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# POLYMORPHISMS IN THE GENES RELATED TO XENOBIOTIC METABOLISM, OXIDATIVE STRESS, AND DNA REPAIR AND THEIR ASSOCIATION WITH THE OUTCOME OF BREAST CANCER

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# *Polymorphisms in the genes related to xenobiotic metabolism, oxidative stress, and DNA repair and their association with the outcome of breast cancer*

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## ABSTRACT

Breast cancer is the most common cancer suffered by women all around the world. The cornerstone of breast cancer treatment is surgery.  However, despite radical surgical treatment, recurrence of the disease occurs in some patients. The adjuvant treatments for breast cancer, e.g. postoperative radiotherapy and medical treatments aim to abolish microscopic disease and decrease recurrences of breast cancer and the resulting mortality.

Genetic factors may influence the effectiveness of breast cancer treatments. The aim of this thesis was to explore single nucleotide polymorphisms (SNPs) in genes related to xenobiotic metabolism, oxidative stress, DNA repair and their association with the outcome of breast cancer in different adjuvant treatment groups.  The study involved 442 women who participated in the Kuopio Breast Cancer Project (KBCP) during the years 1990-1995. The data on survival and adjuvant treatments were merged with data on the studied genetic polymorphisms. Survival analyses were conducted using Kaplan-Meier statistics and Cox regression analysis.

In the first study, the sulfotransferase 1A1 (*SULT1A1*) rs9282861 variant AA genotype predicted improved overall survival (OS) in the cohort of patients treated with adjuvant chemotherapy or tamoxifen.  In addition, the rs9282861 variant AA genotype associated with inferior relapse-free survival (RFS) and OS in the analysis of untreated patients.

In the second study, the variant alleles of nuclear factor erythroid 2-related factor 2 (*NRF*) rs2886162, rs1962142, and rs6721961 were detected to associate with a low level of cytoplasmic NRF2 expression. A statistically significant increase for the risk of breast cancer was detected with the *NRF2* rs6721961 TT, *NRF2* rs2706110 AA and sulfiredoxin (*SRXN1*) rs6053666 CA and CC. The *NRF2* 2886162 variant AA genotype was associated with poorer survival in patients treated with adjuvant chemotherapy or radiotherapy. In addition, the analyses conducted in patients treated with postoperative radiotherapy showed that the *SRXN1* genotypes rs6116929 GG, rs7269823 AA, and rs6085283 CC were associated with statistically significantly improved RFS and breast cancer specific survival (BCSS).

In the third study, the manganese superoxide dismutase (*MnSOD*) rs4880 variant GG genotype and the xeroderma pigmentosum group D (*XPD*) rs13181 variant allele C carriage were found to relate to poorer RFS and BCSS in tamoxifen treated patients.

In the fourth study, the homozygous X-ray repair cross-complementing protein 1 (*XRCC1*) rs25487 variant AA genotype was observed to associate with worse BCSS in patients treated with either adjuvant chemotherapy or radiotherapy. Moreover, in the radiotherapy treated patients, this translated into a significant difference in OS.

In conclusion, the polymorphisms in the genes related to mechanisms of action of cancer therapies may modify the individual response and thus influence the patient outcome.

## TIIVISTELMÄ

Rintasyöpä on maailmanlaajuisesti naisten yleisin syöpä. Paikallisen rintasyövän hoidon kulmakivi on leikkaushoito. Rintasyövän radikaalista leikkauksesta huolimatta sairaus uusii osalla potilaista. Liitännäishoidoilla eli postoperatiivisella sädehoidolla ja lääkehoidoilla pyritään tuhoamaan mikroskooppiset syöpäpesäkkeet ja näin vähentämään syövän uusimisriskiä ja rintasyöpäkuolleisuutta.

Perinnölliset tekijät saattavat vaikuttaa rintasyöpäriskiin sekä rintasyövän hoidon tehoon. Tämän väitöskirjan tavoitteena oli selvittää vierasainemetaboliaan, oksidatiiviseen stressiin ja DNA:n korjausmekanismeihin liittyvien geenien yksittäisten emästen monimuotoisuuden eli polymorfismin vaikutusta rintasyövän ennusteeseen eri liitännäishoitoryhmissä. Tutkimuksen kohteena oli 442 naista, jotka osallistuivat Kuopion Rintasyöpäprojektiin vuosina 1990–1995. Potilaiden elossaolo- ja hoitotiedot yhdistettiin tutkittavien geenien polymorfismitietoihin, ja elossaoloanalyyseissä käytettiin Kaplan-Meierin menetelmää sekä Coxin regressioanalyysiä.

Ensimmäisessä osatyössä havaittiin yhdistetyssä solunsalpaaja- tai tamoksifeenihoitoa saaneiden potilaiden kohortissa tilastollisesti merkitsevästi pidempi kokonaiselossaoloaika homotsygootin *SULT1A1* rs9282861 variantin genotyypin (AA) kantajilla. Lisäksi rs9282861 genotyyppi AA liittyi huonompaan tautivapaaseen elossaoloon ja kokonaiselossaoloon potilailla, jotka eivät saaneet mitään liitännäishoitoja.

Toisessa osatyössä todettiin, että *NRF2*:n variantit alleelit rs2886162, rs1962142 ja rs6721961 liittyivät matalaan sytoplasmiseen NRF2:n ekspressioon. Tilastollisesti kasvanut rintasyöpäriski todettiin genotyypeissä *NRF2* rs6721961 TT, *NRF2* rs2706110 AA sekä *SRXN1* rs6053666 CC ja CT. Homotsygootti *NRF2* rs2886162 variantti genotyyppi (AA) liittyi huonompaan ennusteeseen solunsalpaaja- tai sädehoitoa saaneilla potilailla. Lisäksi sädehoidetuilla potilailla *SRXN1*:n genotyypit rs6116929 GG, rs7269823 AA ja rs6085283 CC olivat yhteydessä pidempään tautivapaaseen ja tautispesifiseen elossaoloon.

Kolmannessa osatyössä havaittiin, että tamoksifeenihoidetuilla potilailla homotsygootti *MnSOD* rs4880 variantti genotyyppi (GG) ja *XPD* rs13181 variantti alleeli C:n kantajuus liittyivät huonompaan tautivapaaseen ja tautispesifiseen elossaoloon.

Neljännessä osatyössä todettiin homotsygootin *XRCC1* rs25487 variantin genotyypin (AA) olevan yhteydessä huonompaan tautispesifiseen elossaoloon solunsalpaajahoitoa tai sädehoitoa liitännäishoitona saaneilla potilailla. Lisäksi sädehoidetuilla potilailla havaittiin merkitsevä ero kokonaiselossaolossa.

Yhteenvetona voidaan todeta, että syöpähoitojen vaikutusmekanismeihin liittyvien geenien monimuotoisuus saattaa muokata yksilöllistä vastetta hoitoihin ja vaikuttaa siten potilaiden ennusteeseen.

To my family

x

# Acknowledgements

# List of the original publications

This dissertation is based on the following original publications:

I    Tengström M, Mannermaa A, Kosma V-M, Hirvonen A, Kataja V. *SULT1A1* rs9282861 polymorphism–a potential modifier of efficacy of the systemic adjuvant therapy in breast cancer? *BMC Cancer 12: 257, 2012.*

II    Hartikainen J M, Tengström M, Kosma V-M, Kinnula V L, Mannermaa A, Soini Y. Genetic polymorphisms and protein expression of NRF2 and sulfiredoxin predict survival outcomes in breast cancer. *Cancer Research 72: 5537-5546, 2012.*

III    Tengström M, Mannermaa A, Kosma V-M, Soini Y, Hirvonen A, Kataja V. *MnSOD* rs4880 and *XPD* rs13181 polymorphisms predict the survival of breast cancer patients treated with adjuvant tamoxifen. *Acta Oncologica 53: 769-75, 2014.*

IV    Tengström M, Mannermaa A, Kosma V-M, Hirvonen A, Kataja V. *XRCC1* rs25487 polymorphism predicts the survival of patients after postoperative radiotherapy and adjuvant chemotherapy for breast cancer. *Anticancer Research 34: 3031-7, 2014.*

This thesis also includes previously unpublished data. The publications were adapted with the permission of the copyright owners.

# Contents

# Abbreviations

| | | | |
|---|---|---|---|
| •OH | Hydroxyl radical | CAT | Catalase |
| 2-Cys | 2-Cysteine | CDK | Cyclin dependent kinase |
| 4-OH-TAM | 4-hydroxy-tamoxifen | CEU | Central European |
| 8-oxo-dG | 8-hydroxydeoxyguanine | CGHFBC | Collaborative Group on |
| A | Adenosine | | Hormonal Factors in |
| ABCC2 | ATP-binding cassette sub- | | Breast Cancer |
| | family C member 2 | CHEK2 | Checkpoint kinase 2 |
| ADP | Adenosine diphosphate | CI | Confidence interval |
| AI | Aromatase inhibitor | CMF | Cyclophosphamide, |
| AKT1 | V-akt murine thymoma viral | | methotrexate, and |
| | oncogene homolog 1 | | 5-fluorouracil |
| Ala | Alanine | CNF | Cyclophosphamide, |
| ANOVA | Analysis of variance | | mitoxantrone, and |
| AP-1 | Activator protein 1 | | 5-fluorouracil |
| APE1 | Apurinic/apyrimidinic | CNV | Copy number variation |
| | endonuclease 1 | CSC | Cancer stem cell |
| APTX | Aprataxin | Cul3 | Cullin 3 |
| ARE | Antioxidant response element | CuZnSOD | Copper zinc SOD |
| Arg | Arginine | CYP450 | Cytochrome P450 |
| ATM | Ataxia telangiectasia mutated | C10orf11 | Chromosome 10 open reading |
| | gene | | frame 11 |
| ATP | Adenosine triphosphate | DFS | Disease-free survival |
| BCSS | Breast cancer specific survival | DMF | Dimethyl fumarate |
| BER | Base excision repair | DMFS | Distant metastasis-free |
| BMI | Body mass index | | survival |
| bp | Base pair | DNA | Deoxyribonucleic acid |
| BRCA | Breast cancer gene | DSB | Double strand break |
| BRCT | BRCA1 carboxyl-terminal | EBCTCG | Early Breast Cancer Trialists' |
| C | Cytosine | | Collaborative Group |

| | | | |
|---|---|---|---|
| EFS | Event-free survival | GWAS | Genome-wide association studies |
| EGFR | Epidermal growth factor receptor | Gy | Gray |
| EM | Extensive metabolizer | HER2 | Human epidermal growth factor receptor 2 |
| EMA | European Medicines Agency | | |
| ER | Estrogen receptor | HIF-1 | Hypoxia-inducible factor 1 |
| ERCC1 | Excision repair cross complementing group 1 | His | Histidine |
| | | $H_2O$ | Water |
| ESE | Exonic splicing enhancer | $H_2O_2$ | Hydrogen peroxide |
| ESMO | European Society for Medical Oncology | HO-1 | Heme oxygenase 1 |
| | | HOCl | Hypochlorous acid |
| F | Forward primer | hOGG1 | Human glycosylase 1 |
| FDA | Food and Drug Administration | HR | Hazard ratio |
| | | HRR | Homologous recombination repair |
| FeSOD | Iron SOD | | |
| FGFR1 | Fibroblast growth factor receptor 1 | HRT | Hormone replacement therapy |
| G | Guanine | HWE | Hardy-Weinberg equilibrium |
| G0 | Quiescent period of cell cycle | IDH1 | Isocitrate dehydrogenase 1 |
| G1 | Growth 1 phase of cell cycle | IGF-1R | Insulin-like growth factor receptor 1 |
| G2 | Premitotic phase of cell cycle | | |
| G6PD | Glucose-6-phosphate dehydrogenase | IHC | Immunohistochemistry |
| | | IKK | IκB kinase |
| Gln | Glutamine | IM | Intermediate metabolizer |
| GPX | Glutathione peroxidase | IMRT | Intensity modulated radiotherapy |
| GSH | Glutathione | | |
| GSK3β | Glycogen synthase kinase 3 beta | ISH | In situ hybridization |
| | | Jak1 | Janus kinase 1 |
| GST | Glutathione-S-transferase | KBCP | Kuopio Breast Cancer Project |
| GSTA2 | GST A2 | | |
| GSTP1 | GST P1 | Keap1 | Kelch-like erythroid cell-derived protein with |

|         | cap'n'collar type homology-associated protein 1 | MS      | Multiple sclerosis |
|---------|--------------------------------------------------|---------|---------------------|
| LD      | Linkage disequilibrium                           | mTOR    | Mammalian target of rapamycin |
| LE      | Linkage equilibrium                              | NADPH   | Nicotinamide adenine dinucleotide phosphate |
| Leu     | Leucine                                          | ND-TAM  | N-desmethyl-tamoxifen |
| LIG3    | Ligase III                                       | NER     | Nucleotide excision repair |
| L-PAM   | L-phenylalanine mustard                          | NF-κB   | Nuclear factor-kappaB |
| Lys     | Lysine                                           | NHEJ    | Non-homologous end joining |
| M       | Mitotic phase of the cell cycle                  | NQO1    | NADPH dehydrogenase quinone 1 |
| Maf     | Musculoaponeurotic fibrosarcoma protein          | NRF2    | Nuclear factor erythroid 2-related factor 2 |
| MAPK    | Mitogen activated protein kinase                 | OFS     | Ovarion function suppression |
| Maspin  | Mammary serine protease inhibitor                | OS      | Overall survival |
| MCF-7   | ER+,PR+, HER2- breast cancer cell line           | $O_2\bullet-$ | Superoxide anion |
| MCF-7-BK | MCF-7 breast cancer cell line sensitive to tamoxifen | p62 | Sequestome 1 |
| MCF-7-BK-TR | MCF-7 breast cancer cell line resistant to tamoxifen | PALB2 | Partner and localizer of BRCA2 |
| MCF-10A | ER-, PR-, HER2- breast cancer cell line          | PARP    | Poly(ADP-ribose)polymerase |
| Mdm2    | Murine double minute                             | PCNA    | Proliferating cell nuclear antigen |
| MDR     | Multidrug resistance                             | PCR     | Polymerase chain reaction |
| ME1     | Malic enzyme 1                                    | PERK    | Protein kinase RNA-like endoplasmic reticulum kinase |
| MMR     | Mismatch repair                                  | PFS     | Progression free survival |
| MnSOD   | Manganase SOD                                     | PI3K    | Phosphoinositide-3-kinase |
| MPO     | Myeloperoxidase                                  | PM      | Poor metabolizer |
| MRI     | Magnetic resonance imaging                       | PNKP    | Polynucleotide kinase 3'-phosphatase |
| MRP     | Multidrug resistance protein                     |         |  |

| | | | | |
|---|---|---|---|---|
| Pol(β/δ/ε) | Polymerase (beta/delta/epsilon) | | TKT | Transketolase |
| | | | TP53 | Tumor protein p53 |
| PR | Progesterone receptor | | Trp | Tryptophan |
| PRX | Peroxiredoxin | | TX | Taxanes |
| PTEN | Phosphatase and tensin homolog gene | | Ub | Ubiquinol |
| | | | UGT | Uridine diphosphate glucuronosyltransferase |
| R | Reverse primer | | UICC | International Union Against Cancer |
| RFLP | Restriction fragment length polymorphism | | | |
| RFS | Relapse-free survival | | UM | Ultrarapid metabolizer |
| RNA | Ribonucleic acid | | V | Vinca alkaloids |
| ROS | Reactive oxygen species | | Val | Valine |
| RPA | Human replication protein A | | VMAT | Volumetric modulated arc therapy |
| RSI | Radiosensitivity index | | | |
| S | Synthesis phase of the cell cycle | | vt | Variant |
| | | | wt | Wild type |
| SERM | Selective estrogen receptor modulator | | XP (A/B/ D/G/F) | Xeroderma pigmentosum (group A/B/D/G/F) |
| siRNA | Small interfering RNA | | XRCC1 | X-ray repair cross-complementing protein 1 |
| SNP | Single nucleotide polymorphism | | | |
| SOD | Superoxide dismutase | | | |
| SRXN1 | Sulfiredoxin | | | |
| SSB | Single strand break | | | |
| SSRI | Selective serotonin uptake inhibitor | | | |
| SULT1A1 | Sulfotransferase 1A1 | | | |
| T | Thymine | | | |
| TagSNP | Tagging SNP | | | |
| TALDO1 | Transaldolase 1 | | | |
| TFIIH | Transcription factor IIH | | | |

# 1 Introduction

Breast cancer poses a substantial burden to all societies: approximately 1.7 million women worldwide are diagnosed with breast cancer every year. In addition, over 500,000 women die every year from breast cancer. The primary curative treatment and staging of early breast cancer comprises breast-conserving surgery or mastectomy and sentinel node biopsy (SNB) or axillary evacuation. The majority of patients are treated postoperatively with adjuvant therapies, e.g. radiotherapy, chemotherapy, hormone therapy, and targeted therapies. The ultimate goal of adjuvant treatments is to eradicate microscopic residual disease, to prevent relapses, and thus to increase breast cancer specific survival (BCSS) and overall survival (OS). However, these adjuvant treatment modalities may reduce the quality of life and cause serious acute and late adverse effects including febrile neutropenia, pulmonary, cardiac, and thromboembolic complications, and even secondary malignancies (Early Breast Cancer Trialists' Collaborative Group [EBCTCG], 1998; Deitcher and Gomes, 2004; Senkus-Konefka and Jassem, 2007; Yi et al., 2009; Rayson et al., 2012).

Despite the convincing evidence that adjuvant treatments significantly improve the prognosis of breast cancer sufferers, all too many patients experience a recurrence of their disease. In Finland, for example, the five-year and twenty-year up-to-date estimates of relative survival from breast cancer at the time of this study were 83.4 % and 61.8 %, respectively (Brenner and Hakulinen, 2001). Nowadays, the relative five-year survival is 90 % (Finnish Cancer Registry). There is growing evidence that interindividual genetic diversity may partly explain these patterns in survival and as well as the differences in response to adjuvant treatments (Yu et al., 2012a; Jamshidi et al., 2013; Seibold et al., 2013). Therefore, it would be of great importance to identify predictive factors in order to find the most appropriate and effective treatment for each individual patient.

A single nucleotide polymorphism (SNP) is the most common type of genetic variation. An SNP occurs when a single nucleotide is substituted by another in the deoxyribonucleic acid (DNA) sequence. If this takes place in a coding region or in a regulatory area near a gene, SNPs may alter also the expression of proteins and modify the response to both internal and external pathogens and chemicals, including cancer treatments. SNPs have also been associated with increased susceptibility to breast cancer (Garcia-Closas et al., 2008). Genetic variation has earlier been related to survival differences in breast cancer patients receiving adjuvant therapies (Nowell et al., 2002; Ambrosone et al., 2005; Jaremko et al., 2007). The identification of more accurate markers of an increased risk of recurrence as well as better prediction of response to therapy might assist in allocating the optimal adjuvant treatments to those patients that would most likely receive a real benefit. The other side of the coin would be that certain patients could be spared from ineffectual and potentially harmful therapies.

The present work investigated the predictive value of polymorphisms in genes known to be involved in the response to the DNA damage and drug metabolism, namely sulfotransferase 1A1 (*SULT1A1*), manganese superoxide dismutase (*MnSOD*), xeroderma pigmentosum group D (*XPD*), X-ray repair cross-complementing protein 1 (*XRCC1*), nuclear factor erythroid 2-related factor 2 (*NRF2*), and sulfiredoxin (*SRXN1*). The influence of selected SNPs in the aforementioned genes on breast cancer outcome was analyzed in a cohort of Finnish women with local breast cancer. The survival differences were studied in

the total study population as well as subdivided according to the adjuvant treatments the patients received. In addition, the association of *NRF2* and *SRXN1* SNPs with the breast cancer risk was evaluated.

# 2 Review of the literature

## 2.1 BREAST CANCER

### 2.1.1 Epidemiology

Breast cancer is the most common cancer in females with nearly 1.7 million new cases globally occurring in 2012 (http://www.cancerresearchuk.org/cancer-info/cancerstats/). The global differences in incidence are nearly four-fold. The lowest rate is in Central Africa, 27 cases per 100, 000, whereas in Western Europe the rate is 96 per 100,000 (Bray et al., 2013). In 2013, 4831 Finnish women were diagnosed with breast cancer (Finnish Cancer Registry).

### 2.1.2 Etiology

Innumerable studies have been conducted to elucidate the molecular and cellular mechanisms leading to carcinogenesis. Estrogens and progesterone have a significant role on the growth and differentiation of several tissues and organs, including the mammary gland (Ceriani et al., 1972; Brisken et al., 1998). Exogenous estrogen has been demonstrated to induce breast cancer in rodents (Noble et al., 1975; Highman et al., 1980). The concept that the development of breast cancer is often dependent on the action of steroidal sex hormones is further supported by the observation that oophorectomy is associated with improved recurrence-free survival and OS in patients with early breast cancer (EBCTCG, 1996). In addition, the incidence of breast cancer can be reduced by blocking the action of estrogen with tamoxifen or aromatase inhibitors (AIs) (Cuzick et al., 2007; Goss et al., 2011).

One of the major pathways for hormone mediated carcinogenesis is the nuclear estrogen receptor (ER)-mediated signaling that enhances cell proliferation, thus leading to an increased risk of mutations in genes associated with tumour suppression, DNA repair, oncogene, and endocrine functions. There are also nongenomic, 17β-estradiol-initiated pathways, which after exposure to estradiol rapidly activate signaling molecules such as insulin-like growth factor receptor 1 (IGF-1R), epidermal growth factor receptor (EGFR), and mitogen-activated protein kinase (MAPK) (Migliaccio et al., 1996; Filardo et al., 2000; Kahlert et al., 2000). A third potential mechanism in the carcinogenesis of the mammary gland has been postulated to be attributable to the genotoxic effects of cytochrome P450 (CYP450) mediated estrogen metabolism. The metabolic oxidation of estrogen leads to the formation of estrogen-catechol complexes; these then produce electrophilic estrogen o-quinones and reactive oxygen species (ROS), which can generate DNA lesions (Zhang et al., 1999; Chen et al., 2000).

The factors related to lifelong exposure to estrogen, i.e. early age at menarche, late age at first birth, nulliparity, no or little breast feeding, and late age at menopause, are associated with elevated risk of breast cancer (Hsieh et al., 1990; Collaborative Group on Hormonal Factors in Breast Cancer [CGHFBC], 2002; Garcia-Closas et al., 2006). Moreover, higher levels of endogenous estrogen, testosterone, and prolactin have been linked with an increased risk (Key et al., 2002; Tworoger et al., 2007). The use of hormonal contraceptives has been associated with an increased risk for breast cancer (CGHFBC, 1996; Gierisch et al., 2013; Soini et al., 2014). In patients with the breast cancer gene 1 (BRCA1) mutation, the

use of oral contraceptives before the age of 25 has been shown to increase the risk of early-onset breast cancer (Kotsopoulos et al., 2014). Furthermore, the use of hormone replacement therapy (HRT) was associated with an increased risk of breast cancer (Beral and Million Women Study Collaborators, 2003). This risk seemed to be associated with the duration of HRT and the time since cessation of HRT (CGHFBC, 1997; Beral and Million Women Study Collaborators, 2003). Five years or more after discontinuing HRT use there did not seem to be any excess breast cancer risk (CGHFBC, 1997).

The density of the breast tissue is also a risk factor: the risk is elevated up to four-fold in females with more dense breast tissue (McCormack and dos Santos Silva, 2006). In addition, women with a previous history of breast cancer (Colzani et al., 2014) or a clear family history of this disease are at an increased risk (Pharoah et al., 1997). For the carriers of certain high-risk susceptibility genes, BRCA1 and BRCA2, the life-time risk of breast cancer has been estimated to be 45-85 % (Antoniou et al., 2003; King et al., 2003). In addition, hereditary syndromes like Cowden disease and Li-Fraumeni which are caused by mutations in the phosphatase and tensin homolog gene (*PTEN*), and tumor protein p53 *(TP53)*, respectively, have been found to be associated with a high genetic predisposition to breast malignancy (Frebourg et al., 1995; Liaw et al., 1997). Moderate-penetrance breast cancer susceptibility genes such as checkpoint kinase 2 (*CHEK2*), ataxia telangiectasia mutated gene (*ATM*), and partner and localizer of BRCA2 (*PALB2*) confer a modestly increased risk for breast cancer (Meijers-Heijboer et al., 2002; Bernstein et al., 2006; Erkko et al., 2007).

