Henri Sova

OXIDATIVE STRESS IN BREAST AND GYNAECOLOGICAL CARCINOGENESIS
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Abstract

Cancer is the leading cause of death worldwide. Despite the significant research effort, underlying mechanisms of carcinogenic processes are still poorly understood. In recent decades, a group of extremely reactive oxygen metabolites, reactive oxygen species (ROS), have been linked closely to carcinogenesis. Levels of ROS are constantly controlled by antioxidants to ensure stable redox balance in our cells. An aberrant cellular redox balance is thought to be connected to carcinogenesis by inflicting damage to cellular macromolecules and disturbing normal cellular signalling.

In this work, the role of ROS in carcinogenesis was studied by observing the ROS-derived DNA damage marker 8-hydroxydeoxyguanosine (8-OHdG) in breast cancer and endometriosis-associated ovarian cancer. This marker was also measured in connection with endometriosis and PCOS to study the early stages of the carcinogenic process. In addition, peroxiredoxin antioxidant enzymes were studied in endometriosis-associated ovarian cancer to explore their impact on the carcinogenic process and relationship with ROS-derived DNA damage.

There seems to be a decreasing trend in the expression of 8-OHdG in the development of breast cancer and endometriosis-associated ovarian cancer. In breast cancer, low levels of 8-OHdG in serum and in tumour tissue were found to be associated with more aggressive disease. In endometriosis-associated ovarian cancer, 8-OHdG and Prx II expressions in tissue decreased with malignant transformation from benign endometriosis tissue to ovarian cancer. Patients with PCOS were found to have lower levels of 8-OHdG in serum compared with healthy controls and metformin treatment further decreased 8-OHdG levels in obese patients.

These results, together with observations is previous studies indicate that in breast cancer and endometriosis-associated ovarian cancer, a high level of ROS-derived DNA damage could be significant factor in the initiation stage of carcinogenesis, whereas in later stages carcinomas benefit from lower ROS levels that support tumour growth and survival via cellular signalling. In endometriosis, there seem to be high amounts of ROS-derived DNA damage, which could explain the increased ovarian cancer risk, while in PCOS, aberrant ROS levels could contribute to the pathogenesis of the disease itself and also to possible cancer incidence by inducing abnormal cellular signalling.

Keywords: 8-hydroxydeoxyguanosine, antioxidant enzymes, breast cancer, endometriosis, ovarian cancer, Oxidative stress, polycystic ovary syndrome, reactive oxygen species
Sova, Henri, Oksidatiivinen stress rintasyövää ja gynekologisten syöpien karsinogeneesissä.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteet laitos, Synnytys ja naistentaudit; Syöpätaudit ja sädendoito; Diagnostiikan laitos, Patologia; Oulun yliopistolinnon sairaala

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Tiivistelmä


Tässä väitöskirjatutkimuksessa tutkittiin happiradikaalien yhteyttä syövän kehittymiseen tarkastelemalla niiden aiheuttaman DNA-vaurion merkkia, 8-hydroksideoksiguanosiinia (8-OHdG), rintasyövässä ja endometrioosin liittyvässä munasarjasyövässä sekä peroksiredoksiniiperheen antioksidantientsyymejä endometrioosin liittyvässä munasarjasyövässä. 8-OHdG:n avulla selvitettiin myös munaarjosten monirakkulaoireyhtymän (PCOS) ja endometrioosin yhteyttä syövän kehittymiseen. Lisäksi tutkittiin metformiinin vaikutusta happiradikaalien aiheuttamaan DNA-vaurioon.


Asiakirjat: 8-hydroksideoksiguanosiini, antioksidatiiviset enzyymit, endometrioosin, munasarjasyöpää, munaarjosten monirakkulaoireyhtymä, Oksidatiivinen stress, reaktiiviset happiradikaalit, rintasyöpä
Acknowledgements

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Foundation, The Finnish Medical Foundation and The Medical Research Foundation of Oulu.
Abbreviations

$^{1}$O$_2$ singlet oxygen
8-OHdG 8-hydroxy-2’-deoxyguanosine
AMH anti-Müllerian hormone
AMPK adenosine monophosphate-activated protein kinase
Ang-2 angiopoietin 2
AP-1 activator protein 1
ARID1A AT-rich interactive domain 1A
ASK1 apoptosis signal-regulating kinase 1
ATM ataxia telangiectasia mutated
ATP adenosine triphosphate
BE benign endometriosis
BER base-excision repair
BRAF v-raf murine sarcoma viral oncogene homologue B
CAE ovarian cancer-associated endometriosis
CAT catalase
cortical inclusion cyst
cyclooxygenase
copper/zinc superoxide dismutase
cysteine
ductal carcinoma in situ
deoxyribonucleic acid
dual oxidase
endometriosis-associated ovarian cancer
extracellular SOD
epidermal growth factor
enzyme-linked immunosorbent assay
epithelial ovarian cancer
oestrogen receptor
extracellular signal-regulated kinase
forkhead box
follicle-stimulating hormone
gonadotrophin-releasing hormone
glutathione peroxidase
 glutathione
 glutathione disulphide
GST glutathione transferase
H+ hydrogen ion
H2O2 hydrogen peroxide
HER-2 human epidermal growth factor receptor 2
HIF hypoxia-inducible factor
HNE 4-hydroxy-2-nonenal
HNF-1β hepatocyte nuclear factor 1β
HNPCC hereditary nonpolyposis colorectal cancer
HOCI hypochlorous acid
IFN interferon
IGF-1 insulin-like growth factor 1
IL interleukin
IRP iron regulatory protein
Keap1 kelch-like ECH-associated protein 1
KRAS kirsten rat sarcoma viral oncogene homologue
LCIS lobular carcinoma in situ
LH luteinising hormone
LIP labile iron pool
LOX lipoxigenase
MAPK mitogen-activated protein kinase
MDA malondialdehyde
Mn-SOD manganese superoxide dismutase
mTOR mechanistic target of rapamycin
NAD+ nicotinamide adenine dinucleotide, oxidized form
NADH nicotinamide adenine dinucleotide, reduced form
NADP+ nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH nicotinamide adenine dinucleotide phosphate, reduced form
NER nucleotide excision repair
NF-κB nuclear factor-κB
'NO nitric oxide
'NO2 nitrogen dioxide radical
NO2− nitrite
NOS nitric oxide synthase
NOX NADPH oxidase
Nrf2 nuclear factor erythroid 2-related factor 2
NSAID non-steroidal anti-inflammatory drug
O2 oxygen molecule
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>O$_3$</td>
<td>ozone</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-oxoguanine-DNA glycosylase</td>
</tr>
<tr>
<td>'OH</td>
<td>hydroxyl radical</td>
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<tr>
<td>ONOO$^-$</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>OSE</td>
<td>ovarian surface epithelium</td>
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<td>PCOS</td>
<td>polycystic ovary syndrome</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
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<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>RNS</td>
<td>reactive nitrogen species</td>
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<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour-node-metastases</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein p53</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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List of original articles

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.


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1 Introduction

Reactive oxygen species (ROS) are a group of highly reactive oxygen metabolites that are constantly produced in our cells to meet the needs of several crucial physiological processes such as cellular signalling, immune responses, hypoxia adaptation, aging and wound healing (Brieger et al. 2012). Endogenous sources such as the mitochondrial electron transport chain and specific ROS-producing enzymes are the main origin of cellular ROS, although some exogenous sources such as ionizing radiation and various xenobiotics can contribute significantly to the total cellular ROS levels (Brieger et al. 2012, Nathan and Cunningham-Bussel 2013). Antioxidants are responsible for the catabolism of ROS in our cells and their adequate function is highly important for the maintenance of a strict cellular redox balance which is required to prevent the unwanted effects of ROS (Valko et al. 2006). Enhanced ROS production and/or disturbed antioxidant function can lead to a state of oxidative stress, where ROS escape the control of antioxidants and are free to cause damaging reactions with cellular macromolecules such as proteins, lipids and DNA (Valko et al. 2006). In addition, a disturbed cellular redox balance can cause dysfunction in several ROS-mediated physiological processes, most importantly in cellular signalling (Forman et al. 2010, Ray et al. 2012). Modern research has linked the aberrant cellular balance between oxidizing and reducing agents to the pathogenesis of several diseases such as Alzheimer’s disease, Parkinson’s disease, cardiovascular diseases and cancer (Hekimi et al. 2011, Brieger et al. 2012). Interest in ROS has been increasing significantly, especially in the fields of cancer research, since it would seem that ROS-mediated cellular damage and abnormal signalling contribute to the initiation, promotion and progression of the carcinogenic process (Fiaschi and Chiarugi 2012).

Breast cancer and ovarian cancer are the most common and most lethal malignancies in women, respectively (GLOBOCAN 2008, Ferlay et al. 2010). Both of these malignancies have a highly heterogeneous nature and although several risk and genetic factors have been identified, researchers still struggle to explain the underlying mechanisms behind the pathogenesis of breast and ovarian cancer. Endometriosis and polycystic ovary syndrome (PCOS) are two of the most common gynaecological disorders linked to the increased risk of developing gynaecological cancers (Giudice and Kao 2004, Bulun 2009, Goodarzi et al. 2011). The pathogenesis and precise mechanisms behind the increased risk of these gynaecological disorders are also still relatively poorly understood. Recent
research results have suggested that defective ROS signalling and oxidative stress could have significant roles in the carcinogenesis of breast and ovarian cancer as well as in the pathogenesis of endometriosis and PCOS (Valko et al. 2006, Karihtala and Soini 2007, Karihtala and Puistola 2011, González 2012, Worley et al. 2013). Oxidative stress-derived DNA damage is thought to be one of the key factors in the initiation stage of carcinogenesis and could therefore be the linking mechanism of the increased cancer risk observed in endometriosis and PCOS (Fiaschi and Chiarugi 2012). In this thesis the significance of ROS-mediated DNA damage in the pathogenesis of breast cancer, endometriosis-associated ovarian cancer and PCOS is evaluated.
2 Review of the literature

2.1 Reactive oxygen species

2.1.1 Background

The term “reactive oxygen species” (ROS) covers a group of highly reactive oxygen metabolites that have a pivotal role in many physiological processes as well as in the pathogenesis of several diseases. Reactive oxygen species can be further divided into extremely reactive free radicals and to more stable non-radicals, depending on whether or not they have unpaired electrons on their orbitals. In mammalian cells, most ROS are formed in step-by-step reactions that begin from reduction of an oxygen molecule ($O_2$) to a superoxide anion ($O_2^{-}$).

