

**OXIDATIVE DAMAGE AND
COUNTERACTING MECHANISMS
IN BREAST CARCINOMA**

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Abstract

Breast cancer is the leading cause of death from cancer among Finnish women, but the ultimate causation of carcinogenesis still remains unclear. Reactive oxygen species (ROS) is a collective term for several types of reactive oxygen metabolites that are continuously generated in human cells mainly as by-products of aerobic respiration. ROS, including nitric oxide and its derivatives, play highly important roles in cell physiology. If ROS production exceeds the capacity of detoxification systems, principally antioxidant enzymes, oxidative stress is said to occur. This state is known to contribute to all stages of carcinogenesis.

To explore the widely unstudied role of ROS and cell redox state modulating enzymes in breast carcinomas, the extent of ROS-derived macromolecule damage and the expression of the vast majority of known antioxidant enzymes were assessed in a large series of breast carcinomas, and the results were compared to the patients' clinicopathological parameters. The results were also compared to angiogenesis, DNA repair enzymes, cell proliferation, NF- κ B, p53 expression, and survival. Immunohistochemistry was the main method applied, but western blotting and immunoelectron microscopy were also used.

There is extensive oxidative damage in breast carcinomas, which seems to associate with tumor development. Oxidative macromolecule damage is notable even in stage I tumors. Cell redox state regulating enzymes, such as peroxiredoxin V, thioredoxin, thioredoxin reductase, and glutamate-cysteine ligase, associate with more aggressive phenotypes of tumors, including larger primary tumors, growth of metastases, increased cell proliferation, and poor differentiation. This indirectly suggests that cell redox state modulating enzymes may be inductive of tumor promotion in an oxidated environment. The results of this thesis support the importance of ROS in all stages of carcinogenesis. These observations are largely in line with the previous studies on different carcinomas, but there seem to be certain carcinoma type specific differences in the expression of these enzymes. Since the expression of given cell redox state modulating enzymes distinctly associates with clinicopathological parameters, these enzymes may be useful as prognostic indicators and facilitate the choice of appropriate treatment in the future.

Keywords: angiogenesis, antioxidant enzymes, breast cancer, immunohistochemistry, mismatch repair, nitric oxide, oxidative stress, reactive oxygen species

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Abbreviations

·	unpaired electron
·OH	hydroxyl radical
¹ O ₂	singlet oxygen
8-OHdG	8-hydroxydeoxyguanosine
8-OHG	8-hydroxyguanosine
AOE	antioxidant enzymes
AP-1	activator protein 1
ATP	adenosine triphosphate
BMI	body mass index
BRCA	breast cancer susceptibility gene
BSA	bovine serum albumin
BSO	buthionine sulfoximine
erbB2	erythroblastic leukemia viral oncogene homolog 2
Cat	catalase
cNOS	constitutive nitric oxide synthase
Cu/ZnSOD	copper zinc superoxide dismutase
Cys	cysteine
Cyt C	cytochrome C
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECSOD	extracellular superoxide dismutase
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
Fe ²⁺	ferrous ion
Flk-1	fetal liver kinase 1
Flt-1	FMS-related tyrosine kinase 1
Flt-4	FMS-like tyrosine kinase 4
GLCL-c	glutamate-cysteine ligase, catalytic subunit
GLCL-r	glutamate-cysteine ligase, regulatory subunit
Gly	glycine

GPx	glutathione peroxidase
GSH	glutathione
GSSG	oxidized form of glutathione
GST	glutathione-S-transferases
H ₂ O ₂	hydrogen peroxide
HE	haematoxylin-eosin
HNE	4-hydroxy-2-nonenal
HNPCC	hereditary nonpolyposis colorectal carcinoma
IκB	inhibitory subunit κB
iNOS	inducible nitric oxide synthase
kDa	kilodalton
L-NAME	N ^o -nitro-L-arginine methyl ester
Lys	lysine
MDA	malondialdehyde
MLH1	mutL homolog 1
MLH3	mutL homolog 3
MMP	matrix metalloproteinase
MMR	mismatch repair
MnSOD	manganese superoxide dismutase
MSH2	mismatch repair protein 2
MSH6	mismatch repair protein 6
mRNA	messenger ribose nucleic acid
MRP	multidrug resistance protein
MSI	microsatellite instability
MVD	microvascular density
MW	molecular weight
NADP ⁺	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	nuclear factor- κB
nNOS	neuronal nitric oxide synthase
NO [•]	nitric oxide
NOS	nitric oxide synthase
NOS1	neuronal nitric oxide synthase
NOS2	inducible nitric oxide synthase
NOS3	endothelial nitric oxide synthase
NS	no significance
ONOO ⁻	peroxynitrite
O ₂ ^{-•}	superoxide anion
Prx	peroxiredoxin
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PMS1	postmeiotic segregation increased 1
PR	progesterone receptor
Pro	proline
ROS	reactive oxygen species

Ser	serine
SOD	superoxide dismutase
TNM	tumor-node-metastases
TopBP1	DNA topoisomerase binding protein 1
Top II	DNA topoisomerase II
Trx	thioredoxin
TrxR	thioredoxin reductase
Trp	tryptophan
VEGF	vascular endothelial growth factor

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

- I Karihtala P, Mäntyniemi A, Kang SW, Kinnula VL & Soini Y (2003) Peroxiredoxins in breast carcinoma. *Clin Cancer Res* 9:3418-3424.
- II Turunen N, Karihtala P, Mäntyniemi A, Sormunen R, Holmgren A, Kinnula VL & Soini Y (2004) Thioredoxin is associated with proliferation, p53 expression and negative estrogen and progesterone receptor status in breast carcinoma. *APMIS* 112:123-132.
- III Soini Y, Karihtala P, Mäntyniemi A, Turunen N, Pääkkö P & Kinnula V (2004) Glutamate-L-cysteine ligase in breast carcinomas. *Histopathology* 44:129-135.
- IV Karihtala P, Kinnula VL & Soini Y (2004) Antioxidative response for nitric oxide production in breast carcinoma. *Oncol Rep* 12:755-759.
- V Karihtala P, Winqvist R, Syväoja JE, Kinnula VL & Soini Y (2005) Increasing oxidative damage and loss of mismatch repair proteins during breast carcinogenesis. (submitted)

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1 Introduction

Breast cancer is the leading cause of death from cancer among Finnish women. It has been estimated that 12% of all women will develop breast carcinoma during their lifetime. Breast cancer incidence is growing fast in the Western countries. Although many risk factors, such as genetic factors, hormonal status, viruses, radiation, and environmental influences have been implicated in the pathogenesis of breast cancer, the ultimate etiology still remains unclear. During the past decade, the formation of free radicals has been proposed as a linking factor between certain potential carcinogens, such as hormonal factors, alcohol, microbes, and radiation.

Reactive oxygen species (ROS) are highly reactive molecules, including free radicals, which have an unpaired electron on their outermost orbital, and also other reactive oxygen metabolites, such as hydrogen peroxide. The term ROS can also be taken to cover nitric oxide-derived reactive molecules, such as peroxynitrite. Low levels of ROS are continuously formed during physiological cellular metabolism, and they also play an important role in cellular defence, intercellular signaling, and apoptosis. However, if the amount of the ROS exceeds the capacity of the ROS-suppressing machinery, oxidative stress is said to occur. This imbalanced redox status is potent enough to induce DNA, lipid, and protein damage. ROS damage is known to accumulate into the genome, and on the other hand, a large extent of carcinogenesis can be considered a degenerative disease of DNA that increases with age. ROS are nowadays widely recognized as a significant class of carcinogens.

To protect against the toxic effects of oxygen, cells from prokaryotes to primates have developed various efficient defense mechanisms against ROS damage. To prevent the accumulation of already damaged DNA, there are a variety of DNA repair enzymes, but a more specific and efficient way to protect the body from radicals consists of antioxidants. Antioxidative and other cell redox state modulating enzyme systems act as first-line defense against ROS in various cellular compartments and also extracellularly. The most important of these enzymes include superoxide dismutases, which decompose superoxide radicals to hydrogen peroxide (H_2O_2). H_2O_2 is further decomposed to molecular oxygen and water by glutathione peroxidases, catalase, and peroxiredoxins.

It has been suggested that some antioxidant enzymes or oxidative stress metabolites could be used in the future as markers when evaluating the prognosis of breast carcinoma

patients. On the other hand, high antioxidant levels enhance the resistance of cancer cells to therapeutic regimens aimed to counteract increased ROS formation. These methods include radiotherapy and chemotherapy. The role of oxidative stress and especially the role of antioxidant and other cell redox state modulating enzymes are still somewhat unclear in the initiation, promotion and progression of breast cancer. However, *in vivo* studies have shown high ROS concentrations to be detrimental to DNA and therefore to result in a risk for cancer development. This thesis was undertaken primarily to study the role of oxidative stress and its connections to redox-regulating enzymes in a large series of breast carcinomas.

2 Review of the literature

2.1 Breast carcinoma

2.1.1 Epidemiology

Breast carcinoma is the most common cancer in women in Finland and other developed countries (The Finnish Cancer Registry 2005a, Bray *et al.* 2002), as more than every tenth woman will develop this disease during their lifetime (The Finnish Cancer Registry 2005a). The risk of breast cancer has been increasing during the past decades and will continue to do so in the near future, especially in the developing countries (Key *et al.* 2001, Botha *et al.* 2003, The Finnish Cancer Registry 2005b). The prognosis of breast cancer has improved significantly particularly during the last 10 years, due to both efficient population screening and more effective treatment (Sasco *et al.* 2003). In Finland, the predicted 5-year relative survival rate for breast carcinoma patients diagnosed in 1999-2001 is 85% (The Finnish Cancer Registry 2005c), compared to 67.4% among the Finnish breast cancers diagnosed in 1975-1977 (Brenner & Hakulinen 2001). Mortality rates have also decreased in most other Western countries (Peto *et al.* 2000, Sasco *et al.* 2003, Kobayashi 2004). Breast carcinoma prognosis is even better when the disease is detected at an early stage. Breast cancer is uncommon before the age of 30, but these patients tend to have a more aggressive carcinoma phenotype (Shannon & Smith 2003, Zhou & Recht 2004).

2.1.2 Pathogenesis

The pathogenesis of breast carcinoma is still unknown. As is the case with most malignancies, age has been recognized as a major risk factor also for breast cancer. However, the causes of breast cancer are multifactorial and probably partly still unknown (Table 1.). Exposure to female hormones, especially estrogen and, to a certain extent, progesterone, has been considered the most potent breast cancer risk factor along with

age. This may explain why early menarche, late menopause, high endogenous sex hormone level, oral contraceptives at the time of the use, and hormonal replacement therapy significantly increase the probability of breast cancer development (Kelsey *et al.* 1993, Nandi *et al.* 1995, Bernstein & Ross 1993, Key *et al.* 2001, Lancet 350 (1997), pp. 1047–1059, Sasco *et al.* 2003). High alcohol consumption, low vegetable intake, and previous proliferative benign breast lesions also increase the breast carcinoma risk (Hunter & Willett 1996, Key *et al.* 2001). High body mass index (BMI) associates with increased endogenous estrogen production, which may explain the association between obesity and breast cancer (Berclaz *et al.* 2004).

Mutations in the BRCA genes 1 and 2 are the major genetic risk factors for breast cancer development. These patients have an up to 8-fold breast carcinoma risk by 70 years of age, depending on the diagnostic methods used and the type of population studied (Struewing *et al.* 1997, Easton *et al.* 1998, Ford *et al.* 1998). Mutations in the p53, ATM, and PTEN genes also expose their carriers to the disease (Bennett *et al.* 2000, Key *et al.* 2001).

Table 1. Risk factors for breast carcinoma (for reference, see text and The American Cancer Society 2005).

Risk Factor	Comments
Gender	Females have approximately 100-fold incidence
Age	Mean age at the time of diagnosis is around 60 years, with the incidence increasing exponentially along with age
Genetics	Mainly BRCA 1, BRCA 2, and p53 mutations
Previous breast cancer	Increases the risk 3- to 4-fold
Previous proliferative benign breast lesion	Atypic ductal or lobular hyperplasia increases the risk by four to five times
Thoracic radiation	The younger the patient, the higher the risk
Early menarche and late menopause	
Nulliparity	
Oral contraceptives	At the time of use
Hormone replacement therapy	Especially combined estrogen and progesterone therapy
High alcohol consumption	
High-fat diet	
Low vegetable intake	
High BMI	Associates with high serum estrogen levels

2.1.3 Histological classification

The main histological types of breast cancer are ductal and lobular carcinoma (Table 2). Despite this somewhat inappropriate terminology, both types arise from the terminal duct's lobular unit. If cancer cells remain within the basement membrane of the neoplastic duct, the disease is classified as *in situ* (non-invasive) carcinoma. Correspondingly, if

cells disseminate through the basement membrane, the carcinoma is considered invasive (infiltrating).

2.1.3.1 *In situ* carcinomas

More than half of all *in situ* breast carcinomas are classified as ductal carcinoma *in situ*. Ducts are filled by neoplastic epithelial proliferation, which may further be divided to cribriformic, papillary, micropapillary, apocrine, comedocarcinoma, or solid types (Lester & Cotran 1999). Depending on the cytomorphological and some histological features, such as the presence of necrosis, ductal *in situ* carcinomas of breast can be graded into three groups. Lobular carcinoma *in situ* is another main group of *in situ* breast carcinomas. It is considered a breast cancer risk factor (Sewell 2004).

2.1.3.2 *Invasive* carcinomas

Infiltrating or invasive ductal carcinomas account for approximately 70% of all female breast cancers (Berg & Hutter 1995). Morphologically, they form cell islets, which may develop tubular structures or be composed of cell cords (Maluf 2004). Surrounding desmoplasia is often seen (Hasebe *et al.* 2000). Invasive ductal carcinomas are graded into three groups according to the degree of tumor tubule formation, cellular mitotic activity, and nuclear pleomorphism of tumor cells (Elston & Ellis 1998).

Lobular invasive carcinoma is the second most common breast carcinoma, accounting for about 6% of all female invasive carcinomas (Berg & Hutter 1995). The incidence of lobular carcinoma incidence seems to be increasing (Li *et al.* 2003). Medullary, papillary, mucinous, and tubular carcinomas are the other invasive carcinomas types (Table 2).

Table 2. Types and incidence of some histological invasive breast carcinomas according to WHO (Tavassoli & Devilee 2003).

Histological diagnosis	Incidence
Invasive ductal carcinoma	50-80%
Invasive lobular carcinoma	5-15%
Tubular carcinoma	2%
Invasive cribriform carcinoma	0.8-3.5%
Medullary carcinoma	1-7%
Mucinous carcinoma	2%
Neuroendocrine carcinoma	2-5%
Papillary carcinoma	less than 1-2%
Micropapillary carcinoma	less than 1-2%
Apocrine carcinoma	0.3-4%
Metaplastic carcinoma	less than 1%

2.1.4 Prognostic factors

2.1.4.1 TNM classification

Tumor size is a very powerful predictor of both survival and recurrence, and it is also positively associated with metastasis (Carter *et al.* 1989, Fitzgibbons *et al.* 2000, Sainsbury *et al.* 2000). Patients with a primary tumor of less than 1 cm have five-year overall survival up to 99% compared with 89% of patients with tumors between 1 cm and 3 cm and 86% of those with tumors between 3 cm and 5 cm (Carter *et al.* 1989). This trend also seems to continue in 20-year recurrence-free survival charts (Rosen *et al.* 1993).

However, most studies clearly show that the presence or absence of axillary lymph node metastasis is an even more powerful predictor of disease-free survival and overall survival in breast carcinomas (Russo *et al.* 1987, Chua *et al.* 2001, Singletary *et al.* 2003). The number of lymph node metastases is also considered an essential risk factor of distant recurrence (Gianfrocca & Goldstein 2004). Furthermore, tumor grade and the presence of lymphatic or vascular invasion have been demonstrated to be important prognostic factors of breast cancer aggressiveness (Fitzgibbons *et al.* 2000, Subramaniam & Isaacs 2005).

TNM (tumor node metastases) classification is the most widely used staging system in Finland. The 6th revised edition of the TNM classification was published recently (Singletary *et al.* 2003, see Table 3).

Table 3. 6th TNM classification of breast tumors (Sobin & Wittekind 2002).

Category	Description
Primary tumor size (T)	
T _x	Primary tumor cannot be assessed.
T ₀	No evidence of primary tumor.
T _{is}	Carcinoma <i>in situ</i> : Ductal or lobular carcinoma <i>in situ</i> , or Paget's disease of the nipple with no tumor.
T ₁	Tumor 2 cm or less in greatest dimension.
T _{1mic}	Largest focus of invasion ≤ 0.1 cm in greatest dimension. (Do not sum up individual foci.)
T _{1a-c}	Tumor more than 0.1 cm but not more than 2 cm in greatest dimension
T ₂	Tumor more than 2 cm but not more than 5 cm in greatest dimension.
T ₃	Tumor more than 5 cm in greatest dimension.
T _{4a-c}	Tumor of any size with an extension to skin or chest wall, not including pectoralis muscle.
T _{4d}	Inflammatory carcinoma: When pathologically staging a clinical inflammatory carcinoma, if the skin biopsy is negative and there is no localized, measurable primary cancer, the category is pTx.
Regional lymph nodes (N)	
pN _x	Regional lymph nodes cannot be assessed.
pN ₀	No regional lymph node metastasis.
pN _{1mi}	Micrometastasis (more than 0.2 mm, but not more than 2 mm in greatest dimension)
pN _{1a-c}	Metastasis in 1-3 ipsilateral axillary lymph node(s) and/or in ipsilateral internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent.
pN _{2a-b}	Metastasis in 4-9 ipsilateral axillary lymph nodes or in clinically apparent ipsilateral internal mammary lymph node(s) in the absence of axillary lymph node metastasis.
pN _{3a-c}	Metastasis in 10 or more axillary lymph nodes, in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of 1 or more positive axillary lymph nodes; or in more than 3 axillary lymph nodes; or in ipsilateral supraclavicular lymph nodes.
Distant metastasis (M)	
M _x	Distant metastasis cannot be assessed.
M ₀	No distant metastasis.
M ₁	Distant metastasis.

2.1.4.2 Other prognostic factors

The rarest histological breast carcinoma types have generally better prognosis than ductal and lobular carcinomas (Mansour *et al.* 1994). Estrogen and progesterone receptor status are routinely determined immunohistochemically from breast cancer lesions, and they also affect cancer treatments (Molino *et al.* 1997, Harvey *et al.* 1999, Fitzgibbons *et al.* 2000). Tumors with positive hormone receptor status have better prognosis, partly because of their good response to hormone therapy (Pertschuk *et al.* 1985).

The most widely used cell proliferation markers include S-phase fraction, mitotic index, and immunohistochemical antibodies such as proliferating cell nuclear antigen (PCNA) and Ki-67. Breast tumors with high proliferation indexes predict considerably

higher risks of both disease recurrence and death (Cianfrocca & Goldstein 2004). Ki-67 is a widely used immunohistochemical marker of cell proliferation, the levels of which associate with the cell proliferation rate measured by other methods, enlarged primary tumor size, as well as decreased survival and disease-free survival (Railo *et al.* 1993, Gasparini *et al.* 1994, Brown *et al.* 1996, Trihia *et al.* 2003, Urruticoechea *et al.* 2005). Instead, the usage of PCNA as a marker of cell proliferation is decreasing. Low PCNA levels have been demonstrated to predict both 20 years' disease-free survival and overall survival, (Kato *et al.* 2002, Horiguchi *et al.* 1998), but results without any association with these figures have also been published (Cummings *et al.* 1993, Gasparini *et al.* 1994)

20-30% of breast cancers overexpress the transmembrane tyrosine kinase receptor c-erbB-2 (also known as HER-2, neu, or epidermal growth factor-2 (EGFR-2)) proto-oncogene, which has become the most widely used tumor marker in breast cancers (Yamauchi *et al.* 2001, Hayes & Thor 2002). c-erbB-2 gene amplification results in poor prognosis, resistance to hormonal therapies, and generally more aggressive phenotype (Slamon *et al.* 1987, Gusterson *et al.* 1992, Ravdin & Chamness 1995, Thor *et al.* 1998, Fitzgibbons *et al.* 2000, Kato *et al.* 2002). However, trastuzumab, a specific monoclonal antibody against HER-2, is increasingly used in breast cancer chemotherapy, and it significantly prolongs the survival of c-erbB-2-positive patients, when administered either as monotherapy or combined with conventional chemotherapeutic drugs (Vogel *et al.* 2001, Slamon *et al.* 2001, Emens 2005, Marty *et al.* 2005).