The risk for breast cancer increases with age, and the incidence of breast cancer is higher in women with high socioeconomic status (Braaten et al., 2004). There are also racial differences in the incidence and mortality of breast cancer, with African-American women having a worse prognosis than white women (Silber et al., 2013). Alcohol consumption (Zhang et al., 2007) and a previous history of chest irradiation are also risk factors for breast cancer (Travis et al., 2003), whereas physical exercise has been shown to reduce the risk (Steindorf et al., 2013). Several studies have reported a significant association between elevated body mass index (BMI) and the risk of breast cancer (Cecchini et al., 2012; Gaudet et al., 2014). In addition, some breast cancers may arise in a setting of random mutations.

### 2.1.3 Clinical features
The most common symptom of breast cancer is a lump in the breast or axilla. Other symptoms are changes in the size and density of the breast, local pain, retraction of the nipple, rash and ulceration of the skin, and discharge from the nipple. Those patients who present with an advanced disease may complain of skeletal pain, dyspnea, and neurological symptoms caused by distant metastases.

### 2.1.4 Diagnosis
The diagnosis of breast cancer is based on the so-called triple assessment including clinical examination, radiological evaluation and pathological assessment by needle biopsy (Senkus et al., 2013). A diagnostic mammography remains the primary tool for radiological imaging of the breast, complemented by ultrasound for the evaluation of the axillary nodes. Preoperative magnetic resonance imaging (MRI) is recommended in cases where there is a discrepancy between the clinical examination and findings in the mammogram or ultrasound, if the breast density hinders reliable mammogram interpretation, or in cases with lobular carcinoma planned to be operated by resection (Sardanelli et al., 2010). A substantial proportion of breast cancer cases are detected by screening mammography (Miller et al., 2014). In 2012, 34 % of new breast cancer cases in Finland had been detected by screening mammography (Finnish Cancer Registry).

### 2.1.5 Pathology

### 2.1.5.1 Histopathology
The histopathologic classification of breast cancer is based on the morphological features of the tumour (WHO Classification of Tumours, Volume 4). Approximately 80 % of breast cancers are of ductal or lobular origin (Sihto et al., 2008). There are also less common tumour subtypes such as mucinous, tubular, medullary, or papillary carcinoma.

### 2.1.5.2 Histological grade and Ki-67
The widely used histologic grading system of breast tumours, Nottingham Grading System, is based on the assessment of three morphological features: First, the degree of gland formation, second, nuclear pleomorphism, and third, mitotic activity (Elston and Ellis, 1991). Grade is divided into three classes: grade 1 represents well differentiated (low grade) cells; grade 2 is associated with moderately differentiated (intermediate grade) cells, and grade 3 features poorly differentiated (high grade) cells. In addition, the proliferative fraction can be assessed by immunohistochemical staining of the Ki-67 antigen. There is some controversy about which value represents the reliable cut-off points for low and high values for Ki-67, however, often the "low" value for Ki-67 is considered as a level of <14% (Cheang et al., 2009).

### 2.1.5.3 Hormone receptors
Estrogen and progesterone receptors (PRs) are examined with immunohistochemical staining. The threshold for receptor positivity has been lowered to tumours containing 1 % or more of immunoreactive cells (Goldhirsch et al., 2009).

### 2.1.5.4 Novel molecular classification of breast cancer
The molecular classification of breast cancer with a hierarchical clustering analysis of gene expression profiling has revealed the heterogeneity of breast cancer (Perou et al., 2000; Sorlie et al., 2003). The molecularly defined subtypes, luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and basal, have been demonstrated to be of both prognostic and predictive value and have therapeutic implications (Coates et al., 2015). It has been shown that there is concordance between classification defined by molecular evaluation of genetic subtype and traditional immunohistochemical markers ER, PR, HER2, and Ki-67 (Fan et al., 2006). Nowadays, tumours are recommended to be grouped into surrogate intrinsic subtypes for prognostication and treatment decisions (Table 1). However, these subtypes and markers do not overlap completely. For example, not all basal-like cancers fall within the classification of triple negativity (Banerjee et al., 2006).

   Approximately 40 % of tumours are of Luminal A type. HER2 gene amplification or over-expression is found in approximately 10 % of luminal B tumours (Cheang et al., 2009). Luminal A tumours tend to have a lower histological grade than luminal B tumours (Engstrom et al., 2013). Approximately 15 % of breast cancers are HER2 enriched with a high expression of HER2, and they are likely to present with high grade and node positivity (Pathmanathan et al., 2012). Tumours of the basal subtype are often triple negative with neither hormone receptors nor HER2 overexpression, and they may display a high expression of basal keratins (Perou et al., 2000). BRCA1 associated breast cancer is often basal-like (Laakso et al., 2005).

   A novel strategy to combat cancer involves the DNA sequencing of the tumour. One of the major goals of next generation sequencing (NGS) is to offer broad personalized genomic data which can help in clinical decision-making. Breast cancer is a highly

heterogeneous disease and the individual germline and somatic mutations may affect the prognosis and the treatment. The first report of sequencing of the breast cancer genome sequencing appeared in 2009 (Shah et al., 2009). In breast cancer, there are already several actionable mutations that permit targeted therapies including poly(ADP-ribose)polymerase (PARP) inhibitors, Janus kinase (JAK1) inhibitor ruxolitinib, mammalian target of rapamycin (mTOR) inhibitor everolimus, v-akt murine thymoma viral oncogene homolog 1 (AKT1) inhibitor MK-2206, and fibroblast growth factor receptor 1 (FGFR1) (Fong et al., 2009; Crown et al., 2012; Andre et al., 2014; Piccart et al., 2014).

However, there are several challenges in implementing NGS profiling into clinical practice. There may be significant genetic variation between the primary tumour and the metastases as well as between the metastases in different sites. Furthermore, more knowledge is needed to assess the causative and functional role of specific mutations. At the moment, genomic testing is usually performed with a limited approach, directed at the specific alterations which can be targeted by the approved drugs whereas NGS is mainly applied in the trial settings.

*Table 1.* Surrogate definitions of intrinsic subtypes of breast cancer. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor. Modified from Maisonneuve et al., 2014 and Coates et al., 2015.

| Intrinsic subtype | Clinicopathologic surrogate definition |
|---|---|
| Luminal A | 'Luminal A-like' |
| | ER+, HER2-, Ki-67 < 14 % or ER+, HER2-, PR ≥ 20 %, Ki-67 14-19 % |
| Luminal B | 'Luminal B-like (HER2-negative)' |
| | ER+, HER2-, Ki-67 ≥ 20 % or ER+, HER2-, PR< 20 %, Ki-67 14-19 % |
| | 'Luminal B-like (HER2-positive)' |
| | ER+, HER2+, any PR, any Ki-67 |
| HER2 overexpression | 'HER2-positive (non-luminal)' |
| | ER-, PR-, HER2+ |
| 'Basal-like' | 'Triple-negative' |
| | ER-, PR-, HER2- |

**2.1.6 Staging**
Staging of breast cancer is based on the UICC TNM-classification which describes the size of the tumour (T), lymph node involvement (N), and presence of distant metastasis (M). T and N are assessed in the pathological examination of a surgical specimen. Asymptomatic distant metastases are infrequent. In our Kuopio Breast Cancer Project (KBCP) material, only 3.4 % of patients presented with distant metastases at the time of breast cancer diagnosis. Radiological staging with thoracic X-ray, an abdominal ultrasound, and a bone scan or body computed tomography scan are recommended in patients with ≥ 4 metastatic axillary nodes, large tumour (T3-4) or symptoms suspicious of distant metastasis. The current clinical classification and stage grouping are depicted in Table 2.

*Table 2*. International pTNM pathologic classification and staging of breast tumours

**Primary tumour (T)**

| | |
|---|---|
| TX | Primary tumour cannot be assessed |
| T0 | No evidence of primary tumour |
| Tis | Carcinoma in situ |
| T1a | Tumour > 1 mm but ≤ 5 mm in greatest dimension |
| T1b | Tumour < 5 mm but ≤ 10 mm in greatest dimension |
| T1c | Tumour > 10 mm but ≤ 20 mm in greatest dimension |
| T2 | Tumour > 20 mm but ≤ 50 mm in greatest dimension |
| T3 | Tumour > 50 mm in greatest dimension |
| T4 | Tumour of any size with direct extension to chest wall and/or to skin |

**Regional lymph nodes (N)**

| | |
|---|---|
| NX | Regional lymph nodes cannot be assessed |
| N0 | No regional lymph node metastasis |
| N1 | Micrometastases or metastasis in 1-3 axillary or internal mammary lymph nodes |
| N2 | Metastasis in 4-9 axillary or internal mammary lymph nodes |
| N3 | Metastasis in ≥ 10 axillary lymph nodes or in infraclavicular or ipsilateral supraclavicular lymph nodes |

**Distant metastasis (M)**

| | |
|---|---|
| M0 | No distant metastasis |
| M1 | Distant metastasis |

**Stage grouping**

| | | | |
|---|---|---|---|
| Stage 0 | Tis | N0 | M0 |
| Stage IA | T1 | N0 | M0 |
| Stage IB | T0, T1 | N1mi | M0 |
| Stage IIA | T0, T1 | N1 | M0 |
| | T2 | N0 | M0 |
| Stage IIB | T2 | N1 | M0 |
| | T3 | N0 | M0 |
| Stage IIIA | T0, T1, T2 | N2 | M0 |
| | T3 | N1,N2 | M0 |
| Stage IIIB | T4 | N0, N1, N2 | M0 |
| Stage IIIC | Any T | N3 | M0 |
| Stage IV | Any T | Any N | M1 |

Modified based on TNM Classification of Malignant tumours, 7th edition, International Union Against Cancer, 2009.

**2.1.7. Prognosis**

The prognosis of breast cancer depends on the histopathological and clinical variables including tumour stage (Jatoi et al., 1999; Elkin et al., 2005), age, (Host and Lund, 1986; Kroman et al., 2000) and comorbidities (Land et al., 2012). In addition, histopathological and molecular features such as grade (Rakha et al., 2008), Ki-67 (de Azambuja et al., 2007), HER2 overexpression (Slamon et al., 1987; Ross and Fletcher, 1998), and lymphovascular invasion (Lee et al., 2006; Song et al., 2011) have an influence on the outcome. The effect of hormone receptor status seems to vary over time. During the first five years after diagnosis, those patients with ER positive (ER+) disease have a smaller risk of recurrence than patients with ER negative (ER-) breast cancer, but subsequently, i.e. 5-9 years from diagnosis the risk becomes higher in the ER+ population (Bentzon et al., 2008; Yu et al., 2012b).

It has also been noted that the histological type of breast cancer may associate with the prognosis. For example, tubular and cribriform carcinomas often have a favorable outcome (Colleoni et al., 2012), whereas metaplastic carcinomas tend to behave aggressively (Bae et al., 2011). Molecular subtyping with gene expression profiles also offers prognostic information (Wirapati et al., 2008). The poorest survival is found in basal and HER2 enriched subtypes, whereas the luminal A subtype has the most favourable outcome. Multi-gene assays such as Oncotype DX (Genomic Health) and Mammaprint (Agendia) have been developed to assist in tailoring treatments to each individual patient, mainly aiming to avoid chemotherapy in ER-positive breast cancer subjects. Their role in routine practice will become clearer when the results of ongoing prospective trials are published.

**2.1.8 Treatment strategies of breast cancer**

The primary treatment of choice for breast cancer is surgery, either breast-conserving surgery or mastectomy. For patients operated with mastectomy, immediate or delayed breast reconstruction should be available. In addition, some axillary nodes or all nodes are removed by sentinel node biopsy or axillary node dissection in order to investigate the nodal involvement (Senkus et al., 2013).

Moreover, the majority of the patients are allocated to adjuvant therapy, e.g. chemotherapy, postoperative radiotherapy, hormone therapy, or targeted therapy. The purpose of the adjuvant therapies is to eradicate microscopic residual disease and ultimately to improve relapse-free survival (RFS), BCSS, and OS. The established clinical or biological characteristics that predict the response to adjuvant therapies, i.e. predictive factors, include age, tumour size, axillary node status, tumour grade, and comorbidities (Ravdin et al., 2001; Land et al., 2012).

Furthermore, hormone receptors and HER2 amplification are factors that predict the response to hormonal therapies and anti-HER2 drugs, respectively. Traditionally, breast cancer has been divided into three main classes according to ER and PR status, i.e. highly endocrine responsive, those not endocrine responsive, and those with uncertain endocrine responsiveness.

The molecular profiling of breast cancer with genomic expression analyses has created a new classification of breast cancer subtypes which is described in the chapter 2.1.5.4. (Perou et al., 2000). Due to the uneven access to genomic profiling, the cost of these analyses, and variability in the current levels of evidence, it has been suggested that hormone receptors, HER2 status, and Ki-67 could act as surrogate markers for intrinsic subtypes of breast cancer, and be applied in choosing the most appropriate adjuvant therapies (Senkus et al., 2015).

At the moment, patients with ER and/or PR-positive breast cancer are treated with adjuvant hormone therapy with a minimum duration of 5 years. For premenopausal patients, the drug of choice has traditionally been tamoxifen (Goldhirsch et al., 2013). However, recent results suggest that suppression of ovarian function combined with either tamoxifen or an AI may be an option for young premenopausal patients (Pagani et al., 2014; Francis et al., 2015).

In postmenopausal patients, the choice can be made between tamoxifen or an AI. Tamoxifen and AI can also be used sequentially, 2-3 years of each for a total of five years. After 5 years of tamoxifen therapy, it is recommended to continue hormonal treatment beyond 5 years in cases of nodal positivity (Coates et al., 2015).

Patients with breast cancer overexpressing HER2 benefit from adjuvant trastuzumab therapy (Dahabreh et al., 2008). The St Gallen International Expert Consensus in 2015 recommended adjuvant chemotherapy for patients with grade 3 tumours, high Ki-67, low hormone receptor staining, extensive lymphovascular invasion, HER2 overexpression, triple negativity, more than three positive nodes, and a high-risk grouping in gene profiling tests (Coates et al., 2015).

## 2.2 GENETIC VARIATION

### 2.2.1 Definition of genetic polymorphism
The genetic code for producing proteins in living organisms resides in the DNA, packed in codons formed by three nucleotides. A nucleotide consists of a five-carbon sugar, one or more phosphate groups and a nucleobase. The nucleobases are divided into two groups: the purines, adenosine (A) and cytosine (C), and the pyrimidines, thymine (T) and guanine (G). Each sequence of the three nucleotides specifies a single amino acid. Since there are 64 different possible codons and only 20 different amino acids, some amino acids are coded by several different codons. In addition, there are specific start and stop codons that signal the initiation and termination of translation.

Mutations result from random changes in the sequence of base pairs in the DNA. These changes include substitution of a single base pair, insertion, deletions, or relocation of a segment of base pairs. The substitution, insertion, or deletion of a single base is also called a point mutation.

The most common form of genetic variation among humans is a single nucleotide polymorphism, SNP, where one single nucleotide is substituted by another. The more frequent form is usually referred to as a common or wild type allele, and the more uncommon allele is assigned as the uncommon or variant allele. By definition, a SNP is a genetic allelic variation that is present in ≥ 1 % of the population (Brookes, 1999). Thus, a point mutation presenting with a smaller frequency is not considered a SNP. According to the NCBI dbSNP Build 146 database updated in November 2015, approximately 32 million SNPs exist in the human genome. The frequency of a particular SNP may differ between populations, however within a population, the frequency tends to remain unchanged.

### 2.2.2 Tagging SNP
Two loci are in linkage equilibrium (LE) when they are inherited independently. If their inheritance is a non-random event, the loci are said to be in linkage disequilibrium (LD). A group of alleles of different loci on a single chromosome in high LD is called a haplotype. A haplotype can be recognized by a representative SNP, termed a tagging SNP (tagSNP). This enables identifying genetic variation without genotyping every SNP in a

chromosomal region. Haplotypes and patterns of LD may vary between different populations, and this should be taken into account when performing association studies and selecting tagSNPS (Teo and Sim, 2010). There are several methods for haplotype and tagSNP selection (Hung et al., 2015).

### 2.2.3 Effects of SNPs

Currently the specific functional impact remains undetermined for the majority of the SNPs. However, several SNPs have been shown to cause functional changes *in vitro* or *in vivo*. A SNP may occur in a coding region of a gene, in the non-coding sequence of a gene, or in the area between genes. A synonymous SNP produces the same polypeptide sequence. Non-synonymous SNPs are of two types: missense or nonsense. A missense change generates a distinct amino acid, whereas a nonsense change leads to a termination codon and a prematurely abbreviated protein. SNPs that are not within protein-coding regions may still affect gene splicing, transcription factor binding, messenger ribonucleic acid (RNA) degradation, the sequence of non-coding RNA, or other regulatory elements.

SNPs may lead to altered stability or function of the proteins for which they code. These changes may modulate susceptibility to diseases and exert an influence on the drug metabolism and response towards internal and external agents including pathogens, xenobiotics, and radiation. The efficacy of cancer therapies depends on several genetic and non-genetic factors, and distinct SNPs may make their own contribution, either negatively or positively, to the outcome. The field of pharmacogenomics studies investigates how genetics influences drug metabolism and regulates the absorption, distribution, and excretion of pharmaceutical compounds.

There are also several studies suggesting that polymorphisms may lead to altered efficiency of cancer treatments or increase the risk of adverse effects, such as neuropathy and myelosuppression (Azzato et al., 2010; Baldwin et al., 2012; Custodio et al., 2014; Tulsyan et al., 2014).

## 2.3 TAMOXIFEN

### 2.3.1 Mechanism of action

Tamoxifen is a selective estrogen receptor modulator (SERM) that has been used in the treatment of breast cancer for decades (Cosman and Lindsay, 1999). There are two types of estrogen receptors: ERα and ERβ. The human ERα gene codes for a 595 amino acid protein composed of six domains (Kumar et al., 1987), whereas the human ERβ is 530 amino acids long (Ogawa et al., 1998a). ERs are ligand-activated transcription factors and members of the family of nuclear receptors, which regulate the transcription of their target genes.

ERα is considered to be the main target for endocrine breast cancer therapies, whereas the role of ERβ in breast cancer still remains unclear. ERβ has several isoforms (Mosselman et al., 1996; Ogawa et al., 1998b), which have been studied principally in cultured cell lines and animal models. The putative proapoptotic and antiproliferative properties of ERβ have been difficult to elucidate, perhaps because the presence or absence of ERα seems to influence the function of ERβ (Tonetti et al., 2003; Paruthiyil et al., 2004). The current ER testing in breast cancer is based on the expression of the ERα.

As a SERM, tamoxifen has distinct, tissue-specific effects. In breast tissue tamoxifen acts as an antiestrogen but behaves as an agonist in the uterus and bone. The cellular responses to SERMs are determined by the cell type- and promoter-specific factors, as well as the availability of co-activators and co-repressors (Shang and Brown, 2002). Tamoxifen

competitively inhibits the binding of estrogen to the ER and evokes a reversible blockade at the G1 phase, thus slowing cell proliferation (Osborne et al., 1984). Tamoxifen causes disturbance in the ligand-binding domain of ER, resulting in an abnormal conformation of the receptor and disrupting the binding of the coactivators to the domain (Shiau et al., 1998). Co-repressor molecules are subsequently enrolled to the receptor and these maintain the receptor in an inactive form (Shibata et al., 1997).

Originally, the therapeutic action of tamoxifen was thought to be mediated solely by blocking the binding of estrogen to the ER. Interestingly, tamoxifen has been associated with antitumour activity also in cancers not expressing ERs, including ovarian, pancreatic, and breast cancer, malignant glioma, and melanoma (Gelmann, 1997). There are several mechanisms which may be involved with this ER-independent antitumour activity of tamoxifen: up-regulation of *c-myc* expression (Kang et al., 1996), inhibition of protein kinase C (PKC) (O'Brian et al., 1985), and secretion of IGF-1 (Huff et al., 1988), reduction in plasma levels of IGF-1 (Corsello et al., 1998), calcium channel blocking activity (Lopes et al., 1990), or ROS-associated apoptosis.

Tamoxifen increases ROS production and induces apoptosis in ER negative T-leukaemic Jurkat and ovarian cancer cells *in vitro* (Ferlini et al., 1999). However, in another *in vitro* experiment tamoxifen was found to stimulate ROS formation only in the ER+ cell lines (Razandi et al., 2013). Tamoxifen was shown to be genotoxic in ER+ MCF-7 cells by generating oxidized purines and pyrimidines through the production of ROS (Wozniak et al., 2007). Tamoxifen may also promote cellular senescence by ROS production as well as stabilizing tumour suppressor protein p53 (Lee et al., 2014).

### 2.3.2 Metabolism of tamoxifen

Tamoxifen is converted via a cytochrome P450 (CYP) pathway into a series of compounds that bind with varying affinities to the ER. The primary phase I metabolites are 4-hydroxy-tamoxifen (4-OH-TAM), N-desmethyl-tamoxifen (ND-TAM), and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). The N-demethylation of tamoxifen is mainly catalyzed by cytochrome CYPA4 and CYP3A5, whereas 4-hydroxylation of tamoxifen takes place predominantly via the CYP2D6 enzyme (Desta et al., 2004). 4-OH-TAM and ND-TAM are further converted into endoxifen by CYP3A4 and CYP2D6, respectively (Stearns et al., 2003) (Figure 1). Endoxifen and 4-OH-TAM are the most potent metabolites of tamoxifen, with endoxifen being present at greater concentrations than 4-OH-TAM (Lim et al., 2005).

The effect of tamoxifen is not only dependent on the phase I metabolism but also on its phase II metabolism, namely glucuronidation and sulfation reactions. 4-OH-TAM and ND-TAM are conjugated by the uridine diphosphate glucuronosyltransferases (UGTs), whereas human SULT1A1 is mainly involved in the elimination of 4-OH-TAM (Nishiyama et al., 2002; Sun et al., 2007) (Figure 1). The activity of tamoxifen and its metabolites is modified by the coordinated actions of the several enzymes involved in this metabolic pathway.

### 2.3.3 Pharmacogenomics of tamoxifen therapy

#### 2.3.3.1 *CYP2D6*

There are several studies exploring the pharmacogenomics of tamoxifen, most of them investigating the role of the highly polymorphic enzyme CYP2D6. Based on the genotypes and their ability to metabolize CYP2D6 substrates there is a current division into four CYP2D6 phenotypes: extensive metabolisers (EMs), intermediate metabolisers (IMs), poor metabolisers (PMs), and ultrarapid metabolisers (UMs) (Table 3). In Caucasians, the frequency of PMs is approximately 3-7 % (Sachse et al., 1997; Gjerde et al., 2008).

Approximately 6 % of the Finnish population lack totally or have a very weak activity of CYP2D6 (Hirvonen et al., 1993), whereas some 1 % are in the UM category (Ingelman-Sundberg, 2005). It has been shown that poor metabolisers have lower serum levels of endoxifen (Murdter et al., 2011).

However, the results from the clinical studies investigating the outcome of tamoxifen therapy according to the allelic variants of *CYP2D6* have been conflicting (Goetz et al., 2005; Schroth et al., 2009; Rae et al., 2012; Regan et al., 2012). These discrepancies may be explained by differences in study populations and end points, length of follow-up, and DNA sources. A meta-analysis of 25 studies investigating the CYP2D6 genotype and breast cancer outcomes did not support the hypothesis that CYP2D6 was a predictive factor for tamoxifen efficacy (Lum et al., 2013). At the moment, the scientific and clinical communities are still debating about the relevance of *CYP2D6* testing for the prediction of tamoxifen treatment efficacy.
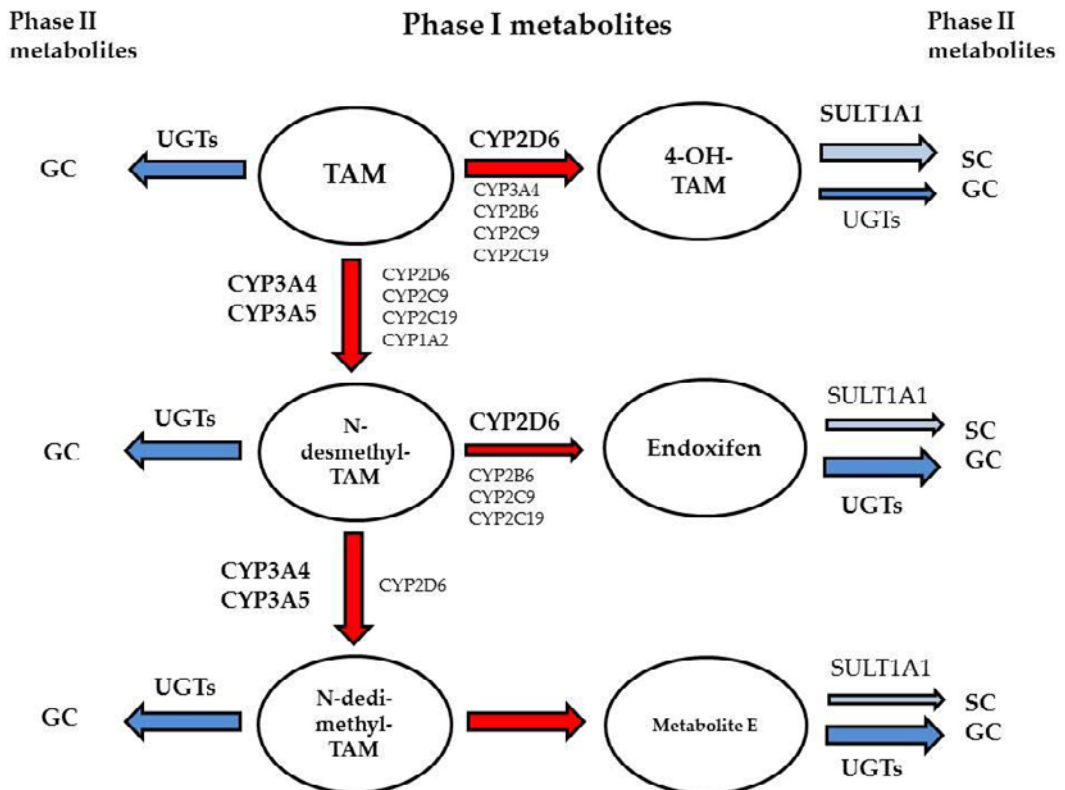


*Figure 1*. Metabolism of tamoxifen (TAM) illustrating the pathways and enzymes involved. GC, Glucuronide; 4-OH-TAM, 4-hydroxy-tamoxifen; SC, Sulphate conjugate; SULT1A1, sulfotransferase 1A1; UGT, Uridine diphosphate glucuronosyltransferase. Modified from Gjerde et al. 2008.

