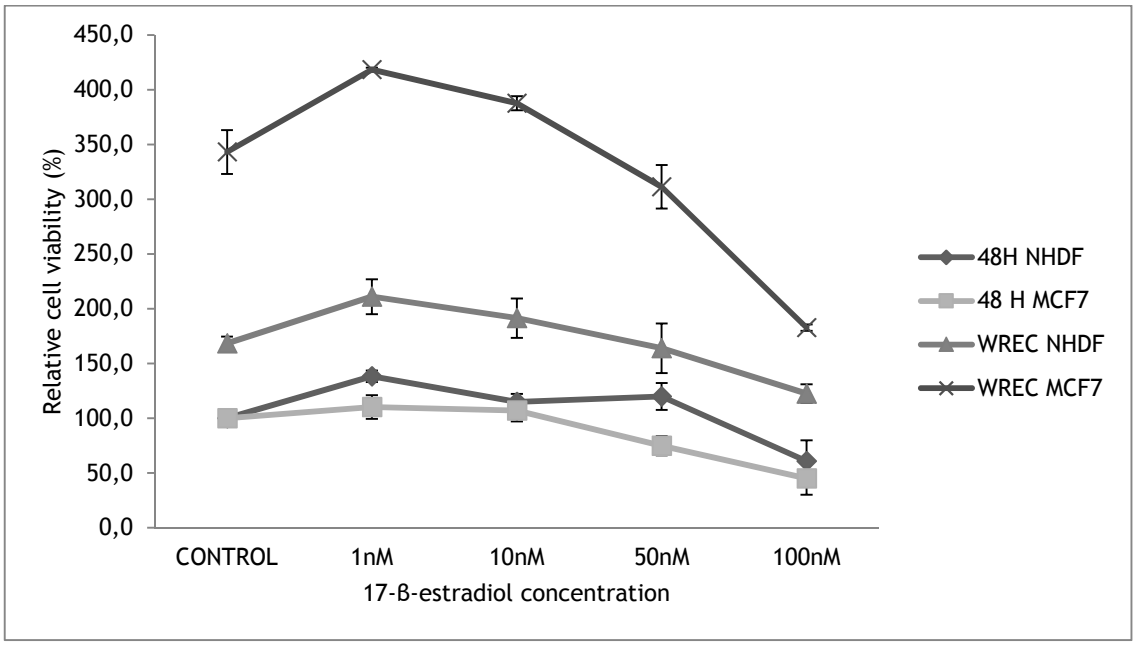

Figure 7 – Relative cell viability of NHDF and MCF7 48 hours of 17β-estradiol exposure (48H) and after 48 hours of recovery post 17β-estradiol exposure (WREC).

Higher proliferation rates were found for MCF7 cells when compared with NHDF cells, after recovery post 17β-estradiol exposure.

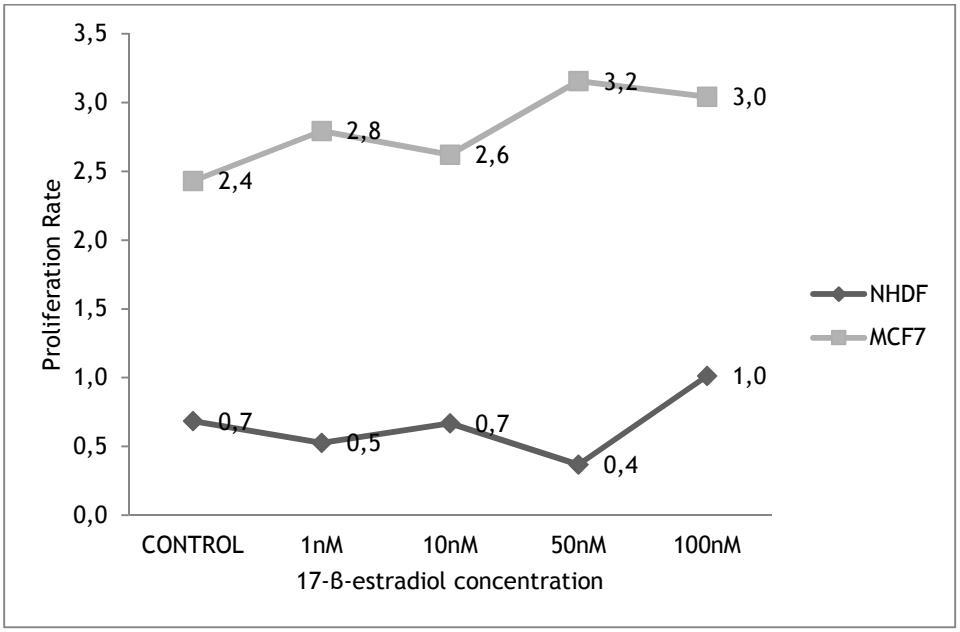

Figure 8 - Proliferation rates of MCF7 and NHDF cells after recovery post 17β-estradiol exposure.

Flow cytometry assays of early apoptosis and cell death by necrosis were performed to evaluate the effect of intermediate concentrations of 17β-estradiol (50nM) in the studied cell lines (Figure 9 and Figure 10). In NHDF control cells, we found higher rate of cell death by necrosis (9.4%) than early apoptosis (0.90%). After stimulation of NHDF with intermediate concentrations of 17β-estradiol, we found a reversed tendency: early apoptosis rate is higher (3.6%) than cell death by necrosis rate (1.7%).

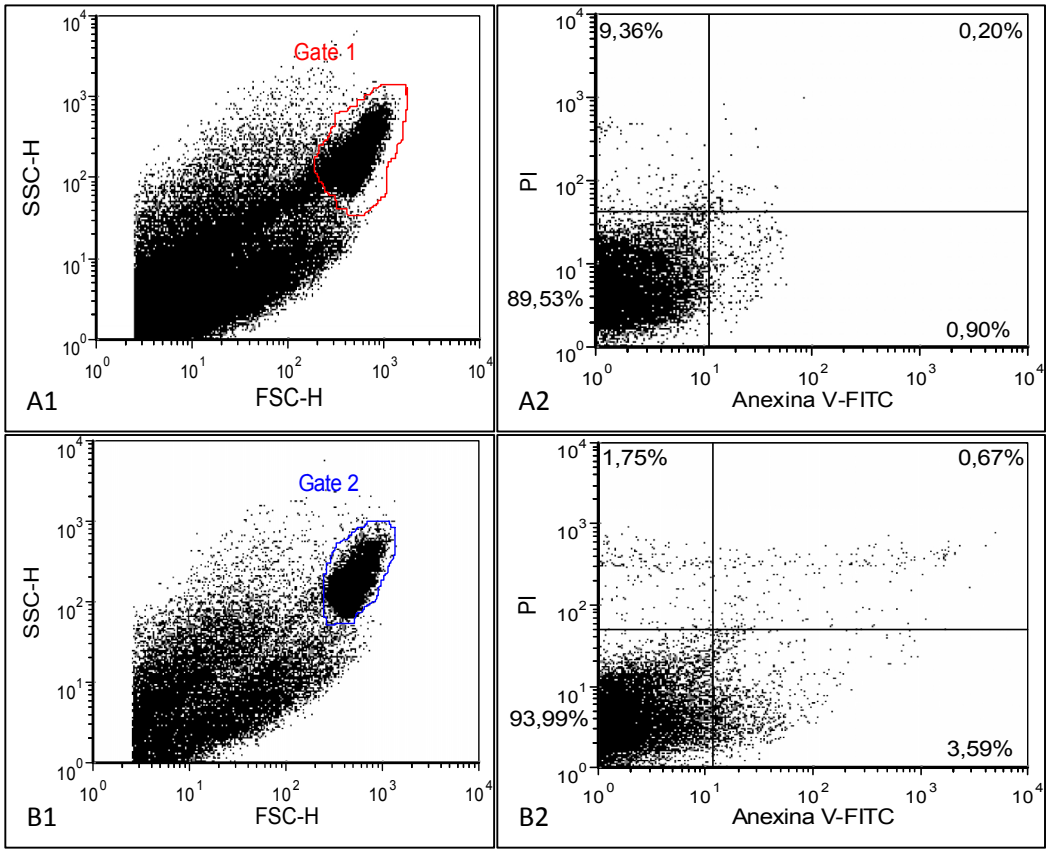

Figure 9 – Flow cytometry of NHDF cells. A1 – control. B – After stimulation with 17β-estradiol for 48 hours. 1 – Selected population. 2 – Annexin V / Propidium Iodide staining.

Regarding not stimulated with 17β-estradiol MCF7 cells, higher rate of cell death by necrosis was found (7.2%) in comparison with early apoptosis (2.8%). After stimulation with 17β-estradiol, and similarly to what was found for NHDF cells, this tendency was reversed, and early apoptosis rate was found to be higher (4.2%), when compared to cell death by necrosis (2.5%).

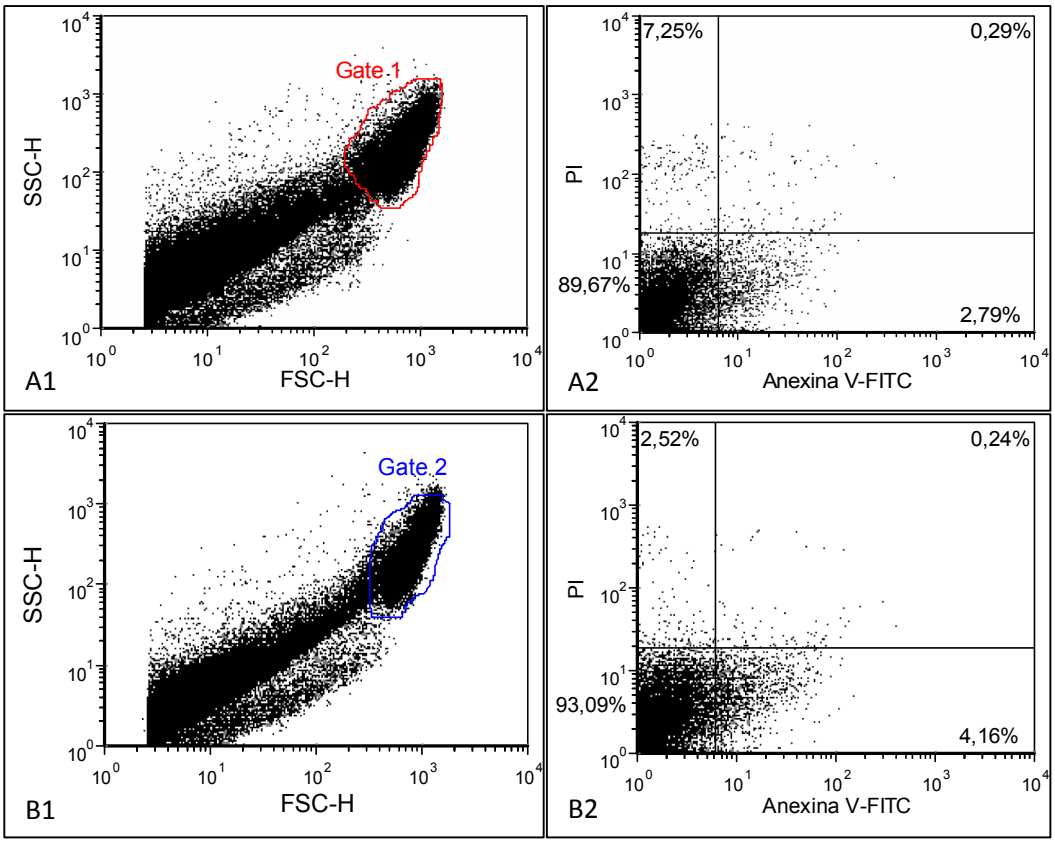

Figure 10 – Flow cytometry of MCF7 cells. A1 – control. B – After stimulation with 17β-estradiol for 48 hours. 1 – Selected population. 2 – Annexin V / Propidium Iodide staining.