Free radicals, like $O_2^{-}$, tend to react extremely rapidly with surrounding molecules, usually by either reducing/oxidising them or by forming a covalent bond with them, leading to the formation of new radicals and thus initiating chain reactions. ROS include the superoxide anion ($O_2^{-}$), hydrogen peroxide ($H_2O_2$), the hydroxyl radical (\(^{\cdot}\)OH), singlet oxygen (\(^{1}\)O_2), ozone (O_3), hypohalous acids and organic peroxides (Nathan and Ding 2010). The most reactive ROS is \(^{\cdot}\)OH, potent in inflicting non-specific damage to cellular components, and therefore also considered to be the most dangerous to our cells.

Interest in ROS in medical science commenced in the early 1950’s, when Gerschman et al. suggested that the increased formation of oxidizing free radicals could be behind the lethal effects of oxygen poisoning and ionizing radiation (Gerschman et al. 1954). Identification of the first mammalian cell-produced ROS (hydrogen peroxide, $H_2O_2$) in 1961 and discovery of the antioxidant enzyme superoxide dismutase (SOD) in 1969 further encouraged research into ROS (Nathan and Cunningham-Bussel 2013). At first, practically all ROS were thought to be harmful by-products of aerobic metabolism, but modern research has proven that ROS are required as signalling molecules in several essential physiological processes such as gene transcription, apoptosis and the immune response and for these purposes ROS are required and generated in mammalian cells (Lambeth 2004, Nathan and Cunningham-Bussel 2013). Hydrogen peroxide is considered to be the most important ROS in cellular signalling since it is the only ROS that properly fulfils the properties of a second messenger (Forman et al. 2010).
2.1.2 Generation and catabolism of ROS

Since ROS participate in several important cellular functions, low to moderate concentrations of ROS are constantly needed in human cells (Brieger et al. 2012). However, exposure to excessively high concentrations of ROS can result in damage through unwanted reactions between ROS and cellular components such as proteins, lipids and DNA (Valko et al. 2006). Therefore, a strict balance between ROS production and catabolism is vitally important for our cells. Simplistic model of ROS, enzymatic antioxidants and few damage markers of oxidative stress are illustrated in Figure 1. For more specific information and references see chapters below.

Sources of ROS

Introduced as early as in 1966, mitochondrial oxidative phosphorylation is probably one of the most essential and familiar endogenous sources of ROS (Murphy 2009). Oxidative phosphorylation is a two-step process that takes place in the inner mitochondrial membrane and it is used by aerobic organisms to store the energy gained from nutrients in the form of quickly utilizable ATP. In the first step, called the electron transport chain, energy gained from oxidation of the electron donor NADH to NAD⁺ is used by enzyme complexes I, III and IV to pump hydrogen ions (H⁺) into the mitochondrial inter-membrane space, thus generating an electrochemical gradient across the inner membrane. The electron transport chain is formed by static electron carriers (complexes I, III and IV) and mobile electron carriers (coenzyme Q and cytochrome c). Donated electrons are...
passed through these carriers to complex IV, where they are used to reduce oxygen and water is produced. Potential energy stored across the inner membrane in the form of a H⁺ concentration gradient is then used by ATP synthase (complex V) in chemiosmosis to form ATP. However, not all electrons taking part in the electron transport chain are used by complex IV to form water. In particular, complexes I and III are known to use some of the donated electrons in the transport chain to generate O₂⁻ (Valko et al. 2007). Mitochondria also contain their own forms of superoxide dismutase in the mitochondrial matrix (Mn-SOD) and in the inter-membrane space (Cu-SOD, Zn-SOD), which further convert O₂⁻ to H₂O₂ (Murphy 2009). Research on isolated mitochondria has shown that mitochondrial ROS production is enhanced when ATP production is low and thus there is either a high NADH/NAD⁺ ratio in the matrix or a high proton motive force accompanied by a reduced coenzyme Q pool (Murphy 2009). It has been estimated that 0.12–2% of O₂ consumed by mitochondria in vitro forms O₂⁻, whereas in vivo this amount is expected to be much lower (Murphy 2009 Brown and Borutaite 2012).

Other significant endogenous sources of ROS are NADPH oxidases (NOX) and dual oxidases (DUOX) (Lambeth 2004). The discovery of this enzyme family revised the perception of ROS, since these enzymes produce ROS naturally, which strengthened the idea that ROS are not solely harmful molecules (Lambeth 2004). NOX and DUOX enzymes are usually membrane-bound proteins consisting of a catalytic subunit and several regulatory subunits which assemble into multi-subunit enzyme complexes when activated by specific stimuli. Activated enzymes produce O₂⁻ by transferring electrons donated by NADPH to molecular oxygen (Jiang et al. 2011). So far, seven NOX/DUOX enzyme isoforms have been identified, which all share similar ROS production principles but differ greatly in expression tissue and activation mechanisms (Brieger et al. 2012). The first NOX enzyme (NOX2) was found in phagocytic cells such as neutrophils and macrophages, where it produces O₂⁻ to kill bacteria (Brieger et al. 2012). Later these enzymes were indentified in a wide range of tissue types such as colon and smooth muscle (NOX1), inner ear (NOX3), kidney, ovary and eye (NOX4), spleen and testis (NOX5), thyroid and lungs (DUOX1 and DUOX2) (Lambeth 2004, Brieger et al. 2012). NOX/DUOX enzymes are activated by various chemical, physical and inflammatory cellular stress stimuli, which has led to the idea that NOX/DUOX-mediated ROS production could act as a stress signalling mechanism in cells (Jiang et al. 2011). Utilizing ROS as signalling agents, NOX/DUOX enzymes are considered to take part in several important
physiological functions such as host defence, apoptosis, cellular growth and angiogenesis (Lambeth 2004).

In addition to mitochondria and plasma membranes, ROS are also produced by several other cellular organelles and enzymes, usually as metabolic by-products. The endoplasmic reticulum is a notable source of ROS via several contributors such as the flavoenzyme Erö1, diamine oxidase, cytochrome p-450 and b5 enzymes (Brown and Borutaite 2012). Protein oxidation during protein folding in the endoplasmic reticulum is considered to produce ROS as a by-product (Malhotra and Kaufman 2007). Peroxisomes constantly produce H₂O₂ in diverse redox reactions where they oxidize various metabolites such as fatty acids, purines, amino acids and polyamines with the aid of several specific oxidative enzymes and donate electrons directly to O₂, reducing it to H₂O₂. Peroxisomes keep ROS production and catabolism in balance by using a wide variety of antioxidants, most importantly catalase, which converts H₂O₂ to O₂ and H₂O (Bonekamp et al. 2009). Furthermore, extracellular spaces and cytosol also contain ROS-producing enzymes such as xanthine oxidase and lipoxygenase, respectively (Brown and Borutaite 2012).

Most of the endogenous sources of ROS produce either O₂⁻ or H₂O₂, from which other ROS are mainly derived through specific reactions with other molecules. In addition, the majority of O₂⁻ is dismutated to H₂O₂ by SOD near the production site, since only a small proportion of extremely reactive O₂⁻ is able to cross membranes, whereas more stable H₂O₂ is free to do so (Brown and Borutaite 2012). If O₂⁻ and H₂O₂ are not scavenged properly, they can form extremely reactive ·OH through the Haber–Weiss reaction, which is catalyzed by free transition metal ions such as iron, copper, chromium and cobalt (Valko et al. 2006). Under normal conditions in vivo, free ions of these metals are extremely rare, since they are usually bound to specific binding proteins (Wiseman and Halliwell 1996). However, it has been shown that O₂⁻ and H₂O₂ are capable, for example, of releasing iron from ferritin and haemprotein, thus making free metal irons available for the Haber–Weiss reaction (Wiseman and Halliwell 1996). In addition, as a result of a continuous demand for iron for normal cellular functions, a “labile iron pool” (LIP) is constantly present in our cells (Kruszewski 2003). It has been estimated that 3–5% of total cellular iron could form a LIP in the form of iron ions associated with various low-affinity ligands (Kruszewski 2003). A labile iron pool could be one of the main contributors in the formation of ·OH when there is excessive O₂⁻ and H₂O₂ present. The Haber–Weiss reaction
consists of two consecutive reactions (Kehrer 2000). In the first step, $O_2^-$ reduces ferric ion ($Fe^{3+}$) to more reactive ferrous ion ($Fe^{2+}$):

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$  \hspace{1cm} (1)

In the second reaction, called the Fenton reaction, $Fe^{2+}$ reduces $H_2O_2$:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$  \hspace{1cm} (2)

The net Haber–Weiss reaction is:

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$$  \hspace{1cm} (3)

Hypohalous acids such as hypochlorous acid (HOCl) are formed when halides are oxidized in reactions catalyzed by peroxidases (Birben et al. 2012):

$$H_2O_2 + Cl^- \rightarrow HOCl + H_2O$$  \hspace{1cm} (4)

Peroxyl radicals are formed in vivo particularly in fatty acid peroxidation. $\cdot OH$ can abstract a hydrogen atom from methylene carbon, forming an alkyl radical which can further react with $O_2$ to form a peroxyl radical (Valko et al. 2004):

$$\cdot CH_2^- + \cdot OH \rightarrow \cdot CH^- + H_2O$$  \hspace{1cm} (5)

$$\cdot CH^- + O_2 \rightarrow \cdot CHOO^-$$  \hspace{1cm} (6)

Peroxyl radicals can commence chain reactions by abstracting hydrogen atoms from other lipid molecules (Valko et al. 2004):

$$\cdot CHOO^- + \cdot CH_2^- \rightarrow \cdot CHOOH + CH^-$$  \hspace{1cm} (7)

Several exogenous sources also contribute to the total amount of ROS to which our cells are exposed. Ionizing radiation can cause covalent bonds of intracellular $H_2O$ to split, forming hydrogen radicals ($\cdot H$) and $\cdot OH$ (Kohen and Nyska 2002). Ultraviolet radiation also has the ability to produce ROS (Birben et al. 2012). Various xenobiotics such as drugs, air pollutants and chemicals are known to increase our exposure to ROS either by directly containing them or by producing them as metabolic by-products (Kohen and Nyska 2002). Interestingly, food also contains significant amounts of oxidized molecules (Kohen and Nyska 2002).