*Table 3*. CYP2D6 alleles and their predicted phenotypes. Modified from Lum et al. 2013.

| CYP2D6 alleles | Predicted CYP2D6 phenotype |
|---|---|
| >2 copies of any of the following functional alleles: *1, *2, *33, *35 | Ultrarapid metabolizer (UM) |
| 2 copies of any of the following functional alleles: *1, *2, *33, *35 | Extensive metabolizer (EM) |
| 2 copies of any of the following reduced function alleles: *9, *10, *17, *29, *36, *41  OR  1 copy of any of the following non-functional alleles: *3, *4, *5, *6, *7, *8, *11, *12, * 13, *14, *15, *16, *19, *20, *21, *38, *40, *42 AND 1 copy of any of the functional alleles: *1, *2, *33, *35  OR  1 copy of any of the following non-functional alleles: *3, *4, *5, *6, *7, *8, *11, *12, * 13, *14, *15, *16, *19, *20, *21, *38, *40, *42 AND 1 copy of any of the following reduced function alleles: *9, *10, *17, *29, *36, *41 | Intermediate metabolizer (IM) |
| 2 copies of any of the following non-functional alleles: *3, *4, *5, *6, *7, *8, *11, *12, * 13, *14, *15, *16, *19, *20, *21, *38, *40*42 | Poor metabolizer (PM) |

### 2.3.3.2 *SULT1A1*

SULT1A1 enzyme is a member of the sulfotransferase family mainly expressed in the liver (Zhu et al., 1993) which sulfates exogenous phenolic compounds and endogenous estrogen (Falany et al., 1993; Ozawa et al., 1995). A functional polymorphism (rs9282861G>A) in exon 7 of *SULT1A1*, results in an arginine to histidine (Arg213His) amino acid change at position 213. The variant A allele is associated with lower catalytic activity and poorer thermostability compared with the wild type G allele (Raftogianis et al., 1999). Hence, it has been hypothesized that the elimination of 4-OH-TAM could be slower in individuals carrying the variant A allele and this might lead to better response. Nevertheless, clinical studies on patients receiving adjuvant tamoxifen have shown the opposite results (Table 4). There are also studies showing no association between the *SULT1A1* rs9282861 genotype and the outcome of breast cancer patients.

Moreover, SULT1A1 is capable of generating reactive species by sulfation of N-hydroxy heterocyclic and aromatic amines (Williams et al., 2001) which may lead to DNA injury and carcinogenesis. Meta-analyses on the *SULT1A1* rs9282861 and breast cancer risk have yielded discordant results: two meta-analyses found no overall association, while a third meta-analysis suggested that carrying the variant homozygous AA genotype slightly increased the breast cancer risk (Jiang et al., 2010; Wang et al., 2010; Lee et al., 2012). In a case-control study this effect was synergistically increased by a high intake of smoked meat (Lee et al., 2012). The variant A allele has also been associated with an increased risk of male breast cancer (Ottini et al., 2014). The hyperestrogenism due to the decreased elimination of circulating estrogen might lead to an increased risk of breast cancer.

**2.3.3.3 Other SNPs associated with clinical activity of tamoxifen**

CYP2C19 enzyme is involved with N-demethylation and 4-OH-hydroxylation of tamoxifen (Crewe et al., 2002). A polymorphism in the *CYP2C19* gene (rs4244285G>A) was associated with survival of postmenopausal breast cancer patients treated with adjuvant tamoxifen for 1-3 years (Table 4). In addition, the rs3740065 in the adenosine triphosphate (ATP)-binding cassette sub-family C member 2 (*ABCC2)* gene and the rs10509373 in the chromosome 10 open reading frame 11 (*C10orf11)* gene, have also been associated with the clinical outcome in breast cancer patients receiving tamoxifen (Table 4).

*Table 4*. SNPs associated with outcome of breast cancer patients treated with adjuvant tamoxifen

| Gene, SNP | Functional consequences | Prognostic/predictive impact | References |
|---|---|---|---|
| *SULT1A1*, rs9282861G>A | Variant allele A associated with lower catalytic activity and poorer thermostability | Variant AA genotype associated with poorer OS | Raftogianis et al., 1999; Nowell et al., 2002 |
| | | Trend of association of the variant A allele with poorer RFS | Wegman et al. 2005 |
| | | No association with survival | Choi et al. 2005, Knechtel et al. 2010 |
| *CYP2C19*, rs4244285G>A | Lack of enzyme activity within the variant allele | The variant A allele associated with improved RFS | Scott et al, 2012; Beelen et al., 2013 |
| *ABCC2*, rs3740065A>G | Not known | Variant GG genotype associated with improved RFS | Kiyotani et al., 2010 |
| *C10orf11*, rs10509373T>C | Not known | Variant CC genotype associated with improved RFS | Kiyotani et al., 2012 |

**2.3.4 Tamoxifen in the clinical setting**

Tamoxifen has been used in the clinical treatment of ER+ breast cancer since the early 1970s. In the United States, tamoxifen is also approved by the Food and Drug Administration (FDA) for chemoprevention of breast cancer in high-risk women, likewise by the National Institute for Health and Care Excellence (NICE) in the United Kingdom for moderate and high risk women with certain conditions. Due to its mechanism of action, tamoxifen can be used in premenopausal, perimenopausal, and postmenopausal ER+ women, and in men, in contrast to AIs, which can only be used in the postmenopausal hormone environment.

It has been estimated that 5 years of adjuvant tamoxifen reduces the 15-year breast cancer recurrence and mortality by at least a third (EBCTCG, 2011a). The benefit of extended adjuvant tamoxifen for up to 10 years was shown in the Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial. The patients continuing tamoxifen after 5 years had improved RFS, BCSS, and OS compared with the patients stopping at 5 years (Davies et al., 2013). The Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial compared 5 years of adjuvant tamoxifen and the AI, anastrozole, in postmenopausal breast cancer. Anastrozole was more favorable in terms of disease-free survival (DFS), but there were no

significant differences in OS after a median follow-up of 10 years (Cuzick et al., 2010). However, a recent meta-analysis of randomized studies indicated that 5 years of AI or switching to AI after 2-3 years of tamoxifen was more efficient than 5 years of tamoxifen in terms of 10-year breast cancer mortality. In addition, the OS favored 5 years of AI compared with 5 years of tamoxifen (EBCTCG, 2015).

Tamoxifen has long been the primary adjuvant endocrine therapy for premenopausal women (Goldhirsch et al., 2013) and male breast cancer patients (Fentiman et al., 2006). However, recent studies have revealed that ovarian function suppression (OFS) combined with an AI or tamoxifen may also be an option especially in young premenopausal women (Pagani et al., 2014; Francis et al., 2015). The St Gallen International Expert Consensus recommends AI/tamoxifen and OFS for premenopausal patients ≤ 35 years, with N2-3 nodal status, or premenopausal estrogen levels persisting after adjuvant chemotherapy (Coates et al., 2015). However, due to the contradictory results from studies in which premenopausal patients had received a combination of AI and OFS, the current Clinical Practice Guidelines of European Society for Medical Oncology (ESMO) take a more cautious stance and recommend the combination of AI and OFS only in cases where tamoxifen is contraindicated (Gnant et al., 2009; Pagani et al., 2014; Senkus et al., 2015).

In postmenopausal women, tamoxifen alone is a suitable option for some patients. However, inclusion of an AI at some point is preferable in patients with N2-3 nodal status, high Ki-67, or grade 3 tumour (Coates et al., 2015). In node-positive disease, it is recommended to continue the endocrine therapy for 10 years after 5 years of tamoxifen regardless of menopausal status (Coates et al., 2015). The choice between different endocrine therapies is also influenced by potential adverse effects (Senkus et al., 2015). Tamoxifen is known to increase the risk of endometrial cancer and thromboembolic complications (Deitcher and Gomes, 2004; Chen et al., 2014).

## 2.4 CHEMOTHERAPY

### 2.4.1 Cell cycle

A normal cell cycle is divided into four phases illustrated in the Figure 2. The first three steps, G1, S, and G2 phases are collectively called interphase. During the G1 phase, the cell grows in size and accelerates intracellular processes before entering the synthetic phase (S). The replication of the genome takes place during the S phase. Between the S phase and mitosis (M), there is a gap, G2, which is required for cell growth and preparing for mitosis in which the cell divides into two daughter cells (Malhotra and Perry, 2003).

The cell cycle is regulated mainly by two classes of molecules, cyclins and cyclin dependent kinases (CDKs). In addition, there are several checkpoints during the cell cycle for monitoring the cellular processes and interrupting the cell cycle if necessary. Entering the next phase is not allowed if the cell does not meet certain requirements, with the main checkpoints being the G1/S and G2/M checkpoints. The G1/S checkpoint, also called the restriction point, verifies intrinsic processes and enables repair of DNA damage. Cells not entering the S phase may move into a quiescent or senescent non-replicating state, G0 phase. The DNA repair processes are completed by the G2/M checkpoint.

The progress of the cell cycle is also influenced by the tumor suppressor proteins, such as p53, and oncogenes. Normally, this complex and explicitly structured network of signals ensures flawless progress of the cell cycle. Dysregulation of the cell cycle may inhibit both cell cycle arrest and apoptosis, leading to the genesis of cancerous cells that replicate interminably. Mitosis results in the formation of two daughter cells which may

continue proliferating and dividing or move into senescence. Some cells are terminally differentiated and non-dividing. These subpopulations are found both in the normal and cancer cells (Hayflick, 1965; Di Micco et al., 2006).
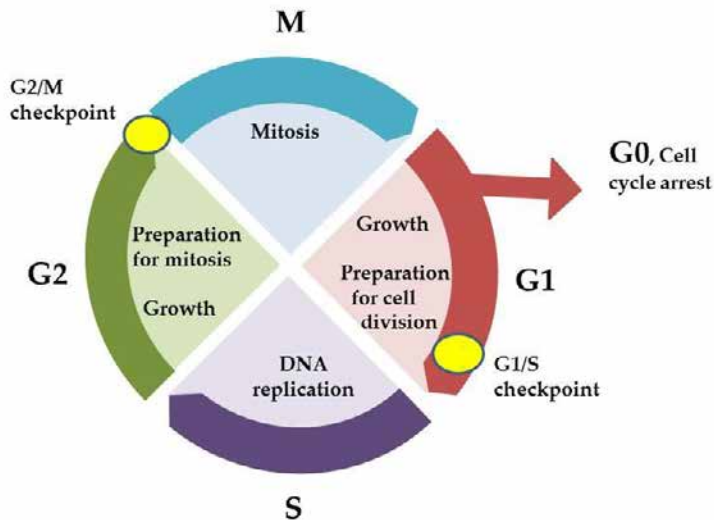


*Figure 2*. The cell cycle and processes involved in the cell division. Modified from http://www.nature.com/scitable/content/the-cell-cycle-14707478.

### 2.4.2 Classical chemotherapy: Mechanism of action

The majority of the chemotherapeutic agents act by hindering mitosis. Cytotoxicity is caused by damage to the DNA or impairment of the machinery for cellular division. Anticancer drugs can be classified by their cell-cycle-phase-specificity or chemical structure and mechanism of action. Classical chemotherapy agents can be categorized as alkylating agents, antimetabolites, anti-microtubule agents, topoisomerase inhibitors, and antitumor antibiotics (Table 5).

### 2.4.3 Chemotherapy in the treatment of breast cancer

In 1946, a study showing effectiveness of nitrogen mustard in treating lymphomas was published (Goodman and Wintrobe, 1946), and soon also patients with advanced solid tumours were treated with gradually evolving chemotherapeutic agents. In the 1960s it was also realized that surgery and radiotherapy alone did not cure all the patients even those with a local malignancy but a remarkable proportion of patients died due to recurrent disease that was most probably resulting from seemingly radical, but microscopically ineffective locoregional treatments. The first studies on the adjuvant chemotherapy of breast cancer were published in 1975 and 1976, using L-phenylalanine

mustard (L-PAM) alone and the CMF regimen (cyclophosphamide, methotrexate, and 5-flurouracil), respectively (Fisher et al., 1975; Bonadonna et al., 1976).

Incorporation of polychemotherapy into postoperative treatment of breast cancer has improved RFS, BCSS, and OS independent of age, nodal status, tumour size, or ER status (EBCTCG, 2012). The standard CMF reduces the 10-year breast cancer mortality 6.1% and the overall mortality by 4.7 %. With the newer taxane-containing regimens, the proportional 10-year risk of breast cancer death has been reduced by roughly a third compared with the patients receiving no chemotherapy. However, the absolute gain depends on the primary absolute risk level. Therefore, the benefits should be weighed against the absolute risks of recurrence or death and the risk of adverse reactions and toxicities should be carefully considered.

*Table 5*. Classification and examples of chemotherapeutic agents according to their mechanism of action. Modified from Payne and Miles, 2007.

| Class of chemotherapy | Mechanism of action |
| --- | --- |
| Inhibitors of DNA, RNA, and protein synthesis<br>Alkylating agents<br>Cytotoxic antibiotics<br>Platinum agents | Form covalent bonds by linking an alkyl group or a heavy metal complex to DNA, RNA, and proteins, thus interfering the replication process. |
| Antimetabolites<br>5-Fluorouracil (5-Fu)<br>Methotrexate (MTX) | Compete with the natural substrate for an essential enzyme or receptor, interfering with DNA synthesis and resulting in cell death. 5-Fu mimics pyrimidine analogues. MTX is a folate antagonist. |
| Microtubule agents<br>Taxanes (TX)<br>Vinca alkaloids (V)<br>Eribulin | Interfere mitosis.<br>Inhibit cell division by stabilizing tubulin in the microtubule (TX).<br>Bind to tubulin and inhibit further assembly of the spindle, resulting in impaired mitotic spindle formation in the M phase (V). |
| Topoisomerase inhibitors<br>Topotecan<br>Irinotecan<br>Anthracyclines | Stabilize the complex between topoisomerase I/II and DNA. This generates breaks in DNA strands and ultimately inhibits DNA replication. |

## 2.5 RADIOTHERAPY

### 2.5.1 Radiotherapy: Mechanism of action

Radiotherapy has been used in the treatment of cancer since the end of the 19th century, shortly after Roentgen discovered X-rays in 1895. The biological basis for the cell death caused by radiotherapy has been studied actively, and the double strand breaks of nuclear DNA are considered as the most important cellular mechanism of radiation induced cellular lethality (Vilenchik and Knudson, 2006). However, the therapeutic action of radiotherapy is largely mediated by the indirect actions of ionizing radiation. The radiolysis of cellular water leads to the formation of free radicals and ROS. These agents disrupt the covalent bonds of the DNA, thus interfering with the cell cycle and cell proliferation. The accumulation of DNA damage may lead to irreversible loss of the reproductive integrity or programmed cell death, apoptosis. The damage can be sublethal, potentially lethal, or lethal.

The classical 5 R's of radiobiology that influence the cell survival are repair, redistribution, reoxygenation, repopulation, and intrinsic radiosensitivity. There are several mechanisms by which cells may repair the damage caused by ionizing radiation. Single strand breaks (SSBs) and injuries to the bases induced directly by radiation or indirectly by ROS are mainly repaired by base excision repair (BER) (Dianov and Lindahl, 1994). Nucleotide excision repair (NER) is activated after the recognition of the distortion caused by DNA damage. Instead of repairing a single base, a short single stranded segment containing the damaged lesion is removed. DNA polymerase uses the undamaged single strand DNA as a template for building a complementary sequence (Josse et al., 1961), and DNA ligase completes the NER by joining the DNA strands together (Olivera and Lehman, 1967).

Homologous recombination repair (HRR) is a repair mechanism in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is used in double strand break (DSB) sites and takes place in the S and G2 phases of the cell cycle. In non-homologous end joining (NHEJ), a similar template is not required but short homologous DNA sequences are used to guide repair (Valerie and Povirk, 2003). NHEJ occurs most actively in the G1 phase of the cell cycle during which homologous templates are not available. Mismatch repair (MMR) system rectifies errors generated during DNA replication (Kunz et al., 2009).

The cellular susceptibility to DNA damage induced by radiotherapy depends largely on the phase of the cell cycle. Cells in the late S-phase are most resistant whereas mitosis is the most sensitive phase for DNA injuries (Biade et al., 1997). In fractionated radiotherapy the time between fractions allows radiation resistant cells to redistribute and synchronize from the S-phase into the more sensitive phases of cell cycle (Withers, 1975).

Malignant tumours are often hypoxic. As irradiation kills cancer cells capillaries reach new areas and hypoxic areas are exposed to oxygen. This reoxygenation process can sensitise the surviving cancer cells to radiotherapy. It is also important to note that the proliferation of malignant cells can continue during irradiation. There is evidence that this process of repopulation can lead to radiation resistance and exert a negative impact on the clinical outcome of radiotherapy (Kim and Tannock, 2005; Gao et al., 2013). Intrinsic radiation sensitivity represents the variability in sensitivity to irradiation in different tumour types (Leith et al., 1994).

There is also evidence that certain gene signatures specific for cellular radiation sensitivity may predict individual radiation sensitivity and clinical outcome in breast cancer subjects (Eschrich et al., 2012). Patients classified as radiation sensitive by the

radiation sensitivity index (RSI) had a superior 5-year RFS or distant metastasis-free survival (DMFS) in two independent datasets in univariate analyses. RSI was an independent predictor of better DMFS in multivariate analysis of radiotherapy-treated ER+ patients in the other of the datasets. The RSI based on an assay of 10 genes (*AR, cJun, STAT1, PKC, RelA,cABL, SUMO1, CDK1, HDAC1, and IRF1*) did not predict survival in the cohort of patients not treated with radiotherapy.

### 2.5.2 Radiotherapy in the treatment of breast cancer

In the early 20th century radiotherapy for breast cancer was delivered mainly to those patients with extensive disease or who were too frail to undergo surgery. Gradually the techniques evolved and trials were conducted comparing the outcome of radical mastectomy against that with conservative surgery and radiotherapy.

Nowadays, postoperative radiotherapy is indicated after breast-conserving surgery. In women treated with breast-conserving surgery, it has been estimated that postoperative radiotherapy nearly halves the risk of any recurrence, and reduces breast cancer mortality by about a sixth (EBCTCG, 2011b). An additional boost of up to 10-16 Gray (Gy) delivered in 2 Gy daily fractions to the tumour bed is recommended for patients with risk factors including age < 50 years, grade 3 tumours, extensive DCIS, vascular invasion or focal non-radical tumour excision. Nodal irradiation is indicated in patients with lymph node involvement (Senkus et al., 2015). Radiotherapy after mastectomy reduces both the risk of recurrence and death from breast cancer in women with nodal positivity, and is also recommended for patients with large T3-T4 tumours irrespective of nodal status or involved margins (EBCTCG, 2014; Senkus et al., 2015). In addition, post-mastectomy radiotherapy should be considered in patients with certain risk factors such as young age or vascular invasion (Senkus et al., 2015).

After breast-conserving surgery radiotherapy is planned to target the whole breast and the scar, and is usually delivered from two tangential fields. An analogical approach is used in post-mastectomy radiotherapy with a slightly different technique. In case there is lymph node involvement, irradiation is also delivered to the regional lymph nodes, e.g. axillary and supraclavicular regions. Three-dimensional planning techniques should be utilized to guarantee safe and anatomically individual implementation of radiotherapy.

The historical standard of fractionation in the radiotherapy of breast cancer, 50 Gy in 25 fractions, is still an option for all patients and it is the recommended fractionation in post-mastectomy radiotherapy. Recent trials of hypofractionated radiotherapy (39-41.6 Gy in 13 fractions over 5 weeks) with nearly 10 years of follow-up have shown that the hypofractionation is as effective as the traditional fractionation in terms of locoregional control (Haviland et al., 2013). Moreover, normal tissue effects, such as telangiectasia, breast oedema, and breast induration were less common in the patients treated with hypofractionated radiotherapy. Hypofractionation is currently widely accepted as the standard of care following breast sparing surgery, especially in patients without mastectomy and/or nodal involvement  (Coates et al., 2015; Senkus et al., 2015).

### 2.5.3 Adverse effects of radiotherapy in breast cancer

Radiotherapy may cause acute, delayed, and late toxicity. In the course of radiotherapy, there may be visible local dermal irritation and redness of the skin which usually resolves within a few weeks. Subacute lung reactions diagnosed by radiological imaging can be detected in approximately 45 % of patients (Jarvenpaa et al., 2006). Symptomatic pneumonitis with cough, mild fever, and dyspnea is a fairly rare event in breast cancer patients, with an incidence ranging from 1 to 13 percent (Lingos et al., 1991; Kwa et al., 1998).

The chronic complications of radiotherapy of breast include cardiac toxicity, lung fibrosis, and arm lymphoedema. There is evidence that the risk of major cardiac events increases linearly with the mean dose delivered to the heart, beginning within 5 years of treatment and continuing for at least twenty years (Darby et al., 2013). The risk seems to be accentuated in patients with preexisting cardiac risk factors, e.g. history of ischemic heart disease, diabetes, chronic obstructive pulmonary disease, smoking, and among women with high BMI values. The rate of cardiac complications is also higher in women with irradiation to the left breast than in women having the right breast irradiated (Darby et al., 2013).

Secondary malignancies after radiotherapy for breast cancer are fortunately rare but should they occur, they are recognized as being severe late adverse effects. They usually arise in the organs that lie in the close vicinity of radiation fields. Breast irradiation has been associated with an increased risk of lung cancer and angiosarcoma of the breast or thoracic region, (Huang and Mackillop, 2001; Darby et al., 2005). Special techniques have been developed and evaluated to decrease the doses and volumes of irradiation of adjacent organs (Aziz et al., 2011; Pignol et al., 2011). For example, intensity modulated radiotherapy (IMRT), respiratory gating, and volumetric modulated arc therapy (VMAT) have already been implemented in clinical practice in order to reduce the doses delivered to cardiac and pulmonary tissues.

### 2.5.4 Radiation genomics

Radiation genomics studies the associations between germ line genotypic variations and the toxities caused by radiation. A substantial proportion of radiotherapy-treated cancer patients are long-term survivors. Hence, it would be important to find individual predictive factors especially for late and serious adverse effects. The dosing and fractionation schedules of radiotherapy are usually designed in order to minimize the risk of severe toxicities in all patients. Usually the radiotherapy dosing is based on the risk level of the patients who are radiosensitive. Consequently, patients who have a low risk of adverse effects may be undertreated. However, the radiosensitivity of normal tissues may not be related to the radiosensitivity of the tumor (Park et al., 2012).

Several candidate genes and their SNPs have been postulated to be involved with variability in radiation sensitivity (Rosenstein, 2011). However, the results have been difficult to replicate (Barnett et al., 2012a; Barnett et al., 2012b). Lately, genome-wide association studies (GWAS) with large patient datasets have reported some associations between common genetic variants and late adverse effects (Barnett et al., 2014).