From our results of real time PCR experiments, we verified that expression of GSTM1 gene was higher for estradiol concentration of 50 nM, and significantly lower for 100 nM estradiol. This effect may be due to cellular death induced by high concentrations of estradiol rather than by an actual decrease in the gene expression.

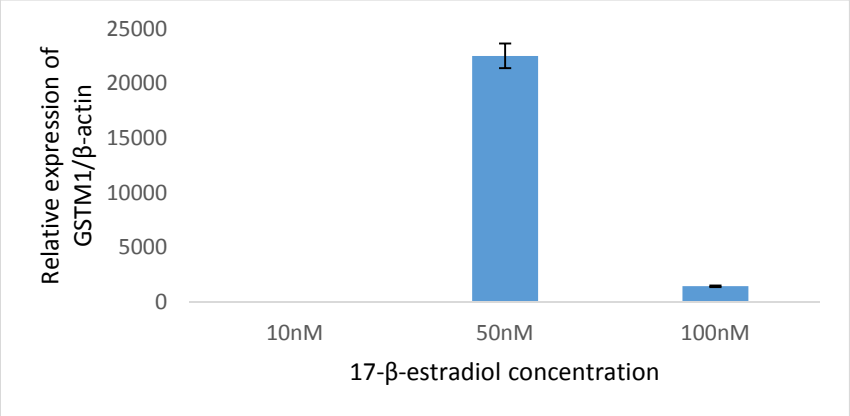

Figure 11 – Relative quantification of GSTM1 in NHDF cells, in comparison with β-actin housekeeping gene.

Establishment of cultures of human dermal fibroblasts from explants

Because of its accessibility, skin is one of the best studied organs in terms of fibroblast diversity, and due to the facility with which a skin sample can be obtained, it can be used as an easy source of human fibroblasts. With our procedure, cells needed seven to fifteen days to emerge from skin sample and attach to the dish (Fig. 11A).

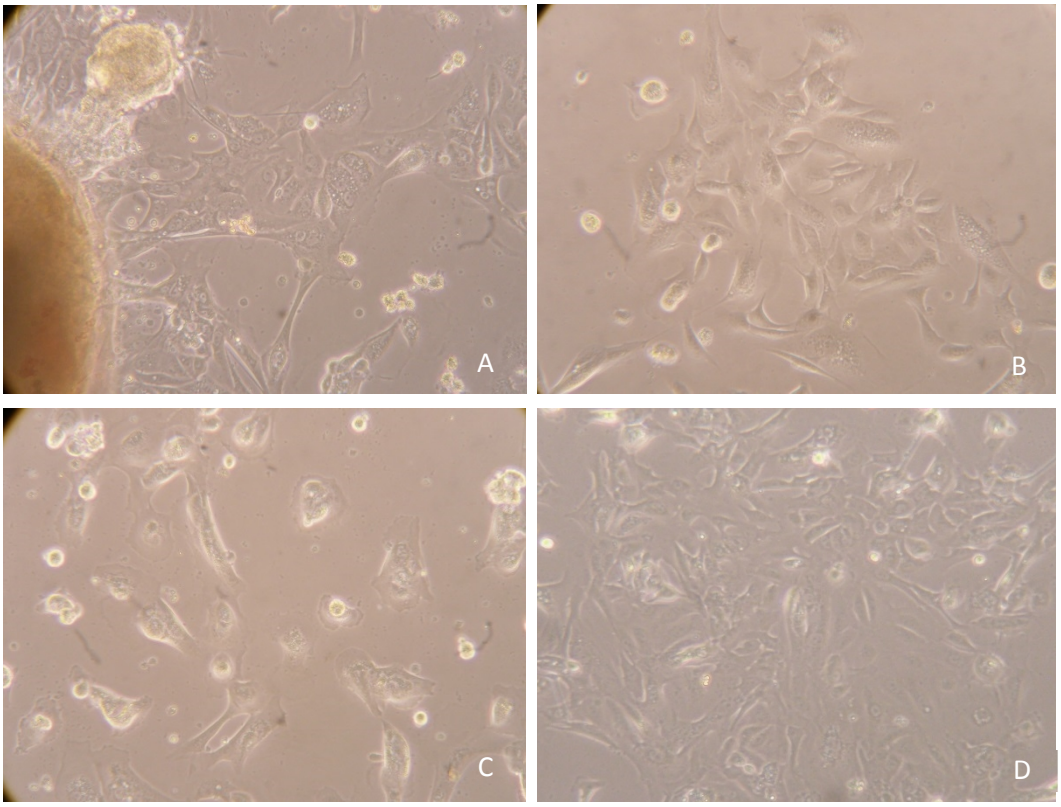

Figure 11 – Primary culture of fibrobrasts from human dermis (200×). A – Skin explant and the emerged dermal fibroblasts from skin (outgrowth). B – Fibroblasts with characteristic fusiform shape. C – Round shaped cells, with expanded cytoplasm and membrane, which resisted in culture until first subculture was performed. D – Primary culture of human dermal fibroblasts after eight days of *in vitro* growth.

The majority of isolated cells had the elongated, fusiform shape characteristic of fibroblasts (Fig. 11B). However, some isolated cells presented rounded shape, with an expanded cytoplasm and membrane (Fig. 11C), but only survived in culture until first subculture was performed. After subculture, 80% confluence was achieved after six to eight days (Fig. 11D).

Characterization of cell cultures of human dermal fibroblasts obtained from explants

Immunofluorescence was used to characterize dermal fibroblasts isolated from human dermis

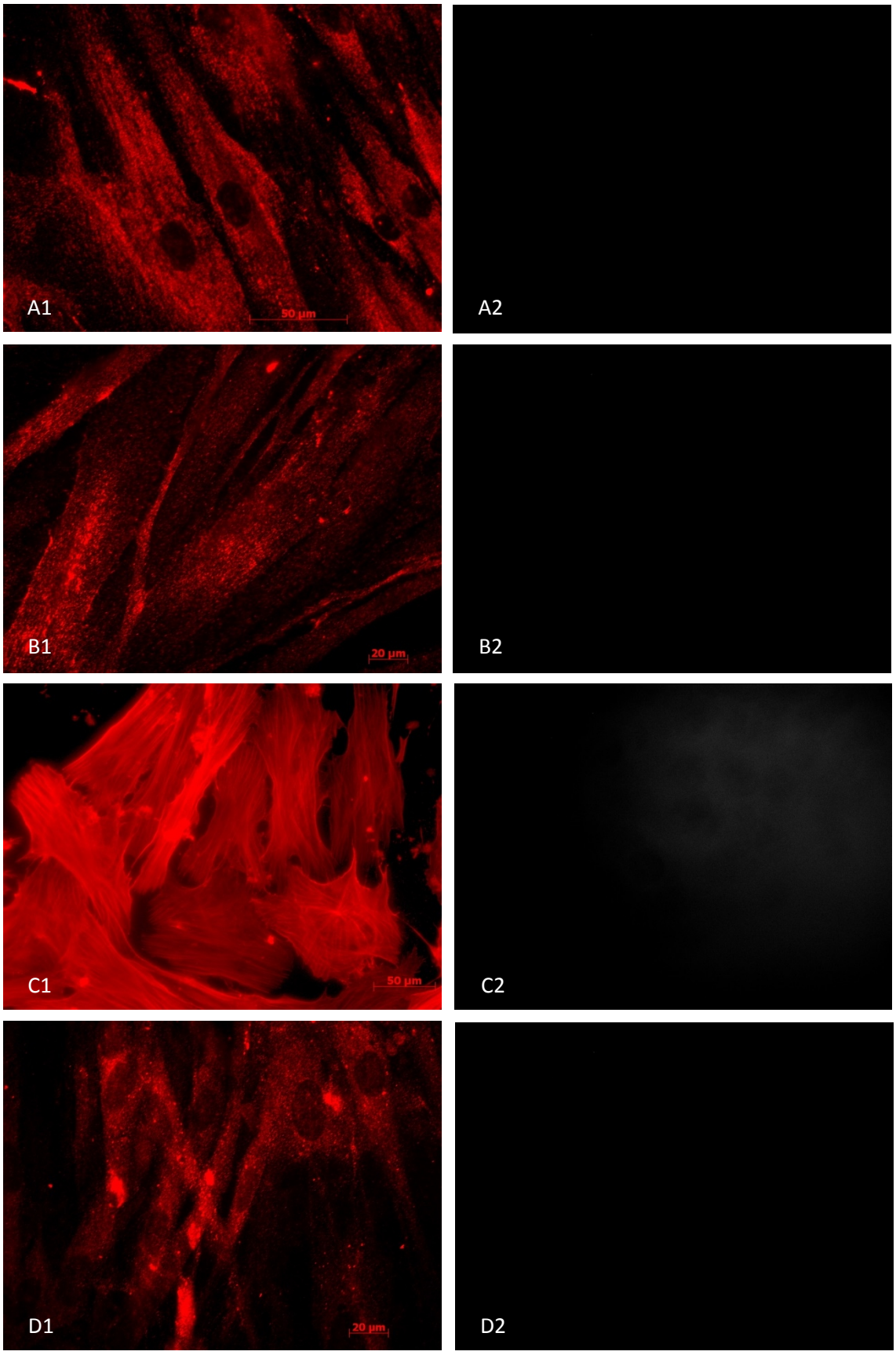

Figure 12 – Immunofluorescence for characterization of human dermal fibroblasts isolated from human dermis explant. A – Staining with antibodies for fibroblast-specific surface antigen (A1) and negative control (A2). B – Staining with antibodies for ICAM 1, a fibroblast marker (B1) and negative control (B2). Human umbilical artery smooth muscle and endothelial cells were run as positive controls for smooth muscle marker (C1) and endothelial marker (D1), respectively. Fibroblasts did not stain for smooth muscle alpha-actin (C2) nor Von Willebrand factor (D2).

We analyzed the presence of characteristic proteins of human fibroblasts, by incubating cells from the third passage with specific antibodies anti ICAM 1 and anti-fibroblast specific surface antigen. Human dermal fibroblasts positively stained for both ICAM 1 and the fibroblast-specific surface antigen (Figure 12A1 and 12B1). Also, human dermal fibroblasts did not express vWF nor smooth muscle alpha-actin, that were expressed in endothelial cells from human umbilical artery and in human umbilical artery smooth muscle cells, respectively (Figure 12C and 12D). These data indicate that human dermal fibroblasts isolated by this method are not contaminated with endothelial cells, which are the major source of putative contaminating cells in dermal cell cultures.