Reactive nitrogen species (RNS) are another extremely reactive group of molecules closely related to ROS. This group consists of nitric oxide (\'NO) and its derivatives, nitrogen dioxide radical (\'NO2), nitrite (NO2^-) and peroxynitrite (ONOO^-) (Nathan and Ding 2010). \'NO is formed when the amino acid L-arginine is oxidized in a reaction catalyzed by nitric oxide synthase (NOS). Our cells have three different NOS enzymes: neuronal, endothelial and inducible. Similarly to ROS, low to moderate levels of RNS, especially \'NO, have several
important physiological functions (Eiserich et al. 1998). Nitric oxide is a important signalling molecule, modulating processes such as neurotransmission, blood pressure regulation, the immune response and smooth muscle relaxation (Valko et al. 2006). However, overproduction of \(^{15}\text{NO}\) contributes to the formation of more reactive and therefore more harmful RNS (Eiserich et al. 1998). Peroxynitrite is produced in the reaction between \(^{15}\text{NO}\) and \(^{15}\text{O}_2^-\) and it is considered to be the most harmful nitrogen species, since ONOO\(^-\) and its protonated form (OONOH) are extremely potent oxidants (Kohen and Nyska 2002). Like \(^{15}\text{OH}\), ONOO\(^-\) and OONOH are also able to inflict significant damage via their non-specific reactions with cellular molecules (Kohen and Nyska 2002). In the process, reactions of OONOH can also produce \(^{15}\text{OH}\) (Nathan and Ding 2010).

**Catabolism of ROS (antioxidants)**

If catabolism of ROS is not controlled properly, extremely reactive radicals such as \(^{15}\text{OH}\) are free to have non-specific reactions with random surrounding molecules, potentially leading to cellular damage. Therefore, our cells harbour and utilize several enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include SOD, catalase (CAT), thioredoxin (Trx), peroxiredoxin (Prx), glutathione peroxidase (GPx) and glutathione transferase (GST) (Table 1) (Birben et al. 2012). Non-enzymatic antioxidants include low-molecular-weight compounds such as vitamins C and E, glutathione (GSH), carotenoids and flavonoids.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Location</th>
<th>Reaction</th>
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</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>CAT</td>
<td>Peroxisomes, cytoplasm</td>
<td>$2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$</td>
</tr>
<tr>
<td>Glutathione peroxidases (1–8)</td>
<td>GPx</td>
<td>Cytoplasm, mitochondria, gastrointestinal epithelium, plasma, membranes</td>
<td>$\text{ROOH} + 2\text{GSH} + \text{H}_2\text{O} \rightarrow \text{ROH} + \text{GSSH} + 2\text{H}_2\text{O}$</td>
</tr>
<tr>
<td>Peroxiredoxins (1–6)</td>
<td>Prx</td>
<td>nucleus, cytosol, mitochondria, peroxisomes, endoplasmic reticulum, extracellular space, Golgi apparatus, lysosomes</td>
<td>$2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$</td>
</tr>
<tr>
<td>Superoxide dismutases (1–3)</td>
<td>SOD</td>
<td>Mitochondria, cytoplasm, nucleus, lysosomes, extracellular fluids</td>
<td>$\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$</td>
</tr>
<tr>
<td>Thioredoxins (1–2)</td>
<td>Trx</td>
<td>Cytoplasm, mitochondria</td>
<td>Trx-(SH)₂ + Protein-S₂ \rightarrow Trx-S₂ + Protein-(SH)₂</td>
</tr>
<tr>
<td>Thioredoxin reductases (1–2)</td>
<td>TrxR</td>
<td>Cytoplasm, mitochondria</td>
<td>Trx-S₂ + NADPH + H⁺ \rightarrow Trx-(SH)₂ + NADP⁺</td>
</tr>
</tbody>
</table>

**Superoxide dismutase**

Superoxide dismutase was the first antioxidant enzyme identified. It was isolated in 1939 and proven to have antioxidant activity in 1969 (Valko et al. 2006). Proper function of SOD is highly important for us, since it is the only antioxidant capable of catalyzing the dismutation of $\text{O}_2^{\cdot-}$ to $\text{H}_2\text{O}_2$:

$$\text{O}_2^{\cdot-} + \text{O}_2^{\cdot-} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$ \hspace{1cm} (8)

The activity of SOD is based on continuous oxidation and reduction of its active site transition metal ion. It is induced in response to several stress conditions such as high oxygen tension, ozone, cigarette smoke, hypoxia and radiation (Kinnula and Crepo 2004). Human cells have three isoforms of SOD which differ from each other mostly by active site metal, subcellular location and reaction rate constants (Kohen and Nyska 2002, Valko et al. 2006). SOD1 and SOD3 utilize copper and zinc at their catalytic centres whereas SOD2 has a manganese cofactor (Zelko et al. 2002). SOD1 (CuZn-SOD) is localized mainly in the cytoplasm but small amounts have also been detected in the nucleus, mitochondria and lysosomes (Zelko et al. 2002). SOD3 is a glycosylated form of CuZn-SOD and is more familiarly called extracellular SOD (ECSOD) according to its localization (Holley et al. 2012). SOD2 (MnSOD) is exclusively expressed in the mitochondrial matrix of aerobic cells where it has a crucial role dismutating the constant production of $\text{O}_2^{\cdot-}$ by mitochondria. Among the SOD isoforms, MnSOD
seems to be the most vitally important, since knock-out mice lacking MnSOD suffer lethal mitochondrial injury in central nervous system neurons and cardiac myocytes (Lebovitz et al. 1996). In addition, knock-out mice with 50% reduction in MnSOD activity have been shown to have significantly increased ROS-derived DNA damage in mitochondrial and nuclear DNA, leading to a 100% increase in cancer incidence during the lifetime of these mice (Van Remmen et al. 2003). MnSOD has also been studied widely in human malignancies and the majority of results indicate that MnSOD activity has suppressive effects on tumour growth and differentiation (Kinnula and Crapo 2004, Holley et al. 2012). Mutations in genes coding SODs have been linked to several diseases such as amyotrophic lateral sclerosis (CuZn-SOD), progeria, sporadic motor neuron disease, cardiomyopathy and breast cancer (MnSOD) (Zelko et al. 2002).

**Catalase**

Catalase is one of the most important antioxidant enzymes scavenging H₂O₂. It is formed from four identical subunits that each contain a haem group at the active site (Birben et al. 2012). Catalytic breakdown of H₂O₂ is a two-step reaction where H₂O₂ first oxidizes the haem iron of catalase to form compound I, which is then used to oxidize another molecule of H₂O₂ (Kohen and Nyska 2002). The net reaction is:

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]  

Most of the catalase in our cells is located in peroxisomes where it is responsible for the decomposition of excessive production of H₂O₂. Peroxisomes can also use catalase and H₂O₂ to detoxify various substrates such as phenols, alcohols, formic acid and formaldehyde (Nordberg and Arnér 2001). Catalase gene mutations are known to be associated with diabetes, hypertension and vitiligo (Góth et al. 2004). In addition, total deficiency of catalase brings about oral gangrene and various metabolic alterations (Góth et al. 2004). Many studies have revealed decreased catalase activity or expression in connection with various carcinomas (Glorieux et al. 2011, Karihtala and Puistola 2011).

**Peroxiredoxins**

The enzyme family of human peroxiredoxins (Prxs) consists of six (I–VI) distinct enzyme isoforms which all are capable of reducing peroxides such as H₂O₂ and
alkyl hydroperoxides (Karihtala and Soini 2007). Although Prxs do not possess a catalytic efficiency as great as that of catalase or glutathione peroxidase, they are comprehensively spread throughout the subcellular space, which makes them important detoxifiers of peroxides (Wood et al. 2003). In addition to peroxide-scavenging properties, peroxiredoxins take a part in several important cellular functions such as apoptosis, proliferation, differentiation and the immune response (Wood et al. 2003). They have also a central role in ROS-mediated cellular signalling, since they can directly control cellular levels of the most important second-messenger ROS, H$_2$O$_2$ (Forman et al. 2010). Studies on Prx-knock-out mice have shown that loss of Prx I and Prx II causes intravascular haemolysis and anaemia and loss of Prx IV causes severe lung injuries (Neumann and Fang 2007). Peroxiredoxin expression has been detected in the nucleus (I, II), cytosol (I–VI), mitochondria (III, V), peroxisomes (I, IV, V, VI), endoplasmic reticulum (IV), extracellular space (I, II, IV), Golgi apparatus (IV) and lysosomes (IV, VI) (Wood et al. 2003, Karihtala et al. 2007, Bonekamp et al. 2009, Ishii et al. 2012). Cellular Prx expression is induced in response to increased production of ROS (Zhang et al. 2009). Human Prxs can be divided into three classes depending on the number and location of their active site cysteines (Cys) (Wood et al. 2003). Prxs I–IV represent the typical 2-Cys class where the enzyme structure is homodimeric with two cysteines in identical active sites (Wood et al. 2003). In contrast to typical 2-Cys Prxs, Prx V is called atypical 2-Cys Prx, since its functional unit has a monomeric structure where two cysteines are bound to the same polypeptide (Wood et al. 2003). Prx VI has only one active site cysteine and is therefore a 1-Cys Prx (Wood et al. 2003). The catalytic cycle of Prxs has three steps. The first step is identical for all peroxiredoxins, where peroxidatic cysteine reacts with a peroxide substrate such as H$_2$O$_2$, resulting in the oxidation of cysteine to cysteine sulphenic acid (Wood et al. 2003, Poole et al. 2011). The second reaction varies depending on the location and number of cysteines. Previously formed cysteine sulphenic acid reacts with a thiol group, forming a disulphide bond (Poole et al. 2011). 2-Cys Prxs use their own thiol group for this reaction from the second cysteine residue, called the resolving Cys (Poole et al. 2011). 1-Cys Prx has to use a thiol group from a non-Prx molecule such as glutathione (Poole et al. 2011). In the final step of the catalytic cycle, Prx is recycled to return to its peroxide-reactive state by reduction of the disulphide bond by specific electron donors such as thioredoxin (Wood et al. 2003, Poole et al. 2011).
While peroxiredoxins are known to be vitally important for healthy tissues, they are also considered to play a significant role in the development and progression of cancer. Loss of Prx I is known to lead to a significantly increased cancer incidence in mice (Neumann and Fang 2007, Karihtala and Soini 2007). The majority of studies have revealed Prx over-expression to be associated with several types of malignancy, while only a few studies have shown down-regulation of Prxs in this regard (Table 2) (Karihtala and Soini 2007, Karihtala et al. 2009, Zhang et al. 2009, Pylväs et al. 2010, Basu et al. 2011, Ummannip et al. 2012, Tehan et al. 2013). For example, all Prx isoforms have been reported being over-expressed in breast carcinoma (Karihtala and Soini 2007). It would seem that healthy tissue requires Prxs to maintain a ROS balance at physiological levels, where they are unable to inflict significant cellular damage. However, Prx over-expression connected to malignancies might be an adaptive response to increased oxidative stress in tumours and this could lead to enhanced proliferation and survival of tumour tissue via protection against ROS-induced cell death.