## 2.6 OXIDATIVE STRESS

### 2.6.1 Oxidative stress and cancer

Reactive oxygen species, ROS, are highly reactive molecular endogenous products of normal aerobic metabolism. ROS are ions or molecules that have a single unpaired electron in their outermost shell of electrons. They include the superoxide anion ($O_2\bullet-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($\bullet OH$). Oxidative stress and its implications in human diseases have been the subject of expanding research since the free radical theory of aging was first presented in the 1950s (Harman, 1956).

The most important intrinsic sources of ROS are mitochondrial aerobic metabolism and the extramitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway (Cadenas and Davies, 2000; Arnold et al., 2001). It has been estimated that 1-2 %

of the total oxygen consumed during normal mitochondrial respiration is not converted to water (Cadenas and Davies, 2000). Ionizing radiation, several chemotherapeutic drugs, tobacco smoke, and other air pollutants serve as exogenous sources of ROS (Kovacic and Osuna, 2000; Spitz et al., 2004; Huang et al., 2005; Lodovici and Bigagli, 2011). Mammals have developed various antioxidant enzyme systems like catalase (CAT), glutathione peroxidase (GPX), peroxiredoxins (PRXs), and superoxide dismutases (SODs) in order to combat excess oxidative stress and maintain the cellular redox balance.

ROS play an important role in cell signaling (Forman, 2009), but they are also able to kill cancer cells by triggering apoptosis (Ozben, 2007). On the other hand, oxidative stress predisposes DNA to mutations and alters gene expression, thus exposing cells to carcinogenesis (Klaunig et al., 2010). In addition, oxidative stress is responsible for protein and lipid oxidation which may also play a role in the pathogenesis of malignancies including breast cancer (Mannello et al., 2009; De Luca et al., 2010).

ROS promote tumorigenesis by regulating several signaling cascades including MAPK, phosphoinositide-3-kinase (PI3K)/Akt, and IκB kinase (IKK)/nuclear factor-kappaB (NF-κB) pathways (Burdick et al., 2003; Ruffels et al., 2004; Ruiz-Ramos et al., 2009). Moreover, alterations in mitochondrial DNA, oxidative phosphorylation, and energy metabolism result in constitutively expressed levels of ROS in tumour cells and a shift to a pro-oxidative state. This has been demonstrated in *in vitro* studies comparing ROS levels of human tumour cells and non-transformed cells (Szatrowski and Nathan, 1991; Okamoto et al., 1994). Since a drastic increase in oxidative stress might be harmful, also cancer cells have to manage ROS levels carefully in order to survive.

Breast cancer patients have been reported to have elevated levels of 8-hydroxydeoxyguanine (8-oxo-dG), the most abundant and studied oxidative DNA lesion (Malins and Haimanot, 1991). Furthermore, metastatic tumour DNA from breast cancer patients has been reported to exhibit > 2-fold increase in the amount of •OH damage compared with nonmetastastic tumour DNA, suggesting that the DNA phenotypes generated by ROS could have increased the potential for metastases (Malins et al., 1996).

### 2.6.2 Keap1-NRF2 pathway

Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcriptional factor that plays a crucial role in activating expression of several cytoprotective genes in response to oxidative stress. Kelch-like erythroid cell-derived protein with cap'n'collar type homology-associated protein 1 (Keap1) is crucially involved in regulating the activity of NRF2. NRF2 and Keap1 form a complex that controls cellular responses against oxidative stress mediated by extrinsic and intrinsic insults. Under basal conditions, two Keap1 molecules bind to NRF2 which is subsequently polyubiquitylated by the Cullin 3 (Cul3)-based E3 ligase complex. As a result, NRF2 is rapidly degraded by the proteasome (Sekhar et al., 2002).

Under stress conditions, Keap1 is inactivated and consequently, newly synthetized NRF2 is stabilized and translocated from cytoplasm into the nucleus where it binds to the antioxidant response element (ARE) and induces the expression of detoxification and antioxidant genes (Itoh et al., 2004) (Figure 3). Thus, the balance between degradation and stabilization of NRF2 has a substantial influence on the reactions combatting oxidative and electrophilic stress.

However, NRF2 seems to have a dual role in cancer. In normal and premalignant cells, NRF2-Keap1 pathway yields protection from carcinogenic stimuli. Animal studies investigating the chemopreventive features of NRF2 revealed that NRF2 deficiency was associated with larger gastric and colorectal tumours in mice exposed to various carcinogenic agents compared with the wild type mice (Ramos-Gomez et al., 2001; Khor et

al., 2008). A human SNP of *NRF2*, rs6721961, has been predicted to lead to attenuation of antioxidant responses (Marzec et al., 2007) and has been associated with an increased risk of non-small cell lung cancer in carriers of homozygous variant genotype (Suzuki et al., 2013).
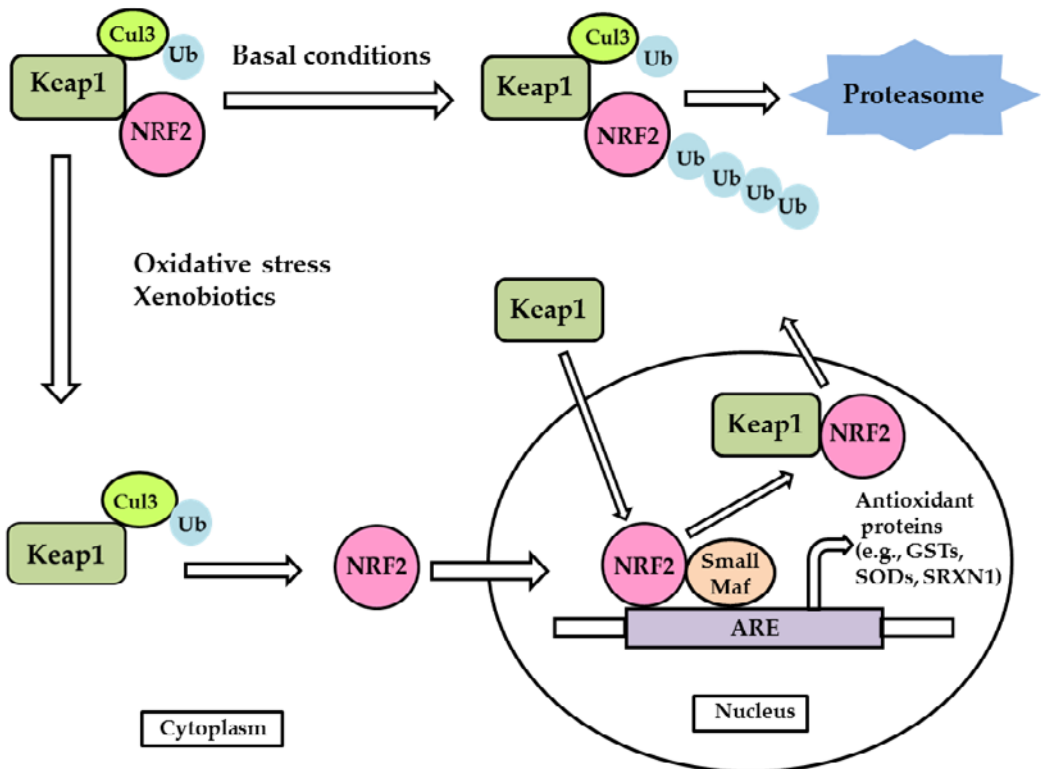


*Figure 3*. The Keap1-NRF2 signaling pathway. Under normoxic conditions NRF2 and Cullin3 (Cul3) bind to the Keap1 in the cytoplasm, resulting in the ubiquitination (Ub) of NRF2. Proteasome 26S further degrades the NRF2. Upon exposure to reactive oxygen species (ROS), NRF2 is released from Keap1 and translocates into the nucleus. NRF2 forms heterodimer with small musculoaponeurotic fibrosarcoma proteins (Mafs) and binds to the antioxidant response element (ARE). Subsequently, ARE-driven gene expression of antioxidative and detoxifying genes is upregulated, including glutathione-S-transferases (GSTs), superoxide dismutases (SODs), and sulfiredoxin (SRXN1). Upon restoration of redox homeostasis, Keap1 moves independently into the nucleus and mediates postinduction repression of NRF2 by dissociating NRF2 from ARE. The Keap1-NRF2-complex is then transported out of the nucleus. Modified from Lau et al. 2008.

On the other hand, there is growing evidence that sustained activation of NRF2 provides a survival advantage to cancer cells and leads to chemoresistance and radiation resistance. In A459 lung cancer cells, NRF2 has been shown to directly activate glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), transketolase (TKT), transaldolase 1 (TALDO1), malic enzyme 1 (ME1), and isocitrate dehydrogenase 1 (IDH1) by binding to their respective AREs (Mitsuishi et al., 2012). These proteins are important in building DNA and RNA and inducing proliferation in malignant cells as they are involved in glucose flux and purine generation. NRF2 has also been shown to promote glutamine (Gln) metabolism, especially in the presence of active PI3K-Akt signaling (Mitsuishi et al., 2012). Reprogramming of these metabolic activities enhance anabolic pathways and contribute to aggressive proliferation of malignant cells.

Several mechanisms have been identified as being involved in the induction of the NRF2 activity in cancer. Somatic mutations in *Keap1* and *NRF2* have been discovered in lung, gallbladder, and breast cancer (Padmanabhan et al., 2006; Sjoblom et al., 2006; Shibata et al., 2008). Furthermore, epigenetic hypermethylation inhibits *Keap1* gene expression, followed by the accumulation of NRF2. Aberrant promoter methylation of *Keap1* gene has been detected in breast cancer patients (Barbano et al., 2013). *Keap1* promoter methylation was associated with inferior OS in patients with triple negative breast cancer, whereas in patients treated with adjuvant epirubicin/cyclophosphamide and docetaxel *Keap1* methylation predicted enhanced apoptosis.

### 2.6.3 SRXN1

Peroxiredoxins (PRXs) are a family of enzymes that catalyze the reduction of $H_2O_2$ to water. A subgroup of PRXs, the typical 2-Cysteine (2-Cys) PRXs, contain a conserved C-terminal cysteine residue which is oxidized to sulfinic acid during enzymatic reduction of $H_2O_2$. The PRXs are inactivated by hyperoxidation in highly oxidative conditions, rendering them resistant to reduction by thioredoxin. This inactivation was considered irreversible until sulfiredoxin (SRXN1) was found and it was shown to have $Mg^{2+}$ and ATP-dependent sulfinic acid reductase activity towards PRXs (Biteau et al., 2003). $H_2O_2$ has been demonstrated to induce the expression of SRXN1, whereas deletion of SRXN1 lead to reduced tolerance to $H_2O_2$ (Biteau et al., 2003). SRXN1 has been reported to be induced by NRF2 in neurons and glia (Soriano et al., 2008) (Figure 3), and it is also a target gene for the activator protein 1 (AP-1), a transcription factor that influences several cellular functions including proliferation and apoptosis.

Glutathione (GSH) is one of the reductants utilized in $H_2O_2$ catalysis. Glutathionylation may lead either to an inhibition or an induction of enzymatic activity (Manevich et al., 2004; Takata et al., 2013). The precise functional consequences of this process in the 2-Cys PRXs have not yet been fully elucidated. However, it has been shown that SRXN1 is capable of catalyzing deglutathionylation of proteins in response to oxidative stress, including 2-Cys PRXs (Findlay et al., 2006; Park et al., 2009). Hence, SRXN1 has been designated as a novel antioxidant that protects cells from the oxidative stress caused by $H_2O_2$ and reverses glutathionylation. On the other hand, there is evidence in experiments on mice that SRXN1 may promote skin and colon tumorigenesis (Wei et al., 2013; Wu et al., 2014c).

### 2.6.4 MnSOD

There are three major families of superoxide dismutases (SODs) in mammals, i.e., manganese SOD (MnSOD), copper zinc SOD (CuZnSOD), and iron SOD (FeSOD). The SODs catalyze the dismutation of $O_2$ to $H_2O_2$, which is subsequently converted to water by CAT. The proper functioning of these scavenging enzymes seems to be an essential

biochemical process. Genetic deletion of *MnSOD* in mice leads to early post-natal lethality associated with neuronal degeneration and severe cardiomyopathy (Lebovitz et al., 1996).

MnSOD has a critical antioxidative role in establishing cellular redox balance. The MnSOD precursor synthesized in the cytosol is imported to the mitochondria (Pfanner and Geissler, 2001) (Figure 4). A substitution of valine (Val) to alanine (Ala) at codon 16 in exon 2 (rs4880A>G) results in a SNP with functional consequences. The 16Val-containing precursor is less abundant in the mitochondria and has 30-40 % reduced enzymatic activity (Sutton et al., 2003).

MnSOD is induced by several transcription factors including NF-κB, and PKC (Xu et al., 1999; Chung et al., 2011). On the other hand, MnSOD in turn affects the activity of several transcription factors, such as mammary serine protease inhibitor (maspin) (Li et al., 1998a), and p53 (Drane et al., 2001). The overexpression of MnSOD inhibits the transcriptional activity of NF-κB in MCF-7 breast cancer cells (Li et al., 1998b).
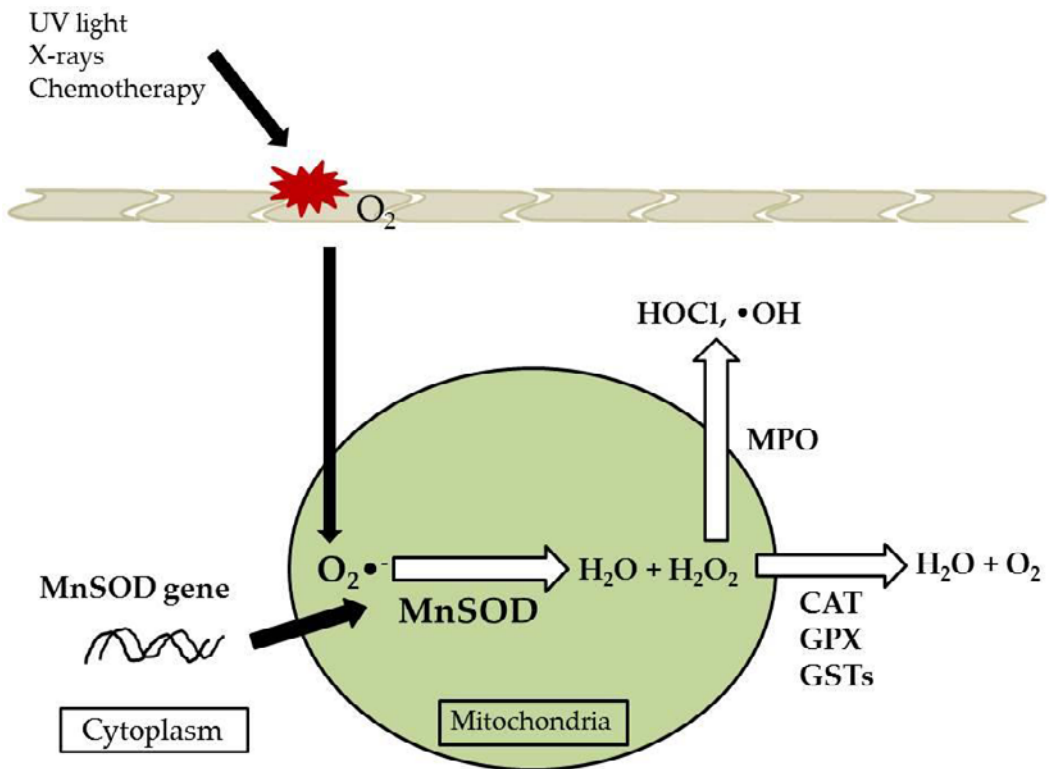


*Figure 4*. The role of MnSOD in the mitochondrial antioxidant defence system. During periods of oxidative stress the MnSOD precursor is transported from the cytosol into the mitochondria where it catalyzes the conversion of superoxide anion ($O_2 \bullet -$) into water ($H_2O$) and hydrogen peroxide ($H_2O_2$). $H_2O_2$ is further detoxified by catalase (CAT), glutathione peroxidase (GPX), or glutathione-S-transferases (GSTs). Alternatively, reactions catalyzed by myeloperoxidase (MPO) may generate highly reactive hypochlorous acid (HOCl) and hydroxyl radicals ($\bullet OH$). Modified from Amaro-Ortiz et al. 2014.

In support of MnSOD's putative activity as a tumor suppressor protein, it has been shown that increased MnSOD expression inhibits breast cancer cell growth *in vitro* and *in vivo* (Li et al., 1995; Weydert et al., 2006). A meta-analysis of fourteen studies investigating the association between the *MnSOD* rs4880 polymorphism and the risk of breast cancer found no significant interaction (Liu et al., 2012). However, a subgroup analysis showed that the risk of breast cancer was increased in premenopausal women carrying the variant G allele. A similar effect was noted in patients with certain lifestyle factors: history of smoking, use of oral contraceptives, and a higher BMI. These results are contradictory to the proposal that the more active rs4880 variant G allele might confer protection from carcinogenic stimuli.

MnSOD activity may also influence tumour cell survival by modulating the response to ROS. For example, breast cancer cells with an increased MnSOD expression have been shown to display resistance *in vitro* to the ROS producing compound, doxorubicin (Park et al., 2004). In view of the functional consequences of the rs4880 polymorphism, it could be postulated that patients carrying the rs4880 A allele might enjoy a survival advantage from an inferior capacity to protect cancer cells from the oxidative stress generated by cancer treatments.

However, the results from clinical studies on the *MnSOD* rs4880 and breast cancer have been conflicting (Ambrosone et al., 2005; Bewick et al., 2008; Glynn et al., 2009; Yao et al., 2010; Hubackova et al., 2012; Cronin-Fenton et al., 2014). Carrying the variant G allele has been associated with worse DFS and BCSS in patients receiving adjuvant cyclophosphamide-containing chemotherapy (Glynn et al., 2009; Yao et al., 2010), whereas no association was found between *MnSOD* genotype and RFS in Danish patients with early breast cancer after cyclophosphamide-based adjuvant chemotherapy (Cronin-Fenton et al., 2014). On the contrary, carrying the homozygous wild type AA genotype predicted worse progression free survival (PFS) and BCSS in patients with metastatic breast cancer treated with high dose chemotherapy and autologous stem cell transplantation (Bewick et al., 2008).

## 2.7 DNA REPAIR GENES *XPD* AND *XRCC1*

### 2.7.1 The role of XPD in DNA repair

The Xeroderma pigmentosum group D (XPD) protein is an essential component in the human NER pathway. XPD functions as a 5'-3' helicase within the transcription factor IIH (TFIIH) complex that is involved in the regulation of transcription and NER pathway (Sung et al., 1993; Hoeijmakers et al., 1996). This unwinding helicase function results in lengthening the unpaired region and allows binding of additional NER factors (Figure 5). In addition, XPD seems to have a substantial role in identifying DNA damage (Mathieu et al., 2013). Mutations in the human *XPD* gene disabling the NER system may result in severe disorders including xeroderma pigmentosum characterized with extreme sensitivity to sunlight and cancer predisposition, and progeroid diseases Cockayne syndrome and trichothiodystrophy (Lehmann, 2003).

### 2.7.2 *XPD* rs13181

One of the best known polymorphisms in the human *XPD* gene is the *XPD* lysine (Lys) 751Gln of exon 23 (rs13181A>C). Data from *in vitro* studies investigating the functional effect of the rs13181 polymorphism have been controversial. A comet assay of human lymphocytes observed significantly higher levels of SSBs in individuals carrying the rs13181 wild type A allele (Vodicka et al., 2004). In a study examining 31 individuals, one

of them with a previous history of breast cancer, the homozygous rs13181 wild type AA genotype was associated with a reduced repair of X-ray induced DNA breaks and gaps in lymphocytes (Lunn et al., 2000). Another study with healthy donors failed to detect any significant association with the correction of X-ray induced DNA injury but revealed an increased rate of DNA damage after exposure to ultraviolet light for the rs13181 AC and CC genotypes (Au et al., 2003). The homozygous rs13181 wild type AA genotype correlated with superior capacity to repair DNA in a host cell reactivation assay obtained from lung cancer patients (Spitz et al., 2001).
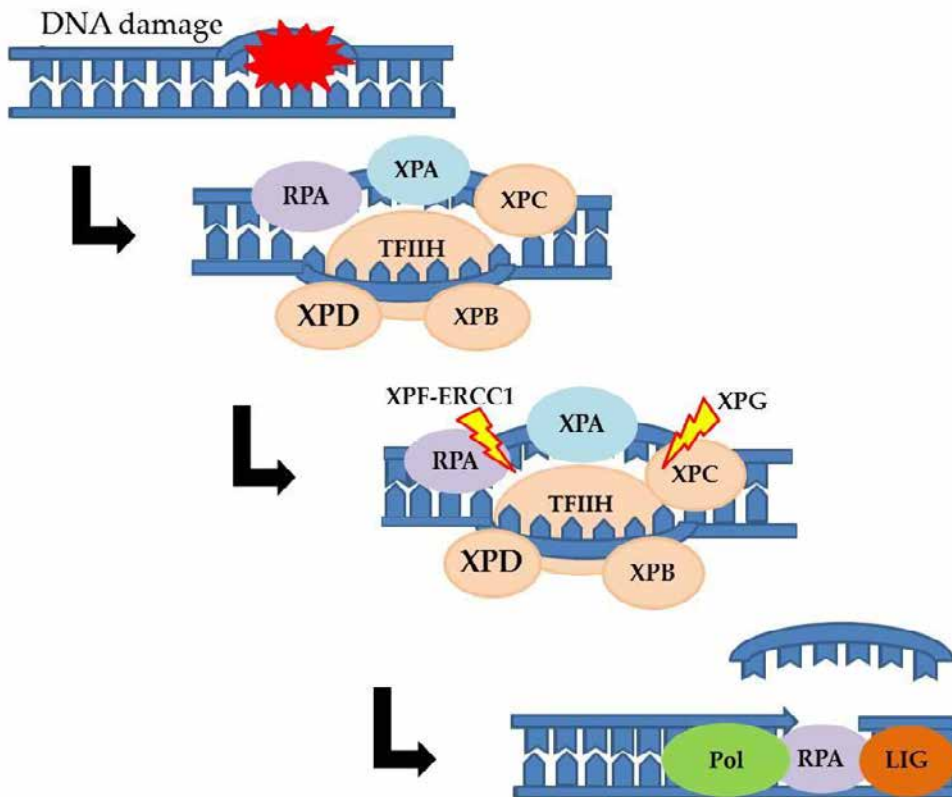


*Figure 5*. Simplified nucleotide excision repair (NER) pathway. Upon identification of DNA damage repair proteins including xeroderma pigmentosum group C and A (XPC and XPA), human replication protein A (RPA), and transcription factor IIH (TFIIH) complex are recruited to the damaged site. Subsequently, helicases XPD and XPB unwind the DNA. The damaged DNA is removed by incisions made by endonucleases XPF, excision repair cross complementation group 1 (ERCC1), and XPG. The gap is filled by DNA polymerases (Pol), and the repair process is completed by DNA ligase (LIG). Modified from Masters et al. 2003.

A meta-analysis of statistically powerful case-control studies showed null associations for the rs13181 polymorphism with risk of breast cancer (Pabalan et al., 2010). However, a subanalysis including studies of individuals exposed to aromatic adducts detected a significant risk for breast cancer in carriers of rs13181 variant C allele (Pabalan et al., 2010).

The *XPD* rs13181 variant CC genotype was associated with a smaller risk of severe erythema after breast conserving radiotherapy in patients with large breasts (Raabe et al., 2012), while several studies have reported no significant influence of the rs13181 genotype on the acute or late dermal side effects of breast irradiation (Chang-Claude et al., 2005; Chang-Claude et al., 2009; Zschenker et al., 2010; Mangoni et al., 2011; Terrazzino et al., 2012).

There are few publications on the *XPD* rs13181 polymorphism and the clinical outcome of breast cancer. Two clinical studies have reported better outcomes for patients with metastatic breast cancer treated with chemotherapy carrying the rs13181 wild type A allele (Chew et al., 2009; Bewick et al., 2011), whereas this SNP did not affect the outcome in early breast cancer patients receiving adjuvant anthracycline-based chemotherapy (Castro et al., 2014).

**2.7.3 XRCC1 function**

X-ray repair cross-complementing protein (XRCC1) is a 70 kilodalton DNA repair protein involved in the BER and repair of SSBs (Figure 6). It has no known enzymatic function of its own but acts as a scaffold protein interacting with the multiple enzymes required for BER and SSB repair such as polymerase β (Pol β), PARP, apurinic/apyrimidinic endonuclease-1 (APE1), human glycosylase (hOGG1), and ligase III (LIG3) (Ginsberg et al., 2011). One important feature of XRCC1 protein is its interaction with two BRCA1 carboxyl-terminal (BRCT) domains, BRCT I and BRCT II (Bork et al., 1997; Callebaut and Mornon, 1997). APE1, hOGG1, and PARP bind to the region of BRCT I domain, whereas LIG3 binds to the C-terminus BRCT II domain (Ginsberg et al., 2011). In addition, XRCC1 seems to participate in DSB repair (Audebert et al., 2004; Levy et al., 2006).