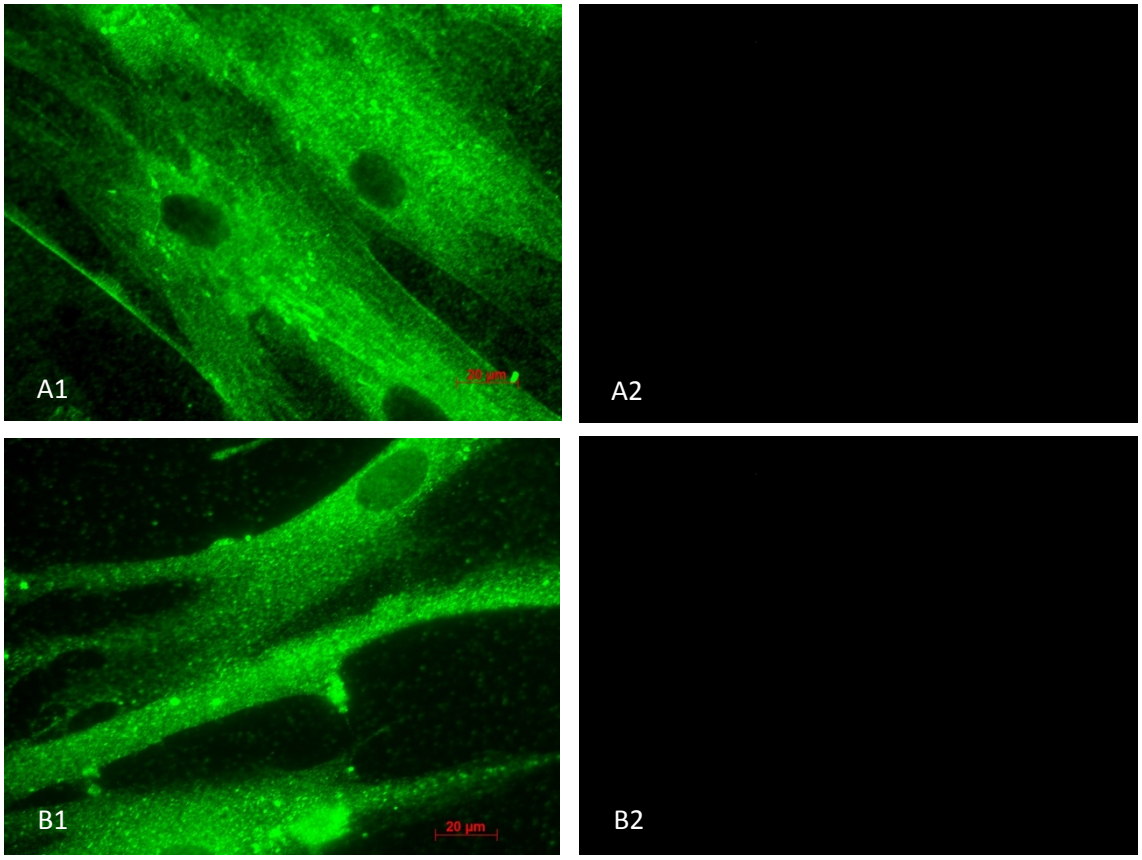

Figure 13 - Immunofluorescence for characterization of human dermal fibroblasts isolated from human dermis explant. A – Staining for estrogen receptor alpha (A1) and negative control (A2). B - Staining for estrogen receptor beta (B1) and negative control (B2).

Immunofluorescence was also used to analyze the expression of ERα and ERβ, and both receptors were expressed in human dermal fibroblasts (Figure 13).

DISCUSSION

Several experimental protocols that describe *in vitro* cell culture assays can be found in literature. In our work, normal human dermal fibroblasts and MCF-7 cells response to 17ß-estradiol was evaluated after 48 hours of exposure. Although lower incubation times were tested (data not shown), considering that NHDF cell population doubling time was approximately 40 hours, we found that 48 hours was the more accurate period of time to accomplish our purposes. Periods of time longer than 48 hours were also tested but an important bias was found: after 96 hours of incubation there was no available space for cells to growth, and that is an essential factor for *in vitro* cell culture assays. These results are in accordance with the ones obtained in previous studies, which reported that when a high number of cells are growing in culture they stay in a stationary *plateau* phase, proliferation stops due to contact inhibition, growth rate nearly comes to zero and arrest of cell cycle can eventually occur (Freshney, 2006). Other tested culture condition was growth media supplementation with FBS. FBS has a crucial importance for *in vitro* cell culture assays because it contains many non-defined growth promoting and survival enhancer factors, thus it has been used in supplementation of human and animal cell culture media (Brunner et al., 2010). FBS also provides components of the extracellular matrix and allows a reduction in the time spent for optimizing the formulation of culture media for each cell type, as it can be used in a broad range of cells (Gstraunthaler, 2003). In most cases, it is used in 10% concentrations. Although all these advantages, FBS also presents some drawbacks, as it can introduce qualitative and quantitative biases in the obtained results, due to presence of steroids in its constitution (Brunner et al., 2010). In our work, in the first phase of the protocol cells were seeded and cultured with FBS for 48 hours. Then, we tested supplementation of cell culture media with FBS in different phases of the protocol, as described in Material and Methods section. The results of cell viability obtained for cells cultured without supplementation with FBS during stimulation with E_2, but with supplementation with FBS in the recovery phase showed that relative viability of these cells was even higher than the one obtained for cells that were cultured with supplementation with FBS during stimulation with E_2 and during the recovery phase. Deprivation of FBS during the period of 48h of E_2 stimulation seemed to induce an enhancement of cell sensibility to FBS steroids, what led to a much emphasized subsequent recovery. These results are in accordance with the ones previously obtained by the group of Santen: they were able to confirm that cells in absence of steroids became more sensible to their effects. The mechanisms underneath this response include a four to ten times elevation of ERα expression levels during long term steroid starvation. The development of hyper sensibility to steroids may also comprise modulation of genomic effects of steroids, acting in transcription, and non-genomic actions involving membrane receptors (Santen et al., 2008).

For cells that were cultured without supplementation with FBS during stimulation with E_2 and during the recovery phase, a decrease in relative viability was found. This result reflects the

absence of steroids and growth factors that are present in FBS, and are needed for the successful growth of cells in culture. Privation of FBS promotes cellular quiescence, and this state can be detected by MTT assay, due to the lowering of mitochondrial activity (Bettuzi et al., 1999).

Ideally, a serum-free, defined medium should be used when the effect of hormones on *in vitro* cell growth is to be studied. Dextran coated charcoal is used to strip hormones from serum and Charcoal/Dextran treatment of FBS was proven to reduce the levels of many components, including steroids and growth factors, and also virus particles. Rapid adaptation of cells from medium with 10% FBS to serum-free or charcoal/dextran treated FBS conditions has been obtained by others; however, in our experiments, this medium did not permit a continuous growth of cells, mainly due to detachment of cells after prolonged culture times. Charcoal-stripping of serum has also been proved to remove stimulators of the MAPK signaling pathway (Dang and Lowik, 2005), and the effects of charcoal/dextran treated FBS per se on steroid modulated pathways have not been addressed.

Thus, we choose to study the effects of E_2 in NHDF and MCF-7 cells cultures always in presence of FBS, and that provided us relative results by comparison with the results obtained for controls, that were cultured in the same conditions, but without stimulation with E_2. This procedure was a guarantee that the influence of serum components in the obtained results was transversal to all the procedures in the study, so our results are all comparable among themselves.

When we tested 1, 10, 50 and 100 nM of 17β-estradiol, we verified that concentrations of 100 nM lead to a diminuished relative cellular viability, whyle 1 and 50 nM induced an increase of fibroblasts relative viability. For 10 nM we did not found a statitically significant difference. These results are in agreement to the ones previously obtained by other authors (Celojevic et al., 2011). We studied the recovery of the cells after 48 hours of stimulation with 17β-estradiol in the different studied concentrations, and found that any cytotoxic effects promoted by 17β-estradiol could be reversed after 48 hours of culture in normal "no-stimulation" conditions. For lower 17β-estradiol concentrations (1 and 10 nM), recovery rate was simmilar to the one obtained for controls. Recovery rate was significantly lower for 50 nM of 17β-estradiol and significantly higher for the highest concentration, 100 nM, a significant higher recovery rate was achieved. Similarly to NHDF, cell viability assays were also performed in MCF7, a commercial breast cancer epithelial cell line, that are well recognized by positively express ERα and ERβ (Papoutsi et al., 2009). We found a significant reduction of relative viability only for the highest concentrations of 17β-estradiol, 50 and 100 nM. Similar results were obtained by other research groups (Chow et al., 2004). 48 hours after exposure to 17β-estradiol, cell recovery rate was analyzed in order to determine if cells were able to recover their viability after the stimulation. Commercial ephitelial MCF7 cells were able to recover their viability despite the concentration of 17β-estradiol, with recovery rates ranging from 200 to 300%. Thus, we can assume that these cells are more resistant to any cytotoxic

effect promoted by the steroid, and its effect is reverted during the onward 48 hours of recovery.

In order to compare the behaviour of both cell lines in reponse to 17β-estradiol, we compared cell viability after 48 hours of 17β-estradiol exposure and after recovery for 48 hours post 17β-estradiol exposure. After 48 hours of 17β-estradiol exposure we did not find statistically significant differences between the two cell lines, but we did find obvious and significant differences when we compared the recovery rates of both cell lines except for the higher concentrations of 17β-estradiol.

We also analyzed the proliferation rate of both cell lines after recovery. Higher proliferation rates were found for MCF7 cells when compared with NHDF cells. MCF7 are derived from human breast adenocarcinoma thus, as being a cancerous cell line, are able to divide much faster than a healthy cell line like NHDF. Also, they can present higher expression of estrogen receptors, what suggests higher response rate to estrogens.

We also performed preliminary flow cytometry analysis to evaluate the effect of intermediate concentrations of 17β-estradiol (50nM) in the studied cell lines. To evaluate differences between the two cell types, we compared rates of early apoptosis and cell death by necrosis. For NHDF control cells, we found higher rate of cell death by necrosis (9.4%) than early apoptosis (0.90%). After stimulation of NHDF with intermediate concentrations of 17β-estradiol, we found a reversed tendency: early apoptosis rate is higher (3.6%) than cell death by necrosis rate (1.7%). Regarding MCF7 cells, we found higher rate of cell death by necrosis (7.2%) in comparison with early apoptosis (2.8%) in control assay. After stimulation with 17β-estradiol, this tendency was also reversed, and early apoptosis rate was found to be higher (4.2%), when compared to cell death by necrosis (2.5%). In summary, for MCF7 cells, 50 nM of 17β-estradiol was not sufficient to promote cell death in those cells. However, concerning NHDF, this concentration of 17β-estradiol was able to promote early apoptosis but did not lead to cell death by necrosis. The differences found are not statistical significant and this assays constitute preliminary results, thus more studies are needed regarding this issue so we can properly delineate any conclusion. With the real-time PCR experiments we proved that normal human dermal fibroblasts express GSTM1 and that this expression is regulated by 17β-estradiol. To our knowledge, this is the first time that dermal fibroblasts have been proven to express GSTM1 gene, in response to 17β-estradiol, and confirms they are a good model for our research.

Normal human dermal fibroblasts have been proven to express estrogen receptors (Haczynski et al., 2002). In our demand to establish *in vitro* dermal fibroblasts cultures as models of study of the action of estrogens, we decided to confirm this expression, and performed immunofluorescence to characterize normal human dermal fibroblasts cultured cells obtained from skin explants primary cultures, obtained from random individuals that accepted to provide a skin sample, in order to validate our culture method. Human dermal fibroblasts obtained by the culture method developed by our group positively stained for both ICAM 1 and the fibroblast-specific surface antigen, and did not express vWF, characteristic of

endothelial cells, nor smooth muscle alpha-actin, characteristic of smooth muscle cells. These data confirms that our method successfully isolates human dermal fibroblasts. Immunofluorescence was also used to analyze the expression of ERα and ERß, and both receptors were shown to be expressed in human dermal fibroblasts obtained by our culture method.