Expression of Prx in breast cancer as been shown to be closely related to tumour cell proliferation and Prx expression can be induced with ROS in vitro (Tehan et al. 2013). In vitro gene silencing of Prx III inhibits cell proliferation in breast cancer (Chua et al. 2010). In addition, a recent in vitro study revealed increased expression of Prx I, II, III and VI genes to correlate with the generation of cisplatin resistance in breast carcinoma, ovarian carcinoma and erythroleukaemia (Kalinina et al. 2012).

<table>
<thead>
<tr>
<th>Prx isoform</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
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<tbody>
<tr>
<td>Prx I</td>
<td>Thyroid tumours</td>
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<tr>
<td></td>
<td>Lung cancer</td>
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<tr>
<td></td>
<td>Bladder cancer</td>
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<td></td>
<td>Breast cancer</td>
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<td></td>
<td>Oesophageal cancer</td>
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<tr>
<td></td>
<td>Pancreatic cancer</td>
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<tr>
<td></td>
<td>Mesothelioma</td>
<td></td>
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<tr>
<td></td>
<td>Tongue cancer</td>
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<tr>
<td>Prx II</td>
<td>Breast cancer</td>
<td>Bladder cancer</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular cancer</td>
<td>Malignant melanomas</td>
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<tr>
<td></td>
<td>Vascular tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mesothelioma</td>
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<tr>
<td></td>
<td>Borderline ovarian tumours</td>
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<tr>
<td>Prx III</td>
<td>Breast cancer</td>
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<tr>
<td></td>
<td>Hepatocellular cancer</td>
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<tr>
<td></td>
<td>Lung cancer</td>
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<tr>
<td></td>
<td>Mesothelioma</td>
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<tr>
<td></td>
<td>Prostate cancer</td>
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<tr>
<td>Prx IV</td>
<td>Breast cancer</td>
<td>Stomach adenocarcinoma</td>
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<tr>
<td></td>
<td>Prostate cancer</td>
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<td></td>
<td>Pancreatic cancer</td>
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<tr>
<td>Prx V</td>
<td>Breast cancer</td>
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<td></td>
<td>Ovarian cancer</td>
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<tr>
<td></td>
<td>Mesothelioma</td>
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</tr>
<tr>
<td>Prx VI</td>
<td>Breast cancer</td>
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<tr>
<td></td>
<td>Ovarian cancer</td>
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<td></td>
<td>Borderline ovarian tumours</td>
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<td></td>
<td>Mesothelioma</td>
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<td></td>
<td>Oesophageal cancer</td>
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<tr>
<td></td>
<td>Oligodendroglioma</td>
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</tbody>
</table>

Glutathione peroxidase (GPx)

Glutathione peroxidases are also an antioxidant enzyme group capable of catalyzing the reduction of \( \text{H}_2\text{O}_2 \) and other hydroperoxides. It has been suggested that the antioxidant function of GPx is crucial only under severe oxidative stress, since knock-out mice develop normally without GPx but are killed by severe
acute oxidative stress (Brigelius-Flohé and Maiorino 2013). In addition to their antioxidant function, GPxs are known to take part in cellular processes such as the inflammatory response and insulin secretion via regulation of ROS-mediated signaling cascades (Brigelius-Flohé and Maiorino 2013). So far, eight different GPxs have been identified in mammals (Brigelius-Flohé and Maiorino 2013). Mammalian GPxs are tetrameric (GPx1, 2, 3, 5, 6) or monomeric (GPx4, 7, 8) enzymes with either selenocysteine (Sec) (GPx1, 2, 3, 4, 6) or cysteine (Cys) (GPx5, 7, 8) at their catalytic centres (Brigelius-Flohé and Maiorino 2013). In addition to structural divergences, GPx isoforms differ in cellular localization; GPx1 in the cytoplasm and mitochondria, GPx2 in gastrointestinal epithelium, GPx3 in plasma, GPx4 in membranes, GPx5 in epididymis and GPx6 in olfactory epithelium (Brigelius-Flohé and Maiorino 2013). The catalytic cycle of selenocysteine-containing GPxs (SecGPxs) starts with the oxidation of selenol by hydroperoxide to form selenic acid. SecGPx is restored to its peroxide-reactive selenol form with the use of two glutathione (GSH) molecules. The first GSH reacts with selenic acid, forming selenadi sulphide, which is then reduced by the second GSH, resulting in regeneration of the selenol form and releasing glutathione disulphide (GSSG) (Brigelius-Flohé and Maiorino 2013). GSSG can also be recycled to GSH via the enzyme glutathione reductase. The net reaction in the catalytic cycle of SecGPx (Nordberg and Arnér 2001) is:

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{GSSH} + \text{H}_2\text{O} \quad (10)
\]

The reaction mechanism of cysteine-containing GPxs is extremely close to that of atypical 2Cys-peroxiredoxins. The significance of GPx in the development of cancer has been studied to some extent and the results indicate that GPxs are likely to have a similar impact on carcinogenesis as peroxiredoxins. GPxs seem to prevent tumour initiation and metastasis, whereas they might support the promotion of initiated tumours (Brigelius-Flohé and Kipp 2009).

**Thioredoxin and thioredoxin reductase**

The maintenance of proper cellular H$_2$O$_2$ reduction by Prxs is highly dependent on the thioredoxin system. Furthermore, thioredoxins (Trxs) are known to have proinflammatory effects, to act as electron carriers for enzymes such as ribonucleotide reductase and methionine sulphotide reductase, to modulate several transcription factors such as p53, NF-kB and AP-1 and to inhibit apoptosis by binding to apoptosis signal-regulating kinase 1 (ASK-1) (Nordberg and Arnér
The activity of Trxs is based on the two thiol-containing cysteines at their catalytic site, which enables Trxs to function as general protein disulphide reductases in our cells (Nordberg and Arnér 2001). Only the reduced, dithiol, form of Trx (Trx-(SH)_2) can reduce other protein disulphides, since the reaction requires oxidation of the catalytic site of Trx, where two cysteine residues form an intramolecular disulphide bond (Trx-S_2) (Arnér and Holmgren 2006):

\[
\text{Trx-(SH)}_2 + \text{Protein-S}_2 \rightarrow \text{Trx-S}_2 + \text{Protein-(SH)}_2 \quad (11)
\]

Thioredoxin reductases (TrxRs) are mainly responsible for the reduction of oxidized Trx back to its reduced form. In addition, mammalian TrxRs can reduce several other oxidized proteins and compounds such as SecGPx, selenite, selenocysteine, dehydroascorbic acid, lipoic acid and ubiquinone (Nordberg and Arnér 2001). TrxRs are NADPH-dependent homodimeric flavoproteins with selenocysteines on their catalytic site. Electrons donated by NADPH are used by TrxR to reduce Trx or other substrates:

\[
\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Trx-(SH)}_2 + \text{NADP}^+ \quad (12)
\]

In mammalian cells, Trxs and TrxRs are expressed in cytosol (Trx1 and TrxR1) and in mitochondria (Trx2 and TrxR2) (Lu and Holmgren 2013). Studies on mice have proven the importance of these enzymes, since knock-out of either Trx1-2 or TrxR1-2 results in death of the embryo (Arnér and Holmgren 2006). In addition to these isoforms, testis-specific thioredoxin reductase has also been identified (Lu and Holmgren 2013). Various malignancies are associated with elevated expression of Trx, including breast and cervical carcinomas (Karihtala and Soini 2007). The thioredoxin system is thought to support tumour progression by promoting cancer cell proliferation, growth and angiogenesis and inhibiting apoptosis (Arnér and Holmgren 2006).