Animal studies have demonstrated the importance of XRCC1 in embryonal development. The embryos of *XRCC1* null mice were unable to survive until midgestation (Tebbs et al., 1999). In addition, *XRCC1* deficient cells were found to have reduced levels of LIG3 activity.

A SNP of the *XRCC1* in codon 399 of exon 10 (rs25487G>A) results in an amino acid change from Arg to Gln. This SNP is located within the BCRT I domain that interacts with PARP (Masson et al., 1998). It has been proposed that the variant rs25487 AA genotype is associated with a 3-4 fold reduction in the DNA repair capacity (Slyskova et al., 2007). In line with this observation, chromosomal deletions have been found to be more frequent in irradiated lymphocytes of nonsmoking individuals carrying the variant A allele compared with the rs25487 GG genotype (Au et al., 2003). Another study compared the homozygous rs25487 wild type and homozygous variant type lymphocytes from two individuals exposed to the reactive intermediate of vinyl chloride capable of generating pro-mutagenic DNA adducts. The repair of DNA adducts was four times more efficient in the homozygous wild type cells compared with the homozygous variant cells (Li et al., 2006). Similarly, the rs25487 wild type GG genotype was associated with a reduced number of DNA breaks in X-ray exposed lymphocytes from colon carcinoma patients (Gdowicz-Klosok et al., 2013). On the contrary, no association between the rs25487 polymorphism and efficiency of DNA damage repair was found in two studies where whole blood samples from breast cancer patients were irradiated *in vitro* (Sterpone et al., 2010; Zschenker et al., 2010).

**2.7.4 *XRCC1* rs25487 and its clinical implications in breast cancer**

Several studies have investigated the *XRCC1* rs25487 polymorphism and risk of breast cancer. The results have been inconclusive as the variant A allele has been associated with an increased risk (Duell et al., 2001; Moullan et al., 2003; Metsola et al., 2005; Sterpone et al., 2010) as well as having no effect on the breast cancer risk (Smith et al., 2003; Zhang et al., 2006; Zipprich et al., 2010; Przybylowska-Sygut et al., 2013).
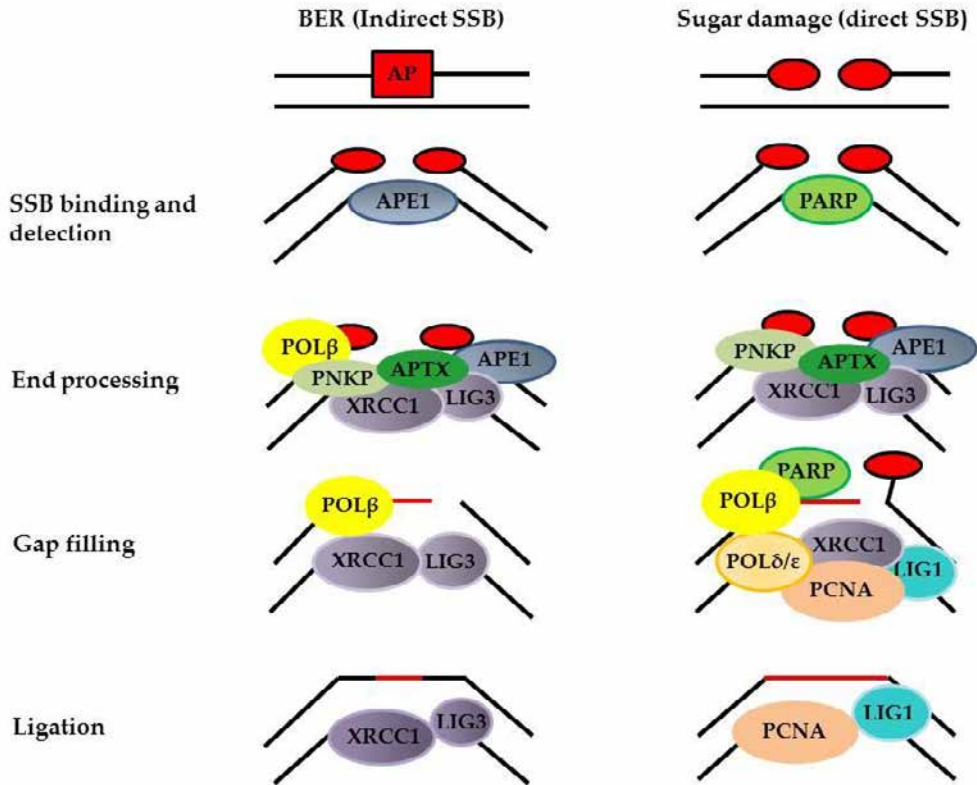


*Figure 6*. Global pathways of single-strand break (SSB) repair pathway. SSBs may arise indirectly during base-excision repair (BER) by enzymatic incision at an apurinic-apyrimidinic (AP) site by AP endonuclease (APE1). Direct SSBs may arise by reactive oxygen species (ROS)-induced sugar damage. Direct SSBs are detected by poly(ADP-ribose) polymerase 1 (PARP). BER-induced SSBs are repaired by APE1, polynucleotide kinase 3'-phosphatase (PNKP), aprataxin (APTX) and polymerase β (POLβ). Direct SSBs are repaired by APE1, PNKP, and APTX. End processing is followed by gap filling. POLβ is essential in the short-patch repair. Sugar damage cannot be repaired by POLβ alone but requires gap filling extended for ∼ 2-12 nucleotides by POLδ and POLε (POLδ/ε). The removal of the damaged 5'-terminus is stimulated by PARP and proliferating cell nuclear antigen (PCNA). Short-patch repair sites are primarily ligated by DNA ligase 3 (LIG3), and long-patch repair sites are mainly ligated by DNA ligase 1 (LIG1). Modified from Caldecott 2008.

The question of whether there is an association between the *XRCC1* polymorphisms and the normal tissue complications in breast cancer patients after postoperative radiotherapy has been addressed in a number of studies. Carrying the *XRCC1* rs25487 wild type G allele in combination with the carriage of *XRCC1* rs1799782 (Arg194tryptophan[Trp]) variant allele was linked with a significantly increased risk of severe acute dermal reactions (Mangoni et al., 2011). The wild type G allele associated with an increased risk of acute skin reactions in patients with normal weight (Chang-Claude et al., 2005), whereas this did not apply for late normal tissue toxicity (Chang-Claude et al., 2009) There are also other studies reporting null effects (Andreassen et al., 2005; Raabe et al., 2012; Terrazzino et al., 2012).

The results from clinical studies exploring the rs25487 and the outcome of breast cancer have been inconclusive. Homozygosity for the wild type G allele has been associated with improved BCSS in a study of 95 patients with metastatic breast cancer treated with chemotherapy (Bewick et al., 2006), and with superior DFS in a study of 84 patients receiving adjuvant chemotherapy  (Castro et al., 2014). On the other hand, possessing the rs25487 AA variant genotype has been correlated with better DFS or OS in early breast cancer patients treated with chemotherapy or radiotherapy (Jaremko et al., 2007; Ye et al., 2012; Przybylowska-Sygut et al., 2013). There are also studies that did not detect any significant differences in survival according to the rs25487 genotype (Costa et al., 2008; Syamala et al., 2009).

# 3 Aims of the Study

The prognosis of breast cancer depends on several tumour-related factors including the stage of the disease at diagnosis, hormone receptor and HER2 status, and the molecular subtype of the tumour. In addition, personal genetic differences in the activity of detoxifying enzymes and DNA repair may influence survival. More precise prognostic and predictive markers are required before one can introduce personalized therapies i.e. finding the most effective and least toxic therapy for each patient.

The aim of this thesis was to investigate the role of genetic components in xenobiotic metabolism, oxidative stress response, and DNA repair on the survival of breast cancer patients. The outcome was specifically assessed according to different adjuvant treatments, e.g. chemotherapy, tamoxifen treatment, and postoperative radiotherapy.

The specific aims of this thesis were:

1. To study the prognostic and predictive value of the *SULT1A1* rs9282861 polymorphism in patients with early breast cancer

2. To assess the influence of polymorphism in ROS-responsive genes *NRF2*, *SRXN1*, and *MnSOD* with the outcome of breast cancer

3. To evaluate the association of *NRF2* and *SRXN1* polymorphisms with the breast cancer risk and to analyze their patterns of protein expression

4. To investigate the association of DNA repair gene polymorphisms *XPD* rs13181 and *XRCC1* rs25487 with the survival of breast cancer patients

# 4 Materials and Methods

## 4.1 CASES AND CONTROLS

### 4.1.1. Breast cancer patients

The Kuopio Breast Cancer Project (KBCP) is a prospective population-based case-control study conducted in 1990-1995. Women entering Kuopio University Hospital due to breast symptoms were invited to take part in the study at their first visit to the hospital. The subjects provided written informed consent for participation in the study. Altogether 520 women out of 1,919 were eventually diagnosed to have breast cancer. Data were collected from the patients with regard to medical history, family history of breast cancer, socioeconomic background, alcohol use, and cigarette smoking. All patients were ethnic Finns. Hospital registries were used to collect information concerning clinico-pathological features of the breast cancer, surgical and oncological treatments, and follow-up.

Only patients who had an operated primary local invasive breast cancer and known TNM status were included in the survival analyses (n=442).

Adjuvant tamoxifen was given to 91 patients, and four of them received also adjuvant chemotherapy. The daily doses of tamoxifen were 20 mg or 40 mg. The median duration of tamoxifen treatment was 36.0 months (range 0.5 -79.0 months). Patients who received tamoxifen for less than 3 months or had negative hormone receptor status were excluded from the tamoxifen subgroup analyses.

The regimens used in the adjuvant chemotherapy were intravenous CMF (cyclophosphamide 500 mg/m2, methotrexate 40 mg/m2, 5-fluorouracil 500 mg/m2) and CNF (cyclophosphamide 500 mg/m2, mitoxantrone 10 mg/m2 and 5-fluorouracil 500 mg/m2). CMF was allocated to 81 patients, whereas 6 patients received CNF. The median number of chemotherapy cycles was six (range 2-8).

A total of 263 patients received postoperative radiotherapy. In 46 patients the target of radiotherapy was the breast, and in 182 patients the radiotherapy was delivered to the breast or chest wall and also to the axillary node region. One patient received irradiation of the regional lymph nodes only. An additional external booster was given to seven patients, and 26 patients were treated with an internal (brachy) booster. One patient was treated with internal booster only. The median dose of external radiotherapy delivered was 50 Gy (range 28-56 Gy). In the radiotherapy subgroup analyses, only patients with ≥ 44 Gy of external radiotherapy to the breast or breast and regional lymph nodes were included. An additional booster was also allowed.

The demographic characteristics of 442 breast cancer patients are depicted in Table 6. Due to the varying availability of genotype data the number of patients in different treatment subgroups varies depending on the polymorphism in question.

The KBCP has been approved by Kuopio University Hospital Board on Research Ethics and the Ethical Committee of the University of Eastern Finland. All patients gave written informed consent to participate in the study.

*Table 6.* Patient characteristics (n=442)

| Characteristics | No. of patients (%) |
| --- | --- |
| Age at diagnosis, years | |
| ≤ 39 | 40 (9.0) |
| 40-49 | 104 (23.5) |
| 50-59 | 114 (25.8) |
| 60-69 | 70 (15.8) |
| ≥ 70 | 114 (25.8) |
| T stage (UICC) | |
| 1 | 233 (52.7) |
| 2 | 172 (38.9) |
| 3 | 25 (15.7) |
| 4 | 12 (2.7) |
| Nodal status | |
| N0 | 263 (59.5) |
| N1 | 166 (37.6) |
| N2 | 13 (2.9) |
| Stage (UICC) | |
| I | 180 (40.7) |
| II | 220 (49.8) |
| III | 42 (9.5) |
| ER status | |
| positive | 325 (73.5) |
| negative | 95 (21.5) |
| unknown | 22 (5.0) |
| PR status | |
| positive | 259 (58.6) |
| negative | 159 (36.0) |
| unknown | 24 (5.4) |
| HER2 status | |
| positive | 51 (11.5) |
| negative | 348 (78.7) |
| unknown | 43 (9.7) |
| Surgery | |
| Mastectomy | 345 (78.1) |
| Resection | 97 (21.9) |
| Adjuvant treatment | |
| Tamoxifen | 91 (20.6) |
| Chemotherapy | 87 (19.7) |
| Radiotherapy | 263 (59.5) |

Abbreviations: UICC, International Union Against Cancer; Fourth, Fully Revised Edition.

**4.1.2 Controls**

The controls (publication II) were selected from of a group of subjects drawn from the National Population Registry living in the same catchment area. The controls were individually matched with the breast cancer cases by age (± 5 years) and the long term area of residence (rural/urban) (Mannisto et al., 1999).

## 4.2 SINGLE NUCLEOTIDE POLYMORPHISM SELECTION FOR *NRF2* AND *SRXN1*

TagSNPs for *NRF2* and *SRXN1* were chosen using the HapMap Genome Browser release 2 as of February 24 and November 8, 2010. TagSNPs for regions chr2:177799989-177853228 and chr20:573580-583579 were selected for the Central European (CEU) population using the Tagger multimarker algorithm with $r^2$ cutoff at 0.8 and minor allele frequency cutoff at 0.05. Two functional polymorphisms located in the *NRF2* promoter region were picked up based on previous publications (Yamamoto et al., 2004; Marzec et al., 2007).

## 4.3 GENOTYPING ANALYSES

**4.3.1 Genotyping of *SULT1A1*, *MnSOD*, *XPD*, and *XRCC1***

One hundred ng of DNA extracted from peripheral blood lymphocytes were used as a template in the genotyping analyses using standard polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assays. Samples with known genotypes and nontemplate samples were used as positive and negative internal controls, respectively. Duplicates of 10 % of the samples were blindly analyzed for quality control with fully concordant results. The primer sequences, restriction enzymes and the allele-specific fragments from each genotyping analysis are shown in Table 7.

*Table 7*. Primers, enzymes, and the allele-specific fragment sizes in the genotyping analyses of *SULT1A1*, *MnSOD*, *XPD,* and *XRCC1*.

| Variation | PCR Primer sequence | Enzyme | Fragment size (wt/vt) |
|---|---|---|---|
| *SULT1A1* rs9282861 | F: GGTTGAGGAGTTGGCTCTGC | *Bsp*143II | 104+77 bp/281 bp |
| | R: ATGAACTCCTGGGGGACGGT | | |
| *MnSOD* rs4880 | F: ACCAGCAGGCAGCTGGCGCCGG | *Ngo*MIV | 107 bp/89+19 bp |
| | R: GCGTTGATGTGAGGTTCCAG | | |
| *XPD* rs13181 | F: ATCCTGTCCCTACTGGCCATTC | *Pst*I | 220+104 bp/157+104+63 bp |
| | R: TGTGGACGTGACAGTGAGAAAT | | |
| *XRCC1* rs25487 | F: TTGTGCTTTCTCTGTGTCCA | *Msp*I | 221+374 bp/615 bp |
| | R: TCCTCCAGCCTTTTCTGATA | | |

wt, wild type allele; vt,variant type allele; bp, base pairs; F, forward primer; R, reverse primer.

**4.3.2 Genotyping of *NRF2* and *SRXN1***

Genomic DNA was isolated from peripheral blood lymphocytes of both cases and controls using standard procedures (Vandenplas et al., 1984). Genotyping of 6 *NRF2* and 8 *SRXN1* TagSNPs and 2 *NRF2* functional SNP was performed by MassARRAY (Sequenom, Inc.) and iPLEX Gold (Sequenom, Inc.) on 384-well plate format. Spectra acquisitions from the

SpectroCHIP were done with MassARRAY mass spectrometer. Data analysis and genotype calling were conducted using TyperAnalyzer Software version 4.0.3.18 (Sequenom, Inc.). A minimum of 8 non-template controls were included in each 384-well plate. Duplicate analysis was performed for 6.7 % of the samples for quality control. Genotyping of the *NRF2* TagSNP rs2886162 was conducted with the 5'-nuclease assay (Taqman) using the Mx3000P Real-Time PCR System (Stratagene) according to the manufacturer's instructions. Primers and probes for the rs2886162 were obtained from Applied Biosystems as TaqMan Genotyping Assays. Reactions were performed in 10-µL volume in 96-well format as previously described (Kauppinen et al., 2010). The primer sequences for other *NRF2* and *SRXN1* genotyping assays are depicted in Table 8.

## 4.4 BREAST CANCER TUMOUR TISSUE MICROARRAY

Paraffin-embedded tumour tissue from the primary tumour was obtained in the breast cancer surgery from all breast cancer patients participating in the KBCP. In the present study, tissue samples from 377 invasive breast carcinomas were available in a tissue microarray format. All the materials had been fixed in 10 % buffered formalin and embedded in paraffin. The array blocks were constructed with an MTA-1 Manual Tissue Arrayer instrument (Beecher Instruments, Inc., Sun Prairie, WI, USA). The sample diameter of the tissue core in the array block was 1000 µm. Each block was produced in triplicate (three biopsies from each sample).

## 4.5 IMMUNOHISTOCHEMISTRY OF NRF2 AND SRXN1

Deparaffinized and rehydrated sections were heated in a microwave oven in citrate buffer (pH 6.0) for 3 x 5 min, incubated in the citrate buffer for 18 min, and washed with PBS for 2 x 5 min. Endogenous peroxidase activity was blocked with 5 % hydrogen peroxide for 5 min and then the sections were washed with PBS for 2 x 5 min. Nonspecific binding was blocked with 1.5 % normal serum in PBS for 25 min at room temperature. The primary antibodies [rabbit polyclonal anti-human NRF2 (sc-722, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal anti-human sulfiredoxin (14273-1-AP, Protein Tech Group, Chicago, IL, USA)] were diluted with 1 % bovine serum albumin in PBS to 1:200 and 1:500 working solutions, respectively, and incubated on the slides overnight at 4°C. The negative control was incubated with 1 % BSA in PBS instead of the primary antibody. The slides were washed with PBS for 2 x 5 min and incubated with the biotinylated secondary antibody (anti-mouse IgG; ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA) for 35 min at room temperature. After this, the slides were washed with PBS for 2 x 5 min, incubated for 45 min in preformed avidin-biotinylated peroxidase complex (ABC Vectastain Elite kit, Vector Laboratories) and washed twice for 5 min with PBS. The colour was developed with diaminobenzidine tetrahydrochloride (DAB) substrate (Sigma, St. Louis, MO). The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with DePex (BDH, Poole, UK). The NRF2 immunostaining was evaluated separately in tumour cell nuclei and cytoplasm. For SRXN1, only the cytoplasmic immunoreactivity was evaluated. The results for NRF2 were semi-quantified as follows: 0 % – 5 %, negative; >5 % to 25 %, weak positivity; >25 % to 75 %, moderate positivity; and >75 % to 100 %, strong positivity. For the analyses, NRF2 expression was dichotimized by the assessment of the pathologist into two groups: low extent (≤25 %) and high extent (>25 %) expression. For SRXN1, the presence (>1 %) or absence of cytoplasmic expression was recorded.

*Table 8*. Primer sequences for genotyping of *NRF2* and *SRXN1*.

| Variation | PCR primer sequence* | Extension primer sequence* |
|---|---|---|
| ***NRF2*** | | |
| rs6721961 | F: 1-CCCTGCCTAGGGGAGATGT | GGGAGATGTGGACAGC |
| | R: 1-GCGTTCAGGGTGACTGCGA | |
| rs1806649 | F: 1-CAGTCTTAGAGGAACTCATA | AGAGGAACTCATATCCTAAG |
| | R: 1-ATCCCCCTTGAGGGACATTt | |
| rs1962142 | F: 1-TTGAGAGCAAAGGCACAGTC | TTTCCTTCTCCAACCC |
| | R: 1-TTCAGAGGACTTTCCTTCTC | |
| rs2364722 | F: 1-CAGAAAACATACCATTAGC | gggGCAATTGAATAAATCTTGGCCTAT |
| | R: 1-TAAGCGCCAACAAACATTt | |
| rs10183914 | F: 1-GTCTTCGTTTATTGCCCAGC | CAGCTGGCTCTTTACT |
| | R: 1-ACCCATTTGCTGCAAGTATC | |
| rs2706110 | F: 1-GTGAGAAATTACAAATTTCA | GTCATGGCATAGTTGAGA |
| | R: 1-CAAGAGAAATGAACACTTGG | |
| rs13035806 | F: 1-AGCCTCCCACCTGGGATTAC | gagaaACCACACCCGGCCCTGAA |
| | R: 1-gacacttgaGAACTTGAAAA | |
| rs6706649 | F: 1-AGCTCGTGTTCGCAGTCACC | CGCAGTCACCCTGAACGC |
| | R: 1-GACCTGAGCTTAGGAGAATG | |
| ***SRXN1*** | | |
| rs6085283 | F: 1-GATCTCATGTGCTCACCTTC | CGGGGGAAGGGGACACAA |
| | R: 1- AGAACAGGTGTCAGGTTAGG | |
| rs13043781 | F: 1- TGAGAAACGACAAGGCAGAG | GAGATAGAAGAGGTTCTCAAC |
| | R: 1- ATCTGCCATGCATGCCCTGT | |
| rs6076869 | F: 1-ATAGACAGGAATGGGTAGAC | cAGGTTACAGAACAAGAGC |
| | R: 1- GAGTCTGGAGTTCAGGTTCT | |
| rs6053666 | F: 1- GCAAAAGGATCCAAGACGTG | TTGGTTCAACAACTCCACG |
| | R: 1- CTGGAGATGGGTGGCTAATG | |
| rs2008022 | F: 1- TAAAACATCCTAGAGGGCTG | CCACTAATGCCCGGTAG |
| | R: 1- CTTAGTGCTCCACTAATGCC | |
| rs6116929 | F: 1- GAAGCTTCTTGGAGGAGGTA | tGAGGAGGTACCACCTTT |
| | R: 1- CCATTCCTGTCTATACTGGG | |
| rs7269823 | F: 1- CAGGTGGAGAGTAGGTCATC | tGAGAGTAGGTCATCCTCTAA |
| | R: 1- TCTCCCCAGAGTGCTCTAC | |
| rs6053728 | F: 1- AACCCTGCAAGGAAGGTATG | ACTGTTATTCCCATTTTACAGA |
| | R: 1- ATCACTTAAACCTGTGTCTC | |

F, Forward primer; R, Reverse primer; 1, non-templated prime tag ACGTTGGATG.
*bases in lower case letters are non-templated bases

## 4.6 DETERMINATION OF HORMONE RECEPTOR AND HER2 STATUS

ER and PR hormone receptors were classified as positive if the percentage of positive cells with nuclear staining was ≥ 10 %, higher than the threshold value today (1 %).

HER2 status assessment was conducted by immunohistochemistry (IHC). Samples with IHC score 2+ or 3+ were classified as HER2 positive (HER2+).

## 4.7 STATISTICAL ANALYSES

Statistical analyses were conducted using SPSS version 17.0. in publications I and III (SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc.), SPSS version 14.0. in publication II (SPSS Inc. Released 2006. SPSS Statistics for Windows, Version 14.0. Chicago: SPSS Inc.), and SPSS version 19.0. in publication IV (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).

RFS was assessed as time from diagnosis to time of first relapse (locoregional relapse, contra-lateral breast cancer or metastatic disease) or the end of follow-up. The cause of death was categorised either as being caused by breast cancer or by other causes. OS and BCSS were computed from the date of diagnosis to the last follow-up date or date of death.

The impact of the studied genotypes on RFS, BCSS, and OS were analyzed by the univariate Kaplan-Meier method with the log-rank test. The P-values, and the hazard ratios (HRs) and their 95 % confidence intervals (CIs) were calculated using Cox proportional hazards models adjusted for potential confounders including age, stage, ER and PR status, and adjuvant treatments. The P-values $\leq 0.05$ were considered to be statistically significant. The survival was estimated by using both the dominant model (the homozygous carriers of the wild type allele compared with the carriers of the variant allele) as well as the recessive model (homozygous carriers of the variant allele compared with the carriers of the wild type allele). All study cohorts and treatment subgroups were tested for Hardy-Weinberg equilibrium (HWE) using a standard $\chi 2$ test.