Assessment of the gene expression profile provides a unique molecular portrait of a single tumor and serves to predict its clinical behavior and resistance to conventional chemotherapeutic drugs (Liu 2003). The recent development in gene expression microarray techniques allows more detailed gene profiling of breast cancer. With these methods, patients with different combinations of genes and clinical subtypes requiring different treatments can be determined, and unnecessary adjuvant chemotherapies can be avoided (Cleator & Ashworth 2004). These patients' subsequent quality of life will improve, financial savings will be accomplished, and most importantly, the patients' prognosis will be improved by more appropriate treatments (Jeffrey *et al.* 2002, Jones *et al.* 2004, Lee & Macgregor 2004). However, it should be pointed out that there are still many pitfalls before gene microarrays can be used in routine diagnostics. The published studies have had relatively small materials, and it is still far from clear which genes should be included in the arrays (Ein-Dor *et al.* 2005). On the other hand, the processing of the material has been suggested to affect the reliability of RNA analyses (Ransosohoff 2005).

2.2 Reactive oxygen species

2.2.1 Background

Reactive oxygen species (ROS) is a collective term for several types of reactive oxygen metabolites, including free radicals, which are defined as any molecule containing an

unpaired electron on its outermost orbital, e.g. superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), and singlet oxygen 1O_2 (Lutgerink *et al.* 1992, Wiseman & Halliwell 1996). The term ROS also encompasses some non-radicals, including hydrogen peroxide (H_2O_2).

Oxygen is a prerequisite to human life, but mainly as a consequence of aerobic respiration, ROS are generated within mammalian cells continuously. It has been estimated that 3×10^{22} free radical molecules are formed per person per hour (Lieber *et al.* 2003). In athletes, this figure may be increased up to 50% (Valko *et al.* 2004). Under physiological conditions, the main endogenous source of ROS is mitochondria, but small amounts of ROS are generated in peroxisomes, the cytochrome P450 system, and inflammatory cells, including neutrophils, eosinophils, and macrophages (Li *et al.* 1992, Kang 2002). There are also some exogenous sources of radicals, such as radiation (ultraviolet, X-ray), ozone, and many xenobiotics, including antiestrogen tamoxifen (Klaunig *et al.* 1998, Ferlini *et al.* 1999).

2.2.2 History

The vital nature of air was suggested as far back as 5000 years ago in Mesopotamian myths, but the disadvantages of oxygen were only discovered 50 years ago. In 1954, Gerschman *et al.* proposed that oxidizing free radicals mediated the toxic effects of oxygen (Gerschman *et al.* 1954). Two years later, Harman suggested the role of oxygen radicals in mutagenesis and cellular damage and as a mediator of biological aging (Harman 1956). Thereafter, milestones in redox research have been marked by the findings of antioxidant enzymes by McCord and Fridovich and the first descriptions of the advantageous effects of free radicals, when it was demonstrated that CuZnSOD inhibits $O_2^{\cdot-}$ production *in vitro* (McCord & Fridovich 1969).

2.2.3 Generation and nature of ROS

2.2.3.1 Oxidative phosphorylation

By means of oxidative phosphorylation, aerobic organisms are able to achieve a far greater energy production efficiency compared to anaerobic organisms. However, as a disadvantage of aerobic metabolism, noxious oxygen species are continuously generated. Peter Mitchell, who was later awarded Nobel Prize for his development of the chemiosmotic theory, was the first to suggest that ATP synthesis in the respiratory chain is ultimately based on proton gradient formation across the inner mitochondrial membrane (Mitchell 1961). According to the theory, three of the respiratory chain complexes, I, III, and IV, operate as proton pumps, pumping H^+ ions out of the mitochondrial matrix (Figure 1.). The energy for proton pumping is gained from electron transfers from more negative to more positive redox potential with the help of special electron carriers. The final electron acceptor of the chain is O_2 , which receives a single electron at a time from cytochrome oxidase. Protons then flow back to the matrix via

ATP-synthase (also called F_0 - F_1 complex or complex V), leading to ATP formation in the F_1 subunit of ATP synthase. (Harper 2000) As a disadvantage, especially the complexes I and III (NADH dehydrogenase and ubiquinone, respectively) convert a fraction of O_2 to $O_2^{\cdot -}$ (Turrens 1997).

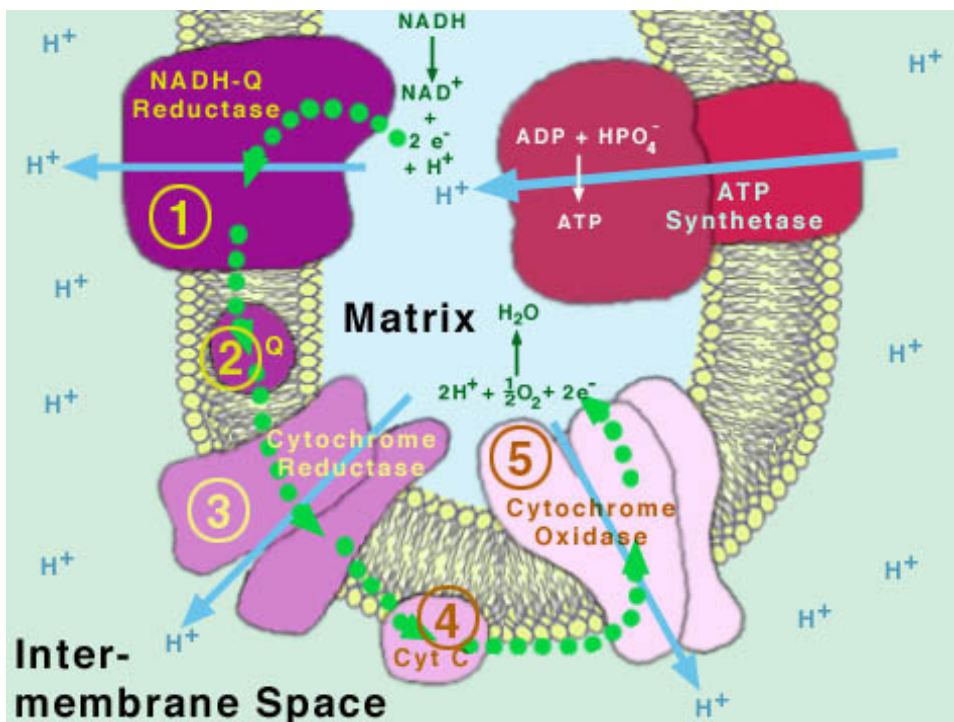


Fig. 1. A schematic diagram illustrating the transfer of electrons from NADH through electron carriers in the electron transport chain to molecular oxygen. NADH-Q reductase (1), cytochrome reductase (3), and cytochrome oxidase (5) are electron carriers as well as proton pumps, which use the energy gained from each electron-transfer step to move protons (H^+) against a concentration gradient from the matrix to the intermembrane space. Ubiquinone (Q) (2) and cytochrome c (Cyt C) (4) are mobile electron carriers. Together, these electron carriers form a "chain" to transport electrons from NADH to O_2 . The path of the electrons is shown with the dotted line. ©Washington University 1999. Reprinted with permission of the author, Regina Frey, Washington University.

2.2.3.2 $O_2^{\cdot -}$

1-5% of the total oxygen consumed in aerobic metabolism produces $O_2^{\cdot -}$, the first reduction product of O_2 (Table 4.) (Dreher & Junod 1996, Loft & Poulsen 1996). The majority of this relatively unreactive radical is generated when the mitochondrial electron transport chain "leaks" one electron to molecular oxygen or, to a lesser extent, via certain enzymes in phagocytic cells (Nordberg & Arnér 2001, Cooke *et al.* 2003). In liver cells,

xanthine oxidase is a significant source of $O_2^{\cdot-}$ (Ray & Husain 2002). $O_2^{\cdot-}$ is one of the most stable free radicals and is further dismutated to H_2O_2 (Pryor 1986). However, if not destroyed properly in mitochondria, $O_2^{\cdot-}$ is able to inhibit mitochondrial function by inactivating the Fe-S centers in the electron transport chain (Wallace 1999). Interestingly, $O_2^{\cdot-}$ has been found to inhibit catalase, an enzyme reducing H_2O_2 , activity *in vitro* (Kono & Fridovich 1982).

2.2.3.3 H_2O_2

H_2O_2 is one of the least reactive ROS. It can be produced either directly from molecular oxygen in peroxisomes or, as usually, from $O_2^{\cdot-}$ by catalytic activity of superoxide dismutases (SOD) (Ray & Husain 2002, Matés & Sanchez-Jimenez 2000). Unlike $O_2^{\cdot-}$, H_2O_2 is able to diffuse throughout mitochondria and cross cell membranes, and it is therefore able to produce many types of cellular injury (Ray & Husain 2002, Matés & Sanchez-Jimenez 2000). Furthermore, being a diffusible, appropriately reactive, and small molecule, H_2O_2 also has a highly important role as an intracellular signal molecule (Sundaresan *et al.* 1995, Rhee 1999). H_2O_2 is further processed by catalase, glutathione peroxidases, or peroxiredoxins to molecular oxygen and water.

2.2.3.4 $\cdot OH$

The main injurious effects of ROS in mammalian cells are mediated by $\cdot OH$. This radical is formed in the presence of reduced transition metals (ions of Fe, Cu, Co or Ni), mainly via a Fenton reaction, when Fe^{2+} comes into contact with H_2O_2 (Chance *et al.* 1979, Dröge 2002). Picograms of ferrous iron are sufficient to catalyze this reaction (Wright *et al.* 1999). Under normal conditions, iron and copper ions are strictly bound to proteins such as caeruloplasmin, ferritin, and transferrin. However, $O_2^{\cdot-}$ is known to release iron ions at least from ferritin, and it hence probably promotes radical-induced DNA damage when present in nucleus (Wiseman & Halliwell 1996, Harper 2000). The half-life of $\cdot OH$ is less than 1 ns, and it is therefore an extremely reactive free radical, which is unable to diffuse more than one or two molecular diameters before it reacts, in practice with any cellular component (Marnett 2000, Valko *et al.* 2004).

Table 4. Formation of the biologically most important reactive oxygen and nitrogen species.

Reaction	Note
$O_2 + e^- \rightarrow O_2^{\cdot-}$	Superoxide formation (various sources, see text)
$2 O_2^{\cdot-} + 2 H^+ \rightarrow H_2O_2 + O_2$	Hydrogen peroxide formation, catalyzed by SODs
$Cu^+/Fe^{2+} + H_2O_2 \rightarrow Cu^{2+}/Fe^{3+} + \cdot OH + OH^-$	Fenton reaction
$O_2^{\cdot-} + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$	Haber-Weiss reaction (iron-catalyzed)
L-arginine \rightarrow NO \cdot + L-citrulline	NO \cdot formation (catalyzed by NOS)
$NO^+ + O_2^{\cdot-} \rightarrow ONOO^{\cdot-}$	Peroxynitrite formation

2.2.4 Reactive nitrogen species

The term reactive nitrogen species (RNS) is used to refer to nitric oxide (NO[•])-derived radicals and certain non-radicals (Wiseman & Halliwell 1996). NO[•] is generated from L-arginine by three NO[•] synthases (NOS) encoded by three highly conserved distinct genes (Nathan & Xie 1994). NADPH is used as an electron donor in the process of synthesis. The majority of NO[•] is synthesized by inducible NOS (iNOS, NOS-2), usually after a challenge by immunological or inflammatory stimuli (Nathan & Xie 1994, Davis *et al.* 2001). Other NOSes include endothelial NOS (eNOS, NOS-1) and neuronal NOS (nNOS, NOS-3), the activity of which is regulated by the intracellular Ca²⁺ concentration. eNOS, and the nNOS are grouped together as constitutive NOSes (cNOS). Nevertheless, cNOSes may also be inducible under certain conditions (Nathan & Xie 1994, Davis *et al.* 2001).

2.2.4.1 NO[•]

The biological effects of NO[•] are complicated and in many respects contradictory. These effects can be roughly divided into three groups (Wink & Mitchell 1998). First, as a highly diffusible lipophilic second messenger, NO[•] is able to affect phenomena far away from its site of production, and it is therefore an important regulator of many physiological functions, including cellular adhesion, neurotransmission, bronchodilatation, and most notoriously, vascular tone and permeability (Moncada *et al.* 1991, Culotta & Koshland 1992, Davis *et al.* 2001). Secondly, NO[•] plays a role in immunological defense, and especially macrophage function is dependent on proper NOS activity (MacMicking *et al.* 1997). Thirdly, NO[•] has many deleterious and carcinogenic effects mainly via its metabolites, such as peroxynitrite.

NO[•] concentration, along with location and timing, is considered one of the principal determinants of the mechanism of these diverse actions (Wink & Mitchell 2003). At low concentrations the direct effects of NO[•] prevail, but at concentrations higher than 1 μM indirect effects, such as nitration, oxidation, and nitrosation, predominate via radical formation (Wink & Mitchell 1998).

2.2.4.2 Peroxynitrite

In contrast to most ROS, NO[•] rarely reacts directly with macromolecules, but instead reacts readily with other free radicals and metals (Nordberg & Arner 2001, Wink & Mitchell 1998). The primary pathway of NO[•] metabolism is the reaction of NO[•] with O₂^{•-}, which yields a powerful oxidant, peroxynitrite (ONOO⁻) (Huie & Padmaja 1993, Beckman & Koppenol 1996). The reactivity of ONOO⁻ is comparable even to that of ·OH (Dempfle & Harrison 1994). The rate-limiting factor of this reaction is the availability of substrates, and SODs therefore play an essential role even here by regulating O₂^{•-} concentration. It has been shown that peroxynitrite has potential for a variety of pathological chemical reactions in cells, including DNA strand breakage, nitration of

tyrosine residues of proteins, and inhibition of mitochondrial electron transport (Radi *et al.* 1994, Inoue & Kawanishi 1995, Maeda & Akaike 1998, Cadenas 2004). Nitrotyrosine is a stable end product of peroxynitrite metabolism, the expression of which has been widely used as an indicator of peroxynitrite formation in various pathological conditions.

2.2.5 Physiological effects of ROS

Although the major focus in radical research has been on the toxic effects of these molecules in numerous pathological conditions, a growing body of evidence suggests physiological roles of ROS and NO[•] in various cellular processes (Finkel 1998, Thannickal & Fanburg 2000, Dröge 2002). One of the most well-known radicals acting as a physiological regulator is NO[•], which affects vascular tone and platelet adhesion (Ignarro *et al.* 1987, Palmer 1987). Less is known about the functions of H₂O₂ and O₂^{•-} in intracellular signaling. During the past decade, however, research in this field has progressed considerably, and especially H₂O₂ is now recognized as a key intracellular messenger in certain important signal pathways, such as epidermal growth factor and NF-κB activation (Schreck *et al.* 1996, Bae *et al.* 1997, Rhee *et al.* 2003).

Both NO[•] and oxygen radicals also function as part of the immune system. Massive O₂^{•-} production, an “oxidative burst”, can be produced by activated macrophages and neutrophils in defense against environmental pathogens, in addition to which mammalian macrophages are able to produce NO[•] (Thomas *et al.* 1988, Demple & Harrison 1994). Other cell types generate continuously lower levels of ROS to regulate redox-responsive signaling pathways (Finkel 1998). ROS and NO[•] are also involved in apoptotic pathways (Jacobson 1996, Kim *et al.* 2001).

2.2.6 ROS and DNA damage

Most of the reactive oxygen and nitrogen species from the above-mentioned physiological sources also interfere naturally with DNA; almost 100 kinds of free radical DNA damage have been described (Demple & Harrison 1994, Wiseman & Halliwell 1996, Marnett 2000). This viewpoint is supported by studies that have found steady-state levels of typical radical-induced damage in DNA bases (Floyd *et al.* 1986, Richter 1992, Wiseman & Halliwell 1996). There is also up to 10⁴-fold DNA base damage in mitochondria compared to nuclear DNA, which is in line with the hypothesis of the mitochondrial electron transport chain as the main source of ROS formation under physiological circumstances (Richter *et al.* 1988, Agarwal & Sohal 1994). However, ineffective DNA repair systems and lack of histones in mitochondria have also been proposed to explain mitochondrial DNA damage (Ljungman & Hanawalt 1992, Wiseman & Halliwell 1996).

Particular types of ROS are responsible for different kinds of DNA damage. ·OH produces the largest variation of mutation types, despite its very short T_{1/2} (Imlay & Linn 1988). When generated from H₂O₂ in the immediate vicinity of DNA, ·OH is able to damage the deoxyribose backbone of DNA and all of the four DNA bases in various

ways, including the generation of 8-hydroxyguanosine (8-OHG), the hydrolytic product of which is the widely studied 8-hydroxydeoxyguanosine (8-OHdG) (Kasai 1997). 8-OHdG has been used as a “fingerprint” of $\cdot\text{OH}$ attack, and it is the most commonly measured and the most widely studied DNA oxidation product (Halliwell & Aruoma 1991, Wiseman & Halliwell 1996, Marnett 2000). $\cdot\text{OH}$ is also responsible for DNA single and double-strand breaks and damage to tumor suppressor genes and other macromolecules (Klaunig *et al.* 1998).

$^1\text{O}_2$ reacts selectively with guanine, whereas H_2O_2 and $\text{O}_2^{\cdot-}$ do not attack DNA bases at all (Halliwell & Aruoma 1991, van den Akker 1994, Wiseman & Halliwell 1996). A wide spectrum of studies show that the central role of NO^\cdot in oxidative damage to DNA. NO^\cdot and its metabolites can deaminate DNA bases, induce transversions and transitions of bases, and inactivate DNA repair proteins (Wink *et al.* 1991, Laval & Wink 1994, Juedes & Wogan 1996). ONOO^\cdot is an especially harmful reactive nitrogen radical, since it is both diffusible and able to penetrate cell membranes and is also an extremely potent DNA oxidant (Marnett 2000).

The chemical modification of DNA is not the only pathway of ROS-induced DNA damage, although it may be the simplest and hence best characterized. The contribution of ROS damage to polymerase-specific “hotspots” and DNA conformation changes due to oxidative damage represent other, as yet less studied, forms of DNA damage by ROS (Feig & Loeb 1993, Wiseman & Halliwell 1996).

2.2.7 ROS and lipid damage

ROS, especially $\cdot\text{OH}$, may attack any cellular macromolecule, not only DNA. It has been proposed that DNA represents a quantitatively less significant ROS target compared to protein and lipid damage *in vivo* (Stadtman 1993, Marnett 2000). All cell membranes contain unsaturated fatty acids, many of them being polyunsaturated and thus susceptible to oxidation when reacting with ROS (Marnett 2000). The formation of a free radical chain reaction in a polyunsaturated lipid layer is characteristic of ROS-induced lipid peroxidation (Marnett 2000). The most important consequences of lipid peroxidation are protein oxidation, loss or weakening of cell membrane structure and function, and generation of aldehyde products such as acrolein, crotonaldehyde, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) (Rice-Evans & Burdon 1993, Klaunig *et al.* 1998, Marnett 2000, Kang 2002). MDA and HNE have been shown to be carcinogenic in animal models (Basu & Marnett 1986, Esterbauer *et al.* 1991), but they also have roles as physiological regulators of especially cell multiplication and differentiation (Dianzani 2003, Folden *et al.* 2003, Leonarduzzi *et al.* 2004).