With this work we can conclude that our group was able to develop and establish a successful method of isolation of human dermal fibroblasts from an explant of dermal tissue, and that the cells obtained by this method express estrogen receptor alpha and beta. We also demonstrated that continuous presence of FBS in the stimulation protocol with 17ß-estradiol allows cell proliferation that is only dependent on cell mechanisms and not in physiological deprivation of growth factors. Exposure to high concentrations of 17ß-estradiol led to a decrease in NHDF and MCF7 cell viability but that effect could be reversed after 48 hours of recovery of the cells in culture. The results obtained with this work are preliminary but confirm that dermal fibroblasts can be used as cell culture models for study of response to estrogens, when the correct culture conditions are defined. In future, we intend to accomplish more studies to improve knowledge about the model and establish primary dermal fibroblasts cultures of single individuals, and evaluate their use as models of inter-individual response to estrogens. To achieve this aim, it is our intention to repeat in cells from primary cultures obtained from different individuals the assays that were performed in cell lines in this work.

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General Conclusions and Future Perspectives

In order to accomplish the main objective of this thesis, the association of low penetrance genes polymorphisms with breast cancer was studied:

- CYP17A1 T27C and CYP19A1 codon 39 Trp/Arg polymorphisms related to estrogen biosynthetic pathway;
- CYP1B1 Val432Leu, CYP1A1 Ile462Val, COMT Val158Met, GTSM1/GSTT1 deletion, GSTP1 Ile105Val and MTHFR C677T polymorphisms related to estrogen metabolic pathway;
- TP53 Arg72Pro polymorphism associated with DNA damage signaling and repair pathway;
- ER alpha XbaI and PvuII polymorphisms related to estrogenic response pathway.

Genotyping was performed for women with and without breast cancer. The population included in the study was analyzed for BRCA1 185delAG, BRCA1 5382insC and BRCA2 6174delT mutations and all participants were found to be negative.

In this work we hypothesize that SNPs in low penetrance genes can exhibit synergistic effects on modulating individual susceptibility to breast cancer. Furthermore, haplotypes associations of polymorphisms in these genes can define a model for determining a risk profile based in genetic characteristics, which is independent of breast cancer risk assessment models based in family and reproductive histories.

Specifically, our results indicate that GSTM1 and GSTT1 null genotypes alone or in association, are associated with increased susceptibility to breast cancer development (Chapter 6). Also C allele in CYP19A1 codon 39, in homozygosity or heterozygosity, is significantly associated with an increased risk of breast cancer in the population studied. Besides, it seems that the effects of CYP19A1 T/C polymorphism in estradiol biosynthesis appear to be modulated by the presence of GSTM1 and GSTT1 in estrogen metabolic pathway, because breast cancer susceptibility is lower in carriers of GSTM1 and GSTT1, independently of CYP19A1 genotype (Chapter 7). Moreover, relatively to ERα gene, a significant reduced risk of breast cancer was verified in carriers of xx genotype but no association of breast cancer risk in relation to PvuII genotypes was found, except for simultaneous carriers of xx and pp genotypes, that were also less susceptible for breast cancer development (Chapter 8). It was also found that TP53 proline allele in homozygosity or heterozygosity (Arg/Pro and Pro/Pro) was associated with a significant increased risk for breast cancer (Chapter 9).

When the distribution of genotypes in women scored as low and high risk by Gail Model was analyzed, it was not found any statistically significant difference. Despite, women affected by breast cancer had significant prevalence of more than one risk genotype when compared to women not affected by breast cancer. Also, the simultaneous presence of three, four and five risk genotypes was more prevalent in breast cancer affected women than in unaffected women. According to these results, it is surely demonstrated, at least in this population, that combinations of two or more polymorphisms of low penetrating genes in the estrogen biosynthesis, estrogen metabolism, DNA damage signaling and repair, and estrogenic response

pathways should be considered important risk factors for breast cancer development in an independent way of Gail Model. These results show that exists a real need to genotype women in this population, because there is a fraction of them that would be classified as "high risk" according to the polymorphisms based model presented in this work, while by Gail Model they would be classified as "low risk". Thus, the combination of the genotype based risk evaluation model with Gail Model in assessing breast cancer risk can promote the inclusion of women in breast cancer clinical screening programs that would be excluded if only Gail Model was used to access their risk (Chapter 10).

From the overall polymorphisms analyzed by us, the results seem to point that the studied single nucleotide polymorphisms of low penetrance genes, alone or in combination, may modulate and predict breast cancer risk. Also, these polymorphisms can be used in an independent way of validated breast cancer prediction models based in clinical parameters like Gail Model. The use of this genetic model based in SNPs, in combination with clinical risk factors, could be used as a screening tool that would improve the accuracy of the existing risk assessment tools. Other authors have tried to compute an estimated risk given by low penetrance polymorphisms, and include it in well-defined models of cancer risk estimation. Face to our results, and to be applied in this specific population, we would suggest to include in Gail Model the risk associated with variants in GSTM1, GSTT1, CYP19A1 and ERα genes.

In the future, similar studies with larger samples sizes are needed to clarify the role of these polymorphisms in the etiology of breast cancer. The influence of the expression of these genes, especially in association with the expression of other known low penetrance genes in defining breast cancer risk, should also be studied. Moreover, further studies in different populations are needed to access the value of the proposed low penetrance polymorphisms model in defining breast cancer risk among the general population.

Regarding the preliminary results of our study on dermal fibroblasts cultures as models of study of estrogenic action, we successfully established optimal conditions for culture of cells for *in vitro* assays with 17β-estradiol and we developed a method for isolation of human dermal fibroblasts, which expressed ERα and ERβ. Our preliminary results showed that exposure to high concentrations of 17β-estradiol led to a decrease of NHDF and MCF7 cell viability but this effect was reversed after 48 hours of recovery of the cells in culture without hormonal stimulus; regarding flow cytometry analysis, we found higher rate of cell death by necrosis than by early apoptosis for NHDF and MCF7 cells not stimulated with 17β-estradiol, but after stimulation with intermediate concentrations of 17β-estradiol, this tendency was reversed. With real time PCR experiments, we verified that expression of GSTM1 gene was higher for estradiol concentration of 50 nM, and significantly lower for 100 nM estradiol, what could be due to cellular death induced by high concentrations of estradiol rather than by an actual decrease in the gene expression.

In summary, our preliminary results indicate that human dermal fibroblasts are responsive to estrogens *in vitro*, and thus they can be used as models of study of estrogenic response (Chapter 11).

In future it is our intention to use primary dermal fibroblasts cultures of single individuals as potential models of study of inter-individual differences in response to estrogens and breast cancer therapeutic agents.

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APPENDIX

Glutathione and Glutathione S-Transferases: Risk Factors in Multifactorial Diseases

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Chapter VIII

Glutathione and Glutathione S-Transferases: Risk Factors in Multifactorial Diseases

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Abstract

Thiol-containing compounds like glutathione (GSH) have a central role in many biochemical and pharmacological reactions due to the ease with which they are oxidized, and the rapidity with which they can be regenerated. It has long been established that the thiol moiety of GSH is important in antioxidant defense, xenobiotic and eicosanoid metabolism, and regulation of cell cycle and gene expression, and that conjugation with GSH is an essential aspect of both xenobiotic and normal physiological metabolism. GSH forms conjugates with a great variety of electrophilic compounds nonenzymatically, when the electrophile is very reactive, or more often through the action of glutathione S-transferases (GST). The role of GSH and GSTs as cell housekeepers engaged in the detoxification of xenobiotics, but also their involvement in stress response, cell proliferation, apoptosis, oncogenesis, tumor progression and drug resistance, along with the interindividual GST variability, has led to associations with several pathologies. This chapter reviews the most recent knowledge about the central role of GSH and GSTs in the pathophysiology of human diseases and focuses on their involvement in cancer cell growth and differentiation, and other multifactorial diseases like diabetes or heart diseases.

Abbreviations

Ala – alanine
Arg – arginine
Asn – asparagine
Asp – aspartic acid
BRCA1 – breast cancer gene 1
BRCA2 – breast cancer gene 2
Cys – cysteine
FAA – fumarylacetoacetate
Glu – glutamic acid
Gly – glycine
GSH – glutathione
GST – glutathione S-transferase
GSTT2P – GSTT2 pseudogene
HDL – high density lipoprotein
Ile – isoleucine
Lys – lysine
MAA - malaylacetoacetate
MAAI – maleylacetoacetate isomerase
MAPEG - membrane-associated proteins in eicosanoid and glutathione metabolism
Met – methionine
NAT – N-acetyltransferase
PAHs – polycyclic aromatic hydrocarbons
Pro – proline
RNS – reactive nitrogen species
ROS – reactive oxygen species
Ser – serine
SLE – systemic lupus erythematosus
Thr – threonine
Tyr – tyrosine
Val – valine
YY1 – Yin Yang 1

1. Introduction

DNA and cells of the human body are constantly exposed to attacks of an oxidative nature. These attacks can be exogenous, like exposure to ionizing radiation, oxidizing chemicals or UVA solar light, and endogenous through cellular signaling, metabolic processes or inflammation [1]. These endogenously induced DNA lesions can often reach a much higher level than the ones induced by environmental factors like ionizing radiation, even if in low doses, contributing significantly in the accumulation of mutations in cells and tissues. The primary damage inductors are reactive oxygen species (ROS) and reactive nitrogen species (RNS) [2]. Glutathione S-transferases (GSTs) are a family of Phase II detoxification enzymes that have co-evolved with glutathione (GSH) and are abundant

throughout most life forms. GSTs detoxify environmental chemicals and are involved in oxidative stress pathways. They catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds. Several allelic variants of polymorphic GSTs, mainly deletion polymorphisms, show impaired enzyme activity and are suspected to increase the susceptibility to various diseases [3].

2. Glutathione S-Transferases

Glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a superfamily of ubiquitous, multifunctional enzymes that play a key role in cellular detoxification, protecting macromolecules from attack by reactive electrophiles [3]. They catalyze nucleophilic attack by reduced glutathione (GSH; g-Glu-Cys-Gly) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom. Their substrates include halogenonitrobenzenes, arene oxides, quinones, and α,β-unsaturated carbonyls like, for example, products of oxidative stress, environmental pollutants and carcinogens [4]. The addition of GSH to the exogenous and endogenous chemicals neutralizes their electrophilic sites, transforming them in products with higher hydrophilicity, what facilitates their elimination from phase III enzymes. GSTs can also function as peroxidases, isomerases and thiol transferases and have non-catalytic functions such as non-substrate ligand binding and modulation of signaling processes [3]. Human GSTs are divided into three distinct super family members: mitochondrial, cytosolic and microsomal, currently named membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) GSTs [5]. Cytosolic and mitochondrial GSTs share some similarities in their three-dimensional fold [6] but have no structural resemblance to MAPEG enzymes [7]. Human cytosolic GSTs represent the largest and most complex family and they are considered the most relevant to disease investigation. They can catalyze thiolysis of 4- nitrophenyl acetate, display thiol transferase activity, reduce trinitroglycerin, dehydroascorbic acid, and monomethylarsonic acid, and catalyze the isomerization of maleylacetoacetate and Δ^5-3-ketosteroids [8]. Seven classes of cytosolic GSTs were created based on amino-acid sequence similarities, physical structure of the genes and immunological crossreactivity, and are termed Alpha (α), Mu (μ), Pi (π), Theta (θ), Omega (Ω), Sigma (ς) and Zeta (ζ). Kappa (κ) class constitutes the mitochondrial family member [9, 10]. GSTs that share more than 60% on the highly conserved N-terminal domain were merged into a class [10]. The active site of a GST protein is formed by the glutathione-binding site, which is preserved in the different classes, and the hydrophobic substrate-binding site, that has variations in the forming residues, leading to wide substrate specificity [11]. Almost all soluble GSTs are active as homodimers or heterodimers of subunits, and each dimmer is encoded by independent genes [5], resumed in Table 1.