### 2.1.3 ROS in physiological processes

Reactive oxygen species participate in the regulation of several essential physiological processes, mainly through cellular signal transduction. Regarding cellular signalling, H_2O_2 is the most important member among ROS, since it fulfils best the properties of a second messenger. In contrast to other ROS, H_2O_2 has certain specificity in its interactions, its enzymatic production and scavenging, and its ability to oxidize thiols (Forman et al. 2010). H_2O_2 has also been shown to
diffuse across membranes with the help of specific aquaporins (Bienert et al. 2007). Owing to a high rate constant for the dismutation of $\text{O}_2^{-}$ by SODs and to the abundance of SOD enzymes in cellular compartments, $\text{O}_2^{-}$ is likely to function merely as a precursor of $\text{H}_2\text{O}_2$ in ROS-mediated signalling (Forman et al. 2010). Furthermore, reactions of $'\text{OH}$ are likely to be too non-specific and rapid for proper signalling purposes (Forman et al. 2010). It has been theorized that cellular signalling of $\text{H}_2\text{O}_2$ is largely based on its ability to oxidize cysteine residues of proteins to sulphenic acid, which can further react with another thiol group, forming a disulphide (Forman et al. 2010). Thiol-containing antioxidants such as Prx, Trx and GPx are therefore likely to regulate the signalling potential of $\text{H}_2\text{O}_2$ and also to mediate some of the signals by acting as second messengers themselves in physiological conditions (Forman et al. 2010). Some of the most important physiological functions of ROS are described in more detail in this section. In addition to these, ROS are also considered to participate, for example, in autophagy, wound healing and in the maintenance of thyroid and cognitive function (Brieger et al. 2012, Bryan et al. 2012, Sena and Chandel 2012).

**Regulation of transcription factors**

The functions of several important transcription factor systems are known to depend on redox reactions (Brigelius-Flohé and Flohé 2011). By modulating the activity of specific transcription factors, ROS participate in gene expression and therefore regulate processes such as cell proliferation (MAPK, PI3K, PTEN, P53), apoptosis (ASK1, P53), metabolism (Shc), antioxidant function (Nrf2, Keap1, Ref-1), the inflammatory response (NF-κP), DNA repair (ATM, Ref-1) and iron homeostasis (IRP) (Brigelius-Flohé and Flohé 2011, Ray et al. 2012). Many of these transcription factor systems are considered to have a crucial role in carcinogenesis, since cellular redox status is usually significantly altered in tumour tissue, leading to abnormal expression of redox-regulated genes and potentially offering tumour survival and growth advantage and also protection against anti-cancer treatments. For instance, Nrf2 over-expression has been observed in several carcinomas and has been linked to doxorubicin and cisplatin resistance in ovarian cancer (Lau et al. 2008, Karihtala and Puistola 2011).
Immune function

Our immune function and response is highly dependent on ROS. Macrophages and neutrophils use NADPH oxidase to generate large amounts of ROS in the form of an "oxidative burst" that is used to eliminate microbes (Nordberg and Arnér 2001). For these antimicrobial purposes, phagocytes are able to produce and utilize the more reactive forms of ROS such as HOCl and 'OH (Nordberg and Arnér 2001). In addition to direct pathogen elimination, ROS also regulate immune responses. ROS participate in the activation and function of T-lymphocytes and inflammasomes, in the regulation of Toll-like and RIG-I-like receptor pathways and in the promotion of NF-κB signalling (Sena and Chandel 2012, Alfadda and Sallam 2012, Brieger et al. 2012). Considering that ROS are responsible for such a large amount of normal immune functions, persistent oxidative stress can cause chronic inflammation, which is known to promote most carcinogenic stages (Reuter et al. 2010).

Adaptation to hypoxia

Growing evidence suggests that the mitochondrial electron transport chain works as an O₂ sensor, utilizing O₂•⁻ and H₂O₂ as signalling molecules to adapt cells to hypoxic conditions. The cellular response to hypoxia is largely mediated via hypoxia-inducible factors (HIFs), which are activated when a cell suffers from oxygen shortage. Activated HIFs attempt to counter low-oxygen conditions by way of various forms of transcriptional modulation such as promotion of red blood cell production (erythropoietin), angiogenesis (vascular endothelial growth factor) and glycolysis. Under conditions of normoxia, HIF-1 is unable to form its heterodimeric structure and translocate to the nucleus to modulate gene transcription, since its first subunit, HIF-1α, is constantly hydroxylated in the presence of oxygen by prolyl hydroxylase, and further degraded (Murphy 2009). In hypoxic conditions, the activity of prolyl hydroxylase is known to decrease, allowing HIF-1α to stabilize and HIF-1 heterodimers to form (Sena and Chandel 2012). H₂O₂ is thought to inhibit prolyl hydroxylase by reacting with its active site non-haem iron (Murphy 2009). Mitochondrial ROS production increases in hypoxic conditions, leading to inhibition of prolyl hydroxylase and activation of HIF-1. It would also seem that chronic hypoxia and active HIF-1 are likely to dampen mitochondrial ROS production by way of various feedback mechanisms to avoid ROS overproduction-induced damage and cell death (Sena and Chandel...
Although adaptation to hypoxia is part of our normal cellular function, activation of HIFs in tumours has several undesirable consequences. Owing to their rapid growth, tumours often suffer from hypoxia and can therefore benefit greatly from the survival promotion and cell death escape offered by HIF activation. Enhanced HIF-1 expression is associated with several malignancies including breast and ovarian cancer (Karihtala and Puistola 2011).

**Aging**

It is interesting that ROS have also been suggested to have a critical role in the aging process. This idea is largely based on the observations that ROS generation and oxidative damage is increased with age and that many age-dependent diseases such as Alzheimer’s, Parkinson’s and carcinomas are associated with ROS-derived damage (Hekimi et al. 2011). In addition, mitochondrial ROS production is also known to increase with aging and to correlate negatively with the length of life (Hekimi et al. 2011). The mitochondrial free-radical theory of aging is that aging could be a consequence of cellular damage inflicted by mitochondrial ROS (Hekimi et al. 2011). However, the latest information regarding the role of ROS in our physiological processes has led to a new hypothesis, i.e. that ROS are more likely to act as mediators of stress responses to age-dependent damage and thus oxidative stress-derived toxicity would increase significantly towards the end of our life spans, where the stress response would be too high for antioxidant systems to counter it (Hekimi et al. 2011).

**2.1.4 Damaging potential of ROS**

If cellular ROS production and scavenging is not maintained within strict boundaries, an unbalanced redox environment will eventually have harmful consequences. Excess ROS production or insufficient antioxidant expression leads finally to the formation of more reactive compounds, such as 'OH and ONOO', which cause damage to DNA, lipids and proteins with their non-specific reactions. Owing to their high reactivity, 'OH and ONOO' have extremely short half-lives ('OH less than 1 ns) and thus they inflict damage in the immediate proximity of their production site. 'OH is able to react and oxidize any component of the DNA molecule including purines, pyrimidines and deoxyribose (Valko et al. 2004). These reactions lead to the formation of oxidized DNA adducts which can be thought of as “footprints” of ROS-mediated DNA damage. These
footprints enable detection of the activity of these extremely reactive radicals, since their life spans are far too short for direct measurement. The most studied oxidized DNA adduct, the nucleoside 8-hydroxy-2’-deoxyguanosine (8-OHdG), is formed when OH oxidizes the C8 carbonyl group of guanine (Klaunig and Kamendulis 2004). Guanine has the lowest oxidation potential among the four main nucleobases and therefore the quantity of 8-OHdG can be considered to reflect the rate of cellular ROS-derived DNA damage fairly well (Peoples and Karnes 2004). The formation of 8-OHdG is known to enable mutations via GC \( \rightarrow \) TA transversions, which are observed, for example, in p53 mutations (Valko et al. 2004). In general, oxidative DNA damage can result in a wide variety of harmful modifications including single- and double-strand DNA breaks, DNA cross-links, deletions, frame shifts, base-free sites and chromosomal rearrangement (Valko et al. 2004). In addition, since low levels of ROS are constantly needed in our cells for normal physiological functions, antioxidants should not neutralize all ROS. Probably due to this fact, our cells suffer continuously from low but persistent oxidative DNA damage and therefore, for example, 8-OHdG is also observable in healthy populations (Sakano et al. 2009). To prevent potential mutagenesis, cytostasis and cytotoxicity caused by oxidative DNA damage, the removal and repair of damaged DNA lesions is extremely important for the integrity of DNA (Evans et al. 2004). Base-excision repair (BER) and nucleotide excision repair (NER) are the two major mechanisms responsible for the repair of oxidatively damaged base lesions (Evans et al. 2004). BER utilizes specific glycosylases that remove the damaged base and leave an abasic site behind, which is further processed by AP endonuclease, deoxyribophosphate lyase, DNA polymerase and DNA ligase to finalize the repair process (Evans et al. 2004, David et al. 2007). 8-Hydroxyguanine-DNA glycosylase (OGG1) is considered to be the most important excision enzyme for oxidatively damaged guanine (Evans et al. 2004, David et al. 2007). After removal, damaged DNA lesions are excreted from the cell, which enables their detection in blood and urine.

Other significant targets of ROS-mediated damage are the polyunsaturated fatty acids in cell membrane phospholipids. By attacking the methyl groups of polyunsaturated fatty acids, OH is able to initiate a chain reaction called lipid peroxidation in cell membranes (Valko et al. 2004). The initial reaction is followed by the formation of lipoperoxyl radicals which can further oxidize other unsaturated fatty acid methyl groups, while reducing themselves to lipid hydroperoxides in the process (Kohen and Nyska 2002). Hydroperoxides form alkoxy and peroxy radicals which finally decompose to end products of lipid
 Peroxidation such as malondialdehyde (MDA) and 4-hydroxy-2-noneal (HNE) (Barrera 2012). MDA and HNE are moderately reactive compounds known to have both carcinogenic and physiological effects in a concentration-dependent manner similar to ROS (Valko et al. 2004, Karihtala and Soini 2007, Barrera 2012). Lipid peroxidation is a significant form of ROS-mediated damage because of the abundance of lipid membranes in our cells and because basically one \( \cdot OH \) is sufficiently potent to cause the peroxidation of all unsaturated fatty acids in a single lipid membrane (Kohen and Nyska 2002). Uncontrolled lipid peroxidation will eventually disturb the normal function and structure of cell membranes and increase the generation of peroxidation end products to injurious levels (Karihtala and Soini 2007).