HNE is one of the main compounds of lipid peroxidation in human tissues, and it is also the most widely studied lipid peroxidation compound along with MDA. Compared to the majority of ROS, HNE is a relatively stable molecule and therefore able to diffuse far from the original site of membrane peroxidation (Uchida 2003). When present at low levels (less than $1\ \mu\text{M}$), HNE has essential physiological roles in cell proliferation, transformation, differentiation, and apoptosis (Awasthi *et al.* 2003, Dianzani 2003, Yang *et al.* 2003). In concentrations higher than $10\ \mu\text{M}$, this α,β -unsaturated aldehyde plays a

role in carcinogenesis by modifying protein and DNA structures in multiple ways and also by suppressing certain enzymatic activities (Dianzani *et al.* 2003).

Both *in vitro* and *in vivo* studies have demonstrated high HNE levels being linked with genotoxicity (Chung *et al.* 1993, Eckl *et al.* 1993, Kowalczyk *et al.* 2004). In animal studies, elevated lipid peroxidation production, including formation of HNE, has been closely associated with carcinogenesis (Feng *et al.* 2004). Lately, formation of HNE has been implicated to inhibit DNA nucleotide excision repair through direct interaction with proteins in DNA repair (Feng *et al.* 2004). HNE-derived p53 mutations have also been widely studied and characterized (Hu *et al.* 2002, Feng *et al.* 2003).

2.2.8 ROS and protein damage

Proteins, including DNA repair enzymes and DNA polymerases, are among the major targets of ROS (Wiseman & Halliwell 1996). Proteins may be damaged either directly or indirectly through lipid peroxidation (Klaunig *et al.* 1998). The consequences of ROS-derived protein damage contain modification of enzyme activity, damage to membrane transporters, and interaction with receptors, all being potentially devastating to normal cell physiology (Bellomo *et al.* 1983, Klaunig *et al.* 1998).

2.2.8.1 p53

P53 was the first tumor suppressor gene discovered in 1979, although initially it was supposed to be an oncogene (DeLeo *et al.* 1979, Jenkins *et al.* 1985). A product of P53, tumor suppressor protein p53, is able to arrest the cell cycle to the G₁ phase if DNA damage has occurred. In addition, p53 may trigger apoptosis if excessive DNA damage is detected, and it is thereby also potent to safeguard genetic integrity (Barnes & Campeljoh 1996). More than 100 different mutations of p53 that lack DNA-binding activity have been characterized in human malignancies (Harper 2000, Swiss Institute of Bioinformatics 2005). The overall frequency of these mutations in human breast tumors is suggested to be approximately 20% according to a wide meta-analysis (Pharaoh *et al.* 1999). Interestingly, in medullary carcinomas, the p53 mutation prevalence seems to be nearly 100% (De Cremoux *et al.* 1999). p53 affects the function of a variety of DNA repair enzymes, including mismatch repair proteins (MSH). p53 activates MSH2 after ultraviolet irradiation, and the MSH2-MSH6 protein complex enhances the DNA binding of p53 up to 4-fold (Subramanian & Griffith 2002, Sengupta & Harris 2005).

2.2.9 ROS and transcription factors

Another both physiologically and pathologically important target of ROS is the direct activation of transcription factors and thus the regulation of gene transcription. The activator protein -1 (AP-1) is a regulator of cell growth, cell proliferation, and cell death,

and ROS is known to induce both the activation and the synthesis of AP-1 (Kerr *et al.* 1992, Shaulian & Karin 2002). Protein kinase C, which participates in a variety of pathways regulating transcription and cell cycle control, is activated by H₂O₂ (Dröge 2002).

2.2.9.1 NF- κ B

NF- κ B is another well characterized H₂O₂-activated transcription factor that involves cell growth control, apoptosis, and inflammatory reactions (Dröge 2002, Schmidt 1996). There is also both *in vivo* and *in vitro* evidence that NF- κ B has a role in breast carcinogenesis etiology (Cao & Karin 2003). The major mechanism of ROS-mediated NF- κ B activation relates to degradation of the inhibitory subunit I κ B after a challenge with oxidants (Toledano & Leonard 1991, Nordberg & Arner 2001, Dröge 2002). Specific NF- κ B inhibitors are being studied as potential anti-breast cancer drugs (Sliva 2004, Wu & Kral 2005).

2.3 ROS in carcinogenesis

Aerobic organisms produce continuously oxidants, which have a potential to disturb cell physiology in various ways. Normally, these oxidants are balanced by synthesis of various defensive proteins. Oxidative stress is said to arise when the amount of ROS exceeds the capacity of the cellular defense mechanisms (Dreher & Junod 1996). In other words, oxidative stress may occur either when ROS are overproduced and/or when reducing equivalents, antioxidants, or repair systems are insufficient for detoxification (Klaunig *et al.* 1998).

High levels of radical generation and persistent oxidative stress are characteristic of carcinoma cells both *in vivo* and *in vitro* (Szatrowski & Nathan 1991, Toyokuni *et al.* 1995, Brown & Bicknell 2001). ROS-producing macrophage infiltration into the tumor and ROS generation during the reperfusion phase following hypoxia in the defective tumor vascular system are most frequently suggested to be the main reasons for the high ROS levels in cancer cells compared to surrounding tissue (Brown & Bicknell 2001, Ray & Husain 2002). The enhanced glycolytic metabolism demonstrated in tumor cells has also been associated with increased O₂^{-•} production and thus with more oxidized redox balance (Spitz *et al.* 2000). O₂^{-•} and H₂O₂ levels have been shown to be elevated in breast cancer patients compared to healthy controls (Ray *et al.* 2000).

These relatively high doses of ROS in tumors are able to cause apoptotic death in a tumor cell population or to induce the kind of potentially carcinogenic alterations described above in cellular macromolecules, especially DNA. However, oxidative stress in tumors *in vivo* is usually not sufficient to induce cytotoxic effects, since tumor cells have developed increased resistance to persistent oxidative stress. Actually, cells with increased chemoresistance often use the same mechanisms (Toyokuni *et al.* 1995, Klaunig *et al.* 1998). Persistent oxidative stress measured in carcinoma cells is able to turn off tumor suppressor genes, activate transcription factors, promote selective cell

death and compensatory proliferation, and therefore possibly lead to initiation of carcinogenesis (Dreher & Junod 1996, Klaunig *et al.* 1998).

2.3.1 ROS and the initiation stage of carcinogenesis

Carcinogenesis initiation involves an inheritable, permanent, and non-lethal mutation of a single cell (Klaunig *et al.* 1998). ROS, especially $\cdot\text{OH}$, are suggested to have multiple effects on cancer initiation, such as inactivation of tumor suppressor genes or activation of oncogenes through point mutations, activation of chemical carcinogens, or prevention of DNA damage repair (Trush & Kensler 1991, Dreher & Junod 1996, Klaunig *et al.* 1998). It is worth bearing in mind that these mutations are, in all likelihood, random and mostly totally irrelevant from the point of view of carcinogenesis or cell survival. Only mutations in very few genes are capable of triggering the development of cancer.

However, some somatic breast cancer-linked mutations such as p53, BRCA1, and BRCA2 have been characterized (Elledge *et al.* 1993, Wright *et al.* 1999, Lou & Chen 2003). These mutations apparently have potential to be carcinogenic only in proliferating cells. It has been recently shown, using DNA microarray and confirmatory reverse transcription-PCR methods, that BRCA1 functions as a protector of cells against oxidative stress by inducing various antioxidant enzymes (Bae *et al.* 2004).

2.3.2 ROS and the promotion stage of carcinogenesis

In the tumor promotion stage, ROS are able to stimulate the expansion of initiated cell clones by modulating multiplication or apoptosis (Cerutti 1985). One possible mechanism of cell proliferation enhancement is the tendency of ROS to induce a large increase in the intracellular Ca^{2+} concentration, which may involve activation of proto-oncogenes such as c-fos, c-jun, and c-myc or activate protein kinase C (Larsson & Cerutti 1988, Maki *et al.* 1992, Dreher & Junod 1995, Dreher & Junod 1996). Activation of several proto-oncogenes has likewise been reported. There is also comprehensive evidence to suggest that promotion-inducing compounds have an ability to generate oxidative stress either via activation of ROS production sources or via direct ROS generation. Again, antioxidant enzymes are shown to inhibit this promotion (Evans *et al.* 2004).

ROS has been strictly implicated in the induction of apoptosis and also further in the apoptotic pathways. For example, anti-apoptotic proto-oncogene bcl-2 has been proposed to regulate antioxidant pathways at the sites of free radical generation (Hockenbery *et al.* 1993, Matés & Sánchez-Jiménez 2000). Nevertheless, if the oxidative stress is severe, it leads to necrosis of the cell (Halliwell 2000). ROS may also activate transcription factor NF- κ B activity and thereby stimulate tumor promotion (Toledano & Leonard 1991).

2.3.3 ROS and the progression stage of carcinogenesis

In the progression stage, the tumor develops uncontrolled growth and is able to invade into surrounding tissue and to develop metastases. One aspect relating to the role of ROS in tumor progression is the chronic inflammation that produces notable amounts of ROS and is also often seen in carcinomas. This inflammation does not eliminate tumor cells but causes more genetic instability (Dreher & Junod 1996). Permanent and high oxidative stress in tumor cells creates selection pressure for such characteristics as accelerated growth, invasion, and metastasis (Toyokuni *et al.* 1995, Cooke *et al.* 2003). The fundamental mechanism of breast carcinoma metastasis is poorly understood, but ·OH-derived DNA damage and subsequent alterations in critical genes are suggested to induce this process considerably (Malins *et al.* 1996). In line with this, the most widely used oxidative DNA damage marker, 8-OHdG, has been reported to be increased 8- to 17-fold in primary breast tumors compared to non-cancerous breast tissue (Malins & Haimanot 1991, Musarrat *et al.* 1996, Matsui *et al.* 2000).

The role of NO· in tumor progression is contradictory in many respects. NO· has been demonstrated to contribute to mutagenesis by damaging nucleic acid bases via various mechanisms or by inactivating DNA repair enzymes (Wink *et al.* 1991, deRojas-Walker *et al.* 1995, Graziewicz *et al.* 1996). Oncogenic p53 mutations are linked to the production of NO· and its metabolites, i.e. ultimately to NOS (mainly iNOS) synthesis (Forrester *et al.* 1996, Chazotte-Aubert *et al.* 2000). Tumor growth and metastasis stimulation, augmented angiogenesis, and invasion have all been implicated in NOS activation (Lala & Chakraborty 2001). The well-known tendency of NO· to increase vascular permeability leads to increased nutritional supply to the tumor and further to accelerated tumor growth (Maeda & Akaike 1998). However, the dual role of NO· in tumorigenesis has been revealed by experiments where tumor cells have been treated with high doses of NOS. In these cases, the effect of NO· seems to be tumoricidal (Xie & Fidler 1998). The studies concerning the role of NOS in tumor progression and cell proliferation are also somewhat contradictory. Even the studies with fundamentally analogous immunohistochemical methods have given rise to rather different conclusions (Thomsen *et al.* 1995, Duenas-Gonzalez *et al.* 1997, Tschugguel *et al.* 1999, Reveneau *et al.* 1999, Martin *et al.* 2000).

2.3.4 ROS – cause or consequence?

One of the main contradictions in the hypothesis of ROS-mediated carcinogenesis is that many other diseases have also been linked with elevated ROS levels, without an association with carcinogenesis. It would also be naïve to generalize that the oxidative damage seen in most malignant tumors has a direct causal connection with the development of cancer. One important hypothesis, suggested by Cooke and co-workers, is that oxidative damage in tumors may, at least to some extent, result from increased metabolism in tumor cells, being thus a consequence rather than cause of carcinogenesis (Cooke *et al.* 2003).

To summarize, the role of ROS in breast carcinogenesis has been convincingly demonstrated in many ways, although not directly shown in humans. ROS damage is known to accumulate into DNA, and on the other hand, carcinogenesis can be considered a degenerative disease of DNA, which increases with age. The cumulative risk to develop cancer is known to increase with the fourth power of age, being at least to some extent caused by toxic endogenous and exogenous ROS exposure during the lifespan (Ames 1989, Wiseman & Halliwell 1996).

2.4 Antioxidant enzymes and cell redox state regulating proteins

To counteract the noxious effects of ROS, cells have developed various defensive or reparative mechanisms to eliminate surplus ROS from all cell compartments (Figure 2.). The most important of these defence systems is the antioxidant system, which includes both endogenous and exogenous and, on the other hand, enzymatic and non-enzymatic antioxidants. As described by Halliwell and Gutteridge, antioxidant is “any substance that delays or inhibits oxidative damage to the target molecule” (Halliwell and Gutteridge 1990). Furthermore, cells have also evolved many specific and non-specific DNA repair systems to prevent the accumulation of already damaged DNA. There are also some other less significant anti-carcinogenic immunological defence systems, including complement, neutrophils, macrophages, and specific antibodies (Jakobisiak 2003).

2.4.1 Superoxide dismutases

Superoxide dismutases (SOD) were the first antioxidant enzymes identified 35 years ago (McCord & Fridovich 1969). There are three types of SOD in human cells: cytosolic copper-zinc SOD (CuZnSOD), extracellular SOD (ECSOD), and manganese SOD (MnSOD). The fourth type of SOD, FeSOD, is present in many aerobic bacteria (Ray & Husain 2002). The SODs have some properties in common, but each SOD also has its own peculiarities (Oberley & Oberley 1984). All SODs are able to dismutate two $O_2^{\cdot -}$ anions to H_2O_2 and molecular oxygen. No other enzymes have activity to this reaction, but, on the other hand, this is the only known activity of the SODs. Induction by various stress conditions is also characteristic of all human SODs (Kinnula & Crapo 2004).

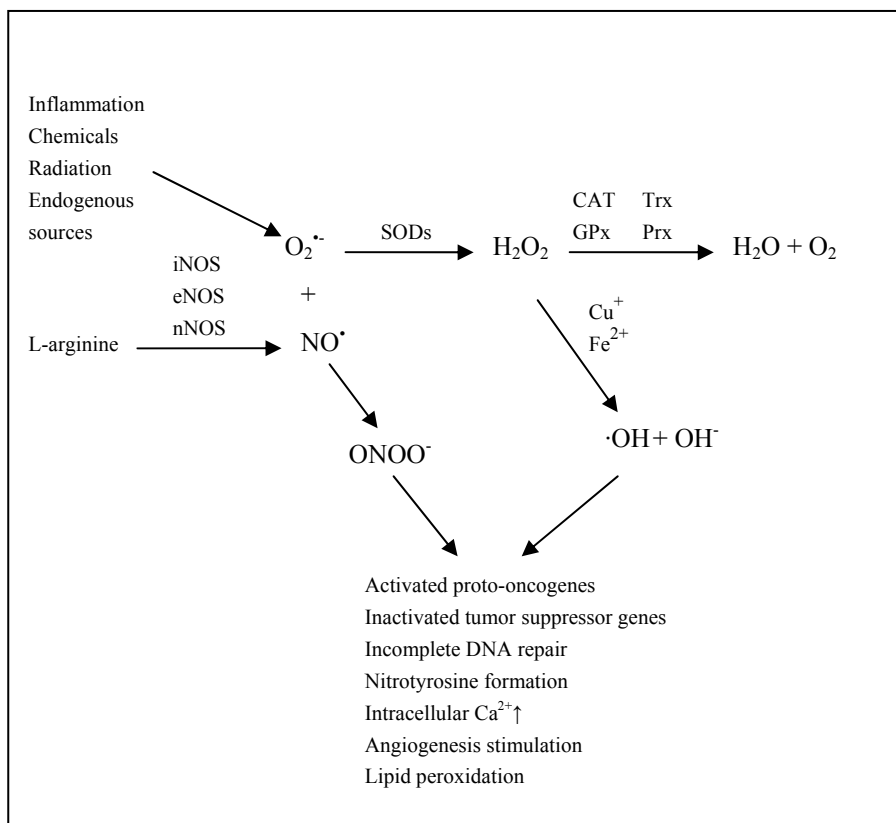


Fig. 2. Relationships between reactive oxygen and nitrogen species and antioxidant enzymes in human tissues. Refer to the text for details.

2.4.1.1 MnSOD

MnSOD is found primarily in the mitochondrial matrix, where most of the cell's oxygen is consumed, and where oxidative stress is most evident. MnSOD without a mitochondrial targeting sequence does not seem to protect cells against radiation (MacMillan-Crow & Cruthirds 2001). MnSOD has been considered as one of the most important antioxidant enzymes in mammalian tissues, and knockout studies have indicated its importance for the vital organs. Mice lacking MnSOD had severe metabolic acidosis and also degeneration in their neurons and cardiac myocytes (Li *et al.* 1995a, Lebowitz *et al.* 1996). These mice died prenatally of dilated cardiomyopathy. For the sake of comparison, neither CuZnSOD nor ECSOD seems to be essential for life (Carlsson *et al.* 1995, Reaume *et al.* 1996). MnSOD is induced after a challenge with ROS, high oxygen tension, radiation, and chronic hypoxia (Sjöström & Crapo 1983, Allen 1991, Clerch *et al.* 1993, Ho *et al.* 1996, Guo *et al.* 2003).

2.4.1.2 SODs and carcinogenesis

The connection between SODs and carcinogenesis is not unambiguous. In general, *in vitro* studies with SOD overexpression tend to suggest suppression of the malignant phenotype. In prostate carcinoma and glioma cell lines, inhibition of cell growth was reported after SOD overexpression (Zhong *et al.* 1996, Li *et al.* 1998). Lung fibroblasts showed a decreased growth rate, lower plating efficiency, and some morphological differences after a 2- to 3.5-fold increase in MnSOD activity, and all of these changes correlated with the MnSOD activity in cells (Yan *et al.* 1996). A rather analogous study with MCF-7 human breast carcinoma cells showed decreased plating efficiency of the cells after MnSOD overexpression (Li *et al.* 1995b). The authors also described inhibited tumor growth when MnSOD-overexpressing cells were inoculated into nude mice. There are also a few promising studies suggesting that increased ROS production may enhance apoptosis selectively in certain malignancies (Huang *et al.* 2000, Ling *et al.* 2003, Pelicano *et al.* 2003). Furthermore, overexpression of MnSOD has been shown also to produce resistance against a few cancer drugs, such as doxorubicin and mitomycin (reviewed in Kinnula & Crapo 2004).

Although the majority of *in vitro* studies have established MnSOD overexpression as a tumor-suppressive factor, the *in vivo* situation is doubtless more complicated and dependent on more than a single overexpressive enzyme. Furthermore, when only one of the antioxidant enzymes is manipulated in the multifactorial and interactive system, the outcome is probably elevation or suppression of the other redox-regulating elements, and this hardly reflects the situation *in vivo*.