In publication II, analysis of variance (ANOVA) was used to compare continuous data. When the ANOVA analyses indicated a difference between groups, 2-tailed tests were used in the *post hoc* comparisons. Categorical data were compared using Fisher's exact test. The significance levels for comparisons of the genotype frequencies between cases and controls and for the association between the genotypes and protein expression and clinical variables (including tumour grade and size, histologic type, nodal status, ER status, PR status, and HER2 status) among the cases were computed using the Armitage trend test.

# 5 Results

## 5.1 GENERAL CHARACTERISTICS OF THE STUDY POPULATION

Information on study subjects' vital status at the data cut-off in February 2011 is provided in Figure 7. The median follow-up in the total study population was 11.8 years (range 0.1-20.4 years). A significant proportion (159) out of the total of 442 subjects experienced a relapse during the follow-up. Of the 28 women who were alive with recurrence of breast cancer, five patients were alive with a locoregional recurrence only, and 14 patients had been diagnosed with a new primary cancer in the contralateral breast. One patient had a locoregional recurrence and a new primary cancer in the other breast. Eight patients were alive with metastatic disease.
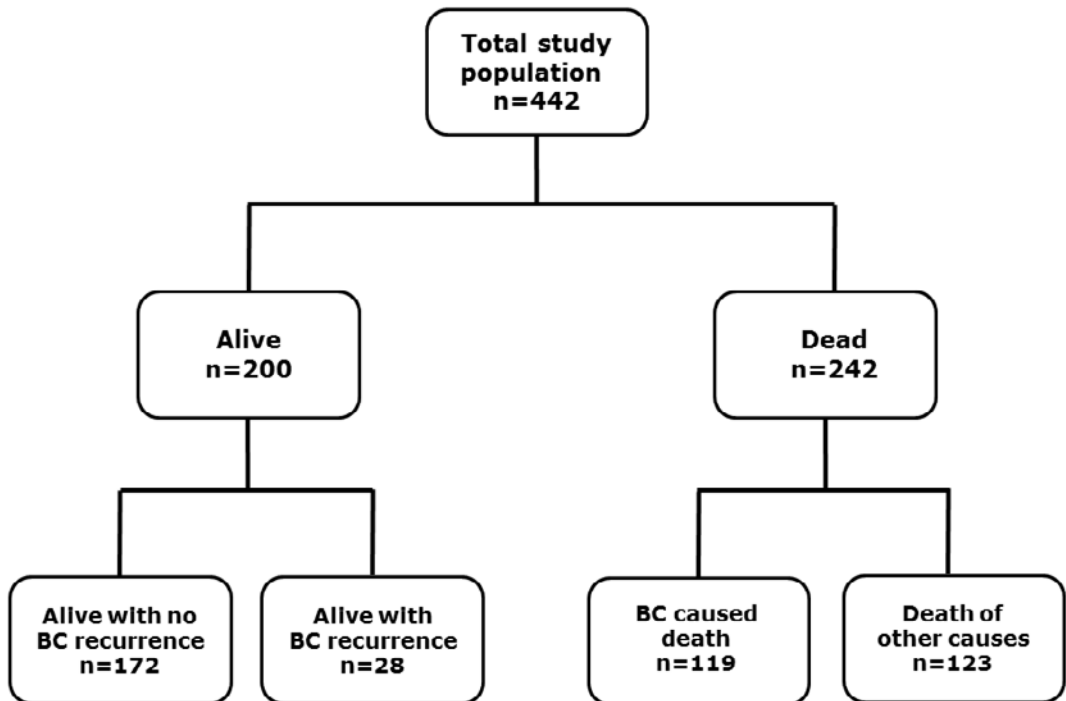


Figure 7. Flow chart describing the study subjects' vital status at the data cut-off in February 2011. BC, breast cancer.

## 5.2 *SULT1A1* RS9282861 AND SURVIVAL OF BREAST CANCER PATIENTS (I)

### 5.2.1 *SULT1A1* rs9282861 is not statistically significantly associated with the survival of patients treated with adjuvant chemotherapy

Adjuvant chemotherapy was the only systemic adjuvant treatment for 76 patients. The chemotherapy regimens used were CMF (n=70) and CNF (n=6). The median number of chemotherapy cycles was 6 (range 2-6). In the univariate Kaplan-Meier analysis, the homozygous rs9282861 variant AA genotype (n=14) was associated with improved OS compared with the AG and GG genotypes (n=62) ($P_{log-rank}$=0.045). The multivariate Cox regression analysis adjusted for age, stage, radiotherapy, and hormone receptor status detected no statistically significant differences in OS (HR=0.33, 95 % CI=0.10-1.09, P=0.068) (Figure 8A). The dominant model did not show any survival differences.

### 5.2.2 *SULT1A1* rs9282861 has no statistically significant influence on the survival of patients receiving adjuvant tamoxifen

Sixty five patients were treated with adjuvant tamoxifen only. The Kaplan-Meier and Cox regression analysis failed to reveal any statistically significant association between the *SULT1A1* rs9282861 genotype and survival in the dominant or recessive model. In the Cox regression analysis there was a non-significant trend for superior OS in 18 patients with the variant AA genotype (HR=0.53, 95 % CI=0.27-1.08, P=0.079) (Figure 8B). Other covariates were age, stage, and radiotherapy.

### 5.2.3 *SULT1A1* rs9282861 genotype is associated with the OS of the combined patient population receiving adjuvant chemotherapy or tamoxifen

There were a total of 145 patients with *SULT1A1* genotype available who were treated with adjuvant tamoxifen (n=65), chemotherapy (n=76), or with both tamoxifen and chemotherapy (n=4). The univariate analysis detected a significant association between the homozygous rs9282861 variant AA genotype (n=33) and superior OS, and this association persisted in the Cox multivariate analysis (HR=0.50, 95 % CI=0.29-0.88, P=0.015) (Figure 8C). There were no statistically significant differences in BCSS (HR=0.53, 95 % CI=0.26-1.05, P=0.069) or RFS (HR=0.50, 95 % CI=0.29-0.88, P=0.091) with respect to the rs9282861 genotype. Adjustments were made for age, stage, radiotherapy, and hormone receptor status.

### 5.2.4 Prognostic significance of the *SULT1A1* rs9282861

In the univariate analysis of the 140 patients who did not receive any kind of adjuvant treatment, there were trends towards statistically significant differences in survival (RFS $P_{log-rank}$=0.053, BCSS $P_{log-rank}$=0.074, and OS $P_{log-rank}$=0.081). In the multivariate analysis adjusted for age and stage, the rs9282861 variant AA genotype was statistically significantly associated with inferior RFS (HR=0.49, 95 % CI=0.24-0.99, P=0.048) (Figure 9A) and OS (HR=0.57, 95 % CI=0.34-0.96, P=0.034) (Figure 9C). The association with the variant AA genotype and inferior BCSS was not statistically significant (HR=0.44, 95 % CI=0.18-1.08, P=0.073) (Figure 9B).

The effect of the rs9282861 genotype on the survival of the total study population (n=412) was also examined. The univariate analyses did not reveal any significant associations in the survival outcomes according to the rs9282861 genotype (RFS $P_{log-rank}$=0.40, BCSS $P_{log-rank}$=0.38, and OS $P_{log-rank}$=0.50). In addition, the rs9282861 genotype did not influence the survival in the patient population not receiving any medical adjuvant

treatment (n=230) (RFS $P_{log-rank}$=0.30, BCSS $P_{log-rank}$=0.35, and OS $P_{log-rank}$=0.32) or in the patients receiving only postoperative radiotherapy (n=90) (RFS $P_{log-rank}$=0.64, BCSS $P_{log-rank}$=0.61, and OS $P_{log-rank}$=0.88).
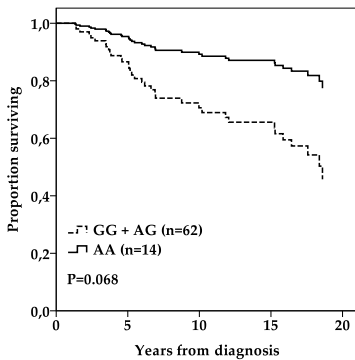
A) OS: Adjuvant chemotherapy          B) OS: Adjuvant tamoxifen
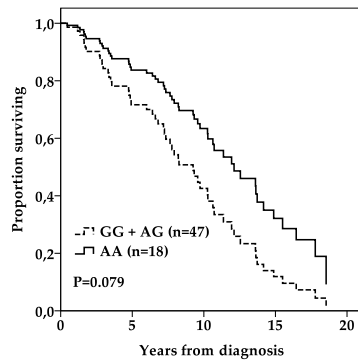
C) OS: Adjuvant chemotherapy or tamoxifen
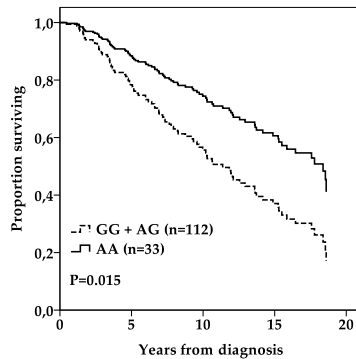


*Figure 8.* The association of *SULT1A1* rs9282861 genotype with the survival in breast cancer patients in the Cox regression analysis. The OS of 76 patients receiving adjuvant chemotherapy (A), 65 patients receiving adjuvant tamoxifen (B), and the combined patient population (n=145) receiving adjuvant chemotherapy or tamoxifen (C).

A) RFS: No adjuvant treatment
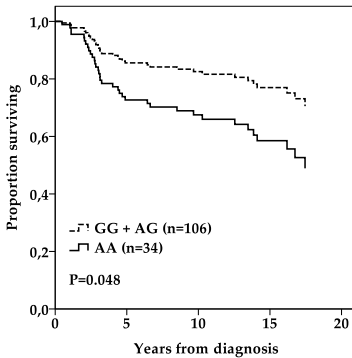
B) BCSS: No adjuvant treatment
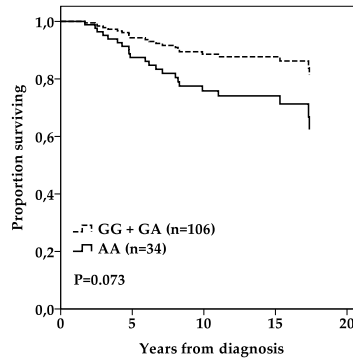


C) OS: No adjuvant treatment
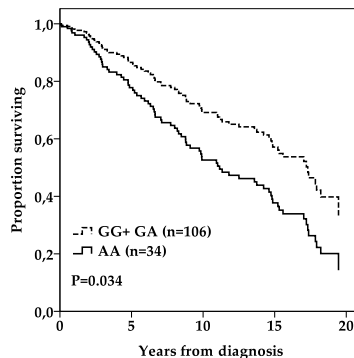


*Figure 9*. The Cox regression models for RFS (A), BCSS (B), and OS (C) according to the *SULT1A1* rs2886162 genotype in 140 patients not receiving any kind of adjuvant treatment.

## 5.3 GENETIC POLYMORPHISMS AND PROTEIN EXPRESSION OF NRF2 AND SRXN1 AND THEIR ASSOCIATION WITH THE RISK AND SURVIVAL OF BREAST CANCER (II)

### 5.3.1 *NRF2* and *SRXN1* genotypes associate with the risk of breast cancer

Genomic DNA was available for genotyping in 452 breast cancer patients and 370 control subjects from the KBCP samples. Two functional SNPs (rs6721961 and rs6706649) and six TagSNPs (rs1806649, rs2886162, rs1962142, rs2364722, rs10183914, rs2706110, rs13035806) were analyzed for the *NRF2* gene. The eight TagSNPs analyzed for *SRXN1* were rs6085283, rs13043781, rs6076869, rs6053666, rs2008022, rs6116929, rs7269823, and rs6053728. The genotypes were in concordance with the HWE except for a slight deviation from HWE of *NRF2* rs6706649 in controls (P=0.029).

An association with breast cancer risk was observed in breast cancer cases with *NRF2* rs6721961 and rs2706110 and *SRXN1* rs6053666 genotypes. The variant homozygous genotypes of *NRF2* rs6721961 (TT) and rs2706110 (AA) were associated with an increased risk of breast cancer whereas carrying the variant allele C was protective in *SRXN1*

rs6053666. There was a trend towards decreased risk of breast cancer for the *NRF2* rs13035806 variant allele G.

### 5.3.2 NRF2 and SRXN1 protein expression

Tumour material for tissue microarrays was available for 373 breast cancer cases. Cytoplasmic NRF2 positivity of high extent (>25 %) was observed in 66 % (237/361) of cases, whereas high extent nuclear positivity was seen in 26 % (96/365) of the samples. The breast tumours with lobular histology were more commonly expressing nuclear NRF2 with high extent (47 %) than ductal breast tumours (20 %) (P=0.001).

Positive cytoplasmic expression of SRXN1 was found in 23 % of breast tumour samples (82/363). The nuclear and cytoplasmic NRF2 expressions were associated with the SRXN1 expression (P=0.003 and P=0.008, respectively).

### 5.3.3 *NRF2* and *SRXN1* SNPs and their association with protein expression

The variant alleles of *NRF2* rs1962142 and rs6721961 were associated with low extent (< 25 %) cytoplasmic NRF2 expression and negative SRXN1 protein expression. In addition, the variant allele of *NRF2* rs2886162 was associated with low extent cytoplasmic NRF2 expression.

The *SRXN1* rs6076869 variant allele T associated with high extent cytoplasmic NRF2 expression (OR=1.927, 95 % CI=1.217-3.051, P=0.005) and with lobular histology (OR=1.83, 95 % CI= 1.092-3.066, $P_{allele-specific}$=0.022).

### 5.3.4 The prognostic value of *NRF2* and *SRXN1* genotypes

In the Kaplan-Meier analyses, the homozygous *NRF2* rs2886162 variant AA genotype, homozygous *SRXN1* rs6116929 variant GG genotype, homozygous *SRXN1* wild type genotypes rs7269823 AA and rs6085283 CC, and *SRXN1* variant allele carriers rs2008022 CA and AA were associated with better survival ($P_{log-rank}$=0.017, $P_{log-rank}$=0.063, $P_{log-rank}$=0.030, $P_{log-rank}$=0.015, $P_{log-rank}$=0.012, respectively). However, only the *NRF2* rs2886162 polymorphism remained as a significant covariant in the multivariate Cox analyses (HR=1.687, 95 % CI =1.047-2.748, P=0.032).

### 5.3.5 The combined *NRF2* and *SRXN1* high-risk genotypes associate with worse BCSS

The influence of the combined effect of the *NRF2* and *SRXN1* genotypes on the prognosis was also examined. Based on the Kaplan-Meier curves in the prognostic survival analyses the *SRXN1* rs6116929, rs2008022, rs7269823, and rs6085283 risk alleles were designated as A, C, G, and T, respectively. The patients were divided into two groups: 0-3 risk alleles and 4-8 risk alleles. The Kaplan-Meier analysis showed that the patients with 4-8 risk alleles had a greater risk of dying from breast cancer than patients with 0-3 *SRXN1* risk alleles ($P_{log-rank}$=0.009).

When also the *NRF2* rs2886162 was included in the univariate combined risk factor analyses, a survival difference emerged between the strata defined by the rs28861612 genotype. Carriage of rs2886162 variant allele A was associated with inferior BCSS in patients with 4-8 *SRXN1* risk alleles ($P_{log-rank}$=0.010) whereas no survival difference was visible among patients homozygous for the wild type G allele.

In the multivariate analysis including also tumour grade, nodal status, ER and PR status, histological type, tumour size, and HER2 status, only nodal and HER2 status and the *NRF2* rs2886162 genotype remained statistically significantly associated with survival. Patients with the homozygous rs2886162 AA variant genotype had an inferior BCSS (HR=1.667, 95 % CI=1.054-2.637, P=0.029). Since also *NRF2* cytoplasmic and nuclear expression and SRXN1 protein expression were included in the multivariate analysis,

similar results were obtained: *NRF2* rs2886162 AA genotype associated with worse BCSS (HR=1.693, 95 % CI =1.040-2.758, P=0.034).

### 5.3.6 The influence of the *NRF2* and *SRXN1* polymorphisms on the survival according to the adjuvant treatment

In the multivariate analyses, the homozygous *NRF2* rs2886162 variant AA genotype was predictive of inferior RFS and BCSS in patients receiving adjuvant chemotherapy (n=16) compared with the carriers of the wild type G allele (n=63) (HR=2.83, 95 % CI=1.43-5.61, P=0.003, and HR=2.43, 95 % CI=1.16-5.08, P=0.019, respectively) (Figure 10A and 10B). A similar effect was seen in 247 patients treated with postoperative radiotherapy as the rs2886162 AA genotype predicted worse RFS (HR=1.68, 95 % CI=1.07-2.64, P=0.025) (Figure 10C). The *NRF2* rs2886162 genotype did not influence survival in patients who did not receive any kind of adjuvant treatment (n=137).
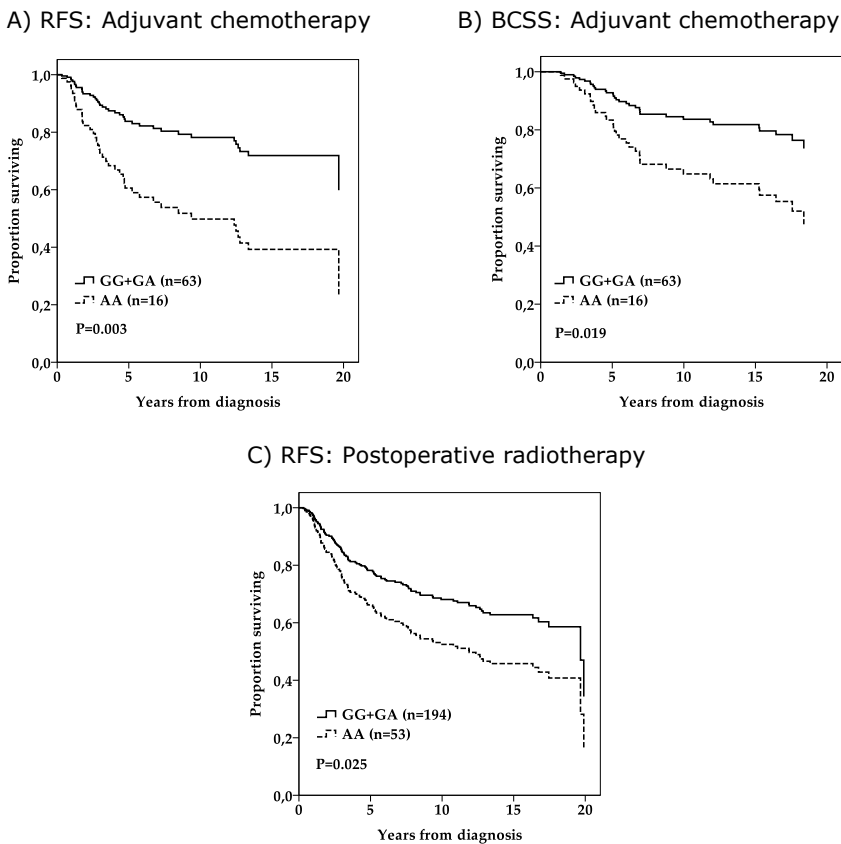


*Figure 10*. The association of the *NRF2* rs2886162 genotype on the RFS (A) and BCSS (B) of patients treated with adjuvant chemotherapy (n=79) and on the RFS (C) of patients treated with postoperative radiotherapy (n=247) in the Cox regression model.

The carriage of the *SRXN1* rs6116929 wild type allele A, or variant allele G of *SRXN1* rs7269823, or *SRXN1* rs6085283 variant allele T predicted a worse RFS (HR=1.96, 95 % CI=1.17-3.27, P=0.010, HR=1.72, 95 % CI=1.16-2.54, P=0.007, and HR=1.72, 95 % CI=1.08-2.75, P=0.022, respectively) and BCSS (HR=1.74, 95 % CI=1.00-3.00, P=0.049, HR=1.56, 95 % CI=1.01-2.41, P=0.045, and HR=1.75, 95 % CI=1.04-2.95, P=0.036, respectively) in patients treated with postoperative radiotherapy. In the same adjuvant treatment group, homozygosity for the variant allele C of *SRXN1* rs6053666 predicted worse RFS (HR=1.62, 95 % CI=1.06-2.46, P=0.026) and the homozygous *SRXN1* rs2008022 wild type CC genotype was associated with inferior BCSS (HR=1.73, 95 % CI=1.09-2.74, P=0.020). The rs2008022 CC genotype was related with the worse BCSS also in patients not receiving adjuvant treatments (HR=3.39, 95 % CI=1.13-10.14, P=0.029).

## 5.4 PREDICTIVE SIGNIFICANCE OF *MNSOD* AND *XPD* POLYMORPHISMS IN PATIENTS TREATED WITH ADJUVANT TAMOXIFEN OR CHEMOTHERAPY (III)

### 5.4.1 *MnSOD* rs4880 genotype and survival after adjuvant tamoxifen
There were 64 patients who received adjuvant tamoxifen in the cohort analyzed for the rs4880 genotype. The univariate Kaplan-Meier survival curves showed better RFS ($P_{log-rank}$=0.014) and BCSS ($P_{log-rank}$=0.026) for patients carrying the wild type A allele. The multivariate analyses adjusted for age, stage, and radiation therapy detected a significant difference in RFS and BCSS favoring patients with the AA or AG genotype (HR=0.36, 95 % CI=0.14-0.91, P=0.030 and HR=0.33, 95 % CI=0.12-0.91, P=0.032, respectively) (Figure 11A and 11B). The *MnSOD* rs4880 had no effect on OS.
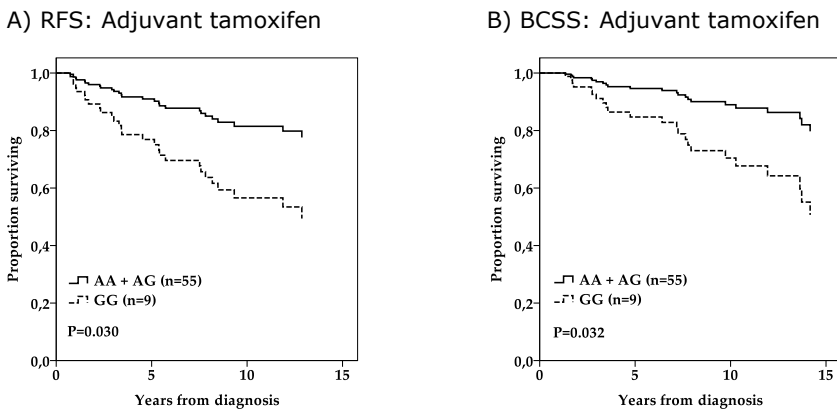


*Figure 11*. The Cox regression model survival curves for RFS (A) and BCSS (B) according to the *MnSOD* rs4880 genotype in 64 patients treated with adjuvant tamoxifen.

### 5.4.2 *XPD* rs13181 genotype associates with survival in patients receiving adjuvant tamoxifen or chemotherapy
In the univariate analysis, the *XPD* rs13181 genotype had a significant effect on OS in 65 patients receiving adjuvant tamoxifen ($P_{log-rank}$=0.036), whereas there was no statistically significant association for RFS or BCSS ($P_{log-rank}$=0.11 and $P_{log-rank}$=0.16, respectively).

In the multivariate analysis of tamoxifen treated patients adjusted for age, stage, and radiotherapy, the homozygous rs13181 wild type AA genotype was predictive for improved RFS (HR=0.36, 95 % CI=0.13-1.00, P=0.049) (Figure 12A), and BCSS (HR=0.30, 95 % CI=0.10-0.95, P=0.040) (Figure 12B). Differences in the OS were nearly statistically significant favoring patients with the rs13181 variant AA genotype (HR=0.48, 95 % CI=0.23-1.00, P=0.051) (Figure 12C).