Proteins are the most abundant macromolecules in our cells and therefore an obvious target for ROS. Even though specific redox reactions are often necessary for normal function of proteins, their backbones and side-chains can be damaged by excessive oxidative stress. Oxidation of a protein backbone leads first to the formation of peroxyl radicals and finally to the fragmentation of backbone either via imine or via alkoxyl radical formation (Davies 2005). Oxidation of side-chains produces a wide variety of distinct compounds (reviewed by Davies 2005) and can lead to dimerisation and aggregation. In addition, since oxidation products of proteins are often also radicals, oxidation of proteins can initiate short chain reactions, similarly to lipid peroxidation. These ROS-mediated modifications to protein backbones and/or side-chains eventually lead to functional and structural abnormalities (Davies 2005). Interestingly, the majority of other forms of oxidative damage and modification to proteins seem to be irreversible, while the oxidation of thiol groups of cys residues in ROS-mediated signalling is easily reducible by thiol-based antioxidants (Davies 2005).

### 2.1.5 ROS in carcinogenesis

Carcinogenesis is thought to be roughly a three-stage process which is first initiated by a non-lethal DNA mutation in a single cell, promoted by proliferative and anti-apoptotic stimuli, finally progressing to a neoplastic tumour with uncontrolled growth and invasion potency (Valko et al. 2006). Although the viewpoint is constantly being adjusted in line with the latest knowledge, modern research has long acknowledged the significance of ROS in the distinct stages of carcinogenesis. Reactive oxygen species are considered to contribute to cancer initiation via damaging reactions with DNA and proteins. Cells suffering from
oxidative stress have either excessive amounts of ROS or diminished antioxidant activity that leads to the formation of more damaging ROS such as 'OH. Reacting with DNA, 'OH can cause point mutations that activate oncogenes such as c-fos, c-jun, c-myc, c-raf1 and K-Ras or inactivate tumour suppressor genes such as p53 (Valko et al. 2006, Karihtala and Puistola 2011). In addition, 'OH can further prevent the proper repair of damaged DNA by reacting with DNA repair and synthesizing enzymes (Liou and Storz 2010). The role of excess ROS-derived mutation potential is supported by the results of several animal knock-out studies where the lack of specific antioxidant enzymes has been shown to lead to an increased incidence of cancer (Halliwell 2007). The mutation potential of oxidative stress is likely to prevail through the different stages of carcinogenesis depending on the redox state of tumour cells, but it is likely that these mutations play their most significant role in the initiation stage.

In the later stages of carcinogenesis, ROS are considered to have important roles as signalling molecules, promoting proliferative and anti-apoptotic pathways. Cancer cells are known to have augmented ROS production that is likely to be due to the cooperative action of several suggested factors such as accelerated metabolism, increased activity of phagocytes (oxidative bursts and cytokine secretion) and peroxisomes, suboptimal vascularization (constant change between reperfusion and hypoxia), mitochondrial dysfunction and deviant growth factor (PDGF, EGF, insulin), cytokine (IFNγ, TNFα, IL-1, TGFβ) and enzyme (NOX, COX, LOX, K-Ras, rac-1) activity (Karihtala and Soini 2007, Liou and Storz 2010 Fiaschi and Chiarugi 2012). In line with augmented ROS production, several studies have revealed ROS-mediated transcription factors such as NF-κB, nrf-2, HIF, Erk1/2, AP-1 and Akt to be up-regulated in malignant tumours, promoting cell growth, cell survival, inflammation and metabolism (Liou and Storz 2010). Reactive oxygen species are also closely related to angiogenesis and metastasis by way of increasing the release of VEGF and angiopoietin, promoting anchorage-independent growth, inhibiting anoikis and regulating matrix metalloproteinases, Snail, src and TGF-β to induce epithelial-mesenchymal transition (Cui 2012, Fiaschi and Chiarugi 2012). However, these tumour-supportive effects of ROS are most likely to be achieved with low to moderate cellular levels of ROS (Liou and Storz 2010). Overly high levels of ROS are known to promote cell cycle arrest and apoptosis through the activity of various proteins such as Shc, FOXO, p53 and Ask-1. An extremely high level of oxidative stress can lead to necrosis (Valko et al. 2006, Liou and Storz 2010). For instance, reduced Trx binds to Ask-1 and inhibits its apoptotic activity. High ROS levels
shift Trxs to an oxidized state, leading to activation of Ask-1 (Liou and Storz 2010). In general, high antioxidant activity is known to prevent ROS-promoted cell death (Valko et al. 2006). Increased antioxidant levels have been observed in several malignancies including breast cancer and ovarian cancer and are often related to the activation of some ROS signalling pathways such as those involving NF-κB, Nrf-2 and HIF (Liou and Storz 2010, Karihtala and Puistola 2011).

Reactive oxygen species are also partly responsible for the shift towards glycolytic metabolism (Warburg effect) in tumour tissues via HIF, ras, myc and p53, which is also known to promote antioxidant production (Fiaschi and Chiarugi 2012). Amplified antioxidant function, as well as the activation of these signalling pathways in tumour tissues, have also been linked to the development of resistance against radiotherapy and chemotherapy. This seems logical, since these therapies are largely based on ROS-mediated cell death (Reuter et al. 2010 and Karihtala and Puistola 2011). Recent results indicate that the high survivability of cancer stem cells, a distinct cancer cell population that has also been isolated from breast and ovarian carcinomas and is suggested to have a huge role in cancer relapse and metastasis, could partly be based on the up-regulation of antioxidants (Reuter et al. 2010). It is probably advantageous for tumour cells to maintain high ROS production in order to up-regulate supportive signalling pathways and simultaneously enhance antioxidant activity to restrain ROS-mediated damage and apoptosis.

2.2 Breast cancer

2.2.1 Epidemiology

Breast cancer is the second most common cancer in the world (GLOBOCAN 2008, Ferlay et al. 2010). Approximately every tenth cancer diagnosed worldwide is a breast cancer. Breast cancer is also the most common cancer and the most common cause of cancer death among women worldwide (Ferlay et al. 2010).

In general the incidence of breast cancer increases quickly until menopause. After menopause the incidence increases at a slower rate or can even decrease slightly (low-incidence countries) (Key 2001, Hulka et al. 2008). The incidence peaks for breast cancer are between 55 and 60 years in developed countries and between 45 and 50 years in developing countries (Leong et al. 2010). For instance, the median age for diagnosis is 61 years in the US population (Siegel et al. 2012)
and 60.7 years in the Australian population (Australian Institute of Health 2012), the while median age for diagnosis is 48.5 years in Arab nations and 50 years in India (Najjar and Easson 2010, Sankaranarayanan et al. 2010). This difference has led to the question of whether or not developed countries and developing countries suffer from different kinds of breast cancer (Leong et al. 2010).

There is still a great variation in breast cancer incidence and mortality throughout the different regions of the world (Key et al. 2001, Boyle and Ferlay 2005, Ferlay et al. 2010). Incidence rates of breast cancer are strongly related to “western lifestyle” since they are a lot higher in developed countries than in developing countries (Ferlay et al. 2010, Jemal et al. 2010). Incidence rates also seem to increase among migrants coming from developing low-risk countries to developed high-risk countries, in time matching the local incidence rates (Ziegler et al. 1993). In recent decades, the incidence of breast cancer has been increasing practically everywhere. However, lately some reports from developed countries have indicated decreased breast cancer incidence rates since the early 2000s (GLOBOCAN 2008). This trend in most reports is linked to decreased use of menopausal hormone therapy and already extremely high mammographic screening rates in these countries (Youlde et al. 2012). At the same time, incidence rates in developing countries have been constantly increasing.

Breast cancer mortality rates have been stable or decreasing during the past decades in developed countries. In most developing countries, however, increased breast cancer incidence has also brought a trend towards increased mortality, since these countries often lack resources for early diagnosis and treatment (Jemal et al. 2010). Most developed countries have breast cancer 5-year relative survival rates of 80–90%, whereas developing countries struggle to keep them between 40–70% (Coleman et al. 2008, Sant et al. 2009, Sankaranarayanan et al. 2010). In Finland, among breast cancer patients diagnosed in 2002–2009, the 5-year relative survival rate is 89% (Finnish Cancer Registry).

### 2.2.2 Pathogenesis

The pathogenesis of breast cancer is still incompletely understood. Modern research has allowed us to identify multiple risk factors which partially shed light on the underlying aetiology of breast cancer.

Hormonal factors, particularly exposure to oestrogen, are known to have a major role in the development of breast cancer. Oestrogen can increase proliferation and inhibit apoptosis in mammary tissue cells (Yager and Davidson
Furthermore, oxidatively active end products of oestrogen metabolism are considered to have carcinogenic potential in several human tissues (Yager and Davidson 2006). Key factors are a woman’s cumulative lifetime exposure to her own endogenous oestrogen and her periodic use of exogenous hormones. Therefore, young age at menarche, delayed menopause, late first pregnancy, nulliparity and use of hormone replacement therapy or oral contraceptives increase the risk of breast cancer, whereas late menarche, early menopause, early first pregnancy, high parity and prolonged lactation seem to have protective effect (Dumitrescu and Cotarla 2005, Yager and Davidson 2006, Reeves et al. 2009, Newcomb et al. 2011). Long lifetime exposure to oestrogen is also largely a reason why aging is a crucial risk factor of breast cancer.

Excessive adipose tissue can also be a notable source of endogenous oestrogen, especially among postmenopausal women (Hulka and Moorman 2001, Yager and Davidson 2006, Lakhani et al. 2012). It has been proven that postmenopausal obesity and weight gain is associated with a higher breast cancer risk. However, breast cancer risk seems to decrease via an unknown mechanism among obese premenopausal women (Dumitrescu and Cotarla 2005, Lakhani et al. 2012).

Benign breast lesions, especially atypical hyperplasias, are associated with higher breast cancer risk (Key et al. 2001, Tice et al. 2013). Additionally, breast cancer risk has been shown to correlate with increased density of the breasts as seen in mammography (Boyd et al. 1995, Brinton et al. 1995, Tice et al. 2013). Dense breast tissue is often characteristic of nulliparous and thin women, which may partly explain why these women have a higher risk of breast cancer (Biglia et al. 2004). Also, most likely because of high oestrogen levels, increased bone density correlates with high breast cancer risk in postmenopausal women (Biglia et al. 2004).