A wide variety of studies have been carried out to measure the *in vivo* levels of SODs, emphasizing MnSOD, in different human carcinomas. The majority of these studies have demonstrated either increased expression or activity of MnSOD in malignant tumors, and high SOD levels have also been linked to poor prognosis and metastasis in many papers. This trend seems to be most evident in gastrointestinal malignancies (Janssen *et al.* 1998, Janssen *et al.* 1999, Toh *et al.* 2000). In pancreatic cancer, high levels of inactivated tyrosine-nitrated MnSOD have been found, suggesting that RNS may act as a MnSOD activity suppressor (MacMillan-Crow & Cruthirds 2001). The tendency is less distinctive in breast carcinomas. Only one small study (n=14) has reached statistical significance, showing both CuZnSOD and MnSOD to be elevated in breast tumor tissue compared to normal breast tissue of the same patients (Bianchi *et al.* 1992). Another breast carcinoma study showed more MnSOD-positive cases among benign hyperplasias and *in situ* lesions than among invasive tumors (Soini *et al.* 2001a). A few recent studies have reported that a valine → alanine mutation at the -9 position of the mitochondrial targeting sequence could increase the breast cancer risk, but some studies with controversial results have also been published (Mitrunen *et al.* 2001, Egan *et al.* 2003, Cai *et al.* 2004, Kocabas *et al.* 2005). One of the most important factors in breast carcinoma metastasis, matrix metalloproteinase 2 (MMP-2), was reported to be activated after MnSOD transfection to MCF-7 cells (Zhang *et al.* 2002).

Theoretically, excess SOD could be used to gain therapeutical effects on tumor growth, invasion, and apoptosis. However, $O_2^{\cdot-}$ also plays important roles as a signal molecule and inhibitor of excess lipid peroxidation, and on the other hand, none of the

human SODs as such have attractive pharmacological properties to be used as clinically useful therapeutic agents (McCord & Edeas 2005). Instead, a few promising small synthetic SOD mimetics have been developed up to date, and they may be used in the future to increase the radiation tolerance of tumors or to suppress tumor incidence in high-risk patients (Vujaskovic *et al.* 2002, Zhao *et al.* 2005).

The difference between the results of *in vivo* and *in vitro* studies is thus obvious. *In vivo* oxidative stress probably results from increased metabolism, and this launches the induction of antioxidant enzymes, as suggested by Kinnula and Crapo in their recent review (Kinnula & Crapo 2004). Paradoxically, antioxidants may enhance tumor promotion by delaying the development of a highly oxidized environment and, hence, oxidant-derived necrosis. It is also possible that SOD mimetics decrease the angiogenesis rate, at least *in vitro*, and therefore suppress tumor growth (Kinnula & Crapo 2004).

2.4.2 Catalase

Catalase is a tetrameric 240 kDa antioxidant enzyme (Matés *et al.* 1999). 80% of catalase is present in peroxisomes, where it decomposes H_2O_2 produced by SODs to water and molecular oxygen (Ray & Husain 2002). In addition, catalase also detoxifies various phenols and alcohols (Nordberg & Arner 2001). A variety of *in vitro* studies report suppressive functions of this heme enzyme against tumor initiation and promotion. For instance, acatalasemic mice had a high incidence of mammary tumors, but the mice were induced similarly to have nutritional vitamin E deficiency (Ishii *et al.* 1996). MacRae and Stich described as early as 1979 that H_2O_2 exposure induced up to three times more sister chromosome exchanges compared to the basic level in Chinese hamsters (MacRae & Stich 1979). Catalase was observed to be potent to prevent these mutations.

2.4.3 Glutathione peroxidases

Glutathione peroxidases (GPx) are another group of enzymes capable of reducing H_2O_2 . GPxs catalyse the oxidation of GSH to its oxidized form of disulfide (GSSG), while H_2O_2 is simultaneously decomposed to water and molecular oxygen. GSSG is again reduced by the specific enzyme, glutathione reductase. Today, four different selenocysteine-containing GPxs have been characterized, in addition to which two GPxs are present in mammalian cells without selenocysteine (Arthur 2000). The role of the leucine to proline mutation at codon 198 of the GPx1 gene has been rather widely studied in different carcinomas. The results have been somewhat contradictory, but at least in bladder carcinoma, it may increase the risk of both primary cancer and recurrence (Hu & Diamond 2003, Ichimura *et al.* 2004, Ahn *et al.* 2005, Zhao *et al.* 2005).

2.4.4 Thioredoxin

Thioredoxins (Trxs) constitute a ubiquitous antioxidant family potent to affect a wide variety of cell functions (Powis & Montfort 2001, Gromer *et al.* 2004). Vertebrates express two different forms of Trx, whereas plants have a much greater diversity of Trxs (Moaueh *et al.* 1998). As an antioxidant, Trx is able to reduce H_2O_2 and therefore further prevents the formation of $\cdot OH$ (Spector *et al.* 1988). Trx contains a conserved Trp-Cys-Gly-Pro-Cys-Lys active site, which is reversibly oxidized to cysteine-disulfide (Trx-S₂) (Mustacich & Powis 2000). Trx-S₂ is further reduced back with the help of thioredoxin reductase (TrxR), and the NADPH molecule is simultaneously oxidized (Powis *et al.* 2000). Trx also functions as an electron donor to Prx I-V and to glutathione peroxidases (Chae *et al.* 1994, Bjørnstedt *et al.* 1994), and the former role has actually been suggested to be the most important antioxidant function of Trx (Powis & Montfort 2001). Furthermore, Trx has been reported to prevent the NO⁻-dependent inhibition of NOS activity (Powis *et al.* 2000). Trx also has a highly important role as a growth factor that sensitizes cells to growth factors produced by the cell itself (Gasdaska *et al.* 1995).

2.4.5 Thioredoxin reductase

Thioredoxin reductase (TrxR) is a selenocysteine-containing enzyme able to reduce disulfides in a variety of oxidized proteins, including NK-lysin, selenite, selenodiglutathione, vitamin K, S-nitroglutathione, lipoic acid, and naturally Trx (Holmgren & Bjørnstedt 1995, Anderson *et al.* 1996, Nordberg & Arner 2001). Three different TrxR isoforms have been characterized in mammalian cells, TrxR1 being extracellular, nuclear, cytoplasmic, and abundant in plasma membranes, while TrxR2 and TrxR3 are only present in mitochondria (Söderberg *et al.* 2000, Oberley *et al.* 2001, Sun *et al.* 2001).

In vitro studies have shown that, although TrxR hyperactivity does not induce cell growth, suppression of TrxR activity below the normal level associates with the inhibition of cell growth (Mustacich & Powis 2000). Significantly higher TrxR concentrations have been measured in cancerous compared to normal tissue in, for instance, malignant melanoma, but were not observed in breast carcinomas (Schallreuter *et al.* 1991, Mustacich & Powis 2000, Lincoln *et al.* 2003).

2.4.6 Peroxiredoxins

The first description of peroxiredoxins (Prx) was published as early as 1988, and at that time it was called a “protector protein” or “thiol-specific antioxidant” (Kim *et al.* 1988, Rhee *et al.* 1999). Later, the Prx family was re-named as thioredoxin peroxidases, until it turned out that not all of these enzymes use thioredoxin as an electron donor (Chae *et al.* 1994). Nowadays, Prxs are known as an important antioxidant enzyme family consisting of at least six different proteins in human tissues. The main function of Prxs is to reduce

peroxides, including H_2O_2 , to the corresponding alcohol or water (Kang *et al.* 1998, Rabilloud *et al.* 2002). However, Prxs have a few features that differentiate them from the other peroxidases. Prxs have both peroxidase and cosubstrate activity; when reducing peroxide, the enzyme itself is oxidized (Rabilloud *et al.* 2002). Prxs are reduced to the initial state by thioredoxin, with the exception of Prx VI, the electron donor of which is supposed to be a cyclophilin A (Lee *et al.* 2001). Whereas most peroxidases have heme or selenocysteine at their active site, Prxs contain one or two cysteines at their active site. The 1-Cys peroxiredoxin subgroup contains only one member, Prx VI (Kang *et al.* 1998). Prxs I-IV have two conserved cysteines and therefore belong to the 2-Cys subgroup (Rabilloud *et al.* 2002). Prx V is classified to the atypical 2-Cys subgroup. It has been suggested that Prx V could be more effective against ROS than the other Prxs (Declercq *et al.* 2001, Wang *et al.* 2001). This is not only because of its subcellular location in places where protection against ROS is mostly needed, but also because of the special features of its catalytic site (Declercq *et al.* 2001). In addition, Prx V also has peroxynitrite-scavenging properties (Bryk *et al.* 2000, Dubuisson *et al.* 2004). A recent study with human lung carcinoma cells suggested that Prxs are reversibly overoxidated after the addition of a high (250-500 μM) H_2O_2 concentration to the cell culture, but more physiological oxidative stress did not modulate Prx proteins (Lehtonen *et al.* 2005).

At the moment, Prxs are regarded as one of the most important and ubiquitous antioxidant enzyme groups. This is supported by reports showing that Prx VI knock-out mice suffer from severe lung injury and show increased sensitivity to hyperoxia (Wang *et al.* 2004). Compared to catalase, which is present almost exclusively in peroxisomes, Prxs are also widely distributed subcellularly. They are located to cytosol (Prxs I, II, III, V and VI), peroxisomes (Prx IV and V), lysosomes, (Prx IV and VI), endoplasmic reticulum, extracellularly, Golgi apparatus (Prx IV), and also to mitochondria (Prxs III and V) (Peri *et al.* 2003, Kang *et al.* 1998, Okado-Matsumoto *et al.* 2000, Declercq *et al.* 2001, Kinnula *et al.* 2002).

The role of Prxs in carcinoma development is not unambiguous, and they are still largely unstudied in human malignancies. In certain carcinoma types, Prxs seem to associate with aggressive phenotypes, whereas in malignant mesothelioma, for example, intensive Prx V expression is associated with longer survival (Yanagawa *et al.* 2000, Kinnula *et al.* 2002, Lehtonen *et al.* 2004).

2.4.7 Other cell redox state modulating proteins

In addition to the previously described antioxidant enzymes, other important antioxidant defenses also exist. Glutamate-cysteine ligase (GLCL; also known as γ -glutamyl cysteine synthetase (γ GCS)), is the rate-limiting enzyme of glutathione synthesis and has therefore been suggested to have a crucial role in cell homeostasis, growth, and chemoresistance (Meister & Anderson 1983, Tew 1994, Kigawa *et al.* 1998, Soini *et al.* 2001b). GLCL consumes a single ATP molecule to catalyze the synthesis of L-glutamate and L-cysteine to L-glutamyl-L-cysteine. Glycine is further added to L-glutamyl-L-cysteine to form GSH. The enzyme named glutathione synthase, which consumes another ATP, is needed to catalyze the latter step.

GLCL consists of two separately encoded subunits, a heavier catalytic and a lighter regulatory or modulatory chain, called GLCL-c and GLCL-r, respectively. In knock-out studies, GLCL-c has been established as essential to life and seems to be the more important subunit for GSH synthesis, but GLCL kinetics is controlled by GLCL-r (Dalton *et al.* 2000, Forman & Dickinson 2003). GLCL is induced by radiation, several toxic agents, anticancer drugs, and oxidative stress (Yamane *et al.* 1998, Morales *et al.* 1998, Tipnis *et al.* 1999, Rahman & McNee 2000, Diaz *et al.* 2001, Seo *et al.* 2004). GLCL overexpression has been found in various carcinomas, and its overexpression has been implicated to associate with increased chemoresistance of tumors (Rahman 1999, Järvinen *et al.* 2002, Tatebe *et al.* 2002, Tiitto *et al.* 2004, Haapasalo *et al.* 2003, Kaartenaho-Wiik & Kinnula 2004).

2.4.8 Small molecular weight antioxidants

The most thoroughly characterized small molecular weight antioxidants are glutathione and the antioxidative vitamins A, C, and E (London *et al.* 1985, Basu *et al.* 1988, Noroozi *et al.* 1998, Bounous & Molson 2003). Furthermore, proteins such as transferrin and ceruloplasmin are involved in the binding of iron and copper ions and therefore play highly important roles in preventing $\cdot\text{OH}$ formation. They can also be considered antioxidants (Gutteridge 1989, Halliwell & Gutteridge 1990).

Similarly to Trx, GSH (L- γ -glutamyl-L-cysteinyl-glycine) is also an easily oxidized and regenerated molecule that participates in the scavenging of H_2O_2 . In addition, it participates in physiological signal transduction by regulating intracellular H_2O_2 concentrations (Forman & Dickinson 2003). GSH is able to quench free radicals, reduce H_2O_2 or lipid peroxidases, and therefore provide defense against the toxic end products of ROS (Hayes & McLellan 1999, Stover *et al.* 2000). GSH is, furthermore, implicated in the synthesis of proteins, nucleic acids, and leukotrienes and in the detoxification of xenobiotics (Rahman *et al.* 1999). GSH overexpression has been associated with multidrug chemoresistance in various types of carcinoma (Blair *et al.* 1997, Yu *et al.* 2000, Friesen *et al.* 2004). Both multidrug resistance proteins (MRPs) and glutathione-S-transferases require GSH for their catalytic activity, and both of these enzyme families have been associated with resistance of breast carcinoma against cytotoxic drugs (Huang *et al.* 2003, Flipits *et al.* 2005, Yang *et al.* 2005).

Table 5. Summary of human antioxidant and other cell redox state modulating enzymes (for references, see text and Human Protein Reference Database 2005).

Abbreviation	Enzyme/protein	Primary antioxidant function	Reaction	MW	Location
CAT	Catalase	Reduces hydrogen peroxide to water and oxygen	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	240 kDa	Peroxisome, cytoplasm
CuZnSOD	Copper/zinc-superoxide dismutase	Dismutates superoxide anions to hydrogen peroxide and molecular oxygen	$2 \text{O}_2^{\cdot -} + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	32 kDa	Cytoplasm
ECSOD	Extracellular superoxide dismutase	Dismutates superoxide anions to hydrogen peroxide and molecular oxygen	$2 \text{O}_2^{\cdot -} + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	135 kDa	Extracellular fluids
GLCL	Glutamate-cysteine ligase	Catalyzes the first production step of GSH	L-Glutamate + L-Cysteine \rightarrow γ -L-glutamyl-L-cysteine	103 kDa	
GSH	Glutathione	See below	See below		
GPx-1	Glutathione peroxidase 1	Reduces hydrogen peroxide (and lipid hydroperoxides), GSH as substrate	$2 \text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}$	84 kDa	Cytoplasm, mitochondrion
MnSOD	Manganese superoxide dismutase	Dismutates superoxide anions to hydrogen peroxide and molecular oxygen	$2 \text{O}_2^{\cdot -} + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	88 kDa	Mitochondrion
Prx I	Peroxioredoxin I	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	22 kDa	Nucleus
Prx II	Peroxioredoxin II	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	22 kDa	Cytoplasm
Prx III	Peroxioredoxin III	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	28 kDa	Mitochondrion
Prx IV	Peroxioredoxin IV	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	31 kDa	Cytoplasm, endoplasmic reticulum, lysosome
Prx V	Peroxioredoxin V	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	22 kDa	Mitochondrion, peroxisome
Prx VI	Peroxioredoxin VI	Reduces peroxides to corresponding alcohol	$2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O}$	25 kDa	Lysosome, cytoplasm
Trx I	Thioredoxin I	Reduces hydrogen peroxide to oxygen and water, electron donor to Prxs I-V and GPx	$\text{Trx}-(\text{SH})_2 + \text{X}-\text{S}_2 \leftrightarrow \text{Trx}-\text{S}_2 + \text{X}(\text{SH})_2$	12 kDa	Nucleus, cytoplasmic, extracellular space
TrxR I	Thioredoxin reductase I	Catalyzes the reduction of oxidized Trx	$\text{Trx}-\text{S}_2 + \text{NADPH} \leftrightarrow \text{Trx}-(\text{SH})_2 + \text{NADP}^+$	55 kDa	Plasma membrane, cytoplasm, extracellular space

2.4.9 Antioxidants in cancer therapy

The current opinion concerning the use of antioxidants in cancer therapy is still very ambiguous. Especially the debate concerning nutritional antioxidant supplementation versus cancer risk is widely discussed. A large body of epidemiological studies suggest an influence of abundant fruit intake in cancer prevention. Nevertheless, these studies have not been able to prove that the specifically high antioxidant contents would actually explain the results. It has been shown that most of the nutritional compounds usually considered antioxidants may actually act as pro-oxidants, depending on the oxidative status of the cell and also on genetic susceptibilities (Schwarz 1996, Palozza 1998, Forsberg *et al.* 2001, Seifried *et al.* 2003). These compounds include β -carotene, selenium, and the vitamins C and E. Although there is a lot of evidence to suggest that, under certain circumstances, antioxidants and conventional cancer therapies may be beneficial to patients, diverse results have also been published in large epidemiological studies (N Engl J Med. 1994; 330:1029-35, Prasad *et al.* 1999, Lamson *et al.* 1999, Richardson *et al.* 2000, Prasad *et al.* 2002). Ninety breast cancer patients treated with conventional cancer therapies and high-dose multivitamin substitution had shorter disease-free survival compared to patients given only standard therapies, but this association did not reach statistical significance ($p=0.08$) (Lesperance *et al.* 2002). In view of the fact that up to 60% of American women who received standard therapy for early-stage breast cancer in the mid-90's also used megavitamin therapy (Burststein *et al.* 1999), the recent results on the pro-oxidative effects of "antioxidants" may have clinical significance. To conclude, epidemiological trials together with a large body of *in vitro* experiments suggest that the optimal approach in preventing ROS-related cancer is to reduce both endogenous and exogenous sources of oxidative stress, rather than to attempt to increase the intake of antioxidants.

2.5 DNA repair mechanisms

Since the integrity of DNA is indispensable to an organism, and because both exogenous and endogenous sources perpetually generate DNA-damaging compounds, aerobic organisms have developed DNA damage repairing enzymes. Despite the multiplicity of potential damage, only a few enzymes are able to cover most oxidative DNA damage because of their multifunctionality (Demple & Harrison 1994, Cooke *et al.* 2003). Nucleotide excision repair and mismatch repair are considered the most important repair systems against ROS-derived damage, but base excision repair and double-strand break repair also play important roles (Harper 2000, Cooke *et al.* 2003).

Under normal circumstances, DNA repair systems are able to detoxify approximately 10,000 oxidative hits per day in a single human cell (Ames *et al.* 1993, Dreher & Junod 1996). The importance of single-enzyme activity is highlighted by several diseases with improperly working DNA repair enzymes. These diseases include xeroderma pigmentosum and Fanconi's anemia, which involve increased susceptibility to develop certain malignancies (Berneburg 2001, Grompe & D'Andrea 2001). It has been

demonstrated that H_2O_2 can prevent the proper functioning of DNA repair systems besides simultaneously bringing out new DNA defects (Hu *et al.* 1995).

2.5.1 Mismatch repair

Mismatch repair (MMR) is a collective term applied to several proteins that remove mispaired nucleotides or extra-helical loops of different sizes while DNA is being copied (Miturski *et al.* 2002, Bernstein *et al.* 2002). First, MMR recognizes the mismatch of a new strand by comparing it to the original, methylated strand. Then, GATC endonuclease cuts the damaged strand and the exonuclease enzyme digests the strand through mutation and thereby removes the defective DNA segment. The defect is then filled, usually by polymerase and ligase (Harper 2000, Miturski *et al.* 2002).

ROS-derived or other defects in MMR proteins give rise to microsatellite instability, which predisposes to cancer (Janatova & Pohlreich 2004). Mismatch repair protein 2 (MSH2) ^{-/-} mice developed normally and were fertile but showed significantly decreased lifespan due to a high frequency of T-cell lymphomas, intestinal tumors, and other malignancies (Reitmair *et al.* 1996). MSH6 ^{-/-} mice were likewise susceptible to lymphomas and gastrointestinal tumors, although they had longer survival than MSH2 mice (Edelmann *et al.* 1997). Hereditary nonpolyposis colorectal cancer syndrome (HNPCC or Lynch syndrome) is a disease caused by mutations in any of the mismatch repair proteins MLH3, PMS1, MSH2, MLH1, or MSH6 (Peltomäki 2001, Umar *et al.* 2004). Consequently, these tumors are genetically highly unstable and likely to acquire genetic mutations (Wei *et al.* 2002). HNPCC patients have a lifetime colorectal carcinoma risk of up to 74% and an endometrial cancer risk of approximately 42% (Watson & Lynch 2001). Breast tumors in HNPCC patients probably do not associate with this disease, although this is still under debate (Risinger *et al.* 1996, Vasen *et al.* 2001, Müller *et al.* 2002).