GSTs expression can be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis including phenobarbital, planar aromatic compounds, ethoxyquin, butylated hydroxyanisole, and trans-stilbene oxide. Some of the compounds known to induce GSTs are themselves substrates of the enzyme [12]. Human cytosolic GSTs display polymorphisms that may contribute to interindividual differences in responses to xenobiotics. Those genotypes, alone or in combination, may identify subjects as “low metabolizers”, and consequently more likely to suffer formation of DNA adducts and/or

mutations that confer major susceptibility to complex multifactorial diseases, with genetic and environmental influences, such as cancer [5]. Despite individuals carrying a variant, or combinations of variants, in these low penetrating genes are estimated to have low risk to develop cancer when compared to carriers of mutations in high penetrating genes like BRCA1 and BRCA2, the high frequency in the population of some of the variants makes the population to be interpreted as being of high risk [13]. The first studies focused individuals carrying the homozygous deletion of GSTM1 and/or GSTT1 genes (null genotype). This genotypes result in total absence of a functional gene product, thus a total absence of the respective enzyme activity [14, 15]. After the discovery of allelic variants of GSTP1 that encode enzymes with altered specific activity and affinity depending on the substrate [16, 17], the hypothesis that combinations of polymorphisms in class Mu, Theta and Pi contribute to diseases, along with an environmental component, was analyzed in many researches. Concerning cancer, GSTs genotypes were investigated in relation to bladder [18], prostate [19], breast [20], colorectal [21], head/neck [22], oral [23] and lung cancer [24]. The best characterized cytosolic classes, Alpha, Mu, Pi, Theta, and the emerging class Zeta will be considered in this chapter due to their relevance in diseases in the general population (Table 2). Furthermore, the influence of GSTs polymorphism will be analyzed in relation to several types of cancer.

Table 1. Most relevant GSTs codifying genes and chromosome localization

Class	Chromosome localization	Gene
Alpha (α)	6p12	GSTA1, GSTA2
Mu (μ)	1q13.3	GSTM1, GSTM2, GSTM3, GSTM4, GSTM5
Pi (π)	11q13.3	GSTP1
Theta (θ),	22q11.23	GSTT1, GSTT2
Omega (Ω)	10q24.3	GSTO1
Zeta (ζ)	14q24.3	GSTZ1

3. Genetic Variants of Cytosolic GST Family

3.1. GST Alpha (α) Class

The human GST α class is encoded by genes clustered within chromosome 6p12. The cluster consists of five genes: GSTA1, GSTA2, GSTA3, GSTA4 and GSTA5 [8]. GSTA1, GSTA2 and GSTA4 are widely expressed in all human tissues but mainly expressed in liver, while expression of GSTA3 is rare and expression of GSTA5 has not been detected in human tissues until now [25]. It has been shown that both GSTA1 and GSTA2 genes, the major hepatic GSTs, are polymorphic and variability is thought to affect the efficiency of detoxification of xenobiotics [26-28]. A genetic polymorphism of GSTA1 is characterized by two alleles, GSTA1*A and GSTA1*B. They contain three linked basis substituted in the proximal promoter region, at positions -567, -69 and -52. GSTA*1 have T, C and G at these positions and individuals with GSTA*B have G, T and A. Specifically, the –52 (G/A) substitution has been shown to increase promoter activity in GSTA1*A, thus making it more

highly expressed [29]. Also, liver from individuals who carried the variant GSTA1*B showed reduced levels of GSTA1 enzyme [28]. GSTA2 has not been extensively studied, but it is known to have five variants (GSTA2*A–E), and they are thought not to affect GSTA2 activity. However, the variant GSTA2E shows reduced catalysis rates when compared to variants A–D [30]. Besides GSTA1 and GSTA2, also GSTA3, a GST selectively expressed in steroidogenic tissues involved in steroid hormone biosynthesis, has been proved to be polymorphic [31]. The polymorphism was found exclusively in African populations and was hypothesized that it could affect steroid biosynthesis through altered protein levels or function [27].

Table 2. Polymorphic human cytosolic GSTs

Gene	Allele	Gene alteration	Effect on protein
GSTA1	GSTA1*A	Wild-type	
	GSTA1*B	Promotor point mutation	Low protein levels
GSTA2	GSTA2*A	Pro110; Ser112; Lys196; Glu210	
	GSTA2*B	Pro110; Ser112; Lys196; ALa210	
	GSTA2*C	Pro110; Thr112; Lys196; Glu210	
	GSTA2*D	Pro110; Ser112; Asn196; Glu210	
	GSTA2*E	Ser110; Ser112; Lys196; Glu210	Reduced catalysis rate
GSTA3	GSTA3*A	Ile71	
	GSTA3*B	Leu71	Reduced activity
GSTM1	GSTM1*A	Lys173	
	GSTM1*B	Asn173	
	GSTM1*0	Gene deletion	No protein
	GSTM1*1x2	Duplication	Protein overexpression
GSTM3	GSTM3*A	Wild type	
	GSTM3*B 3	3 base deletion, intron 6	Unchanged protein
GSTM4	GSTM4*A	Tyr2517	
	GSTM4*B	Cys2517	Unknown
GSTP1	GSTP1*A	Ile105; Ala114	105Val – lower or higher activity and affinity depending on the substrate
	GSTP1*B	Val105; Ala114	
	GSTP1*C	Val105; Val114	
	GSTP1*D	Ile105; Val114	
GSTT1	GSTT1*A	Thr104	
	GSTT1*B	Pro104	Decreased activity
	GSTT1*0	Gene deletion	No protein
GSTT2	GSTT2*A	Met139	
	GSTT2*B	Ile139	Unknown
GSTO1	GSTO1*A	Ala140; Glu155	
	GSTO1*B	Ala140; Glu155 deletion	
	GSTO1*C	Asp140; Glu155	
	GSTO1*D	Asp140; Glu155 deletion	25% reduced activity
GSTO2	GSTO2*A	Asn142	
	GSTO2*B	Asp142	20% reduced expression
GSTZ1	GSTZ1*A	Lys32; Arg42; Thr82	
	GSTZ1*B	Lys32; Gly42; Thr82	
	GSTZ1*C	Glu32; Gly42; Thr82	
	GSTZ1*D	Glu32; Gly42; Met82	Reduced catalytic activity

3.2. GST Mu (μ) Class

The GSTM class has five described isoforms, GSTM1-5, encoded by a gene cluster located on chromosome 1p13.3 and arranged as 5'- GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3'[14]. Concerning GSTM1, three polymorphisms have been identified until now. Deletion of the entire GSTM1 gene, GSTM1*0, frequently affects both alleles, resulting in a lack of functional gene product, the so-called null allele [32]. The other two, a missense single nucleotide polymorphism also occurs in the GSTM1 gene, i.e. nucleotide 534 G/C, resulting in asparagine (Asn) to lysine (Lys) substitution at amino acid 173, corresponding to GSTM1*A and GSTM1*B respectively. No evidence of functional difference between GSTM1*A and GSTM1*B variants was found; thus, these alleles are typically categorized together as a single functional phenotype [33]. Detailed mapping of the GSTM gene cluster revealed that the GSTM1 gene is flanked by two almost identical 4.2-kb regions. The GSTM1*0 deletion is caused by a homologous recombination involving the left and right 4.2-kb repeats and the GSTM1 gene is excised relatively precisely leaving the adjacent GSTM2 and GSTM5 genes intact [32]. This homozygous deletion (GSTM1 null) has been examined extensively in epidemiologic studies. The frequency of the null genotype is around 50% in Caucasians and Asians, but only 27% in Africans [11]. Subjects with a homozygous deletion of the GSTM1 locus have no enzymatic functional activity and several studies suggest that the GSTM1 null genotype can interfere in the drug and carcinogen detoxification [13, 25]. The GSTM3 locus contains two alleles, A and B. The GSTM3*B allele has a three base pair deletion in intron 6 that introduces a recognition motif for the YY1 (Yin Yang 1) transcription factor, which is known to have a fundamental role in normal biologic processes such as embryogenesis, differentiation, replication and cellular proliferation. A linkage disequilibrium has been noted between the GSTM1*A and GSTM3*B alleles [34]. Also, two new polymorphisms have been detected in the GSTM3 gene, a rare p.G147W substitution and a more common p.V224I substitution. These two polymorphisms can combine to form four different isoforms. The p.W147 variant seems to exhibit decreased catalytic and specific activity, whereas the p.I224 variant has shown increased catalytic and specific activity [35].

3.3. GST Pi (π) Class

The GST Pi class is encoded by a single gene, GSTP1, the most studied of GSTs genes, that spans approximately 3 kb and located on chromosome 11q13 [36]. GSTP1 is expressed in many tissues including breast and lung, where it is the predominant GST [37]. GSTP1 is polymorphic with two common functional variants based on substitutions in amino acids 105, Isoleucine (Ile) to Valine (Val), and 114, Alanine (Ala) to Val, demonstrating different catalytic efficiencies due to changes in the active site. Thus, four haplotypes have been identified: the wild-type GSTP1*A (Ile105 + Ala114) and three variant haplotypes, GSTP1*B (Val105 + Ala114), GSTP1*C (Val105 + Val114) and GSTP1*D (Ile105 + Val114). Both amino acids changes lie in close proximity to the hydrophobic-binding site and affect substrate specificity to the point of distinguishing between planar and nonplanar substrates [16]. The Val105 variant has been demonstrated to have either lower or higher specific activity and affinity depending on the substrate. For example, Ile105 seems to have higher catalytic efficiency for 1-chloro-2,4-dinitrobenzene than the Val105 variant, and the

latter seems to confer higher catalytic efficiency to polycyclic aromatic hydrocarbon (PAH) diol epoxide detoxification [5, 38]. The Ala114Val polymorphism seems not to influence the enzyme activity. Only about 5–10% of Caucasians have been shown to be homozygous for the Val allele [39, 40].