Familial burden is also one of the most studied breast cancer risk factors (Hulka and Moorman 2001, Dumitrescu and Cotarla 2005). Breast cancer risk increases gradually with the more affected first-degree relatives a woman has. It has been estimated that approximately 7% of all breast cancers are hereditary (Hulka and Moorman 2001, Collaborative Group on Hormonal Factors in Breast Cancer 2001). Modern research has enabled us to identify various susceptibility genes for breast cancer. It is considered that sporadic breast cancers emerge at a later age as a consequence of piled up risk factors and polymorphism in low-penetrance breast cancer susceptibility genes. On the other hand, hereditary breast cancers usually emerge among younger women (or men) with mutation in one of 40...
the high-penetrance breast cancer susceptibility genes such as *BRCA1*, *BRCA2*, *p53*, *PTEN* and *ATM* (Dumitrescu and Cotarla 2005). Mutations in *BRCA1* and *BRCA2* alone are responsible for roughly 80 to 90% of all hereditary breast cancers and about 5 to 10% of all breast cancer cases (De Jong *et al.* 2002, Martin and Weber 2000).

Lifestyle factors such as alcohol intake, diet and physical activity also have a slight effect on overall breast cancer risk. Excessive alcohol, well-done meat and fat consumption are linked to an increased breast cancer risk, whereas intake of fruits, vegetables and omega-3 fatty acids might protect against breast cancer (Singletary and Gapstur 2001, Dumitrescu and Cotarla 2005, Suzuki *et al.* 2008, Kabat *et al.* 2009, Gonzalez and Riboli 2010). Physical activity in youth reduces breast cancer risk (Lagerros *et al.* 2004, Maruti *et al.* 2008). Genetic, environmental and lifestyle risk factors are most likely to accumulate under the influence of the surrounding population and culture, which is probably the major reason why geographical region has such a high impact on the risk of breast cancer (Dumitrescu and Cotarla 2005, GLOBOCAN 2008).

### 2.2.3 Classification and prognosis

Breast cancer has proven to be a very heterogeneous disease. Therefore, detailed classification of breast cancer is extremely beneficial and invaluable in clinical use to determine the optimal treatment and overall prognosis. Traditionally, breast cancer has been classified mostly on the basis of its histological structure, stage and receptor status. However, modern advances in microarray-based gene expression profiling have led to the identification of novel molecular subtypes which can expand the individualization of breast cancer even further (Perou *et al.* 2000, Malhotra *et al.* 2010, Eroles *et al.* 2012).

**Histology**

Normal functional mammary tissue consists of lobules and ducts which are surrounded by connective tissue. Roughly, milk is secreted from lobules into ducts which drain it to the surface of the nipple. Lobules and ducts are both composed of inner epithelial cells surrounded by myoepithelial cells attached to encircling basement membrane (Underwood and Cross 2009).

Breast carcinomas originate practically always from the epithelial cells of the ducts or lobules. Breast carcinomas are divided into non-invasive (*in situ*) and
invasive carcinomas, depending on whether or not tumour cells penetrate through the basement membrane. Non-invasive carcinomas are further divided into lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS), which is sub-typed into comedo, cribiform, micropapillary, papillary and solid (Malhotra et al. 2010, Lakhani et al. 2012). DCIS can also be graded into high- and non-high-grade lesions according to cellular pleomorphism and mitotic figures (Underwood and Cross 2009, Virnig et al. 2009). Partly as a result of widespread mammographic screening, diagnosis of non-invasive carcinomas has become significantly more common than before 1980. Approximately 25% of all breast cancers diagnosed in the USA are DCIS (Virnig et al. 2009). It has been estimated that a third to a half of cases of DCIS would advance to invasive carcinoma without treatment (Underwood and Cross 2009). LCIS is nowadays considered more as a risk factor of breast cancer rather than a breast cancer precursor lesion.

Invasive breast cancer can be classified into six major histological subtypes; invasive ductal, invasive lobular, mucinous, tubular, medullary and papillary carcinoma (Underwood and Cross 2009, Malhotra et al. 2010, Lakhani et al. 2012). Around 75% of all invasive breast carcinomas are invasive ductal carcinomas (IDCs) and these can be further graded into three differentiation groups depending on the levels of tubule formation, mitotic activity and nuclear pleomorphism (Malhotra et al. 2010, Lakhani et al. 2012). IDC represents highly heterogenous group of breast tumors that do not meet the sufficient histological requirements to be classified as one of the more specific histological type and that’s why the latest name recommendation for this group has been “invasive breast carcinoma of no special type (Lakhani et al. 2012). The second most common type is invasive lobular carcinoma, which represents about 10% of cases (Bertos and Park 2011).

Most of the rarer subtypes of invasive breast cancer (mucinous, tubular, medullary and papillary) have been reported to have slightly better prognosis than invasive ductal carcinoma (Fizgibbons et al. 2000, Bundred 2001, Rampaul et al. 2001, Lakhani et al. 2012). These subtypes are rare and cover only about 5 to 10% of all cases. The grade of the tumour tissue generally describes the aggressiveness of the carcinoma and is known to correlate significantly with long-term prognosis (Rampaul et al. 2001).

Lymphovascular invasion can also be detected histologically from a tumour sample. The presence of a tumour mass in the lymphatic or vascular ducts is a sign of an increased risk of local and distant recurrence (Cianfrocca and Goldstein 2004). This prognostic marker is especially useful when evaluating the recurrence.
risk of lymph node-negative patients (Cianfrocca and Goldstein 2004, Martin et al. 2009).

Molecular subtypes

The most recent advances in breast cancer research have led to the identification of “intrinsic” subtypes which represent different molecular profiles of the tumour tissue as sorted by gene expression microarrays (Eroles et al. 2012). Subtyping is principally carried out according to tumour gene expression related to proliferation, hormone receptor signalling and HER-2 signalling, and interestingly the same subtypes can be detected in both DCIS and metastatic disease (Carey 2010). Six distinct molecular subtypes have been found so far: normal breast-like, luminal A (ER+), luminal B (ER+, aggressive), HER-2-enriched (HER2+, ER-), basal-like (HER2-, ER-) and claudin-low (HER2-, ER-) (Malhotra et al. 2010, Lakhani et al. 2012). Most importantly, these molecular subtypes have been proven to differ as regards overall survival, disease-free survival and response to oncological treatments (Malhotra et al. 2010, Eroles et al. 2012). In addition, there are several other promising microarray-based gene-expression profiling tests for breast carcinomas, such as 70-gene signature, genomic grade index and 21-gene recurrence score (Lakhani et al. 2012). In the near future, genetic subtyping of breast cancer may be an important prognostic and predictive tool for clinical use (Lakhani et al. 2012).

Staging

Major guidelines in breast cancer treatment are mostly determined by the stage of the disease. Stage is determined by combining a few of the most powerful prognostic factors. The TNM system utilizes information on tumour size (T), metastases in regional lymph nodes (N) and distant metastases (M) (Edge et al. 2010). According to these three variables, breast cancers can have four main stages with a few sub-stages. The latest breast cancer TNM classification was published in 2010 in the 7th edition of the American Joint Committee Cancer Staging Manual (Edge et al. 2010) (Table 3).

The most powerful prognostic factor as regards early-stage breast cancer is the status of the axillary lymph nodes (Cianfrocca and Goldstein 2004). Patients with axillary lymph node metastases have significantly poorer prognosis and an approximately 50% higher chance of recurrence within 10 years than node-
negative patients (Fitzgibbons et al. 2001, Rampaul et al. 2001). There is also a linear correlation between the number of affected lymph nodes and poor prognosis (Bundred 2001, Fitzgibbons et al. 2001, Cianfrocca and Goldstein 2004).

Tumour size has a prognostic significance particularly for oestrogen receptor (ER) -positive and node-negative patients (Cianfrocca and Goldstein 2004). Tumour size and axillary lymph node status are in a close relationship, since it is known that larger primary tumours are more likely to develop regional lymph node metastases (Fitzgibbons et al. 2001, Cianfrocca and Goldstein 2004). Tumour size is directly associated with overall long-term survival and it has even been found to predict recurrence-free survival within as long as a 20-year follow-up time (Rosen et al. 1993).

The presence of distant metastases classifies breast cancer to stage IV and is virtually always a predictor of an incurable disease, where treatments given are mostly palliative (Nicolini et al. 2006). Median survival times in cases of breast cancer with distant metastases vary from 24 to 30 months (Nicolini et al. 2006).
Table 3. TNM classification of breast cancer (Edge et al. 2010).