In the multivariate analysis of patients receiving adjuvant chemotherapy (n=74), the *XPD* rs13181 AA and AC genotypes were associated with better RFS (HR=0.42, 95 % CI=0.19-0.94, P=0.034) (Figure 12D). Adjustments were made for age, stage, radiotherapy, and hormone receptor status.
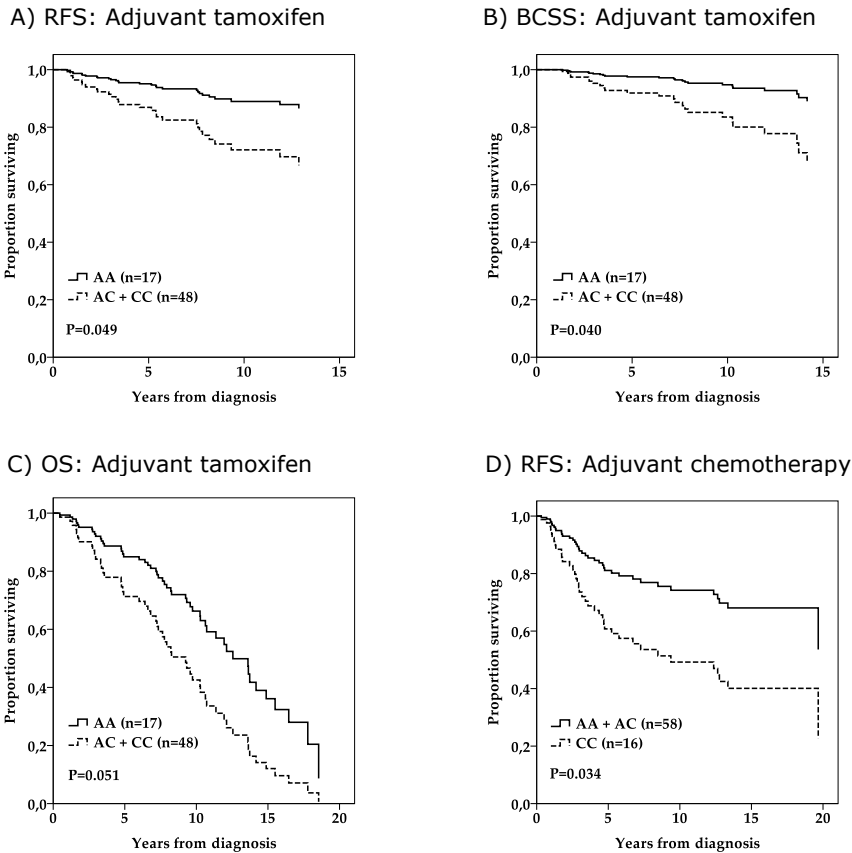


*Figure 12*. *XPD* rs13181 genotype and Cox regression model survival curves for RFS (A), BCSS (B), and OS (C) in 65 patients receiving adjuvant tamoxifen, and for RFS in 74 patients receiving adjuvant chemotherapy (D).

### 5.4.3 The combined *MnSOD* rs4880 and *XPD* rs13181 genotypes influence the survival of patients receiving adjuvant tamoxifen

Based on the analysis of the survival curves, HR's, and 95 % CIs, 58 cases out of 64 tamoxifen treated patients were designated to carry at least one genotype associated with favorable survival outcome, e.g., *MnSOD* rs4880 AA, *MnSOD* rs4880 AG, and *XPD* rs13181 AA. In the multivariate analysis, the carriage of at least one low-risk genotype was

associated with improved RFS (HR=0.20, 95 % CI=0.07-0.58, P=0.003), BCSS (HR=0.20, 95 % CI=0.06-0.65, P=0.008), and OS (HR=0.28, 95 % CI=0.10-0.76, P=0.012) in patients receiving adjuvant tamoxifen (Figures 13A, 13B, and 13C, respectively).

Carrying both the *MnSOD* rs4880 A allele and the *XPD* rs13181 AA genotype (n=14) seemed to be favorable in terms of BCSS, but the difference was not quite statistically significant in the multivariate analysis (HR=0.29, 95 % CI=0.08-1.02, P=0.054) (Figure 13D).

In the tamoxifen treated patients, the median overall survival was 12.3 years in patients with both the *MnSOD* rs4880 and *XPD* rs13181 low-risk genotypes, 8.0 years in patients with one low-risk genotype, and only 3.3 years in patients carrying no low-risk genotypes.

The studied *MnSOD* and *XPD* genotypes did not associate with survival in the total study population (n=396) or in the patients who did not receive adjuvant treatments (n=133).
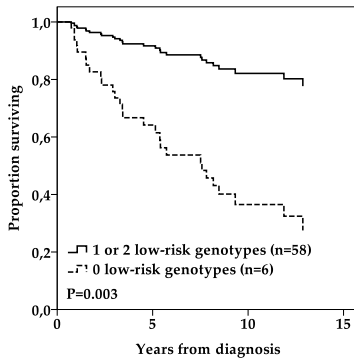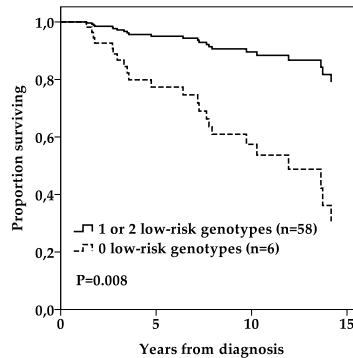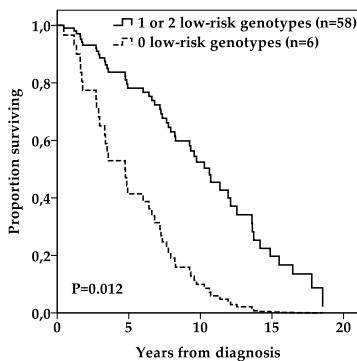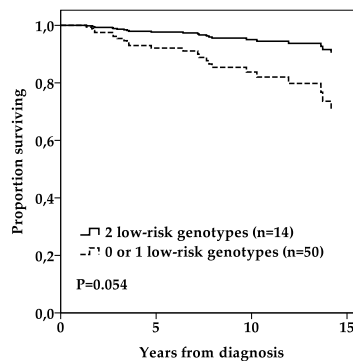


*Figure 13.* The Cox regression model survival curves for the combined *MnSOD* rs4880 and *XPD* rs13181 according to the carriage of low-risk genotypes (rs4880 AA, rs4880 AG, or rs13181 AA) in the tamoxifen treated patient population (n=64). The RFS, BCSS, and OS in patients carrying at least one low-risk genotype were compared with survival of patients carrying no low-risk genotypes (A, B, and C, respectively). (D) The BCSS curves for carrying the low-risk *MnSOD* rs4880 A allele and *XPD* rs13181 AA genotype versus carrying 0-1 low-risk genotypes.

## 5.5 THE EFFECT OF THE *XRCC1* RS25487 POLYMORPHISM ON THE SURVIVAL OF BREAST CANCER PATIENTS (IV)

### 5.5.1 Prognostic significance of the *XRCC1* rs25487

In the cohort of all eligible patients (n=411) the univariate and multivariate analyses detected inferior BCSS for patients carrying the homozygous *XRCC1* rs255487 variant AA genotype (log-rank=0.032, HR=1.95, 95 % CI=1.15-3.32, P=0.014) (Figure 14A). RFS and OS did not differ significantly, neither were there any significant survival differences according to the rs25487 genotype in the subgroup of patients who did not receive any kind of adjuvant treatment.

### 5.5.2 *XRCC1* rs25487 polymorphism predicts the outcome in patients receiving postoperative radiotherapy or adjuvant chemotherapy

The homozygous rs25487 variant AA genotype was associated with worse BCSS (HR=2.03, 95 % CI=1.07-3.85, P=0.031) (Figure 14B) and OS (HR=1.85, 95 % CI=1.06-3.24, P=0.030) (Figure 14C) in the multivariate analysis of 238 patients treated with postoperative radiotherapy. HRs were adjusted for age, stage, chemotherapy, and hormonal treatment.

   In addition, carrying the rs25487 AA genotype emerged as a negative predictor of BCSS (HR=2.79, 95 % CI=1.01-7.67, P=0.047) (Figure 14D) in patients who received adjuvant chemotherapy (n=75). Adjustments in the multivariate analysis were made for age, stage, and radiotherapy. The rs25487 genotype did not influence the survival of patients treated with adjuvant tamoxifen.
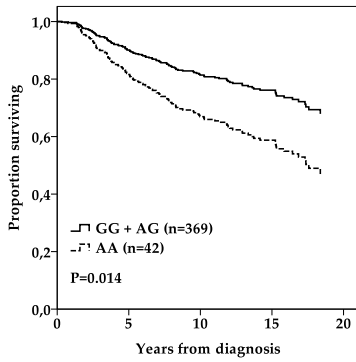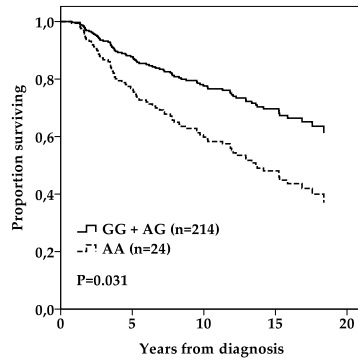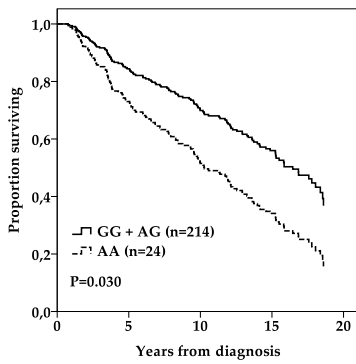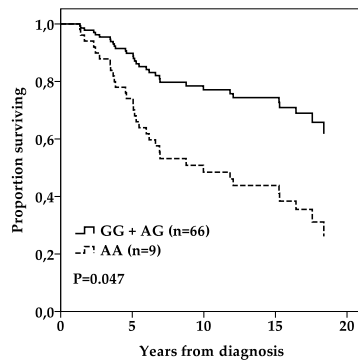
*Figure 14*. The Cox regression model survival curves according to the *XRCC1* rs25487 genotype. (A) The BCSS in the total study population (n=411). (B) and (C): The BCSS and OS in patients receiving postoperative radiotherapy (n=238), respectively. (D) The BCSS in patients treated with adjuvant chemotherapy (n=75).

# 6 Discussion

The purpose of this work was to study polymorphisms in the genes involved in drug metabolism, oxidative stress, and DNA repair and to examine the influence of this genetic diversity on the outcome of patients with early breast cancer. During the median follow-up of 11.8 years 45 % of patients in the study cohort have died due to breast cancer.

It should be borne in mind that 20 years ago the treatment of breast cancer was different from the current practice. In the 1990s, breast cancer was operated most often with mastectomy and adjuvant chemotherapy and hormonal therapies were given more infrequently. In Kuopio University Hospital, the implementation of postoperative radiotherapy was rather similar to the current practice. For example, in the present study, the majority of patients who had been operated with ablative surgery and had N1 status received locoregional radiotherapy (122 out of 142 patients). Even though the prognosis has improved gradually due to more efficient adjuvant therapies, the recurrence of the malignancy still remains a clinical challenge.

## 6.1 ASSOCIATION OF THE *SULT1A1* RS9282861 POLYMORPHISM WITH SURVIVAL OF BREAST CANCER PATIENTS

### 6.1.1 Predictive role of *SULT1A1* rs9282861
SULT1A1 is a phase II enzyme that facilitates the elimination of tamoxifen. Hence, alterations in the excretion of active metabolites of tamoxifen might influence the effectiveness of adjuvant hormonal therapy.

The purpose of the first study (publication I) was to analyze whether the *SULT1A1* rs9282861 polymorphism influences the outcome of breast cancer patients. The results suggest that the homozygous *SULT1A1* rs9282861 variant AA genotype is associated with improved OS of patients treated with adjuvant chemotherapy or tamoxifen when compared with patients carrying the wild type G allele. The result was statistically significant only in the combined analysis of these two adjuvant treatment groups, while separate analyses of patients receiving either adjuvant tamoxifen or chemotherapy did not reveal statistically significant associations. The relatively small number of patients in the separate treatment groups might explain why the difference between OS did not reach statistical significance in these analyses.

### 6.1.2 SULT1A1 as a modifier of tamoxifen metabolism
The present results are consistent with the hypothesis that the homozygous *SULT1A1* rs9282861 variant AA genotype is associated with lower catalytic activity and poorer thermostability of the enzyme compared with the wild type allele G (Raftogianis et al., 1999). Reduced elimination of active metabolites of tamoxifen by phase II metabolism could lead to improved clinical efficacy. However, previous studies have yielded inconsistent results (Nowell et al., 2002; Choi et al., 2005; Nowell et al., 2005; Wegman et al., 2005; Wegman et al., 2007). In the study of Wegman et al., there was a trend towards a lower risk of distant recurrence among carriers of the rs9282861 wild type GG genotype in the group of patients receiving tamoxifen (Wegman et al., 2005). Another study reporting improved OS of breast cancer patients carrying the rs9282861 wild type G allele treated

with tamoxifen suggested this result was attributable to the reabsorption of the sulfated form of 4-OH-TAM in the kidney. Further desulfation of this compound in the breast tumour by steroid sulfatase might extend the duration of action of the active metabolite, 4-OH-TAM (Nowell et al., 2002). Another possible explanation was that the high-activity allele induces global expression of the SULT1A1 enzyme, followed by increased elimination of other potentially harmful substrates, including estrogenic compounds. A subsequent study suggested that these findings, contradictory to the previous hypothesis, might be explained by the observation that sulfated tamoxifen mediates apoptosis in breast cancer cell lines expressing SULT1A1 (Mercer et al., 2010).

The gene copy number variation (CNV) may represent an additional source of variability in the metabolic activity of an enzyme. There are studies reporting that an increasing number of *SULT1A1* copies correlates with elevated SULT1A1 activity (Hebbring et al., 2007; Yu et al., 2013). However, in two studies investigating mainly Caucasian breast cancer patients the SULT1A1 CNV was not found to influence the levels of tamoxifen and its metabolites or to associate with the DFS of patients receiving adjuvant tamoxifen (Gjerde et al., 2008; Moyer et al., 2011).

The outcome of tamoxifen therapy is probably not solely determined by a single SNP but instead by a combination of several genetic factors. In addition to sulfation by SULTs, glucuronidation of tamoxifen is a route of substrate elimination through the bile. Glucuronidation is probably the most effective way to excrete tamoxifen and its derivatives (Lien et al., 1989). In fact, the *UGT2B15* high activity genotype has been associated with an increased risk of recurrence and poorer survival in a group of tamoxifen treated patients (Nowell et al., 2005). Furthermore, several other UGTs (UGT1A4, UGT2B7, UGT1A8 and UGT1A10) have been reported to be active against 4-OH-TAM (Sun et al., 2006; Sun et al., 2007).

It is noteworthy that in the studies of Nowell et al. and Wegman et al. (Nowell et al., 2002; Wegman et al., 2005), the genotyping was made from tumour tissue, which may carry a risk of genotype misclassification. However, the most plausible reasons for the discordant results between different studies are heterogeneity in the study populations and a lack of statistical power due to small sample sizes.

Polymorphisms associated with the CYP genes, especially *CYP2D6*, may also have a substantial impact on the outcome of tamoxifen therapy; CYP2D6 contributes to the formation of 4-OH-TAM in human liver (Dehal and Kupfer, 1997). Moreover, tamoxifen is metabolized to ND-TAM in a CYP-dependent pathway by CYP3A4 and secondarily to endoxifen by CYP2D6, and decreased CYP2D6 enzyme activity has been associated with worse event-free survival (EFS) and disease-free survival in patients treated with adjuvant tamoxifen (Schroth et al., 2009), although contradictory results have also been reported (Wegman et al., 2005). This complexity of tamoxifen metabolism may partly explain the conflicting results in different studies.

There was no specific data on the other medications used by the patients available in our study. For example, concomitant use of CYP2D6 inhibitors, including selective serotonin reuptake inhibitor (SSRI) antidepressants, especially the highly potent CYP2D6 inhibitor, paroxetine, may reduce the efficacy of tamoxifen (Jin et al., 2005; Kelly et al., 2010). However, the influence of this potential confounding factor is likely to be minor since the use of SSRIs was uncommon in the 1990s.

### 6.1.3 SULT1A1 and the pharmacokinetics of chemotherapy

Cyclophosphamide is an alkylating agent metabolized via the CYP450 enzymes into 4-hydroxycyclophosphamide and aldophosphamide (Zhang et al., 2005). These are further β-eliminated by albumin and other proteins to form two active metabolites, acrolein and

phosphoramide mustard. Subsequently, an alkyl group is added to the guanine base of DNA which leads to an inhibition of DNA replication. The cytotoxic effects of cyclophosphamide are in part mediated by ROS generation (Sulkowska and Sulkowski, 1997; O'Toole et al., 2009).

There do not appear to be any studies which have investigated the role of *SULT1A1* polymorphism in the pharmacokinetics of chemotherapeutic regimens, and the mechanism of this potential association is unclear. It is known that heterocyclic amines are activated by SULTs (Glatt, 2000). The sulfonate group is often transferred to oxygen, which is frequently in the form of a hydroxyl group (Glatt, 2000). In theory, 4-hydroxy-cyclophosphamide might serve as a substrate for SULT1A1 and possessing the high-activity *SULT1A1* allele would increase the rate of elimination of cyclophosphamide, thus decreasing the individual's exposure to its cytotoxic effects. However, none of the chemotherapeutic drugs given in the CMF regimen are known to act as substrates to SULT1A1. In addition, to date there are neither pharmacokinetic nor *in vitro* data available to support this hypothesis.

### 6.1.4 *SULT1A1* rs9282861 and radiotherapy
Locoregional radiotherapy was given to 77 patients (95.1 %) receiving chemotherapy and to 47 patients (72.3 %) treated with tamoxifen. In the univariate analysis, the rs9282861 genotype was not associated with any differences in survival among patients who were given adjuvant radiotherapy but no adjuvant chemotherapy or hormonal therapy (n=90).

### 6.1.5 *SULT1A1* rs9282861 as a prognostic factor
The multivariate analyses for survival in patients with no adjuvant treatment suggest that this polymorphism might also have a prognostic effect. There were statistical significant differences in RFS and OS, while the difference in BCSS did not quite reach statistical significance. The rs9282861 variant AA genotype was associated with worse outcome, the opposite of that found in patients receiving adjuvant tamoxifen or chemotherapy.

By a strict definition, a prognostic factor is a measurable clinical or biological characteristic that defines the natural course of a disease in an untreated individual. For example, HER2 amplification is both a prognostic and predictive factor in breast cancer. In untreated patients, HER2-positivity is associated with inferior survival (Slamon et al., 1987). On the other hand, HER2-targeted therapies greatly improved the survival of patients with HER2 amplification (Joensuu et al., 2009; Swain et al., 2015).

In the present study, the improved survival of untreated patients carrying the high-activity wild type G allele might reflect their more efficient capacity to detoxify deleterious chemicals and hormonal compounds, including endogenous estrogen (Falany et al., 1993; Ozawa et al., 1995).

## 6.2 OXIDATIVE STRESS
Oxidative stress seems to play an important role in the origin and progression of cancer. On the other hand, ROS formation is an important mechanism of tumour cell destruction mediated by radiotherapy and other types of cancer treatments. In theory, antioxidative mechanisms may act undesirably in the context of malignant disease as they are able to neutralize the ROS produced by cancer treatments. In the present study, several polymorphisms involved with pathways of oxidative stress were associated with breast cancer risk and survival.

**6.2.1 NRF2: influence on the risk and outcome of breast cancer**

Under basal redox conditions, the antioxidative transcriptional factor NRF2 is bound by the repressor protein Keap1. NRF2 is then polyubiquitylated by Cul3 and is further degraded through the ubiquitin proteasome pathway. In conditions of oxidative stress, Keap1 becomes inactivated and NRF2 will be translocated into the nucleus. Binding of NRF2 to the ARE activates several antioxidant and detoxifying genes, one of which is *SRXN1*, a member of the antioxidant protein family.

NRF2 seems to be an important regulator of the cellular antioxidant defense systems. There are few previous studies examining the *NRF2* and *SRXN1* polymorphisms and their association with breast cancer risk and outcome. It was found that homozygosity for the *NRF2* rs6721961 variant allele T associated with an increased risk for breast cancer. The rs6721961 variant allele T also associated with low-extent cytoplasmic NRF2 protein expression and negative SRXN1 expression. In line with this result, rs6721961 has been predicted to affect ARE-like promoter binding sites and basal level expression of NRF2 which ultimately results in attenuated gene transcription (Marzec et al., 2007). These features coupled with down-regulation of other NRF2 target genes could lead to increased cancer susceptibility. In support of the present findings, this SNP has also been linked with an increased risk of lung cancer (Suzuki et al., 2013).

In addition, the variant homozygous genotype of rs2706110 was associated with an increased risk of breast cancer. The functional consequences of this polymorphism have not been fully clarified. Carrying the variant rs2706110 allele has been associated with reduced Keap1 expression in human olfactory neurosphere-derived (hONS) cells of patients with Parkinson's disease (Todorovic et al., 2015). However, there were no differences in cellular viability between the wild type and variant type cells after exposing these cells to the ROS generating agent, rotenone.

Estrogen exposure is an established risk factor of breast cancer (Yager and Davidson, 2006). Treatment of MCF-10A immortalized breast stem cells with estrogen metabolite 4-hydroxyestradiol has been shown to decrease the NRF2 transcript and induce mutations. This effect was significantly reduced as the cells were treated with NRF2 inducer shikonin (Zhang et al., 2014). In addition, estrogen has been reported to up-regulate NRF2 and heme oxygenase 1 (HO-1) through the PI3K/glycogen synthase kinase 3 beta (GSK3β) pathway in MCF-7 cells (Wu et al., 2014b).

NRF2 is believed to be one of the key regulators of resistance to radiation and chemotherapy. Oncogenic gain-of-function mutations in *NRF2* and loss-of-function mutations in *Keap1* lead to a sustained up-regulation and nuclear accumulation of NRF2. NRF2 subsequently increases the expression of genes known to be involved with radiation sensitivity including *HO-1*, NADPH dehydrogenase, quinone 1 (*NQO1*), *PRX-1*, and murine double minute (*Mdm2*). In addition, cross-talk of NRF2 with other genes related to radiation resistance including hypoxia-inducible factor 1 (*HIF-1*), *NF-κB*, CDK inhibitor *p21[Cip1/WAF1]*, and *ATM* may contribute to the decreased sensitivity to radiotherapy.

It was observed that the *NRF2* rs2886162 variant allele A was associated with low-extent cytoplasmic NRF2 expression. The homozygous rs2886162 variant AA genotype was also associated with worse RFS and BCSS in patients receiving adjuvant chemotherapy and inferior RFS in patients treated with postoperative radiotherapy. As the patients who underwent only the operative treatment of breast cancer were analyzed, there were no differences in survival with respect to the *NRF2* rs2886162 status. At the moment, it is not known what consequences the rs2886162 polymorphism may evoke on the NRF2 activity. In theory, a low cytoplasmic level of NRF2 might reflect the response to oxidative stress as NRF2 moves to the nucleus under conditions of oxidative stress.

One reason for cancer recurrence after surgery and adjuvant therapies is the existence of resistant cancer stem cells (CSCs). In comparison with non-stem cells, CSCs are associated with lower levels of ROS, contributing to radiation resistance in *in vitro* and *in vivo* breast cancer models (Diehn et al., 2009). NRF2 is an essential protein in promoting homeostasis of intestinal and hematopoietic stem cells (Hochmuth et al., 2011; Tsai et al., 2013). The substrate adaptor sequestome 1 protein, p62, is a linker that induces the dissociation of the NRF2-Keap1 complex. In a study investigating the NRF2 pathways in CSC-enriched mammospheres, it was found that silencing of p62 suppressed the NRF2 activation. Moreover, NRF2 knockdown resulted in increased cell death and prevented the development of chemotherapy resistance. It was also observed that attenuation of NRF2 activity led to decreased expression of efflux transporters compared with the control mammospheres (Ryoo et al., 2015). In support of NRF2 involvement in drug resistance, the MCF-7 cell lines resistant to doxorubicin (MCF-7/DOX) have been found to have elevated levels of NRF2, HO-1, and NQO1. This resistance could be partially reversed by the *NRF2* small interfering RNA (siRNA) (Zhong et al., 2013).

Resistance to tamoxifen treatment has been linked with an increased expression of NRF2-dependent antioxidative proteins *in vitro* but this effect was not coupled with the deregulation of the ER (Kim et al., 2008). However, no associations were observed here between the *NRF2* genotype and the outcome of tamoxifen treated patients.

It has also been suggested that there is a direct interaction between HER2 and NRF2. There is evidence that constitutively activated HER2 enhances the NRF2 pathway in MCF-7 breast cancer cells, and HER2 and NRF2 cooperatively up-regulate the expression of various detoxifying and chemotherapy resistant enzymes including glutathione-S-transferases A2 and P1 (GSTA2/GSTP1), CYP3A4, HO-1, and multidrug resistance proteins 1 and 5 (MRP1/MRP5) (Kang et al., 2014).

HER2 status was included in the present multivariate analysis examining the prognostic influence of the studied *NRF2* and *SRXN1* polymorphisms, and it was significantly associated with BCSS. As described previously, the cases with IHC score 2+ or 3+ were regarded as HER2+. However, an IHC score 2+ for HER2 is considered as equivocal and it is recommended to confirm the results by in situ hybridization (ISH) (Wolff et al., 2013). This shortcoming should be taken into account while evaluating the present results. HER2 status was not included in the survival analyses for adjuvant treatment subgroups. In addition, HER2-targeted therapies were not available in the adjuvant setting at the time of this study.