3.4. GST Theta (θ) Class

The Theta class of GSTs consists of two different subfamilies: GSTT1 and GSTT2. Genes encoding both proteins are co-localized on chromosome 22q11.2 and are separated by 50 kb [41]. This class is considered the most ancient of the GSTs, and θ-like GSTs are found in almost all the investigated organisms [5]. Both GSTM1 and GSTM2 genes have five exons with identical intron/exon limits but share only 55% amino acid identity. Among the GSTT substrates, there are several environmental carcinogens found in food, air or medications, such as polycyclic aromatic hydrocarbons (PAHs), found in combustion products, diet and tobacco smoke [42]. GSTT1 is expressed in human erythrocytes, and various tissues including liver, but no expression in breast tissue has been reported [41]. Polymorphisms exist within both genes. Similar to GSTM1, homozygous deletion exists in GSTT1 gene and results in the lack of the active codified enzyme [43]. Large inter-ethnic differences have been reported in the frequencies of the GSTT1 null genotype; the prevalence of GSTT1 null genotypes is significantly lower among Caucasians (10–20%) compared to Asians (50–60%) [44, 45]. Another less common polymorphism results in a threonine (GSTT1*A) to proline (GSTT1*B) substitution at amino acid 104. The GSTT1*B allele shows a decreased catalytic activity when compared to the GSTT1*A allele, which could be attributed to the conformational change induced by the proline substitution [46]. Regarding GSTT2 gene, the transition of guanine to adenine in intron 2 gives rise to a pseudogene (GSTT2P). Evidence points to transcription of this pseudogene, although the protein product is thought to be inactive [25].

3.5. GST Omega (Ω) Class

In humans, the Omega class of GSTs contains two members (GSTO1 and GSTO2) and a pseudogene (GSTO3p). This class has different characteristics in structure and function from the other members of GST superfamily; for example, these enzymes have a cysteine residue in their active site in contrast to serine or tyrosine that is in the active sites of other subfamilies. Also, X-ray crystallography shows a unique 19-residue N-terminus extension that forms a structural unit unlike any other found in other classes. Furthermore, these GSTs exhibit poor activity with common GST substrates (such as 1-chloro-2,4-dinitrobenzene) but exhibit novel GSH dependent thioltransferase, dehydroascorbate reductase and monomethylarsonate reductase activities, and are able to modulate Ca^{2+} release by ryanodine receptors [47]. Expression of GSTO1 is abundant in a wide range of normal tissues, including liver, colon, heart, ovary, pancreas, prostate, spleen, macrophages, endocrine and glial cells. The widespread distribution of GSTO1 suggests that it has important biological functions, although they remain undefined [48]. To date, four polymorphisms have been identified, GSTO1*A–D. Among the Australian, African and Chinese populations, GSTO1*A was the

most prevalent haplotype and demonstrated a GSH-dependent reduction of dehydroascorbate, a characteristic function of glutaredoxins rather than GSTs [47]. This allele was first described as the human monomethylarsenic acid reductase, and is the rate-limiting enzyme of inorganic arsenic metabolism [49]. GSTO1*C results in an alanine (Ala) to aspartic acid (Asp) substitution at amino acid 140, which creates a non-conservative amino-acid change from hydrophobic to hydrophilic residue [47]. Thioltransferase activity differs among the GSTO1*A–C polymorphisms.

Thioltransferase activity of GSTO1*C was verified to be 75% of the wild type, reflecting that it may result in defective protection against cellular oxidation stresses and may contribute to individual capacity to metabolize arsenic [50]. GSTO2 is separated from GSTO1 by 7.5 kb on chromosome 10 and shares 64% amino-acid identity [48]. Like GSTO1, GSTO2 is ubiquitously expressed and shares GSH-dependent dehydroascorbate reductase activity. However, GSTO2 has a high catalytic activity toward chlorodinitrobenzene, and its overexpression induced apoptosis, suggesting a possible role in cell signaling. In GSTO2 gene, a transition of adenine to guanine at nucleotide position 424 in exon 4 (GSTO2*C) was reported, which results in an amino-acid difference from asparagine (Asn) to aspartic acid (Asp) in codon 142. It was reported that the GSTO2 Asp142 variant allozyme showed 20% reduction in level of expression when compared with the level of the GSTO2 wild-type (Asn142) allozyme [51].

3.6. GST Zeta (ζ) Class

GSTs Zeta class (GSTZ1) is constituted by a single gene located on chromosome 14, spanning 10.9 kb, and encoding for a 29 kDa protein [52]. The cytosolic GST Zeta class has been identified as a maleylacetoacetate isomerase, and therefore catalyzes the penultimate step in the catabolism of phenylalanine and tyrosine [8]. GSTZ1 is preferentially expressed in hepatocytes and renal proximal tubule cells where phenylalanine and tyrosine are catabolized [53].

Although literature is restrictive for this class, GSTZ1 polymorphisms have been identified (GSTZ1*A–D). The isozyme GSTZ1*A has the highest catalytic activity toward dichloroacetic acid, an investigational drug for certain metabolic disorders, and a nephrotoxic metabolite of industrial solvents [52]. In contrast, GSTZ1*D (p.T82M) has a reduced catalytic activity and has been associated with innate errors in tyrosine metabolism, although the disorders have also been attributed to mutations in other enzymes. Rodent models deficient for GSTZ1 provide insight into its role in metabolic deficiencies. GSTZ1/maleylacetoacetate isomerase (MAAI) converts maleylacetoacetate (MAA) to fumarylacetoacetate (FAA).

GSTZ1- deficient mice have an elevated urinary excretion of FAA and were subject to renal injury following phenylalanine and tyrosine overload [53]. In fact, four families have been identified that have GSTZ1-deficient members that have died within the first year of life. Although clinical data for GSTZ1 are insufficient to deduce a role for GSTZ1 in inherited genetic disease, it is plausible that a perturbation in GSTZ1-mediated tyrosine metabolism is contributory to the described pathology [25].

4. GSTs Polymorphisms and Predisposition to Multifactorial Diseases

4.1. Cancer

In the last twenty years an increasingly higher number of cancer susceptibility genes were known. At this point, the role of low penetrance genes has not been completely understood. Cancer is a multifactorial complex disease resulting from environmental/lifestyle and genetic influences. Germline mutations in the so-called high penetrance genes to cancer susceptibility appear to account for the majority of hereditary breast cancer, but they represent only a small part of all cancer cases. Thus, low penetrance genes, acting together with endogenous or life-style risk factors, can be associated with a significant percentage of cancer cases. Low penetrance genes can be found in several pathways like detoxification of environmental carcinogens, steroid hormone metabolism and DNA damage repair pathways. GSTs became interesting in the investigation of a potential association with cancer risk due to the wide range of compounds, including several environmental and endogenous carcinogens, they can detoxify. As discussed earlier, inherited genetic traits co-determine the susceptibility of an individual to metabolize toxic endogenous or exogenous chemicals. Studies regarding this issue, and relation with several types of cancers will be further discussed.

4.2. The Significance of GST Null Genotypes in Cancer

Cytosolic GSTs display polymorphisms in humans and this is likely to contribute to interindividual differences in responses to xenobiotics. GSTM1 and GSTT1 null genotypes have been the focus of several studies because of their special condition: the majority of polymorphisms affecting genes involved in carcinogen metabolism are single nucleotide polymorphisms, deletions are less common, and the complete absence of a gene in form of a null allele is rare. Deletion of the GSTM1 and GSTT1 genes results in a 'null' genotype characterized by a general deficit in enzymatic activity. GSTs are phase II detoxifying enzymes and the polymorphisms in the codifying genes may be associated with the increased susceptibility to cancer, as normal or increased GSTs activity may facilitate detoxification of electrophilic carcinogens, protecting susceptible tissues from somatic DNA mutations [13]. Thus, population with reduced GSTs enzyme activity, as result of homozygous deletions of GSTM1 or GSTT1, may be at greater risk for malignancies due to their impaired ability to metabolically eliminate carcinogenic compounds and reduced detoxification efficiency [8]. Thus, the earliest studies in this area addressed the question of whether individuals lacking GSTM1 and/or GSTT1, i.e., are homozygous for GSTM1*0 and/or GSTT1*0 alleles, have a higher incidence of bladder, breast, colorectal, head/neck, and lung cancer [25]. This phenotype has been studied as a predictive factor for cancer prognosis or response to therapy and the results achieved have been dependent upon tumor type and population. It has been found that GSTs genes do not make a major contribution in susceptibility to cancer, at least by themselves. GSTM1*0 seems to have a modest effect on lung cancer [55], GSTM1*0 and GSTT1*0 seem to have a modest effect on the incidence of head and neck cancer [56]. Also, GSTM1*0 and GSTT1*0, alone or in combination, seem to be associated with higher

susceptibility to breast cancer development [13]. It is important to note that a possible limitation of many studies into the biological effects of GSTM1*0 and GSTT1*0 is that only individuals who are homozygous nulled for these genes (−/−) have been identified, due to the PCR method used in the analysis. Generally, the absence of a PCR product indicates the GSTM1 or GSTT1 null/null genotype and individuals are categorized as either "present" (wild-type) or "null" genotypes. This analytical approach does not positively identify the null allele and, therefore, cannot distinguish homozygous wild-type from heterozygous present/null individuals but it conclusively identifies the null/null genotypes [13]. Consistently, individuals who are heterozygous (−/+) or homozygous (+/+) for the functional allele are not distinguished and are not analyzed separately. As a consequence, the significance of being homozygous wild type for GSTM1 and GSTT1 is rarely addressed and the benefit of such a genotype is probably underestimated in the literature because it is grouped together with the heterozygote genotype [8]. New assays that differentiate between −/−, −/+, and +/+ genotypes at the GSTM1 and GSTT1 locus have been developed, and revealed significant protection against breast cancer in homozygous GSTM1 +/+ individuals [57, 58]. Besides the effects in tumorigenesis, several studies indicate that loss of these genes increase susceptibility to inflammatory diseases, such as asthma and allergies, atherosclerosis, rheumatoid arthritis, and systemic sclerosis [59, 60, 61].

4.3. Breast Cancer

Breast cancer is the prevailing cancer among women in industrialized countries [11]. Although many risk factors for the development of breast cancer have been identified, the molecular mechanisms related to breast carcinogenesis remain unclear. Estrogens have been clearly identified has carcinogens, by inducing aneuploidy and structural chromosomal changes and stimulation of breast-cell proliferation has been proposed as the main effect of estrogens in breast carcinogenesis: as more rapidly cells proliferate, greater the chance of acquiring a potentially cancer-causing mutation. Also, metabolic by-products of estrogens are responsible for free-radical-mediated DNA damage, single-strand breaks, estrogen–DNA adducts formation, protein oxidation and lipid peroxidation, what triggers genetic instability and cellular damage [62, 63]. Inter-individual variability has been observed in estrogens biosynthesis and metabolic pathways, namely in genes encoding for proteins involved in estrogen biosynthesis, and in genes encoding for xenobiotic metabolizing enzymes, like GSTs. The results regarding the genotypes distribution of GSTM1, GSTT1, and GSTP1 in cases and controls in the Portuguese population were published by our group, as well as the results of the association in two and three-way combinations to evaluate the impact of gene-gene interaction [13]. We found that GSTM1 null genotype was significantly more common among breast cancer cases compared to controls (OR 2.592; 95% CI 1.432–4.690;P = 0.002), so as GSTT1 (OR 3.597; 95% CI 1.849–6.999; P = 0.0001). We did not find any significant increase of breast cancer risk associated with GSTP1 genotypes (OR 1.103; 95% CI 0.611–1.989; P = 0.765) when compared Val allele carriers in homozygosity or heterozygosity with Ile/Ile carriers. We also found an 8-fold increased breast cancer risk in women who carry null genotype both in GSTM1 and GSTT1 (OR 8.287; 95% CI 3.124–21.980; P = 0.0001). After analyzing the three-way combination of GSTM1, GSTT1, and GSTP1 polymorphisms we found that Ile/Ile genotype, so as presence of Val allele, seemed to be associated with risk of