2.5.2 DNA topoisomerases

DNA topoisomerases (Top) are ubiquitous and highly conserved ATP-dependent enzymes able to cleave and re-ligate double-stranded DNA and thereby to catalyze changes in DNA topology (Kellner *et al.* 2002, Walker *et al.* 2004). Two topoisomerase isoforms, encoded by two different genes, have been characterized from human cells. Top type I cuts one strand of DNA, whereas top II cuts both strands to relax the coil and extend the DNA molecule. The topoisomerases II α and II β have high-degree homology, with some divergence in the C-terminal region. Since DNA replication and further cell proliferation are dependent on these enzymes, top II inhibitors have been under extensive research with regard to primary and adjuvant chemotherapy of breast cancer, although their side effects have restricted their use (Isaacs *et al.* 1995, Järvinen & Liu 2003).

Human DNA topoisomerase II-binding protein 1 (TopBP1) is a DNA damage response gene weighing 161 kDa, which binds the C-terminal region of TopII β . Following exposure to ionizing radiation, TopBP1 is phosphorylated and colocalizes at the sites of

DNA breaks (Xu *et al.* 2003). TopBP1 protein expression shows a peak in the S-phase, suggesting that TopBP1 is involved in the S-phase checkpoints and possibly also in DNA damage recognition (Yamane & Tsuruo 1999, Mäkineniemi *et al.* 2001).

2.6 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is the most active, specific, and potent mitogen for vascular endothelium (Gasparini 2000), and its expression correlates with high microvessel density (MVD) in breast cancer (Toi *et al.* 1996), which has been shown to be an independent prognostic factor for both disease-free survival and overall survival of breast carcinoma patients in many studies (Gasparini 2001). Apart from its vasculoproliferative effects, VEGF also inhibits apoptosis, regulates the tone of great vessels, and increases the production of proteolytic enzymes (Gasparini 2001). Six VEGF isoforms through different types of mRNA splicing have been identified up till now (Robinson & Stringer 2001).

2.6.1 VEGF receptors

VEGF binds three tyrosine-kinase receptors, Flk-1 (KDR/VEGFR2), Flt-1 (VEGFR1), and Flt-4 (VEGFR3), which play indispensable roles in angiogenesis and especially Flt-4 in lymphangiogenesis (Larrivee & Karsan 2000). Knock-out studies have demonstrated the crucial role of both of these receptors (Fong *et al.* 1995, Shalaby *et al.* 1995). Two clinical breast cancer studies of Flk-1 and Flt-1 inhibitors are currently in phase II and III trials (Schneider & Miller 2005). Especially Flt-1 is upregulated in many malignancies, including non-small cell lung carcinoma, pulmonary adenocarcinoma, prostate carcinoma, breast carcinoma, colon carcinoma, hepatocellular carcinoma, glioblastoma, multiple myeloma, and nephroblastoma (Volm *et al.* 1997, Takanami *et al.* 1997, de Jong *et al.* 1998, Hahn *et al.* 2000, Andre *et al.* 2000, Stefanik *et al.* 2001, Lin *et al.* 2002, Ghanem *et al.* 2003).

2.6.2 Angiogenesis and NO[•]

There is increasing evidence to suggest that NO[•] is the key mediator of tumor angiogenesis, i.e. the development of new blood vessels from pre-existing ones. Physiologically, angiogenesis occurs during wound healing, embryogenesis, and reproductive events (Ferrara 2000). However, angiogenesis is also an absolutely essential process for tumor progression, invasion, and metastasis. Breast and other tumors are unable to develop beyond the size of 1-2 mm³ without proper neovascularization (Zhang *et al.* 1995, Gasparini 2001), and there are a number of investigations showing that neutralization of angiogenic factors leads to efficient inhibition of tumor growth and metastasis (Hori *et al.* 1991, Kim *et al.* 1993, O'Reilly *et al.* 1997).

Ziche *et al.* have demonstrated inhibition of NOS by N^o-nitro-L-arginine methyl ester (L-NAME), leading to complete inhibition of angiogenesis in VEGF-transfected MCF-7 breast carcinoma cells, pointing out the fundamental role of NOSes in the VEGF pathway (Ziche *et al.* 1997). Both eNOS and iNOS knock-out mice showed impaired wound healing (Yamasaki *et al.* 1998, Lee *et al.* 1999), which was repairable by the supplementation of L-arginine or by iNOS gene transfection, respectively (Yamasaki *et al.* 1998, Murohara *et al.* 1998). Furthermore, NO^{*} functions *in vivo* by increasing vascular permeability and is also able to stimulate migration of endothelial cells, which, combined with its vasodilative effects, enables increased metabolism and nutritional supply to the tumor (Doi *et al.* 1996, Noiri *et al.* 1998). Thus, NO^{*}, or actually NO^{*} synthases, are indispensable in angiogenesis and consequently also in all steps of carcinogenesis. Many studies have shown positive associations between the levels of either NOSes or nitrotyrosine and vascularization in human breast cancer (Vakkala *et al.* 2000, Samoszu *et al.* 2002).

3 Aims of the study

This thesis was undertaken

1. To investigate Prx I-VI expression in breast carcinoma cells and its correlation with clinicopathological parameters, such as TNM classification, hormone receptor status, and survival in breast cancer patients
2. To find out how nuclear and cytosolic Trx and TrxR expression relates to the expression of NF- κ B, p53, cell proliferation, estrogen, and progesterone receptor status and survival in breast carcinoma patients
3. To evaluate whether *in situ* and invasive breast tumors express GLCL subunits, and whether they associate with the standard clinicopathological parameters of breast carcinoma
4. To study the expression NO \cdot synthesis and the extent of NO \cdot -derived damage in breast carcinomas, and whether elevated NO \cdot levels have an impact on antioxidant enzyme levels
5. To explore the relations between NO \cdot synthesis and angiogenesis in breast cancer and their probable connections with such parameters as TNM classification, patient survival, tumor differentiation, and cell proliferation
6. To elucidate the extent of ROS-derived damage in pre-invasive lesions and early-stage breast carcinomas, and whether this damage associates with the expression of DNA repair enzymes

4 Materials and methods

4.1 Study material

Study I originally included 642 and in study IV 485 breast cancer lesions diagnosed during the years 1979-2001. The samples had been fixed in 10% formalin and embedded in paraffin blocks. From each block, a representative tumor region was chosen and integrated into multitissue microarray blocks with Beecher Instruments Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD, USA). The microarray sample diameter was 1300 μm . Part of the original samples was lost in both studies due to exhaustion of the microarray blocks, detachment of the samples during the staining and presence of non-representative areas in punch samples. This explains the difference in the number of lesions between the studies I and IV, although the same microarray blocks were principally used.

In study II, the material consisted of 303 breast cancer samples in whole paraffin blocks, originally obtained between the years 1981 and 1998. Whole paraffin blocks were also used in study III (n=274), and these tumors derived from the years between 1981 and 1997. The material overlapped partially between studies II and III. The material of study V consisted of 80 T1N0 breast cancer lesions in standard paraffin blocks. These tumors were also included in the microarrays used in studies I and IV. All samples were randomly obtained from the files of the Department of Pathology, Oulu University Hospital.

Histopathological typing for invasive carcinomas was done according to Elston & Ellis and that for *in situ* carcinomas according to Holland *et al.*; the distribution of the cases is summarized in Table 6. When the material was divided into two subgroups, i.e. tumors diagnosed in or before 1987 and tumors diagnosed after 1987, the percentage of T1 tumors was 24.1% in the earlier group and 35.7% in the latter subgroup. The corresponding results for T2 tumors were 47.8 and 44.6%, those for T3 tumors 13.4% and 15.5%, and those for T4 tumors 14.7% and 4.2%, respectively. In study II, 36 of the tumors were *in situ* lesions, while in study III 37 lesions were classified as T_{IS} according to the TNM classification of breast tumors, which was used for tumor staging in all studies (Sobin & Wittekind 2002). There were 26 *in situ* breast carcinomas and 12 benign

breast tissue hyperplasias in material of study V. Twelve of the *in situ* tumors were low grade (gr I-II) and 13 high grade (gr III). 80 T1N0 tumors were also analyzed in study V. All clinical data, such as tumor size, presence of metastases, and patient survival, were obtained from hospital records.

Table 6. Clinical characteristics of patients included in the thesis.

Study	I	II	III	IV	V
Number of patients	642	303	274	485	118
Technique	Microarray	Standard	Standard	Microarray	Standard
Median age	57.7	58.0	58.0	56.0	58.5
Tumor size					
T _{1s}	0	36	37	0	26 *
T ₁	98	63	58	85	80
T ₂	186	106	115	135	0
T ₃	46	34	34	38	0
T ₄	31	14	16	22	0
Nodal status					
N ₀	189	112	150	138	80
N ₁	187	87	98	128	0
N ₂	12	12	10	7	0
N ₃	7	2	2	6	0
Distant metastases					
M ₀	341	200	260	164	80
M ₁	20	13	14	14	0
Histological grade					
I	36	26	26	21	10
II	191	85	96	91	27
III	148	83	77	100	17
Histological diagnosis (invasive lesions)					
Ductal	451	228	204	336	63
Lobular	92	29	32	52	10
Other	40	10	14	33	7

* Additionally, 12 benign breast hyperplasias were included in the material.

4.2 Methods

4.2.1 Assessment of ROS damage

Immunohistochemical antibodies against 8-OHdG, HNE, and nitrotyrosine were used when evaluating the extensiveness of ROS damage in studies IV and V. Four to five μm paraffin sections were cut from the specimens and placed either on SuperFrostPlus glass

slides (Menzel-Gläser, Germany) or on silane-treated glass slides. The slides were deparaffinized in xylene and rehydrated through a descending ethanol series. Sections were then placed in 10 mM citric acid monohydrate and boiled for 10 min in a microwave oven, after which they were properly cooled at room temperature. In order to consume endogenous peroxidase activity, the sections were immersed in 0.1% or 3% hydrogen peroxide in methanol for 15 minutes. A more detailed summary of immunohistochemical antibodies, secondary antibodies, manufacturers, and dilutions is shown in Table 7. The negative controls consisted of phosphate-buffered saline (PBS) (pH 7.2) and mouse/rabbit/goat immunoglobulin isotypes.

The antibodies used in study V, 8-OHdG, HNE, nitrotyrosine, MMR proteins, and TopBP1, were subclassified into four groups according to the extent and intensity of immunostaining in tumor cells.

Table 7. Details of antigens, antibodies, and immunohistochemical methods used in the immunohistochemical studies.

Antigen	Study	Antibody	Dilution	Immunostaining method	Source of primary antibody
Prx I	I	Rabbit polyclonal Prx I antibody	1:1500	Histostain-Plus Bulk Kit	A gift from S.W. Kang (Ewha Women's University, Seoul, Korea)
Prx II	I	Rabbit polyclonal Prx II antibody	1:1000	Histostain-Plus Bulk Kit	- "
Prx III	I	Rabbit polyclonal Prx III antibody	1:500	Histostain-Plus Bulk Kit	- "
Prx IV	I	Rabbit polyclonal Prx IV antibody	1:1000	Histostain-Plus Bulk Kit	- "
Prx V	I	Rabbit polyclonal Prx V antibody	1:2000	Histostain-Plus Bulk Kit	- "
Prx VI	I	Rabbit polyclonal Prx VI antibody	1:2000	Histostain-Plus Bulk Kit	- "
Ki-67	I, IV, V	Mouse monoclonal anti-human Ki-67 antibody	1:50	Avidin-biotin-peroxidase complex method; 3,3'-diaminobenzidine as the chromogen	Zymed Laboratories Inc., South San Francisco, CA, USA
ER	I-IV	Mouse monoclonal estrogen receptor antibody	1:100	Histostain-Plus Bulk Kit	Novocastra Laboratories Ltd, Newcastle upon Tyne, UK
PR	I-IV	Mouse monoclonal progesterone receptor antibody	1:100	Histostain-Plus Bulk Kit	Novocastra Laboratories
c-erbB2	I, IV	Mouse monoclonal anti-human c-erbB2 antibody	1:500	Histostain-Plus Bulk Kit	Novocastra
Trx	II	Goat polyclonal anti-human Trx antibody	1:200	A biotinylated secondary anti-goat antibody; avidin-biotin-peroxidase complex	American Diagnostica, Greenwich, CT
TrxR	II	Rabbit polyclonal anti-human TrxR antibody	1:1000	A biotinylated secondary anti-rabbit antibody; avidin-biotin-peroxidase complex	See Luthman & Holmgren 1982
NF-κB	II	Rabbit polyclonal antibody to the p50 subunit of the protein	1:200	A biotinylated secondary anti-rabbit antibody; avidin-biotin-peroxidase complex	Santa Cruz Biotechnology Inc., CA, USA
p53	II, III	Rabbit polyclonal antibody CM1	1:1000	A biotinylated secondary anti-rabbit antibody; avidin-biotin-peroxidase complex	Novocastra
PCNA	II, III	Mouse monoclonal antibody PC10	1:50	A biotinylated secondary anti-mouse antibody; avidin-biotin-peroxidase complex	Dako, Glostrup, Denmark

Table 7. Continued.

Antigen	Study	Antibody	Dilution	Immunostaining method	Source of primary antibody
Ki-67	I, IV, V	Monoclonal mouse Ki-67 antibody	1:50	A biotinylated secondary anti-mouse antibody; avidin-biotin-peroxidase complex	Zymed Laboratories Inc.
GLCL-c	III	Rabbit polyclonal GLCL-c antibody	1:1000	Histostain-Plus Bulk Kit	A gift of professor T. Kavanagh (University of Ontario, Toronto, Canada)
GLCL-r	III	Rabbit polyclonal GLCL-c antibody	1:1000	Histostain-Plus Bulk Kit	- "
iNOS	IV	Rabbit polyclonal iNOS antibody	1:200	Histostain-Plus Bulk Kit	Santa Cruz
eNOS	IV	Rabbit polyclonal eNOS antibody	1:50	Histostain-Plus Bulk Kit	Santa Cruz
nNOS	IV	Rabbit polyclonal nNOS antibody	1:200	Histostain-Plus Bulk Kit	Santa Cruz
Nitrotyrosine	IV, V	Rabbit polyclonal nitrotyrosine antibody	1:100	Histostain-Plus Bulk Kit	Upstate, NY, USA
MnSOD	IV	Rabbit polyclonal MnSOD antibody	1:1000	Histostain-Plus Bulk Kit	A gift from J. D. Crapo (National Jewish Medical and Research Center, Denver, CO, USA)
Catalase	IV	Rabbit polyclonal catalase antibody	1:200	Histostain-Plus Bulk Kit	A gift from J. D. Crapo
VEGF	IV	Mouse monoclonal VEGF antibody	1:250	Histostain-Plus Bulk Kit	Santa Cruz
Flk-1		Rabbit polyclonal Flk-1 antibody	1:100	Histostain-Plus Bulk Kit	Santa Cruz
Flt-1		Goat polyclonal Flt-1 antibody	1:250	A biotinylated secondary antibody (Zymed), enzyme conjugate by DAKO	Santa Cruz
HNE	V	Rabbit polyclonal HNE antibody	1:250	A biotinylated secondary antibody and enzyme conjugate, both from Zymed	Calbiochem, CA, USA
8-OHdG	V	Mouse monoclonal 8-OHdG antibody	1:125	Dako Envision Kit	Gentaur, Brussels, Belgium
TopBP1	V	Mouse monoclonal TopBP1 antibody	1:100	Dako Envision Kit	Becton, Dickinson and Company Biosciences, NJ, USA
MSH2	V	Mouse monoclonal MSH2 antibody (ab-2)	1:200	Dako Envision Kit	OncogeneScience, MA, USA
MSH6	V	Mouse monoclonal MSH6 antibody	1:250	Dako Envision Kit	BD Biosciences, CA, USA

4.2.2 Detection of antioxidative and other redox-regulating enzymes

In addition to the immunohistochemical methods, Western blotting and immunoelectron microscopy were used in the assessment of antioxidative enzymes. Immunostaining of antioxidative and other redox-regulating enzymes was performed as described in the previous section and in Table 7. In study I, Prx immunostaining reactions were further classified into four subgroups according to the intensity of immunostaining. In study IV, catalase and MnSOD immunostaining was subclassified into three different groups according to intensity.

In studies II and III, the staining intensity of Trx, TrxR, GLCL-c, and GLCL-r in tumor cells was assessed and divided into four subgroups: 1=weak cytoplasmic staining intensity, 2=moderate cytoplasmic staining intensity, 3=strong cytoplasmic staining intensity, and 4=very strong cytoplasmic staining intensity. In study II, the intensity of nuclear immunostaining was similarly evaluated. In these studies, the quantity of the immunostaining was also assessed, and the results were further divided into five subgroups according to the percentage of tumor cells showing positivity to the studied antibody. A combined score for immunostaining based on both qualitative and quantitative immunostaining was then reached by summing up the qualitative and quantitative scores. This total score was finally divided into four main groups in study II and into three main groups in study III.

4.2.2.1 Western blotting

In order to detect GLCL in non-tumorous and tumorous tissues by Western blotting in study III, the tissue samples had been frozen in liquid nitrogen. They were first removed into ice-cold PBS (pH 7.4) and then homogenized, sonicated, and centrifuged at 4000 rpm for 15 min. The protein concentration of the samples was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 75 µg of denatured protein was loaded per lane onto 12% sodium dodecyl sulfate polyacrylamide gel. The gel was then electrophoresed for 1.5 hours at 90 V, and the proteins were transferred (45 min, 100 V) onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom) in a Mini-PROTEAN II Cell (Bio-Rad). The blotted membrane was treated with antibodies against GLCL-c (dilution 1:30000) and GLCL-r (1:20000) followed by incubation with rabbit secondary antibody (Amersham) conjugated to horseradish peroxidase (1:20000). The detection of proteins was done with an ECL system (Amersham), and luminol excitation was imaged on X-ray film (Biomax MR; X-Omat). β -actin was used as control.

4.2.2.2 Immunoelectron microscopy

The Trx and TrxR subcellular locations were investigated with immunoelectron microscopy (unpublished data). Fresh samples from three breast carcinomas were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer with 2.5% sucrose, pH 7.4, for 2 hours.

Small tissue pieces were immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with Leica Ultracut UCT microtome. For immunolabeling, the sections were first incubated in 0.05 M glycine in PBS followed by incubation in 5% BSA with 0.1% CWFS (cold water fish skin) gelatin (Aurion, Wageningen, The Netherlands) in PBS. Antibodies and gold conjugate were diluted in 0.1% BSA-C (Aurion) in PBS. All washings were performed in 0.1% BSA-C in PBS. The sections were then incubated with antibody to Trx and TrxR for 60 min followed by protein A-gold complex (size 10 nm) for 30 min, made according to Slot and Geuze (Slot & Geuze 1985). The controls were prepared by carrying out the labeling procedure without primary antibody. The sections were embedded in methylcellulose and examined under a Philips CM100 transmission electron microscope.

4.2.3 Detection of other studied proteins

Immunostainings of other than redox-regulating enzymes in studies I-V were performed as described above in section 4.2.1 and in Table 7. Additionally, in order to minimize background staining, the NF- κ B (study II) and VEGF (study IV) sections were blocked with 2% non-fat dry milk after citric acid treatment. The presence or abundance of estrogen and progesterone receptors was likewise assessed immunohistochemically in studies I-IV, as above, excluding citric acid treatment. In each case, the percentage of positively stained nuclei was evaluated, after which a percentage of positively stained tumor cells for estrogen and progesterone receptors were obtained.