The NRF2-induced antioxidant machinery is a complex network of genes and proteins. Not only the NRF2 itself but also Keap1, the adaptor protein Cul3, and the reactions mediated by NF-κB, for example, may exert a remarkable influence on the responses to oxidative stress. Loss-of-function mutations in Keap1 may lead to elevations in the cellular antioxidant level (Singh et al., 2006). Indeed, polymorphisms in Keap1 have been associated with risk and survival of breast cancer (Hartikainen et al., 2015).

Overexpression of Cul3 has been reported to associate with depleted levels of NRF2 expression in breast cancer cell lines, and the Cul3-siRNA-silenced MCF-7 cell lines were more resistant to both doxorubicin and paclitaxel (Loignon et al., 2009). However, that study only examined the NRF2 expression from nuclear or cytoplasmic extracts without specifying the messenger RNA (mRNA) and protein level results according to the subcellular location (Loignon et al., 2009).

In the present cohort of breast cancer patients, 66 % of cases had high extent cytoplasmic positivity for NRF2. High nuclear positivity was found in 26 % of cases, this being significantly more common in lobular subtypes. In the literature, there is inconsistency regarding the NRF2 expression level in breast tumour cells (Loignon et al., 2009; Syed

Alwi et al., 2012; Funes et al., 2014). Different methods applied in the immunohistochemical analyses and their interpretation may in part account for the variability in the results of the studies reporting protein expressions.

Several studies have also shown that molecular subtypes of breast cancer have unique patterns and pathways of gene expression. It has been observed that NRF2 is constitutively residing and activated in the nucleus of dedifferentiated, basal type breast cancer cells, leading to increased ROS scavenging and multidrug resistance (MDR) (Del Vecchio et al., 2014). The protein kinase RNA-like endoplasmic reticulum kinase (PERK) seems to activate NRF2 and its downstream signaling even in the absence of oxidative stress. In support of this *in vitro* finding, in xenograft models of basal type tumours with MDR, it has been that the PERK inhibition reduced the expression of antioxidant proteins and significantly reduced the size of the tumours when applied in combination with doxorubicin. On the other hand, the PERK inhibition did not exert any effect on the efficacy of chemotherapy in the tumours of luminal type.

It should also be acknowledged that the protein expression analyses of tumour specimens biopsied at the time of diagnosis or surgery do not necessarily correlate reliably with the protein expression after exposure to ROS generating cancer therapies. Oxidative stress may also modulate the subcellular localization of NRF2 and thereby alter the redox balance.

Considering the mounting evidence that NRF2 has a distinct role both in the evolution of malignancy as well as resistance to cancer therapies, pharmacological modulators have been developed to address these issues. Some NRF2 activators are plant-derived phytochemicals such as curcumin, lycopene, and garlic organosulfur compounds (Kensler and Wakabayashi, 2010). The synthetic NRF2 activators include oltipraz and dimethyl fumarate (DMF). These have already been tested for chemoprevention and treating various diseases in animal models and in humans, but with inconclusive results (Kensler et al., 1998; Kelley et al., 2005; Ashrafian et al., 2012; Gold et al., 2012). DMF has been approved by the FDA and European Medicines Agency (EMA) for the treatment of multiple sclerosis (MS) after it was observed to decrease the annual rate of MS relapses (Gold et al., 2012).

Interestingly, following promising results emerging from a study investigating the activity of DMF in several glioma models (Ghods et al., 2013), a phase I study is recruiting patients with newly diagnosed glioblastoma multiforme to be treated with DMF, temozolomide and radiotherapy (ClinicalTrials.gov Identifier: NCT02337426). In the study of glioma cell lines, it was also observed that DMF suppressed the activation of NF-κB (Ghods et al., 2013). While it could seem unreasonable to expect therapeutic tumour responses by activating NRF2 and other antioxidant proteins, the favorable effects may be explained by NRF2-independent, NF-κB-mediated mechanisms of action.

Several molecules have been found to inhibit NRF2, including brusatol, ascorbic acid, and all-trans retinoic acid (Tarumoto et al., 2004; Wang et al., 2007; Ren et al., 2011). Brusatol, a component of *Brucea javanica* seeds, has been shown to sensitize cancer cells to several chemotherapeutic agents both in vivo and in vitro (Ren et al., 2011). However, it should be taken into account that while NRF2 inhibitors might enhance the destruction of tumour cells, they also may increase the vulnerability of the non-target normal cells.

### 6.2.2 The association of the *SRXN1* polymorphisms on the risk and outcome of breast cancer

SRXN1 is considered mainly as an antioxidant. In the present analyses for breast cancer risk, the *SRXN1* rs6053666 variant allele C was protective. In addition, carrying the rs6053666 variant allele C was associated with worse RFS in patients receiving adjuvant

radiotherapy. The rs6053666 has been predicted to participate in splicing regulation. No exonic splicing enhancer (ESE)-binding sites have been predicted for the wild type T allele, whereas three ESE-binding sites are predicted for the variant allele C (FastSNP). In theory, defects in splicing might disturb the correct translation of RNA for a normally functioning SRXN1 protein. As SRXN1 has anti-oxidative actions, a reduced capacity to tolerate oxidative stress caused by radiation might explain the superior RFS of breast cancer patients carrying the rs6053666 wild type TT genotype compared with patients carrying the variant C allele.

Four *SRXN1* polymorphisms (rs6085283, rs2008022, rs6116929, and rs7269823) associated with BCSS in all patients with invasive breast cancer in the univariate analysis. The same polymorphisms were independently associated with BCSS in the Cox regression analysis of patients treated with postoperative radiotherapy. When analyzing the patients who did not receive any kind of adjuvant treatment, only the rs2008022 associated statistically significantly with a lowered risk of breast cancer death. Thus, the influence of *SRXN1* 2008022 on BCSS appeared to be prognostic whereas the improved BCSS associated with the other *SRXN1* genotypes in the whole study population might reflect the tumour response to radiotherapy. Interestingly, an *in vivo* study has shown that proton-irradiation of mice significantly up-regulated the SRXN1 expression in liver whereas no changes were discovered after electron therapy (Gridley et al., 2011). However, the biological functions of the studied *SRXN1* polymorphisms still remain to be elucidated.

### 6.2.3 Predictive significance of *MnSOD* rs4880 in breast cancer patients treated with adjuvant tamoxifen

MnSOD is one of the most important defense enzymes combatting oxidative stress. As far is known, there were no previous clinical studies investigating the influence of *MnSOD* rs4880 genotype on the survival of breast cancer patients treated with tamoxifen monotherapy. In the present study, tamoxifen treated patients carrying the rs4880 wild type AA genotype had improved RFS and BCSS compared with the patients carrying the rs4880 variant G allele. These results have been corroborated by a subgroup analysis of Norwegian patients receiving adjuvant cyclophosphamide-based chemotherapy. The rs4880 wild type A allele was associated with improved 10-year BCSS in ER+ patients receiving both adjuvant chemotherapy and tamoxifen (Glynn et al., 2009). These parallel findings are supported by the data showing that the G-MnSOD precursor results in 30-40 % higher activity of human MnSOD in comparison with the A-MnSOD precursor (Sutton et al., 2003), although no significant differences in survival were observed in the other treatment subgroups.

In addition to its antiestrogenic effects in breast tissue, tamoxifen has been shown to induce ROS formation in ER+ breast cancer cells in vitro (Kallio et al., 2005). It has also been postulated that ERβ engagement by tamoxifen may regulate the intrinsic apoptosis process (Razandi et al., 2013). Tamoxifen is known to bind to the mitochondrial ERβ as an antagonist in MCF-7-BK (tamoxifen sensitive) cells and to increase ROS production and apoptosis. Interestingly, tamoxifen seemed to act as an agonist at mitochondrial ERβ in MCF-7-BK-TR (tamoxifen resistant) cells. It is unclear why tamoxifen acts as an ERβ agonist in these cells. Furthermore, MnSOD knockdown in the presence of tamoxifen caused a 15-fold increase in the number of cells undergoing apoptosis in MCF-7-BK-TR cells, indicating that MnSOD activity can be associated with tamoxifen resistance.

Inhibition of MnSOD could offer a compelling way to overcome resistance against tamoxifen. Indeed, in a study investigating silencing of MnSOD by siRNA-delivering nanoparticles and tamoxifen treatment, the combination of gene silencing and hormonal

therapy restored cellular apoptosis induced by tamoxifen *in vitro* and attenuated tumour progression in an *in vivo* model of nude mice (Cho et al., 2013). Tamoxifen has also been demonstrated to target liver mitochondria in an ER-independent manner (Moreira et al., 2006) and it has induced ROS production and apoptosis in ER- ovarian cancer cells (Ferlini et al., 1999).

MnSOD does not work alone but is a part of a chain of antioxidative enzymes. As MnSOD catalyzes $O_2$ into the less toxic $H_2O_2$, this compound's effects may be still be damaging unless $H_2O_2$ is rapidly removed by CAT or GPX. On the other hand, $H_2O_2$ can be converted into highly deleterious hydroxyl radicals via myeloperoxidase (MPO). Thus, MnSOD seems to have a pivotal role as it both generates and catabolizes ROS and the balance between these opposite actions may be affected by the activities of CAT and MPO. Interestingly, the *MnSOD* GG genotype has been observed to increase breast cancer risk alone or in combination with the *GPX* homozygous variant TT genotype (leucine198leucine) (Cox et al., 2006; Liu et al., 2012).

The effect of *MnSOD*, *GSTP1*, and *MPO* polymorphisms on the outcome was studied in 95 patients with metastatic breast cancer (Bewick et al., 2008). The *MnSOD* rs4880 wild type AA genotype was associated with worse PFS and BCSS, and this effect was further accentuated when the *GSTP1* rs1695 was included in the analyses. It was postulated that the more favourable outcome in patients with the variant G allele might derive from the increased DNA damage caused by $H_2O_2$ and hydroxyl radicals. In another study evaluating the association between *MnSOD* rs4880, *CAT* rs1001179, and *MPO* rs2333227 polymorphisms and the outcome of 279 women including also patients with metastatic breast cancer, there was a non-significant trend towards better OS among patients with the homozygous rs4880 GG genotype carriage compared with those with at least one A allele (Ambrosone et al., 2005). Moreover, the patients with combined homozygous *MnSOD* rs4880 GG and high-activity *MPO* GG genotypes and thereby capable of generating higher levels of ROS had a significantly reduced risk of death compared with patients carrying other genotype combinations.

Resistance to anoikis (apoptosis resulting from loss of cell-matrix interactions) has been proposed to be one of the mechanisms promoting metastases. The glucose consumption was decreased and levels of ROS were elevated in human mammary epithelial cells cultured under non-adherent conditions (Schafer et al., 2009). These changes leading to cell death could be prevented by anti-oxidants. Interestingly, MnSOD expression has been found to be higher in human breast cancer metastases compared with primary tumours (Kamarajugadda et al., 2013). Matrix detachment of suspended human mammary epithelial cells was shown to activate NF-κB which in turn induced MnSOD expression, whereas inhibition of NF-κB or MnSOD enhanced anoikis (Kamarajugadda et al., 2013). It was hypothesized that detachment-induced MnSOD was counteracting the accumulation of ROS in the mitochondria and inhibiting anoikis.

The present study did not include analyses of MnSOD protein expression. However, in two previous reports, the *MnSOD* transcript levels or MnSOD protein levels did not associate with PFS or OS of breast cancer patients treated with adjuvant chemotherapy (Sgambato et al., 2009; Hubackova et al., 2012).

The influence of tamoxifen on the apoptotic process may depend on the concentration of tamoxifen. Tamoxifen has been reported to induce either proliferation or apoptosis in ER-negative cells in vitro, depending on the dose (Ferlini et al., 1999). Low concentrations (0.1 μM) of tamoxifen resulted in a slight proliferative effect whereas a high concentration (10 μM) evoked increased cell death. An increase in ROS production was observed at both concentrations. In addition, tamoxifen has been shown to induce apoptosis in both ER-positive and ER-negative breast cancer cell lines at a concentration of 5 μM (Mandlekar et

al., 2000). Mean intra-tumoral concentrations of tamoxifen in patients treated with 20 mg/day for at least three months have been reported to be approximately 4 µM (MacCallum et al., 2000). The clinical benefit of adjuvant tamoxifen of 20 mg/day has been shown to be equivalent to higher doses (30-40 mg/day) (EBCTCG, 1998).

## 6.3 DNA REPAIR MECHANISMS – XPD AND XRCC1

### 6.3.1 *XPD* rs13181 as a predictive factor for the efficacy of adjuvant tamoxifen and chemotherapy

XPD is involved in the transcription-coupled NER and identifies damaged DNA (Sung et al., 1993; Hoeijmakers et al., 1996; Mathieu et al., 2013). It was demonstrated that homozygosity for the *XPD* rs13181 wild type allele A associated with better RFS and BCSS in patients treated with tamoxifen. In addition, carrying the rs13181 wild type A allele predicted improved RFS in chemotherapy treated patients, suggesting that the homozygous rs13181 variant CC genotype results in a more efficient repair of DNA damage generated by cancer therapies. These findings are parallel with the results of two studies on metastatic breast cancer patients which both detected better survival for patients with the *XPD* rs13181 wild type A allele compared with those carrying the variant C allele in patients treated with chemotherapy (Chew et al., 2009; Bewick et al., 2011). On the other hand, the rs13181 genotype was not found to influence DFS or OS in breast cancer patients receiving adjuvant anthracycline-based adjuvant chemotherapy (Castro et al., 2014).

NER is the main repair pathway for removing the bulky DNA adducts formed by cisplatin and cyclophosphamide (Andersson et al., 1996; Altaha et al., 2004). In patients with metastatic pancreatic cancer treated with cisplatin-gemcitabine, the *XPD* rs13181 variant CC genotype associated with worse PFS and OS. Furthermore, an *in vitro* analysis of lymphocytes from healthy volunteers revealed that the DNA repair was significantly more efficient in the rs13181 CC genotype after exposure to cisplatin or cisplatin-gemcitabine (Avan et al., 2013). NER also participates in the repair of DNA damage induced by oxidative stress (Gopalakrishnan et al., 2010).

The DNA adducts formed by tamoxifen have been shown to act as substrates for the human NER pathway (McLuckie et al., 2005). Tamoxifen is able to generate DNA adducts in human endometrium (Hernandez-Ramon et al., 2014). This may contribute to the risk of endometrial cancer associated with the use of tamoxifen. However, a comparison of breast tissue biopsies from a breast cancer patient who had taken tamoxifen for 24 months with another woman with no breast cancer did not detect any evidence of DNA adducts in either breast tissue samples (Beland et al., 2004). Even though the effects of tamoxifen are most likely tissue specific, no definitive conclusions can be drawn based on this study with only two individuals.

The influence of genetic polymorphisms of the *XPD* gene remains a subject of some controversy. *In vitro* assays conducted with various methods have generated conflicting results (Lunn et al., 2000; Spitz et al., 2001; Au et al., 2003; Laine et al., 2007; Wlodarczyk and Nowicka, 2012). It has also been predicted that the *XPD* rs13181 is located outside the catalytic sites and a regulatory domain (Bienstock et al., 2003; Dubaele et al., 2003). Thus, the rs13181 may not be detrimental for the helicase activity or stability of TFIIH complex required in the repair process. However, experimental *in vitro* assays do not necessarily represent the processes of repair *in vivo*, where multiple transcriptional factors and structural DNA modifications may alter the consequences.

**6.3.2 Association of the *XRCC1* rs25487 with the outcome of breast cancer**

XRCC1 protein does not possess any enzymatic activity on its own but is thought to act by recruiting and coordinating other DNA repair enzymes (Ginsberg et al., 2011). It is crucially involved in the processes of SSB repair and BER. XRCC1 is also involved with the NHEJ repair of DSBs as it reinforces the function of Lig3 but it is dispensable for this process (Soni et al., 2014).

It was found that homozygosity for the *XRCC1* rs25487 variant A allele associated with worse BCSS and OS in patients treated with postoperative radiotherapy. Likewise, the rs25487 variant AA genotype was associated with worse BCSS in patients who received adjuvant chemotherapy as well as in the analyses conducted in the whole study population. However, this particular SNP did not emerge as a significant factor for outcome in patients who did not receive any adjuvant treatments.

The present findings are contradictory to the *in vitro* study reporting that the variant AA genotype would be associated with reduced capacity to repair DNA damage (Slyskova et al., 2007). In addition, an evaluation of isolated lymphocytes irradiated with γ-rays revealed that the irradiation-specific DNA repair rate defined as the number of SSBs was significantly lower in subjects carrying the rs25487 variant A genotype (Vodicka et al., 2004). These findings have been further corroborated in an *in vitro* study exploring the effect of *XRCC1* polymorphisms on the kinetics and dissociation after micro-irradiation (Hanssen-Bauer et al., 2012). That report suggested that the rs25487 variant has a reduced ability to remain at sites of damage.

It has been estimated that irradiation of cells with 1 Gy leads to 1,500-2,500 base damages, 1000 SSBs, and 40 DSBs per cell (Ward, 1986). Of these injuries, DSBs are most lethal to cells. In the case when the SSBs or damaged bases are located close to each other in the opposite DNA strands, the repair process may lead to a new DSB due to the cleavage of both backbones (Ward, 1988). It has been proposed that a deficiency in BER might actually result in increased cell survival after treatment with ionizing radiation due to the decreased production of DSBs (Sak et al., 2005). This might explain why the rs25487 variant genotype was associated with inferior survival in the present study.

*In vitro*, cells expressing the rs25487 variant AA genotype were found to be more resistant to many chemotherapeutic agents including several alkylating regimens and 5-fluorouracil (Yarosh et al., 2005). There have been different approaches applied to evaluate the influence of rs25487 on the outcome of various cancer treatments. For example, an extensive meta-analysis of esophageal and gastric cancer patients treated with neoadjuvant chemoradiotherapy found that the rs25487 wild type GG allele was associated with better pathologic complete response (Findlay et al., 2015). On the other hand, in a meta-analysis of advanced or metastatic gastric or colorectal cancer, the rs25487 polymorphism did not have any predictive value for DFS or OS (Wu et al., 2014a).

Similarly, results from clinical studies in breast cancer patients are inconsistent. Three studies have reported an association between carriage of the rs25487 variant A allele and better EFS, BCSS or OS in early breast cancer patients receiving adjuvant treatment (chemotherapy and radiotherapy, or chemotherapy alone) (Jaremko et al., 2007; Ye et al., 2012; Przybylowska-Sygut et al., 2013). It is worth noting that in the study of Przybylowska-Sygut et al., the OS was defined as the time between the surgery and the death caused by cancer, and they reported only univariate survival analyses. Furthermore, there was inconsistency in the numbers of patients reported and analyzed in the different treatment subgroups (Przybylowska-Sygut et al., 2013).

Two studies found no associations between polymorphism in rs25487 on the survival of breast cancer patients (Costa et al., 2008; Syamala et al., 2009). The study by Syamala et al. included also patients with stage IV disease. One study with stage IV breast cancer

patients treated with chemotherapy found a correlation between carrying the rs25487 variant A allele and poor BCSS (Bewick et al., 2006), and another study similarly reported an association with the rs25487 A allele carriage and inferior DFS in early breast cancer patients receiving adjuvant chemotherapy (Castro et al., 2014).

Results from trials investigating the influence of the rs25487 on the *XRCC1* gene or XRCC1 protein expression have been inconclusive. In an analysis of lymphocytes from 30 healthy subjects, the *XRCC1* gene expression was higher in those individuals carrying the variant A allele but there was no difference in the repair activity between the rs25487 genotypes (Zipprich et al., 2010). Moreover, carriage of the rs25487 variant A allele associated with higher XRCC1 protein expression in patients with cervical carcinoma (Cheng et al., 2009). On the other hand, an evaluation of breast tissue samples from 39 breast cancer patients did not reveal any relationship between the rs25487 polymorphism and XRCC1 protein expression (Rybarova et al., 2011). It has been suggested that the rs25487 might influence the DNA repair capacity by altering the conformation of other structures within the BRCT I domain (Monaco et al., 2007).

Two studies evaluating the XRCC1 protein expression in a total of 2,700 cases of early breast cancer indicated that negative or low XRCC1 expression was an independent predictive factor for worse outcome in terms of DFS and BCSS (Sultana et al., 2013; Abdel-Fatah et al., 2014). In the study of Sultana et al., this effect was visible both in ER+ and triple-negative cases, whereas the study of Abdel-Fatah et al. included only ER+ patients. The absence of XRCC1 expression also associated with more aggressive biological subtypes and loss of other DNA repair factors including BRCA1. The authors suggested that the XRCC1 deficiency leads to a mutator phenotype i.e. enhancing mutation rates in other loci, and subsequently results in chemotherapeutic agent resistance. Data on the *XRCC1* genotypes was not available in these studies.

One explanation for the discordant results might be allelic imbalance between the germline cells and the tumour. This question was addressed in a study on patients with esophageal adenocarcinoma treated with chemo-radiotherapy (Yoon et al., 2011); no significant allelic imbalance was detected at the *XRCC1* rs25487 SNP. As far as is known, there are no published studies which have investigated the rate of allelic imbalance in breast cancer at this particular locus.

# 7 Conclusions

Breast cancer is a complex disease that presents in several different molecular subtypes with their own distinct prognostic and predictive features. In addition, the inherited genetic capacity to respond to oxidative stress, to metabolize drugs, and repair DNA damage can modify the outcome of adjuvant treatments in each individual breast cancer patient. This thesis examined the prognostic and predictive significance of the genetic polymorphisms in SULT1A1 gene which is involved in phase II drug metabolism, the antioxidant response genes *NRF2*, *SRXN1*, and *MnSOD*, and the DNA repair genes *XPD* and *XRCC1*.

The following conclusions can be drawn from the individual studies (I-IV) of this thesis:

I    The homozygous *SULT1A1* rs9282861 variant genotype emerged as a predictive factor in a group of breast cancer patients treated with either adjuvant tamoxifen or chemotherapy, associating with improved OS. In addition, the rs9282861 variant genotype was observed to be a negative prognostic factor for RFS and OS in patients who did not receive any kind of adjuvant treatment.

II    The nuclear and cytoplasmic expression of NRF2 associated with the SRXN1 protein expression. The rare homozygous genotypes of *NRF2* rs6721961 and rs2706110 and the homozygous *SRXN1* rs6053666 wild type genotype were related to an elevated risk of breast cancer. The *NRF2* rs2886162 variant genotype was predictive of worse RFS and BCSS in patients who received adjuvant chemotherapy and of worse RFS in patients treated with postoperative radiotherapy. In the *SRXN1* analyses, carriage of the rs6116929 wild type allele, rs72699823 variant allele, or rs6085283 variant allele associated with better RFS and BCSS. The *SRXN1* rs6053666 variant genotype predicted inferior RFS in patients receiving postoperative radiotherapy. The *SRXN1* rs2008022 variant allele was prognostic for better BCSS.

III    Carrying the *MnSOD* rs4880 wild type allele or *XPD* rs13181 wild type genotype was related to better RFS and BCSS in tamoxifen treated patients. Carriage of the *XPD* rs13181wild type allele also associated with better RFS in patients treated with adjuvant chemotherapy. In addition, in the combined analysis of the *MnSOD* rs4880 and *XPD* rs13181 genotypes, the increasing number of low-risk genotypes (rs4880 AA, rs4880 AG, or rs13181 AA) predicted superior RFS, BCSS, and OS in tamoxifen treated patients.

IV    The homozygous *XRCC1* rs25487 variant genotype predicted worse BCSS and OS in breast cancer patients treated with postoperative radiotherapy, and associated with inferior BCSS in patients receiving adjuvant chemotherapy.

It should be noted that the allele frequencies and linkage disequilibrium patterns may vary considerably between different ethnic populations. In addition, environmental, cultural and dietary factors may cause variability in the gene-gene and gene-environment interactions. Furthermore, also chance may play a role especially in studies with small sample sizes. While the influence of a single polymorphism may be subtle, a combination

of high- or low-risk genotypes may result in a more significant effect on the disease risk or treatment efficacy.

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*This prospective study examined the predictive value of single nucleotide polymorphisms related to drug metabolism, oxidative stress, and DNA repair in breast cancer patients receiving adjuvant chemotherapy, radiation therapy, or hormonal treatments. Polymorphisms in genes coding for SULT1A1, NRF2, SRXN1, MnSOD, XPD, and XRCC1 were shown to predict the response to adjuvant therapies. A better understanding of genetic characteristics could help in tailoring treatment strategies for breast cancer.*

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