breast cancer when combined with both GSTM1 and GSTT1 null genotypes (OR 12.600; 95% CI 2.358–67.315; P = 0.001 and OR 5.040; 95% CI 1.392–18.248; P = 0.016), so it appears that the increase of breast cancer risk was mainly given by GSTM1*0/GSTT1*0 genotype, rather than by any genotype of GSTP1. An investigation conducted by Mitrunen and collaborators [64] analysed the association of GSTM1, GSTM3, GSTP1 and GSTT1 polymorphisms with the risk of breast cancer development in 483 patients. They observed a positive association with GSTM1 null genotype (OR 1.49; 95% CI 1.03 – 2.15) and GSTM3*B allele. Also, the risk was significantly higher in women with GSTT1 null (OR 9.93; 95% CI 1.10 – 90). For GSTP1 Ile/Ile genotype they observed an OR of 2.07 (95% CI 1.02 – 4.18). These authors found that in the combination GSTM1*0-GSTT1*0-GSTP1 Ile105/Ile105, the OR was of 3.96 (95% CI 0.99 – 15.8). Another group analyzed seven studies of GSTM1, GSTT1 and GSTP1*B polymorphisms in 2048 breast cancer patients and 1969 controls [65]. The authors obtained no significant association of these polymorphisms and breast cancer development. As was described, and as is observed in other types of cancer, the majority of the studies concentrated on GSTM1, GSTT1 and GSTP1 genes. Although, other genotypes were analyzed, reaching the same conflictuous conclusions. Recently, the group of Andonova [66] did not observe any breast cancer risk associations with GSTA2, GSTM2, GSTO1, GSTO2, and GSTZ1polymorphisms. Regarding GSTA and GSTO, most of the evidence for GSTA1*B polymorphism has a negative association with breast cancer, and the same occurs with GSTO2*B [67].

4.4. Prostate Cancer

Prostate cancer is the most common cancer among men in industrialized countries. Prostate cancer is uncommon in men younger than 45, becomes more common with increasing age and the main risk factor is being the age of over 50 [68]. Increased exposure to carcinogens is implicated in multistage carcinogenesis, thus the differences in the effectiveness of detoxification of carcinogens could possible help to understand why one man may be at higher risk than another. As in the other types of cancer, it was much speculated that GSTM1, GSTT1 and GSTP1 polymorphisms could be associated with the carcinogenic development in the prostate. The results regarding this issue were, once again, inconclusive. A meta-analysis of 29 studies, comprising 4564 cases and 5464 controls concluded that GSTM1 null genotype increased prostate cancer risk (OR 1.3; 95% CI 1.15 – 1.55). In the same report, 22 studies including 3837 cases and 4552 controls were analyzed, and association of prostate cancer with GSTT1 null genotypes was not verified [69]. Another meta-analysis conducted to analyze several studies of GSTM1, GSTT1 and GSTP1 polymorphisms and prostate cancer did not obtain correlation between GSTM1 and GSTT1 null genotypes, and GSTP1*B genotype, and risk for breast cancer [70]. Concerning GSTA, it was found an association with prostate cancer risk in Japanese that carried GSTA1*B polymorphism and also an increase of risk in carriers of the combined GSTA1*AB or BB and GSTT1 “present” genotypes [71]. However, another study evaluating various GSTA1 and GSTA2 genetic polymorphisms did not find any type of association with prostate cancer risk [30]. A study performed in a southern European population found no association of prostate cancer risk with GSTM1 or GSTT1 null genotypes (OR 1.20; 95% CI 0.75 – 1.90; p = 0.420).

However, they found a 5.5-fold increased risk of breast cancer associated with GSTM3*BB genotype (OR 5.50; 95% CI 1.2 – 25.8; p = 0.016) [72].

4.5. Lung Cancer

Coal, wood, biomass smoke, and cooking oil fumes have been associated with a variety of health outcomes, namely lung cancer [72]. The associations observed between air pollution and lung cancer risk are not surprising since fuel combustion products are known to contain carcinogens. Smoky coal combustion increases levels of sulfur dioxide, carbon monoxide, fluorine, and known carcinogens such as PAHs, benzene, arsenic, and formaldehyde. Genetic variation in enzymes responsible for activating and detoxifying PAHs or other carcinogens may alter susceptibility of individuals exposed to these environmental pollutants. GSTs are involved in the metabolic detoxification of reactive electrophilic compounds, such as PAHs, that are formed during incomplete combustion of carbon-based fuels such as coal and wood [72].

As denoted previously, GSTP1 is the GST isoform most expressed in lung, having among its main metabolic agents the PAHs [37]. A meta-analysis of six studies comprising 560 cases and 635 controls evaluated GSTM1, GSTT1 and GSTP1 genotypes and their association with risk of lung cancer in Asians populations [73]. They found that carriers of GSTM1 null genotype had a borderline significant increased lung cancer risk (OR 1.31; 95% CI 0.95-1.79; p=0.10). The GSTT1 null genotype was also associated with an increased lung cancer risk (OR 1.49; 95% CI 1.17-1.89; p = 0.001) but association was observed for the GSTP1 105 Val allele. A larger meta-analysis of 27 studies (8322 cases and 8844 controls) analyzed the association of GSTP1*B (105 Val) and lung cancer risk and, once more, no significant association was found [74]. The same work included a pooled analysis of 15 studies (4282 cases and 5032 controls), in which was found a slightly increased risk of lung cancer associated with Val 105 allele, compared with Ile 105 (OR 1.11; 95% CI 1.03-1.21). Several studies focus on associations between tobacco habits, development of lung cancer, and GSTM1 and GSTT1 genotypes. A meta-analysis that included 34 studies, with 7629 cases of lung cancer and 10087 controls suggested a positive association for GSTT1 null in Asians (OR 1.28; 95% CI 1.10 – 1.49) but not in Caucasian individuals (OR 0.99; 95% CI 0.87 – 1.12). No significant interaction was observed between GSTT1 and smoking on lung cancer susceptibility [75]. In another study that enrolled 1921 lung cancer cases and 1343 healthy Caucasians the association of GSTP1*B genotypes and risk of lung cancer was not verified (OR 1.02; 95% CI 0.78 – 1.34) [76].

4.6. Gastric Cancer

Gastric cancer is the fourth most common cancer and the second most frequent cause of cancer death all over the world. It is widely accepted that gastric carcinogenesis is a multilevel multifactorial process. Despite several studies showing that infection by Helicobacter pylori is the cause of most gastric cancer, genetic factors are believed to play an important role in the development of gastric cancer.

Interindividual variations in the genetic and cellular mechanisms of activation and detoxification of cancer causing chemicals could confer different degrees of susceptibility to gastric cancer. Studies investigating the association between genetic polymorphisms of GSTs and gastric cancer risk have reported conflicting results [77]. A meta-analysis comprising eighteen case-control studies (2508 cases and 4634 controls) detailing a possible association between the GSTT1 null genotype and gastric cancer, observed a non-statistically significant OR for gastric cancer risk associated with GSTT1 deficiency (OR 1.09; 95% CI 0.97-1.21). By pooling data from seven studies (319 cases and 656 controls) that considered combinations of GSTT1 and GSTM1 genotypes, a statistically significant increased risk for gastric cancer (OR 1.95, 95% CI 1.42-2.67) was detected for individuals with deletion mutations in both genes compared with wild-types, suggesting that the GSTT1 null genotype may slightly increase the risk of gastric cancer [78]. Another meta-analysis enrolled 4357 gastric cancer cases and 9796 controls, and the combined results based on all studies showed there was a significant link between GSTT1 null genotype and gastric cancer risk (OR 1.14, 95% CI 1.01–1.28). In the subgroup analysis stratified by ethnic group, it was observed a positive association between GSTT1 polymorphism and gastric cancer risk among Caucasians, but not among East Asians [77]. In a European case-control study, including 304 gastric cancer cases and 427 control subjects, a 1.48-fold increased risk was found in carriers of GSTT1 null genotype, but not with GSTM1, GSTM3 or GSTP1 genotypes [79].

4.7. Hepatocellular Carcinoma

Hepatocellular carcinoma accounts for 85 to 90 percent of all primary liver cancers. Genetic variation has been postulated to influence risk for hepatocellular carcinoma and currently the most extensively studied inherited genetic risk factors for hepatocellular carcinoma are variants of GSTs [80]. Interestingly, liver and kidney are two organs that express the highest level of GSTT in the human body [5]. Recently, a meta-analysis of 24 individual case-control studies, involving a total of 3349 hepatocellular carcinoma and 5609 controls, investigated the associations between GSTs genetic polymorphisms and this disease. The results revealed an increased risk for hepatocellular carcinoma significantly associated with null genotypes of GSTM1 (OR 1.26; 95% CI 1.03-1.54, p=0.027) and GSTT1 (OR 1.28; 95% CI 1.09-1.51, p=0.002). Also, the GSTM1-GSTT1 interaction analysis showed that the dual null genotype of GSTM1/GSTT1 was significantly associated with increased hepatocellular carcinoma risk (OR 1.89, 95% CI 1.38-2.60, p<0.001) [81]. Another meta-analysis in 15 studies associated hepatocellular carcinoma risk with GSTA1, GSTA4, GSTM1, GSTM2, GSTM3, GSTT1, GSTP1, GSTO1 and GSTO2 polymorphisms in Asian, African and European populations. The authors observed that only GSTT1 (OR 1.19; 95% CI 0.99-1.44) and GSTM1 (OR 1.16; 95% CI 0.89-1.53) null carriers showed a positive association [82].

A study demonstrated that GSTT1*A carriers have an elevated risk for hepatocellular carcinoma development when exposed to halogenated solvents [83]. However, another study found that GSTT1 null individuals have an increased risk for hepatocellular carcinoma, and GSTM1 null genotype presented a protective effect [84].

4.8. Colorectal Cancer

Colorectal cancer is the third most common cancer and is globally the fourth most common cause of cancer. Large variations in rates have been found among different regions, with the lowest rates in Africa and Asia, and the highest in Europe, North America, and Australia [85]. Controversy exists about whether GST polymorphisms (GSTM1 null/present genotype, GSTT1 null/present genotype, GSTP1 Ile105Val and GSTA1*A/*B) represent risk factors for colorectal cancer. A meta-analysis involving forty-four studies, with 11998 colorectal cancer cases and 17552 controls, examined the associations between the above-mentioned polymorphisms and colorectal cancer. GSTM1 null allele carriers exhibited increased colorectal cancer risk in Caucasian populations (OR 1.150; 95% CI 1.060–1.248), and no significant association was detected for Chinese subjects (OR 1.025; 95% CI 0.903–1.163). In the same study, the authors found that GSTT1 null allele carriers exhibited increased colorectal cancer risk in Caucasian populations (OR 1.312; 95% CI 1.119–1.538). These associations were not found in the Chinese populations. Concerning GSTP1 Ile105Val and GSTA1*A/*B, no significant associations were demonstrated in either ethnic group [86]. Another meta-analysis of 36 case-control studies (10009 cases and 15070 controls) analyzed the association of GSTM1 polymorphisms and colorectal cancer. The combined data showed that GSTM1 deficiency is associated with a marginal effect on colorectal cancer risk (OR 1.13; 95% CI 1.03–1.23; $p<0.001$). When stratified by ethnic group and tumor site, significant results were only observed in Caucasians (OR 1.14, 95% CI 1.01–1.27; $p<0.001$), whereas no increased risk was detected in other subgroups, suggesting that GSTM1 polymorphism is associated with an increased risk of colorectal cancer, especially in the Caucasian population [85].