Monoclonal mouse anti-human Ki-67 antibody (Zymed Laboratories Inc.) was used to study cell proliferation in studies I, IV and V (Gerdes *et al.* 1983). Immunostaining was performed as described above, except that the avidin-biotin-peroxidase complex method was used, and 3,3'-diaminobenzidine was used as the chromogen. The results were evaluated as a percentage of positive cells of the whole cell population.

NF- κ B immunoreactivities in the samples were assessed semiquantitatively, as previously described, for Trx and TrxR by grading both the staining intensity of tumor cells and the extent of positive staining of tumor cells. The combined score was then divided into four main groups: negative (-), weak (+), moderate (++), and strong (+++). The p53 staining reactions were divided into two groups; less than 5% positive and more than 5% positive. Similarly, PCNA stainings were divided into less than 10% positive and more than 10% positive. c-erbB2 immunostaining was classified as either positive or negative.

In study IV, the staining reaction of nitrotyrosine, NOS, VEGF, Flt-1, and Flk-1 in tumor cells was classified into three subgroups: no immunostaining present, weak or strong immunostaining.

4.2.4 Statistical analysis

SPSS versions 10.1.4 and 12.0.0 for Windows (Chicago, IL, USA) were used for statistical analysis. The significance of the associations was determined using Fisher's

exact probability test, two-tailed *t*-test, correlation analysis, and Cox multivariate regression analysis. Levene's test was used to determine when the Mann-Whitney test was required. In survival analysis, Kaplan-Meier curve was used and significance was measured by the log-rank, Breslow, and Tarone-Ware tests. Probability values $p \leq 0.05$ were considered to be statistically significant.

5 Results

5.1 Staining patterns of the studied antigens

In study I, the highest Prx levels in non-malignant tissue were observed in acinar and ductal cells. Cells of larger ducts were mainly negative for all Prx isoforms. In tumor cells, Prxs showed mainly cytoplasmic immunoreactivity, but nuclear positivity was also seen. Prx I-VI stainings were considerably stronger in malignant tumors than in benign lesions. This trend was most noticeable with Prx IV, where no staining in healthy tissue could be found. In malignant tissue, however, the vast majority (93.8%) of the samples stained positively.

In study II, Trx and TrxR staining was both cytoplasmic and nuclear. The expression seen in benign tissue varied and was much stronger in some places than in neighboring neoplastic tissue. Leukocytes, fibroblasts, and endothelial cells did not express Trx, whereas macrophages showed occasional positivity. Endothelial cells, reactive fibroblasts, and macrophages were sometimes positive to TrxR. *In situ* carcinomas expressed nuclear Trx more than invasive lesions. Trx staining was significantly stronger in ductal carcinomas than in lobular carcinomas ($p=0.03$). No differences emerged between TrxR expression and ductal, lobular, or *in situ* carcinomas.

In study III, sclerosing adenosis and stromal fibroblasts had a weaker staining pattern than neoplastic cells. Apocrine metaplasia and endothelial cells were usually strongly positive for both GLCL-c and GLCL-r. The cytoplasm of tumor cells showed diffuse positivity for GLCL-c and GLCL-r. Both GLCL-c and GLCL-r overexpressed in lobular tumors compared to other histological diagnoses ($p=0.050$ and $p=0.046$, respectively).

In study IV, the expression of NOSes and catalase was mainly cytoplasmic in tumor cells, as could be expected. As a mitochondrial enzyme, MnSOD showed granular cytoplasmic immunostaining. Nitrotyrosine and HNE showed diffuse cytoplasmic expression in both neoplastic and non-neoplastic cells, whereas 8-OHdG had a nuclear staining pattern. TopBP1 and MMR proteins were found predominantly in nuclei in both invasive and pre-invasive lesions.

5.2 Immunoelectron microscopy

Immunoelectron microscopy was studied in three breast carcinoma samples to reveal the subcellular distribution of Trx and TrxR (unpublished data). Two of the samples were ductal and one lobular. Two carcinomas expressed simultaneous labeling for both Trx and TrxR, while one was negative. In immunoelectronmicroscopy, labels for Trx and TrxR could be seen in the cytoplasm but also in the nuclei of neoplastic breast carcinoma cells, which is in line with their immunohistochemical localization. Cytoplasmic Trx labeling was frequently seen in association with cytoplasmic filaments, while TrxR labeling was preferentially observed near cellular junctions. In some cells, extracellular Trx reactivity was also observed, suggesting some kind of secretory activity of tumor cells for Trx. No labeling was seen in mitochondrial structures with either Trx or TrxR.

5.3 Immunoreactivity for studied antibodies

In study I, non-malignant lesions expressed lower levels for all Prx isoforms than invasive lesions. The highest Prx levels in non-carcinomatous tissues were detected in acinar and ductal cells. Prx IV was completely undetectable in healthy breast tissue, whereas invasive lesions stained most intensively for it in study I, 93.7% of studied carcinomas being at least weakly positive (n=447). Prxs I (83.2%; n=475), III (89.0%; n=446), and V (79.8%; n=481) were also expressed in invasive lesions to a distinctly higher degree than Prx II and VI, of which only 59.0% and 52.6% showed positivity (n=458 and 380, respectively). There was a significant association between the expression rates of Prx III and Prx IV ($p=0.00003$) as well as between Prx III and VI ($p=0.002$).

Trx was present in cytoplasm in 75% and in nucleus in 67% of the *in situ* tumors. The respective figures for TrxR were 50% and 4% (n=28). Trx was expressed in cytoplasm in 67% and in nucleus in 59% of the studied invasive lesions (n=192). TrxR was expressed in cytoplasm in 55% of the carcinoma cases, but in nucleus only in 6% (n=221). Cytoplasmic and nuclear Trx immunoreactivity as well as cytoplasmic Trx and cytoplasmic TrxR immunoreactivity were associated when all studied lesions were combined ($p<0.001$ and $p=0.003$, respectively). Both cytoplasmic and nuclear NF- κ B was seen in 42% of carcinoma cases (n=105). They correlated significantly with each other ($p<0.001$).

78% of *in situ* carcinomas expressed GLCL-c and 51% GLCL-r (n=33 and n=35, respectively). Moreover, it is worth noting that all studied LCIS lesions expressed GLCL-c, and 7/9 were also positive for GLCL-r. 50% of the studied invasive lesions were GLCL-c-positive (n=260) and 44% GLCL-r-positive (n=272), and they also associated significantly with each other ($p=0.002$). GLCL expression was also investigated with Western blot analysis, using a single sample of each mucinous, medullary, lobular, and ductal carcinoma. Four cases of healthy breast tissue were also included. The intensity of both GLCL subunits appeared to be stronger in carcinomas compared to normal tissues in three out of four cases in study III. The mean immunoreactivity of GLCL-c (n=4) was

32% higher and that of GLCL-r (n=4) 14% higher in cancer tissue than in control samples.

Only invasive lesions were included in study IV. cNOSes (eNOS 71.8%, n=305; nNOS 43.7%, n=270) were expressed with a higher intensity than iNOS (24.1%, n=307). Nitrotyrosine expression was observed in 56.1% of cases (n=319). MnSOD and catalase were expressed in 34.0% (n=259) and in 50.7% (n=357) of the lesions. Expression of VEGF (n=236), Flk-1 (n=215), and Flt-1 (n=231) was seen in 52.4%, 84.1%, and 4.3% of the lesions, respectively. The three NO^{*} synthases associated significantly with each other (iNOS vs. eNOS, p=0.003; iNOS vs. nNOS, p=0.003, eNOS vs. nNOS p<0.0005), and furthermore, the associations between NOSes and nitrotyrosine were very strong (for all p<0.0005). MnSOD associated with nitrotyrosine (p=0.04) and eNOS (p=0.001) and catalase (p=0.002). Instead, catalase had strong associations with all NOSes (with iNOS p=0.01, nNOS p<0.0005, eNOS p=0.002) and also with nitrotyrosine (p<0.0005). Catalase was also overexpressed in the presence of Flk-1 (p=0.01). There was a general tendency for NOSes and nitrotyrosine to be overexpressed in VEGF and Flk-1-positive cases, although in the case of VEGF, the associations were not statistically significant. However, Flk-1 positivity was convincingly connected with the presence of eNOS (p=0.03) and nNOS (p=0.004), and nitrotyrosine also tended to be overexpressed in Flk-1-positive cases, although not significantly (p=0.06). VEGF expression associated positively with Flk-1 (p=0.02) and negatively with Flt-1 (p=0.03) expression.

Oxidative damage markers in study V, 8-OHdG, nitrotyrosine, and HNE showed increasing expression from benign hyperplasias through *in situ* carcinomas to T1N0 tumors. TopBP1 expression also increased with cancer development. However, due to the relatively small material in the non-invasive groups, some of these differences failed to reach statistical significance. *In situ* tumors showed significantly more intensive staining for nitrotyrosine than hyperplasias (p=0.011), and T1N0 lesions expressed more nitrotyrosine than hyperplasias, respectively (p=0.003). HNE expression was seen in 91.8% of T1N0 tumors (n=80). Nitrotyrosine was also expressed widely, 93.5% of invasive lesions being positive. Topoisomerase B1 binding protein immunostaining in the nuclei of invasive carcinomas was observed in 77.2% and 8-OHdG in 67.4%, with only one sample showing strong positivity.

MSH2 was constantly positive in the nuclei of hyperplasias and *in situ* tumors. MSH6 was present in 82.8% of hyperplasias and 84.6% of all *in situ* tumors. In stage I tumors, MSH2 and MSH6 showed positivity in 59.5% and 56.5% of the studied lesions. Expression of MSH6 was significantly (p=0.014) increased in *in situ* compared to T1N0 tumors.

In the T1N0 cohort, TopBP1 expression associated with 8-OHdG (p=0.008) but not with nitrotyrosine or HNE. 8-OHdG also associated with nitrotyrosine positivity (p=0.041). HNE and nitrotyrosine had a very convincing association, as 88% of nitrotyrosine-negative lesions did not express HNE, either, whereas 61% of nitrotyrosine-positive lesions were also HNE-positive (p<0.0005). MSH2 and TopBP1 co-expressed significantly (p=0.039), and the MSH2 and MSH6 immunostaining results also associated convincingly (p<0.0005).

5.4 Clinicopathological parameters versus expression of studied antibodies

Prxs I and II had no associations with the clinicopathological parameters covered in study I (Table 8.). However, Prx II tended to be more intensively expressed in poorly differentiated tumors (grade III), although this association did not reach statistical significance ($p=0.07$). Prxs III, IV, and V (for all $p=0.03$) were overexpressed in high-grade tumors. Furthermore, Prx V correlated significantly with larger tumor size ($p=0.05$) and positive nodal status ($p=0.04$). Prxs III, IV, and VI associated with progesterone receptor positive cases, the corresponding p -values being 0.02, 0.009 and 0.036. Prx III also showed stronger expression in estrogen receptor-positive tumors ($p=0.033$). Prxs III and IV associated with prolonged survival ($p=0.0005$ for Prx III and $p=0.01$ for Prx IV). Patients with Prx V immunonegativity had better prognosis than those with Prx V expression ($p=0.04$). In Cox regression analysis, no Prx showed any significant independent prognostic value. The ages of the patients did not associate with Prx expression. No associations between Prx immunostaining and c-erbB2 or cell proliferation rate were observed.

The same material was used in studies I and IV, which made it possible to study the associations between Prx family and catalase, MnSOD, VEGF and its receptors, NOSes, and nitrotyrosine (unpublished data). Prx I had a significant connection with higher MnSOD levels ($p=0.02$). Prx V was overexpressed in catalase-positive lesions, the p -value being 0.04. Prxs II and VI correlated inversely with catalase expression ($p=0.006$ and $p=0.03$, respectively). Prx II immunostaining associated with Flt-1 ($p=0.01$) and Prx IV with both Flt-1 ($p=0.04$) and Flk-1 ($p=0.008$). No connections between Prxs and VEGF were found. Finally, Prx III had a strong association between nitrotyrosine and nNOS expression ($p=0.003$ and $p=0.007$, respectively).

Nuclear Trx positivity associated with larger primary tumor size ($p=0.027$). It also was connected with positive hormone receptor status (with ER $p=0.001$ and with PR $p=0.05$). Cytoplasmic ($p=0.026$) and nuclear ($p=0.007$) Trx expression associated with accelerated cell proliferation rate, as measured in study II with PCNA. Furthermore, nuclear Trx and TrxR expression was stronger in p53-positive cases ($p=0.028$ and 0.021, respectively). No associations between Trx or TrxR and NF- κ B or survival were found in study II. However, TrxR-positive patients had a shorter disease-free interval (for cytoplasmic TrxR $p=0.016$ and for nuclear TrxR $p=0.003$). Nuclear and cytoplasmic NF- κ B expression associated inversely with p53 expression and nuclear NF- κ B with reduced cell proliferation.

In the GLCL study (III), GLCL-c showed significantly stronger expression in invasive carcinomas with negative nodal status ($p=0.013$). No other associations with TNM-classification, histological grade, survival, hormone receptor status, cell proliferation, or p53 positivity were observed. Instead, nuclear NF- κ B was strongly overexpressed in GLCL-c-positive invasive tumors ($p=0.004$) (unpublished result). GLCL-c and Flk-1 also had a significant association ($p=0.028$) (unpublished result). Invasive carcinomas showing either GLCL-c or GLCL-r positivity or both had better survival than those without GLCL expression ($p=0.037$, log rank; $p=0.041$, Breslow; $p=0.037$, Tarone-Ware). GLCL-r or GLCL-c immunoreactivity did not associate with disease-free interval of the

patients ($p=0.28$ and $p=0.55$, respectively). According to the Cox regression model, GLCL-c and GLCL-r expression did not have independent prognostic significance.

eNOS was the only NOS associated with the examined clinicopathological variables. It was overexpressed in tumors of at least T2 size ($p=0.005$) and had a near-significant inverse association with nodal status ($p=0.051$). There was also increased nitrotyrosine and VEGF synthesis in the largest tumors (for both $p=0.046$). In the same material, higher Flk expression was observed among cases with N1-3, and Flk-1 also had a near-significant positive association with tumor grade ($p=0.082$; unpublished data). Catalase expression associated inversely with hormone receptor status (for ER $p<0.0005$, for PR $p=0.003$; unpublished data).

8-OHdG had a near-significant association with increased cell proliferation ($p=0.054$). No significant associations with histological diagnosis, hormone receptors, histological grade, survival, or disease-free interval were found in study V in the T1N0 material.

5.5 Oxidative stress versus antioxidant enzyme expression in stage I breast lesions

Sixty-two of the microarray breast samples used in study I overlapped with the T1N0 material used in study V. Therefore, it was possible to compare the immunostainings of HNE, 8-OHdG, nitrotyrosine, TopBP1, and MMR proteins with Prx expression (unpublished data). Of these, an inverse association between Prx V and HNE expression was statistically significant ($p=0.042$, unpublished result). Respectively, 49 of the lesions used in study IV overlapped the T1N0 blocks of study V. No significant differences were found when studying this integrated material. However, it is worth noting that even if there would had been significant associations between these parameters, they would probably not have been manifested in such a narrowly overlapping material. For instance, only 5 of 49 Flk-1 lesions were available in the T1N0 material. This is due to the previously described methodological problems of the analysis of microarray blocks.

Table 8. Studied redox-regulating proteins compared to clinicopathological parameters. Figures represent p-values between studied parameters. Abbreviations: cytopl. = cytoplasmic expression, NS = no significance. * indicates inverse associations; ** indicates when PCNA is preferred in cell proliferation determination.

Study	T	N	M	Hist. grade	ER	PR	Survival (log-rank)	Ki-67/PCNA**	c-erbB2	p53	Disease-free interval	NF-κB cytopl.	NF-κB nuclear
Prx I	NS	NS	NS	NS	NS	NS	NS	NS	NS				
Prx II	NS	NS	NS	NS	NS	NS	NS	NS	NS				
Prx III	NS	NS	NS	0.033	0.033	0.020	<0.0005	NS	NS				
Prx IV	NS	NS	NS	0.027	NS	0.009	0.013	NS	NS				
Prx V	0.051	0.038	NS	0.031	NS	NS	0.04*	NS	NS				
Prx VI	NS	NS	NS	NS	NS	0.036	NS	NS	NS				
iNOS	NS	NS	NS	NS	NS	NS	NS	NS	NS				
eNOS	0.005	NS	NS	NS	NS	NS	NS	NS	NS				
nNOS	NS	NS	NS	NS	NS	NS	NS	NS	NS				
Catalase	NS	NS	NS	NS	0.000*	0.003*	NS	NS	NS				
MnSOD	NS	NS	NS	NS	NS	NS	NS	NS	NS				
Cytopl. Trx	NS	NS	NS	NS	NS	NS	NS	0.026**		NS	NS	NS	NS
Nuclear Trx	0.027	NS	NS	NS	0.001	0.050	NS	0.007**		0.028	NS	NS	NS
Cytopl. TrxR	NS	NS	NS	NS	NS	NS	NS	NS**		NS	0.016*	NS	NS
Nuclear TrxR	NS	NS	NS	NS	NS	NS	NS	NS**		0.021*	0.003*	NS	NS
GLCL-c	NS	0.013	NS	NS	NS	NS	NS	NS**		NS	NS		0.004
GLCL-R	NS	NS	NS	NS	NS	NS	NS	NS**		NS	NS	NS	NS

5.6 Summary of the main results

Several Prx isoforms, most notoriously Prxs III-V, associated with higher histological grade, hormone receptor positivity, and TNM classification. Although Prxs III and IV were significantly connected with improved survival and Prx V with poorer prognosis, these results were not independent prognostic factors.

Both nuclear and cytoplasmic thioredoxin positivity associated with enhanced cell proliferation. Nuclear Trx was also overexpressed in cases with positive hormone receptor status, and it similarly associated with high p53 expression. Both cytoplasmic and nuclear TrxR positive tumors had a shorter disease-free interval than those with negative immunostaining.

GLCL-c and GLCL-r expression was pronounced in *in situ* and lobular invasive carcinomas. Overall GLCL expression associated with prolonged survival, but was not an independent prognostic factor.

The expression rates of the three NOSes and nitrotyrosine were closely associated. Catalase and MnSOD were, for the first time, demonstrated to associate with NO[•]-derived damage.

Oxidative protein damage was found to be significantly higher in invasive lesions than in pre-invasive lesions, and furthermore, 8-OHdG and HNE showed increasing expression from benign hyperplasias through *in situ* carcinomas to invasive stage I tumors. There was a simultaneous loss of both MSH2 and MSH6 in stage I breast carcinoma compared to pre-malignant lesions.

6 Discussion

6.1 Significance of oxidative stress in breast cancer initiation, promotion, and progression

Sufficient understanding of cancer pathogenesis is essential for the development of anti-cancer treatments and efficient preventive methods. Cancer is nowadays widely attributed to DNA degeneration, the incidence of which exponentially increases when the population ages and when damage, principally ROS-derived, accumulates in the genome. It takes approximately 5 to 7 mutations of normal epithelium to develop colorectal carcinoma (Kinzler & Vogelstein 1996), in which area research has been most intensive. However, this hypothesis is probably also applicable to other types of carcinoma, including breast cancer (Ingvarsson 1999, Pavelic & Gall-Troselj 2001). Oxidative or nitrosative stress may play a part in any of these steps, via the mechanisms of increased ROS production, impaired antioxidative systems, or insufficient DNA repair enzyme capacity. On the other hand, elevated ROS levels may mutate the genes of antioxidant enzymes or DNA repair enzymes. When interacting with cellular membranes, ROS produce lipid peroxidation products such as HNE and MDA, which are genotoxic at high levels. ROS may also promote carcinogenesis by inducing angiogenesis or by overactivating transcription factors such as AP-1, NF- κ B, or protein kinase C. ROS-derived induction and activation of matrix metalloproteinases have also been recently observed (Zhang *et al.* 2002, Paquette *et al.* 2005).