4.9. GSTs Polymorphisms and Tumor Drug Resistance

One of the primary causes of cancer treatment failure and patient relapse is an acquired or intrinsic resistance to anticancer therapies. Acquisition of drug resistance can be attributed to various factors that include avoidance of apoptotic cell death, altered expression of multidrug resistance-associated proteins and altered drug metabolism or uptake [25]. Recent studies have shown that the simultaneous polymorphisms of multiple classes of GSTs may correlate with therapeutic. Furthermore, it has been demonstrated that simultaneous polymorphisms in different classes may have more influence than a single gene by its own, maybe because various classes of cytosolic GSTs share overlapping substrate specificities and, therefore, absence of one GST isoform can be compensated by increased expression of other GSTs [88]. In cancer chemotherapy, pharmacogenetic studies have traditionally focused on candidate genes like GSTs, which function to detoxify reactive metabolites, thus interfering on pharmacokinetic characteristics of a specific drug. This polygenetic approach is also due to the combination of different anticancer agents with which patients are frequently treated, that are metabolized by different GSTs [88]. The most focused genotypes in relation to tumor drug resistance are the homozygous deletions of GSTM1 and GSTT1, and also the substitution of Isoleucine to Valine in amino acids 105 of GSTP1 gene. Recently, head and neck squamous cell carcinoma cases carrying the null genotypes of GSTM1 and GSTT1 or variant genotypes of GSTP1 showed a significant and superior treatment response [89].

Regarding colorectal cancer, it was found that mortality is significantly reduced in patients with one GSTM1 copy (HR 0.45; 95% CI 0.23-0.90; p = 0.02) and nonsignificantly reduced in those with the null genotype (HR 0.67; 95% CI 0.35-1.27; p = 0.22), compared with carriers of two copies. In this study, both GSTP1 and GSTT1 genotypes were not associated with survival [90]. In relation to ovarian cancer, was observed a significant survival advantage among carriers of GSTP1 Ile105Val GG/GA genotype (HR 0.77; 95% CI 0.61-0.99; p=0.04) and a non-significant survival advantage among homozygous women for the GSTM1 and GSTT1 deletion variants. There was also evidence of an additive effect, with a stronger survival benefit in women carrying three low function GST genotypes GSTM1 null, GSTT1 null and GSTP1 GA/GG (HR 0.47, 95% CI 0.22-1.02) [91].

4.10. Influence of GSTs Null Genotypes in other Multifactorial Diseases

Inflammation and oxidative stress are implicated in the pathogenesis of several multifactorial diseases such as heart diseases and diabetes mellitus. Oxidative stress is associated with increased generation of reactive oxygen species that leads to enhanced lipid peroxidation, the generation of hydroperoxides and other toxic compounds which may contribute to the development of atherosclerosis. Oxidative stress and inflammation biomarkers differ by GST genotype: the dual deletion, GSTM1-0/GSTT1-0 (null genotype), seems to be associated with higher serum iron, total and LDL-cholesterol concentrations, and lower malondialdehyde concentrations [92-96]. The null GSTM1 genotype has been associated with elevated levels of DNA adducts and besides the fact that the DNA adducts are promutagenic and procarcinogenic, they are detectable in smooth muscle cells of human abdominal aorta affected by atherosclerotic lesions [97]. The same authors evaluated the polymorphic status of GSTM1 and GSTT1 in the population of atherosclerotic patients who had previously been investigated for presence of adducts in smooth muscle cells and they provide evidence that the levels of adducts are consistently increased in patients with GSTM1 null genotype, but no association was established with the GSTT1 polymorphism [98]. With respect to GSTs variant role in relation with heart diseases, literature remains unclear. Research concerning the influence of smoking on cancer risk demonstrated that these enzymes may result in either harmful or protective actions depending on whether GSTs activate or deactivate relevant chemicals [33, 95, 99]. Also, polymorphisms of GSTM1 and GSTT1 may modify the effects of cigarette smoking on risk of coronary heart disease and diabetes [100]. On a recent study, Tang et al [93] showed that the common variants in the GSTM1 and GSTT1 genes are related to markers of oxidative stress and inflammation in patients with coronary artery disease. Surprisingly, authors found a negative correlation between GSH levels and the GSTT1 gene variant. It seems that the reduction of GSTs level may result in the induction of a compensatory process in which other GSTs are overexpressed. GSTM1-0/GSTT1-0 subjects had higher C-reactive protein, fibrinogen and lower plasma total antioxidant status, compared to patients with wild-type GSTM1/GSTT1 genes. Stepwise elevations in age, the incidences of hypertension and diabetes mellitus, and levels of fibrinogen, as well as reductions of total plasma antioxidant status and GSH were associated with increased number of stenosed vessels [33]. Stroke, an interruption or severe reduction of blood flow arteries, is a leading cause of death in the world. Carotid stenosis resulting from atherosclerosis, hypertension, and diabetes are among the several risk factors

for ischemic stroke. Association analysis of GSTT1 and GSTM1 polymorphisms and GST total serum activity with ischemic stroke risk performed by Adah et al showed that GSTT1 and GSTM1 null genotypes, together with hypertension, may play a role in the pathogenesis of ischemic stroke [101]. An association between the GSTT1 variant, markers of inflammation and lipid peroxidation was described on Caucasian subjects with diabetes [94]. Krieger et al established an association between GST polymorphisms, triglycerides and HDL-cholesterol, and by reviewing Morrow et al and Evans et al issues, they hypothesized that GSTs are involved in the reverse cholesterol transport [102-104]. Chronic oxidative stress may also contribute to the increase of intraocular pressure by increasing resistance of aqueous humour outflow through trabecular meshwork [96]. The increase of intra ocular pressure is known to be one of the major risk factors for glaucoma, a progressive neuropathy which has a characteristic pattern of optic nerve and visual field damages. Glaucoma exhibits a heritable susceptibility consistent with a complex trait inheritance. Several studies concerning association of GST genetic polymorphisms with the risk of developing primary open angle glaucoma in different populations have been carried. GSTM1 polymorphism seems to be associated with increased risk of development of primary open-angle glaucoma among Estonians and Brazilians. A possible association of the GSTM1 positive genotype and GSTT1 null genotype, or the combination of both, with the increased risk of development of this disease was found in the Turkish population, however no evident association was found between GSTM1 and glaucoma in the Swedish population [105-108]. Concerning systemic lupus erythematosus (SLE), a chronic autoimmune disease featured by inflammation induced by autoantibodies, there are several studies postulating that GSTT1, GSTM1, and CYP1A1 polymorphisms may be involved in the development of SLE. The results are still not conclusive [109-111]. Glutathione S-transferases have an important role from social point of view as the intake of alcohol and caffeine could have different individual effects associated with the allelic variants of the enzymes. Glutathione S-transferase allelic variants have been associated with alcoholic liver disease. Carriers of GSTM1 null genetic variant or Val/Val genotype of Ile/Val GSTP1 polymorphism may have an increased risk to suffer from alcoholic liver disease and GSTs seem to be a potential therapeutic target in alcoholic liver disease [112]. Obstetric and gynecological diseases also seem to be associated with GSTs polymorphisms. A study performed by Tanaka et al in Japan found that the frequency of individuals with the GSTM1 deletion was significantly higher in cases of recurrent miscarriage compared with the control population among coffee drinkers, and concluded that GSTM1 enzyme activity may represent a risk factor for recurrent miscarriage, especially in the population of coffee consumers, as a result of impaired placental detoxification [113]. Women with the GSTM1 null genotype are reported to have a shorter gestation by almost one week compared to women with the normal genotype. This difference was not observed in the present study for either smokers or non-smokers [114]. However, risk of foetal growth restriction in mothers who smoked during pregnancy was modulated by maternal metabolic gene polymorphisms, as Guiguet et al observed on a case-control study in the Canadian population, where they reported a fourfold increased prevalence for a small-for-gestational-age outcome in homozygous mothers for CYP1A1 with a significant interaction between maternal smoking in the third trimester and CYP1A1, or absence of newborn GSTT1 [115]. These genes are linked to cytochrome P450 enzyme activity which is increased in CYP1A1 and decreased in homozygous deficient GSTT1 genotypes, indicating a genetic influence on cell metabolism which is altered by cigarette smoke exposure during pregnancy [115, 116].

The effect of polymorphic variants of GSTs have also been a study target for drug response; GSTM1 null genotype contributed to the hepatic disorder induced by hydrazine, a metabolic compound originated from isoniazid metabolism [92]. No difference was observed by GSTT1 genotype with respect to pharmacological treatment with ACE inhibitors, statins, aspirin or insulin [94]. Although this chapter focus GSTs genotypes it is not unlikely that one single low penetrance polymorphism is pivotal in carcinogenesis, atheromatosis and even longevity. Most findings regarding phase II genes reported an association between polymorphisms in NAT, GSTM and GSTT and various types of cancer or coronary heart disease. Lacczmanska et al and van Larebeke et al found that polymorphisms in genes encoding for xenobiotic metabolizing enzymes had a greater influence on reaching age 50-65 without severe morbidity than polymorphisms in genes encoding for DNA repair [117,118].

Conclusion

The importance of gluthatione is evident by the widespread expression in plants, mammals, fungi and some prokaryotic organisms. GSH is an important component of antioxidant defenses, and an antioxidant by itself, as the thiol group is a potent reducing agent. As an antioxidant, GSH plays a role in the detoxification of a variety of electrophilic compounds via catalysis by glutathione S-transferases. In addition to detoxification, GSH plays a role in several other cellular reactions, including the glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides, and regulation of protein and gene expression via thiol-disulfide exchange reactions. GSTs are involved in Phase II detoxification, protecting cells from attack by reactive electrophiles or reducing the ability of the cells to metabolize toxins. Due to their relevant functions and since they display interindividual variations, special focus has been put on GSTs, associating them with several multifactorial diseases. In fact, allelic variants of relevant xenobiotic metabolizing enzymes are often considered as a differential risk of developing a disease such as cancer. Although polymorphisms have been described in several GSTs, most studies emphases polymorphisms in class Mu and Theta because GSTM1 and GSTT1 may suffer homozygous deletions. The majority of polymorphisms affecting genes involved in carcinogen metabolism are single nucleotide polymorphisms, deletions are less common and the complete absence of the entire gene in form of a null allele is rare. Thus, population with reduced GST enzyme activity, as result of homozygous deletions of GSTM1 or GSTT1, may be at greater risk for developing diseases due to their impaired ability to metabolically eliminate carcinogenic compounds and reduced detoxification efficiency. As it was extensively discussed in this chapter, the studies performed about this issue report conflicting results. The inconsistent results between studies reflect the complexity in the role of GSTs and might be due, for example, because some GSTs isoforms like GSTP1 may exhibit different activity, affinity, and thermostability according to genotype and substrates. Furthermore, GSTs are known to have overlapping substrate specificities and the absence of GST isoenzymes may be compensated by other isoforms. Also, different populations with different risk agent exposures may explain the differences in the outcomes of the reported studies conducted on this topic. Consequently, further studies with larger samples sizes, and considering gene–environmental and gene–gene interactions analysis should be performed.

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