6.2 Problems in measuring the redox status and antioxidant levels of tumors

Mainly because of the short lifetime of the radicals and their cross-reactions with other redox-regulating components, it is challenging to determine their concentrations directly *in vivo*. Immunohistochemical visualization of oxidative damage “footprints”, such as 8-OHdG and nitrotyrosine, is usable in this respect and also more widely used. However, it

is worth noting that some nitrotyrosine formation also occurs in physiological conditions, and that there are a few ONOO⁻-independent pathways to nitrotyrosine production. This lack of specificity has been considered the main problem when using markers of this type (Allred *et al.* 1998, Davis *et al.* 2001).

Also, the specificity of 8-OHdG formation in DNA damage with the current methods has lately been questioned (Halliwell 2000, Seifried 2003). 8-OHdG formation may partly be an artifact of guanine oxidation during DNA isolation or analysis (Halliwell 2000). Many oncological studies have measured urinary 8-OHdG, which represents the total balance of regular and repaired guanine oxidation in the past, not the real-time redox state of cancer cells (Seifried *et al.* 2003).

Some problems are also implicit in the immunohistochemical assessment of antioxidant enzyme levels. For instance, enzyme expression levels do not necessarily correlate with enzyme activity (Frank *et al.* 2000). Again, it is impossible to visualize the distribution of the enzymes in the different cellular compartments with standard immunohistochemistry, although this would be of great importance in many cases (reviewed by Kinnula & Crapo 2004). However, immunoelectron microscopy methods are increasingly used. Nor do other available methods, such as measurement of antioxidant serum levels, offer any better general view of the tumor redox state. *In vitro* gene transfers into malignant cells are not particularly accurate, either, since a single overexpressive gene probably has an effect on the whole pro-oxidant/antioxidant system (Kinnula & Crapo 2004). Depressed levels of several antioxidant enzymes have been observed in tumor cell cultures after isolating them from the primary tumor, which complicates the generalization of the results from *in vitro* to *in vivo* studies (Sun *et al.* 1989, Kinnula *et al.* 1992). Many other differences should also be taken into account when assessing the *in vitro* results. In laboratories, precisely regulated growing circumstances cause a non-hypoxic, usually monolayer culture with no interactions with surrounding non-malignant cells. Real breast tumors show variable hypoxia in cancer cells, resulting in necrosis and powerful interactions with surrounding malignant and non-malignant cells (Kinnula & Crapo 2004).

6.3 Methodological aspects concerning microarray blocks

The immunohistochemical stainings of microarray block used in the studies I and IV of this thesis have their special pros and cons. Proper selection of even smaller than 1000 μM samples from original blocks is an essential prerequisite to avoid the presence of non-representative areas in punch samples and false negative results. Therefore, much patience, skill, and experience are needed in the preparation of microarray samples. Different parts of the tumor differ in, for instance, cell proliferation rate, apoptosis, and necrosis, and this variation cannot be totally avoided in the construction of microarray blocks. Furthermore, part of the samples is always lost due to detachment especially during the citric acid treatment. Exhaustion of microarray blocks was considered a problem in some samples in this thesis. Since the ages of the samples used in the same study occasionally varied by up to 22 years, it is possible that fixation times may also affect the results.

However, when samples are composed and mounted on glasses, the microarray technique enables the processing of much notably larger materials compared to traditional paraffin blocks. In addition, the microarray technique is especially applicable to breast carcinoma lesions since it enables the examiner to avoid processing unrepresentative adipose tissue areas of the kind frequently seen in the blocks. As reviewed by Simon *et al.*, numerous studies have validated the correlation of immunostaining results between whole blocks and microarray techniques (Simon *et al.* 2004). Furthermore, savings in reagents and technical costs are significant compared to standard immunohistochemical techniques (Van de Rijn & Gilks 2004).

The material used in microarray blocks dated back to 1979-2001 and that used in studies II and III to 1981-1998. The breast cancer screening program in Finland was initiated in 1987. When primary tumor sizes were compared before and after the launching of mammography screenings, the results showed a 1.5-fold increase in T1 tumors and a 3.5-fold decrease in T4 tumors, respectively (Figure 3). These figures reflect the importance of mammographies in the early detection of the smallest, and more operable, tumors.

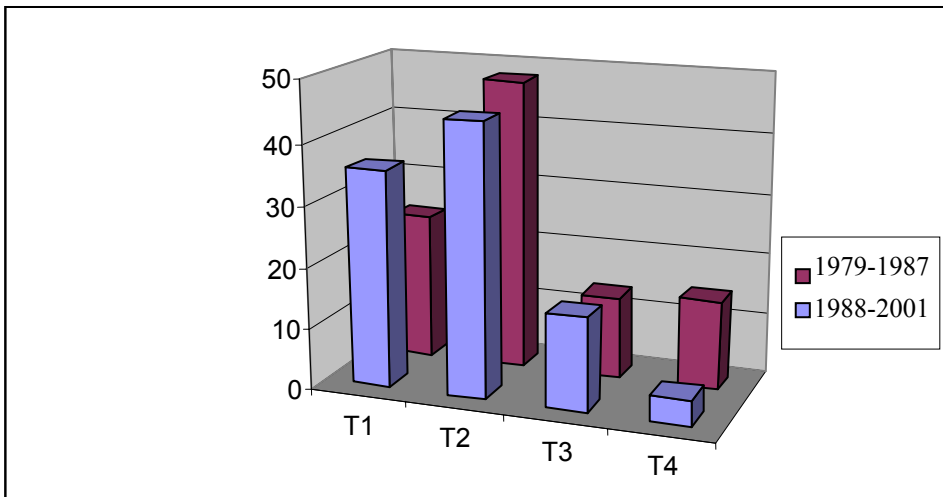


Fig. 3. Bar graph visualizing the decrease of primary tumor sizes after the beginning of breast cancer screenings in Finland.

6.4 Peroxiredoxin I-VI expression in carcinomas

Peroxiredoxins are a family of six ubiquitous thio-protein enzymes, which are encoded by six different genes in human tissues. So far, the majority of research has focused on bacterial Prxs and biochemical characterization of the enzymes. During the past five

years, however, there has been growing interest in the place of the Prx family in carcinogenesis and also in inflammatory diseases.

In study I, three samples of healthy breast tissue, which were used as controls, expressed markedly less Prx than malignant lesions. Since Prxs are a rather novel family of antioxidants, there is only one previous study concerning them in breast carcinomas (Noh *et al.* 2001). Yet, the material in that particular paper was somewhat restricted (n=24), and only the Prx I-III isoenzymes were studied. One of the main findings of that study was overexpression of Prxs II and III compared to benign breast tissue, which is in line with our results. Analogous results with different Prx subtype overexpressions have also been published on other carcinoma types (Yanagawa *et al.* 1999, Yanagawa *et al.* 2000, Chang *et al.* 2001, Choi *et al.* 2002, Kinnula *et al.* 2002, Kim *et al.* 2003, Lee *et al.* 2003, Lehtonen *et al.* 2004, Shen *et al.* 2004).

Although there was a nearly 20-fold number of specimens in study I compared to the breast cancer study of Noh *et al.*, the results of Prx I-III expression in malignant lesions in our study I correlated rather well with theirs (Noh *et al.* 2001). Strikingly weaker expression of the Prx isoforms II and VI compared to the others was seen, and both stained positively only in approximately half of carcinoma cases, whereas Prxs I, III, IV, and V showed reactivity in at least 80% of the studied carcinomas. In the light of the study I of this thesis and previous studies, there seem to be cancer-specific Prx expression types in different malignancies, including breast carcinoma.

6.5 Prxs and clinicopathological parameters

Prx expression has not been previously compared to TNM classification, hormone receptor status, patient survival, or any other clinicopathological parameters in breast carcinomas. In oral squamous cell carcinoma, Yanagawa *et al.* found low Prx I expression levels to be associated with larger tumor masses, lymph node metastases, and poor tumor differentiation (Yanagawa *et al.* 2000). Increased expression of Prxs I and II has been marginally and Prx V significantly connected to longer patient survival in malignant mesothelioma (Kinnula *et al.* 2002), whereas a recent lung cancer study found increased Prx VI expression to associate with high grading and Prx II to associate with advanced tumor stage (Lehtonen *et al.* 2004). In renal cell carcinomas, Prx II associated with better differentiation, absence of distant metastases, and better prognosis (Soini *et al.*, unpublished results).

Antiapoptotic effects of the Prx isoenzymes I, II, III, and V have been observed in human cell lines (Zhang *et al.* 1997, Kim *et al.* 2000, Yuan *et al.* 2004). In a recent paper by Chang *et al.*, Prx III depletion was demonstrated to result in the accumulation of H₂O₂ and, further, in the acceleration of apoptosis (Chang *et al.* 2004). It is therefore possible that the association between Prx V overexpression and augmented tumor size observed in study I may partly be explained by inhibition of apoptosis in breast tumors.

The study I of this thesis showed elevated Prx III and IV levels in patients with better prognosis. This association was especially convincing for Prx III, with the patients with at least moderate expression showing a mean survival of 186 months compared to 140 months among those with weak or absent Prx III expression. Instead, Prx V expression

associated with shorter survival. In mesotheliomas, contrariwise, high Prx V expression was reported to associate with better survival (Kinnula *et al.* 2002).

Since positive receptor status is known as the one of the most important factors of better prognosis, the association of Prxs III and IV, but not V, with receptor expression may explain the discrepancies in patient prognosis between the isoforms. In addition, Prxs III, IV, and VI were all significantly overexpressed in progesterone receptor-positive cases, which may partly explain their tendency to correlate. However, the regulation and even function of Prxs in normal or malignant tissues have still not been characterized sufficiently to explain convincingly these differences in Prx expression and induction in carcinomas.

The results of study I imply that overexpression of Prxs, especially the isoforms III and V, may play a part in breast cancer progression, and Prxs might therefore be used in the future as tumor markers when assessing the prognosis of single breast cancer patients. These Prx isoforms are located subcellularly to mitochondria and peroxisomes, the locations where oxidative stress is most evident. Observations from study I also suggest the inductive nature of Prxs as a response to oxidative stress, which has actually been previously shown *in vitro* with Prxs I-IV (Mitsumoto *et al.* 2001).

6.6 Relations between antioxidant enzyme levels in studies I and IV

Since the same material was used in studies I and IV, it was possible to compare the staining results of the Prx family, MnSOD, and catalase. Both Prxs and catalase are able to reduce H₂O₂ to molecular oxygen and water. Whereas the vast majority of catalase is present in peroxisomes, Prxs are ubiquitous in all cellular compartments and are also expressed extracellularly.

Peroxiredoxin expression has not been previously compared to catalase levels in human tissues. There were convincing inverse associations between catalase and the expression of Prxs II and VI. Therefore, it could be hypothesized that depressed catalase levels, which have previously been observed in several breast carcinoma studies (Punnonen *et al.* 1994, Ray *et al.* 2000, Polat *et al.* 2002), are also balanced with Prx induction in human tissues. Prx V, on the other hand, had a barely significant positive association with catalase expression. As described above, Prx V seems to be an atypical Prx not only because of its structure but also because of its properties as peroxynitrite reductase, location in important sources of ROS, and important role in mtDNA protection (Declercq *et al.* 2001, Wang *et al.* 2001, Dubuisson *et al.* 2004, Banmeyer *et al.* 2005).

Neither MnSOD nor Prx family have been previously assessed in the same study. In study I, MnSOD was significantly overrepresented among Prx I positive cases. It remains unclear whether this arises from synergistic induction of these enzymes as a response to oxidative stress, although it is known that both of these enzymes are inducible under oxidative conditions (Asoh *et al.* 1989, Rabilloud *et al.* 2001, Fajardo *et al.* 2004).

c-erbB2 oncogene expression was immunohistochemically assessed in studies I and IV without any association with antioxidant enzymes, NOSes, or nitrotyrosine. In a study by Preston *et al.*, addition of catalase to fibroblast culture in order to scavenge H₂O₂ levels, did not affect c-erbB2 activity, either (Preston *et al.* 2001). However, H₂O₂ is known to

regulate many other important growth factor receptors, including EGRF-1 (Gamou & Shimizu 1995). In the light of the published studies, it seems that neither ROS nor antioxidant enzymes are involved in the regulation of c-erbB2.

6.7 Subcellular location of Trx and TrxR

Thioredoxin-1 (Trx-1) is the most widely studied human Trx, and it is present in cytosol, nucleus, and also extracellularly, while Trx-2 is located exclusively in mitochondria (Makino *et al.* 1999, Tanudji *et al.* 2003). However, Trx-1 is also able to translocate to nucleus when cells are exposed to oxidative conditions or anti-cancer drugs such as cisplatin (Masutani *et al.* 1996, Hirota *et al.* 1997, Makino *et al.* 1999). This translocation is probably carried out by NF- κ B (Powis *et al.* 2000). In the study II of this thesis, Trx and TrxR staining patterns were found to be mainly cytoplasmic, but nuclear staining was also detected. Congruent results were obtained with immunoelectron microscopy. These observations concerning the subcellular location of Trx are in line with the previous studies on gastric carcinomas, non-small cell lung cancer, malignant mesothelioma, and breast carcinoma (Nakamura *et al.* 1992, Grogan *et al.* 2000, Kahlos *et al.* 2001, Ueno *et al.* 2000).

6.8 Thioredoxin system and NF- κ B

Trx and NF- κ B have dual and opposing roles in cytoplasm and nucleus. In cytoplasm, Trx shows its antioxidative properties and suppresses the activation of NF- κ B, whereas in nucleus, Trx significantly helps NF- κ B to act as a transcription factor (Hirota *et al.* 1999). NF- κ B, for its part, performs many roles in the immune system, cell adhesion, and inflammatory response and also seems to have anti-apoptotic and tumor progression enhancing effects (Hirota *et al.* 1999, Cao & Karin 2003). Regulation of transcription factors has been proposed to be the most important function of Trx (Powis & Montfort 2001). The study II of this thesis was the first to compare NF- κ B and Trx or TrxR expressions in human carcinomas. There were no connections between NF- κ B expression and Trx or TrxR, but instead, nuclear NF- κ B was shown by PCNA staining to significantly associate with reduced cell proliferation. A previous *in vitro* study on MCF-7 breast carcinoma cells suggested that the growth-promoting effects of Trx are mediated by activator protein-1 rather than via NF- κ B activation (Freemerman *et al.* 1999).

6.9 Trx and clinopathological parameters

Trx overexpression has been reported in some human primary tumors, including malignant melanoma, lung, colon, cervix, gastric, liver, and pancreatic carcinomas, and malignant mesothelioma (Fujii *et al.* 1991, Nakamura *et al.* 1992, Gasdaska *et al.* 1994, Berggren *et al.* 1996, Grogan *et al.* 2000, Nakamura *et al.* 2000, Kahlos *et al.* 2001, Soini

et al. 2001c, Lincoln *et al.* 2003). However, most of these studies have had relatively small series of subjects. In study II, nuclear but not cytoplasmic Trx was overexpressed in *in situ* tumors compared to invasive tumors. In addition, the T₁₋₂ group showed a more intensive nuclear Trx staining pattern compared to T₃₋₄ tumors. Ductal carcinomas stained positively for nuclear Trx in 49% of the studied lesions, whereas only 25% of lobular tumors showed expression, and this association was also statistically significant. The results of study II support the hypothesis that nuclear Trx expression could be a more important marker of tumor stage than cytoplasmic Trx. This possibly derives from the diverse roles of Trx in nucleus and cytoplasm, as described above.

Likewise, only nuclear Trx expression associated with negative estrogen and progesterone receptor status. In a previous breast cancer study, no correlation was observed between Trx and ER expression, but Trx expression was associated with a lower mitotic index in an ER-positive and p53-intact cohort (Matsutani *et al.* 2001). Another study with two breast cancer cell lines showed that H₂O₂ treatment of cells significantly increased their estrogen receptor β expression (Tamir *et al.* 2002). The reduced estrogen receptor expression in highly Trx-expressive tumors in study II may be, at least in part, explained by decreased cellular H₂O₂ concentrations and supports the important role of Trx as a regulator of redox status and hormone receptor expression. It is further possible that these results may lead to the development of novel anti-cancer therapies.

Nuclear Trx expression was connected with p53 positivity (meaning mutated p53), whereas nuclear TrxR associated inversely with mutant p53 expression. Pearson and colleagues have previously observed that deletion of the TrxR-encoding gene, TRR1, resulted in *Saccharomyces cerevisiae* in p53 inhibition, although the levels of p53 were not altered (Pearson & Merrill 1998, Merrill *et al.* 1999).

Both cytoplasmic and nuclear Trx associated in study II with increased cell proliferation, which is a prerequisite for cancer promotion and progression. Trx has been reported to be an important growth factor in many types of carcinomas (Powis *et al.* 2000, Kinnula *et al.* 2004). One of the most important experimental studies that have elucidated the carcinogenic properties of Trx was made by Gallegos *et al.* 1996. In that study, markedly depressed cell proliferation and tumor formation were observed in immunodeficient mice after redox-inactive mutant thioredoxin transfection in MCF-7 cells (Gallegos *et al.* 1996). In a previous breast carcinoma study, Trx was found to overexpress in the cohort of most aggressive tumors (Lincoln *et al.* 2003). In the light of a recent study, however, it seems that overexpression of Trx predicts a poorer outcome in breast cancer drug therapies (Iwao-Koizumi *et al.* 2005). The first *in vitro* studies on the novel Trx inhibitor, AW464, yielded encouraging results for further studies on this drug with anti-angiogenic and antiproliferative properties (Mukherjee *et al.* 2005).

6.10 GLCL in breast lesions

At least certain prostate carcinoma, leukaemia, lung adenocarcinoma, and cervix cancer cell lines express GLCL-c mRNA (Bailey *et al.* 1992, Ishikawa *et al.* 1996, Tipnis *et al.* 1999, Järvinen *et al.* 2000). In lung tissue, GLCL expression and regulation have been rather extensively studied (Rahman 1999, Tiitto *et al.* 2004, Kaartenaho-Wiik & Kinnula

2004). Up-regulation of GLCL has been previously reported in colorectal carcinomas, astrocytomas, and malignant mesothelioma compared to less malignant control tissues (Tatebe *et al.* 2002, Järvinen *et al.* 2002, Haapasalo *et al.* 2003).

As one could expect, there was a close connection between CLCL-c and GLCL-r in invasive breast lesions. GLCL-c immunostaining was seen in 50% of lesions and GLCL-r in 44%. Two other studies have been published where GLCL expression has been established in human carcinomas *in vivo*. In non-small cell lung carcinomas, the respective figures were 71% for GLCL-c and 67% for GLCL-r, whereas in astrocytomas, GLCL-c positivity was observed in 73% and GLCL-r in up to 89% of studied immunohistochemical sections (Soini *et al.* 2001b, Haapasalo *et al.* 2003). Diffuse astrocytomas expressed both GLCL subunits more intensively than grade I pilocytic astrocytomas (Haapasalo *et al.* 2003). Rather inconsistently with this, in the breast carcinoma material of study III, GLCL-c expression was more intensive in *in situ* lesions than in invasive carcinomas. In addition, lobular invasive carcinomas showed more intensive GLCL expression than the other histological types. On the basis of the available studies, it seems that GLCL expression has a cancer-specific expression pattern, although both subunits are strongly co-expressed in all published *in vivo* studies.

In addition to the higher GLCL expression in *in situ* lesions, there was also an inverse association between GLCL-c and nodal status. Thirdly, GLCL expression was connected with better prognosis in patients with invasive breast carcinoma if total GLCL expression was considered. These results indirectly suggest that low GLCL levels may lead to H₂O₂ accumulation and, further, to more oxidized tumor redox-state and therefore play a role in breast cancer promotion. Corresponding results have been reported from lung carcinomas, with GLCL-c being overexpressed in low-grade tumors (Soini *et al.* 2001b). However, previous studies have failed to elicit parallel results on GLCL expression and survival (Soini *et al.* 2001b, Haapasalo *et al.* 2003).

GLCL and tumor chemoresistance have been under extensive research during the last five years. There is nowadays plenty of evidence that connects anticancer drug resistance to high glutathione levels and changes in GLCL activity. It has been reported from various cell lines that the potent and specific GLCL inhibitor buthionine sulfoximine (BSO) improves the efficiency of some chemotherapeutic agents by depressing GSH levels (Bailey *et al.* 1994, Soini *et al.* 2001b, Fojo & Bates 2003, Rappa *et al.* 2003). Some studies have reported GLCL overexpression after the administration of anti-carcinogenic drugs or significantly higher GLCL levels in chemoresistant tumor cells (Bailey *et al.* 1992, Oguri *et al.* 1999, Soini *et al.* 2001b). In study III, which was the first study to investigate GLCL in breast lesions, no difference in survival was observed between GLCL- and GLCL+ patients who had received chemotherapy.

6.11 Nitric oxide production and tumor progression

There were very strong associations between eNOS, iNOS, nNOS, and nitrotyrosine in the material of study IV, indicating a near linkage between the expression rates of all three NOSes and tissue damage caused by NO[•]. Previously, iNOS, eNOS, and nNOS have been simultaneously assessed in brain tumors and in certain gastrointestinal

malignancies, but not in breast tissues (Yagihashi *et al.* 2000, Broholm *et al.* 2001, Broholm *et al.* 2003, Wang *et al.* 2005).

Previous breast cancer studies have yielded contradictory results about the role of iNOS in metastasis, cell proliferation, and differentiation, as both negative (Reveneau *et al.* 1999, Tschugguel *et al.* 1999, Loibl *et al.* 2002) and positive (Thomsen *et al.* 1995, Duenaz-Gonzalez *et al.* 1997) correlations have been reported from breast lesions. As regards eNOS, too, inverse connections with grade and metastases have been described previously (Martin *et al.* 2000, Loibl *et al.* 2002). Study IV revealed a significant association between eNOS and larger tumor sizes. The explanation of these discrepancies remains unclear. However, it has been discussed previously that there may be genetic differences between tumor cell populations, which may underlie the different outcomes (Lala & Chakraborty 2001). It has also been postulated that low local NO[•] concentrations may mediate carcinogenic effects, while high NO[•] levels may, conversely, have antitumor activity (Jenkins *et al.* 1995).

Nitrotyrosine has been widely used as a biomarker of oxidative stress and inflammation. In addition, it seems that nitrotyrosine itself induces H₂O₂ formation, which further promotes carcinogenesis (Murata & Kawanishi 2004). Nitrotyrosine overexpression has been observed in retinoblastoma and bladder carcinoma compared to non-invasive lesions (Eshan *et al.* 2002, Adithi *et al.* 2005). There were no benign lesions in the material of study IV, but it is still the only study where nitrotyrosine formation has been assessed immunohistochemically in breast tissues. Nitrotyrosine was markedly overexpressed in the largest primary tumors. It could be hypothesized that increased nitrotyrosine production in the most aggressive tumors results from increased NO[•] and O₂^{•-} production and further peroxynitrite formation, which leads to increased genetic instability in the critical genes of breast cancer progression.

6.12 VEGF, Flk-1, and Flt-1 in breast lesions

It has been shown conclusively that VEGF expression is accompanied by high MVD, which is an important prognostic factor in breast cancer (reviewed by Gasparini 2001), increased tumor growth, differentiation (Gasparini 2001), and metastatic potential (Weidner *et al.* 1991, Toi *et al.* 1995). It was hence expected that VEGF-expressing tumors would be of significantly larger size than VEGF-negative ones. Some previous breast carcinoma studies have reported a similar growth advantage provided by VEGF (Zhang *et al.* 1995, Claffey *et al.* 1996, Mattila *et al.* 2002), including one *in vivo* study (Linderholm *et al.* 1998). Although there was no relationship between VEGF and patient survival or disease-free interval in the material of study IV, there are numerous studies showing such correlations in breast carcinomas, as reviewed by Gasparini (Gasparini 2001).

A single study has previously reported an association between Flt-1 and large tumor size in papillary thyroid carcinoma (Fenton *et al.* 2000). Flt-1 was not expressed in the majority of microarray lesions of study IV, which may explain to some extent the absence of significant associations. Instead, there was significant co-expression between VEGF and Flk-1. Flk-1 has been suggested to have the most significant angiogenic effects of

these receptors (Millauer *et al.* 1993), and its expression has been associated with a high cell proliferation rate in breast carcinoma both *in vitro* (Xie *et al.* 1999) and *in vivo* (Nakopoulou *et al.* 2002). On the contrary, the former study found a reverse correlation between Flt-1 and Ki-67 (Xie *et al.* 1999). A recent epidemiological breast cancer study showed Flt-1 to have prognostic significance for both local and metastatic recurrence risk (Dales *et al.* 2004). However, no significance for Flk-1 or the risks of either survival or recurrence were observed in this study. The increased number of nodal metastases among the patients with positive Flk-1 status is in agreement with the studies describing a linear relationship between metastasis and the degree of vascularization (reviewed by Gasparini 2001) and emphasizes the *in vivo* role of the VEGF system in breast cancer metastasis. Although a single anti-angiogenic drug may not be effective against breast cancer progression, combination with other anti-cancer drugs may lead to a therapeutical response. Phase III studies with a VEGF-specific monoclonal antibody bevacizumab have shown rather promising results (Rugo 2004, Miller *et al.* 2005).

The similarity of Flk-1 and antioxidant enzyme expressions detected in study IV probably indicates that elevated H₂O₂ production, rather than MnSOD and catalase expression in themselves, is a factor involved in the angiogenic process at some level. Parallel results of the angiogenic role of H₂O₂ have been previously reported in *in vitro* studies (Arbiser *et al.* 2002, Cisowski *et al.* 2005). Again, a recent study where the CuZnSOD gene was transferred to mouse fibroblasts reported enhanced intracellular H₂O₂ generation and significant stimulation in both VEGF mRNA and protein expression (Grzenkiewicz-Wydra *et al.* 2004).

6.13 NO[•] production compared to angiogenesis and antioxidant expression in breast lesions

High microvascular density has been implicated to increase NO[•] production, measured as nitrotyrosine expression, in breast and esophageal carcinomas (Kato *et al.* 2001, Samoszu *et al.* 2002). Although, in study IV, there was a general trend toward VEGF being overrepresented in the presence of NOSes and nitrotyrosine, these associations were not statistically significant. Nevertheless, the VEGF receptor Flk-1 was overexpressed along with both cNOSes.

Taking into account their interrelated roles in ROS metabolism, it is not surprising that there was a close connection between MnSOD and catalase levels in study IV. MnSOD has been connected *in vivo* with aggressive tumor phenotype and poor prognosis in most studied malignancies. This association seems to be especially striking in gastric and colorectal carcinomas (Janssen *et al.* 1998, Janssen *et al.* 1999, Toh *et al.* 2000, Kim *et al.* 2002-2003, Tsanou *et al.* 2004, Nozoe *et al.* 2003, Korenaga *et al.* 2003).

Catalase activity has been studied rather extensively in human breast cancer patients. Decreased activity has been found both in blood samples and in tumor tissues of breast cancer patients (Ray *et al.* 2000, Punnonen *et al.* 1994). The maximum catalase depression in plasma was observed in stage II patients, and MDA and O₂^{•-} generation was likewise highest in stage II patients. Catalase was considerably overexpressed in ER and

PR-negative tumors (for both $p \leq 0.003$). No such associations with receptor status have been previously published.

Both catalase and MnSOD showed very close co-expression with NO^{\bullet} production, which indirectly suggests unspecific induction of these antioxidants during nitrosative and probably also oxidative stress. On the other hand, a recent *in vitro* study proposed that catalase induces iNOS expression in macrophage and microglia cells, while the authors did not find a similar association with human colon and lung cancer cell lines (Jang *et al.* 2004). Another paper by MacMillan-Grow *et al.* reported that peroxynitrite-mediated nitrotyrosine formation induces MnSOD in human renal allografts, while enzymatic activity of MnSOD was concurrently decreased (Mac-Millan-Grow *et al.* 1996). It can be speculated that breast carcinoma cells MnSOD also involve inactivation and overexpression of the inactive form, but it is impossible to verify this with immunohistochemical methods.

To summarize, these results obtained from different diseases and with rather diverse methods implicate that antioxidant enzymes and NO^{\bullet} production and further imbalanced redox status are closely linked, although the essential mechanism for this synergic induction/suppression still remains unclear and requires further studies. Since immunohistochemistry does not provide information about enzymatic activity, it would be interesting to test whether nitrotyrosine is also able to block antioxidant enzyme activity in breast carcinoma cells, despite the increase in total expression.

6.14 Oxidatively modified proteins in breast lesions

Apart from the fact that HNE, H_2O_2 , and NO^{\bullet} are important intracellular messengers involved in cell proliferation, transformation, differentiation, and apoptosis, they are also involved in all carcinogenesis stages as higher concentrations either directly or via their metabolites. H_2O_2 , $\cdot\text{OH}$, and NO^{\bullet} are all relatively rapidly reacting molecules, but their metabolites, such as nitrotyrosine, HNE, MDA, and 8-OHdG, can be measured when assessing oxidative and nitrosative damage in tumor cells. The most widely used marker is 8-OHdG. It has been found to be overexpressed 8- to 17-fold in breast primary tumors compared to benign breast tissue (Malins & Haimanot 1991, Musarrat *et al.* 1996, Matsui *et al.* 2000). Breast carcinoma studies reporting no differences between 8-OHdG levels in malignant and non-malignant cohorts have been also published (Nagashima *et al.* 1995).

8-OHdG expression has not been previously assessed immunohistochemically in human breast carcinomas. Hence, the study V of this thesis is the first to show that carcinoma cells themselves, rather than inflammatory, epithelial, or interstitial cells, express high levels of 8-OHdG even in T1N0 tumors. Positivity was observed in two thirds of stage I carcinomas, and the DNA of *in situ* carcinomas also showed, though to a lesser extent, traces of $\cdot\text{OH}$ damage-derived guanosine. Benign breast hyperplasias were all negative for 8-OHdG, excluding one sample with weak immunostaining. Further studies with higher-stage material would be of interest to elucidate whether 8-OHdG expression in tumor tissue could be a useful marker when assessing breast carcinoma prognosis.

Similarly to 8-OHdG, HNE and nitrotyrosine expression also increased from hyperplasias to *in situ* carcinomas and further to the first stage of invasive carcinoma (Figures 4a-c). HNE expression has not been previously assessed with immunohistochemical antibodies in human breast cancers, although this immunohistochemistry has been considered the most reliable and the most specific method for HNE detection (Oberley *et al.* 1999, Uchida 2003). T1N0 carcinomas showed high nitrotyrosine levels in 8-OHdG-positive lesions, and nitrotyrosine also associated distinctly with HNE positivity. Both HNE and nitrotyrosine expression rates were very high in study V. This suggests extensive oxidant-derived macromolecule damage in stage I breast lesions already, and that early stage breast carcinoma cells have coincidentally increased NO^* , $\cdot\text{OH}$, and O_2^* production, which accumulates into cellular macromolecules. In addition, the association between HNE and nitrotyrosine may be explained by one of the most important consequences of lipid peroxidation, protein modification. In study IV, only 56% of invasive stage I-IV lesions in microarray blocks showed nitrotyrosine expression with the same immunohistochemical methods. This probably suggests sensitivity to detect immunostaining between these divergent methods.

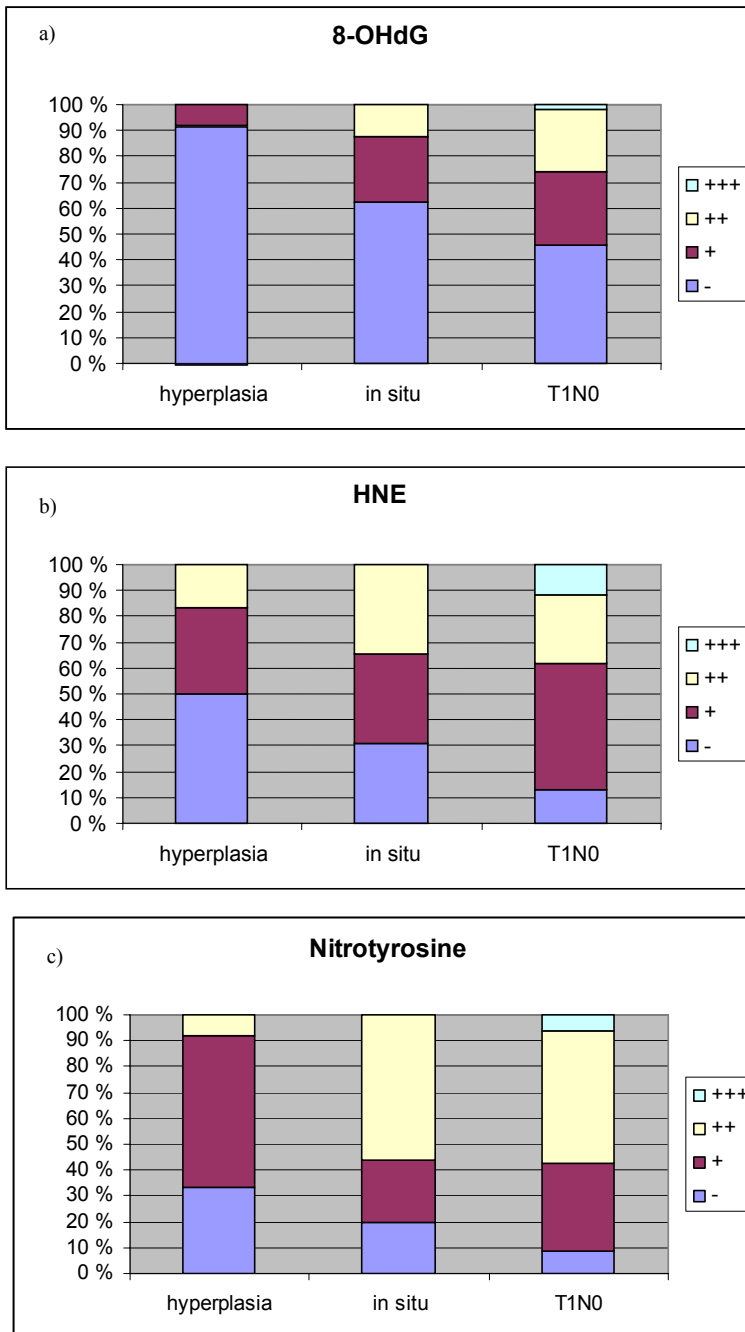


Fig. 4. a-c. Piled bar graphs visualizing the increasing oxidative damage during the early steps of carcinogenesis.

6.15 TopBP1 in breast lesions

Topoisomerase II is upregulated in many malignancies, and its two isoforms, α and β , are important targets of several anti-cancer drugs (Hande 1998, Zhou *et al.* 2001, Sandri *et al.* 1996). TopII β has been previously assessed only in a few human breast carcinoma studies. In the study of Sandri and colleagues, TopII β expression was observed in more than 90% of breast tumor cells, whereas TopII α was markedly less expressed (Sandri *et al.* 1996). In addition, TopII β , but not TopII α , expression associated with decreased survival rates. In study V, there was TopII β -binding TopBp1 expression in 77.2% percent of T1N0 carcinomas, the respective figure being 58.3% for *in situ* carcinomas and 50% for benign hyperplasias. Although the percentage of positive tumor cells was not assessed in study V in the way Sandri *et al.* did, these two studies seem to convincingly indicate that topoisomerase II β expression increases during breast cancer progression.

According to study V, TopBP1 is significantly overexpressed in 8-OhdG-positive T1N0 breast tumors ($p=0.008$). This is in line with the previous hypotheses that TopBP1 localizes to sites of ROS-derived DNA damage and possibly also plays a central role in the recognition of damaged DNA (Yamane & Tsuruo 1999, Xu *et al.* 2003). The results also imply that DNA repair is induced along with \cdot OH-derived DNA damage and gives further support to the role of reactive oxygen species in even the early stages of breast carcinogenesis.

6.16 Mismatch repair proteins in breast lesions

In addition to proto-oncogenes and tumor suppressor genes, a third major group of genes affecting carcinogenesis consists of mismatch proteins (Tamura *et al.* 2004). Currently, six different MSH proteins have been characterized from human tissues. In the light of knock-out studies and clinical observations, one of the most important of these proteins is MSH2 (Reitmair *et al.* 1996, Umar *et al.* 2004). MSH2 can complex with either MSH3 or MSH6. The complex of MSH2 and MSH6 is responsible for recognizing base-base mispairing of DNA and further repairing these coding errors, whereas both MSH3 and MSH6 may contribute to the correction of small insertion-deletion loops (Marsischky *et al.* 1996, Peltomäki 2003).

Microsatellite instability (MSI), which is associated with MMR defects in e.g. HNPCC, is rarely observed in breast carcinomas (Anbazhagan *et al.* 1999, Caldes *et al.* 2000, Tokunaga *et al.* 2000). In addition, the role of MMR proteins in breast tumor development is controversial. Both higher and lower expression rates in invasive carcinomas compared to more benign lesions have been described (Friedrich *et al.* 1999, Bock *et al.* 2000, Balogh *et al.* 2003, Hussein *et al.* 2004). The MSH2 immunostaining results in breast carcinomas have also shown notable discrepancies, and the results have varied from 53% to 100%, partly due to the different methods in immunoscoreing (Friedrich *et al.* 1999, Yang *et al.* 2002, Spagnoletti *et al.* 2004, Son *et al.* 2004). There was at least moderate immunostaining of MSH2 in all pre-invasive breast tumors. MSH6, however, showed loss of expression in 15-20% of these tumors. The discrepancy compared to invasive lesions was still high; there was loss of MSH2 in 55.9% and MSH6

in 56.5% of T1N0 tumors. Highly parallel co-expression between these MMR proteins was seen in the T1N0 material of study V, and this association has not been previously reported or assessed in breast tumors. MSH2 and the DNA damage response protein TopBP1 also associated with each other in the T1N0 cohort. However, MMR proteins did associate with ROS-derived damage markers. To conclude, the significant proportion of both MSH2 and MSH6-negative tumors even in stage I, indirectly suggests their importance in breast cancer suppression.

7 Conclusions

- There seems to be extensive oxidative stress in breast carcinomas, even in stage I tumors. This stress is manifested as increasing formation of carcinogenic compounds such as nitrotyrosine and 8-OHdG in the early steps of carcinogenesis. On the other hand, expression of certain DNA repair enzymes seems to decrease along with breast cancer progression. These results give further support to the hypothesis of persistent oxidative stress in breast carcinomas *in vivo*.
- Redox-modulating thiol proteins, including the previously unstudied Prx family, also show comprehensive expression in breast tumors. Especially the mitochondrial and peroxisomal isoforms of Prxs overexpress in the most malignant tumors, suggesting their inductive nature, although they are overwhelmed by the elevated H₂O₂ levels of breast cancer.
- NO[•] synthesis and oxidative damage are strongly associated with breast carcinomas. There is also a proximate interdependence between MnSOD and catalase levels and NO[•]-derived damage, which is in line with the protective role of these enzymes against ROS-derived damage.
- Cell redox –state-regulating enzymes have a general tendency to be upregulated in breast carcinomas, and most of these enzymes increase their expression along with tumor aggressiveness. However, it is impossible to postulate direct causal connections of the protective or detrimental effects of these enzymes, since the circumstances *in vivo* are highly complicated and depend on the highly regulated balance of several enzymes more than on the overexpression of a single enzyme.

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