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor (T)</td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: Ductal or lobular carcinoma in situ, or Paget’s disease of the nipple not associated with invasive carcinoma and/or carcinoma in situ in the underlying breast parenchyma</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor ≤ 20 mm in greatest dimension</td>
</tr>
<tr>
<td>T1mi</td>
<td>Tumor ≤ 1 mm in greatest dimension</td>
</tr>
<tr>
<td>T1a-c</td>
<td>Tumor &gt;1 mm but ≤20 mm in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor &gt;20 mm but ≤50 mm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor &gt;50 mm in greatest dimension</td>
</tr>
<tr>
<td>T4a-c</td>
<td>Extension to the chest wall (a) or ulceration/ipsilateral satellite nodules/edema of the skin (b) or both (c).</td>
</tr>
<tr>
<td>T4d</td>
<td>Inflammatory carcinoma</td>
</tr>
<tr>
<td>Regional lymph nodes (N)</td>
<td></td>
</tr>
<tr>
<td>pN0</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>pNx</td>
<td>No regional lymph node metastasis identified histologically</td>
</tr>
<tr>
<td>pN1mi</td>
<td>Micrometastases (&gt;0.2 mm and/or &gt;200 cells but none &gt;2.0 mm)</td>
</tr>
<tr>
<td>pN1a</td>
<td>Metastases in 1–3 axillary lymph nodes, at least one metastasis &gt;2.0 mm</td>
</tr>
<tr>
<td>pN1b</td>
<td>Metastases in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected</td>
</tr>
<tr>
<td>pN1c</td>
<td>Both a+b</td>
</tr>
<tr>
<td>pN2a</td>
<td>Metastases in 4–9 axillary lymph nodes</td>
</tr>
<tr>
<td>pN2b</td>
<td>Metastases in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases.</td>
</tr>
<tr>
<td>pN2c</td>
<td>Metastases in ≥10 axillary lymph nodes or metastases to the infradacudicular nodes</td>
</tr>
<tr>
<td>pN3a</td>
<td>Metastases in clinically detected ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes or metastases in ≥3 axillary lymph nodes in internal mammary lymph nodes with metastases detected by sentinel lymph node biopsy but not clinically detected</td>
</tr>
<tr>
<td>Distant metastases (M)</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>No clinical or radiographic evidence of distant metastases</td>
</tr>
<tr>
<td>cM0,i</td>
<td>No clinical or radiographic evidence of distant metastases but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow or other non regional nodal tissue that are ≤0.2 mm in a patient without symptoms or signs of metastases.</td>
</tr>
<tr>
<td>M1</td>
<td>Distant detectable metastases</td>
</tr>
</tbody>
</table>
Other prognostic factors

In addition to histology and staging, several other important prognostic factors as regards breast cancer have been identified. Nowadays, the analysis of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) status in tumour tissue has been set as part of routine breast cancer management. All of these three factors have been proven to possess great prognostic and predictive value (Payne et al. 2008, Martin et al. 2009, Chang and Hilsenbeck 2010, Schiff et al. 2010).

Steroid receptors are proteins found on the plasma membrane, in the cytosol and in the nucleus of target cells (Rampaul et al. 2001, Payne et al. 2008). Prognostically, ER- and PR-positive tumours appear to be associated with lower mortality and fewer recurrences (Cianfrocca and Goldstein 2004, Schiff et al. 2010). Positive steroid receptor status also indicates a high probability that the patient would benefit from the use of hormonal adjuvant therapy via oestrogen agonists (tamoxifen) or aromatase inhibitors (Schiff et al. 2010). There is comprehensive evidence that hormonal adjuvant therapy lowers the risk of mortality and recurrence among steroid receptor-positive breast cancer patients (Lancet 1998, Schiff et al. 2010).

Human epidermal growth factor receptor 2 is a transmembrane glycoprotein whose tyrosine kinase activity is essential for the normal function of epithelial and myoepithelial cells of mammary tissue (Cianfroccan and Goldstein 2004, Payne et al. 2008). The encoding gene for HER-2 is considered to be a proto-oncogene and is found to be amplified in approximately 15% of all breast cancers. This gene amplification results in HER-2 over-expression in cancer cells, which is further known to result in poor overall survival, higher recurrence risk and weaker response to hormonal therapy (Bundred 2001, Payne et al. 2008, Chang and Hilsenbeck 2010). However, early stage as well as metastatic HER-2-positive breast cancers are treated with a specific humanized monoclonal antibody to HER-2 (trastuzumab) which significantly reduces the risk of recurrence and mortality in these patients (Bundred 2001, Cianfrocca and Goldstein 2004, Payne et al. 2008, Chang and Hilsenbeck 2010, Ebstein et al. 2010).

Tumour proliferation is usually assessed by way of Ki-67 immunohistochemistry (Fizzgibbons et al. 2000, Bundred 2001, Cianfrocca and Goldstein 2004, Stuart-Harris et al. 2008). Proliferation has prognostic significance and is of use when evaluating the need for adjuvant therapy (Cianfrocca and Goldstein 2004, Stuart-Harris et al. 2008).
2.2.4 Treatment

Treatment of stage I–III breast cancer is based on optimal surgical removal of the primary tumour and possible metastatic lymph nodes, followed by postoperative radiation therapy and adjuvant therapies based on the risk of relapse and tumour features (Finnish breast cancer group 2013). The indication for adjuvant therapy is at least a 10% estimated relapse risk over 10 years of follow-up. Tamoxifen and aromatase inhibitors can be used as adjuvant therapy for hormone receptor-positive tumours and trastuzumab can be used for HER-2-positive tumours (Finnish breast cancer group 2013). Chemotherapy is most usually based on combinations of docetaxel, cyclophosphamide, epirubicin and 5-fluorouracil (Finnish breast cancer group 2013).

2.3 Ovarian cancer

2.3.1 Epidemiology

Ovarian cancer is the eighth most common cancer type in the world (GLOBOCAN). Annually, approximately 225,500 patients are diagnosed with ovarian cancer and 140,200 patients die from it (GLOBOCAN 2008). Such a high incidence-mortality rate makes ovarian cancer the most lethal gynaecological malignancy in the world. On average, women have an approximately 1% probability in developed countries and a 0.5% probability in developing countries of having ovarian cancer by the age of 75 years (Jamal et al. 2011). The incidence of ovarian cancer peaks at the age of 60 (Holschneider and Berek 2000).

The reason for the high global mortality rate is mainly the lack of proper diagnostic tools for early detection of ovarian cancer. It has been estimated that less than 30% of new ovarian cancer cases are diagnosed in the localized stage of the disease (Jamal et al. 2008). The late detection of the disease leads naturally to poor prognoses and poor survival rates. Overall five-year relative survival rates are slightly above 40% in most developed countries (Klint et al. 2006, Jamal et al. 2008, Sankaranarayanan et al. 2010). In Finland, the five-year relative survival rate is 49% (Finnish cancer registry 2007–2009).

The long-term trend of ovarian cancer incidence seems to be slightly decreasing in the high-incidence developed countries, while minor increases in incidence rates have been observed in the low-incidence developing countries (Bray et al. 2005, Klint et al. 2006, Murthy et al. 2009, Dhillon et al. 2011, Wong
et al. 2012, Yahata et al. 2012). Since there have not been any advances in the early detection of ovarian cancer and no breakthroughs with new therapeutic agents, relative survival rates have improved only modestly in recent decades (Klint et al. 2006, Jamal et al. 2008, Lowe et al. 2013).

2.3.2 Aetiology

Ovarian cancer is a heterogeneous disease that has been thought to arise either from the ovarian epithelial cells, sex cord-stromal cells or germ cells (Chen et al. 2003). For several decades, ovarian epithelial cells are thought to be the most significant origin of ovarian tumours covering about 90% of all ovarian malignancies (Chen et al. 2003). At the same time, the aetiology and the heterogeneity of epithelial ovarian cancer (EOC) has been relatively poorly understood. The latest advances in genetic tumour profiling together with well-known risk-factors and the discovery of the possible precursor lesion for ovarian cancer in fallopian tubes have brought up some interesting models of EOC pathogenesis (Piek et al. 2001, Shih and Kurman 2004, Prat 2012).

Risk factors

The risk factors of EOC are still primarily based on epidemiological factors, with the main elements being hormonal balance, parity, inflammation, lifestyle and hereditary. Women’s hormonal factors are thought to be important in the development of EOC, since the normal ovarian surface epithelium (OSE) is known to be hormonally responsive via its receptors for steroid hormones and gonadotrophins (Hunn and Rodriguez 2012). Oestrogens can stimulate the proliferation and inhibit the apoptosis of OSE cells, raising the possibility of cancer initiation, whereas progesterone counteracts these effects by promoting apoptosis and dampening oestrogen-induced proliferative gene expression (Salehi et al. 2008). In addition, androgens are also considered to have proliferative effects on OSE cells (Salehi et al. 2008). These hormonal effects shed light on most of the hormone- and reproduction-based risk and protective factors of EOC.

Nulliparity, infertility, early age at menarche, late age at menopause and menopausal hormone replacement therapy increase the risk of EOC, whereas multiparity, breastfeeding and use of oral contraceptives provide protection against it (Salehi et al. 2008, Sueblinvong and Carney 2009, Hunn and Rodriguez 2012). Besides the oestrogen–progesterone balance, another element of these risk
and protective factors is their impact on the ovulatory cycle. Traditionally it has been thought that each ovulation damages OSE cells and this persistent damage during a woman’s lifetime exposes OSE cells to the risk of malignant transformation (Karst and Drapkin 2010). Pregnancy interrupts this chain of constant damage momentarily and excess progesterone gives damaged or premalignant OSE cells the opportunity to be removed by apoptosis (Salehi et al. 2008, Sueblinvong and Carney 2009, Hunn and Rodriguez 2012).

The results of recent research also suggest that inflammatory factors play a central role in the development of EOC. Women’s normal ovulation and menstruation seem to maintain a chronic inflammatory environment around OSE and the fallopian tubes (Shan and Liu 2009). This inflammatory environment exposes surrounding cells to cytokines and reactive oxygen species that can cause prominent DNA damage (Karst and Drapkin 2010). In addition, inflammation-related conditions such as pelvic inflammatory disease and endometriosis are known to increase the risk of developing EOC (Hunn and Rodriguez 2012).

Obesity is the only lifestyle factor that has relatively sufficient research evidence for its association with increased EOC risk. Obese women seem to have a moderately increased EOC risk which is likely to be explained by increased androgen secretion and endogenous oestrogen production (Hunn and Rodriguez 2012). Research data concerning EOC risk and other lifestyle factors such as diet, exercise and cigarette smoking have shown conflicting results and therefore these factors have not yet established their position among definite risk factors (Hunn and Rodriguez 2012). There is some evidence linking red meat consumption to increased EOC risk, whereas consumption of fruits, vegetables and vitamins may have a protective effect against EOC (Salehi et al. 2008, Sueblinvong and Carney 2009, Hunn and Rodriguez 2012). Cigarette smoking seems to increase the risk of mucinous ovarian cancer (Salehi et al. 2008, Sueblinvong and Carney 2009, Hunn and Rodriguez 2012).

Familial history and genetic factors can also significantly increase the risk of EOC. It has been estimated that roughly 10% of ovarian cancers are of hereditary origin (Sueblinvong and Carney 2009). A first-degree relative with ovarian cancer increases a woman’s ovarian cancer risk up to 3-fold compared with women with no familial burden of ovarian cancer (Hunn and Rodriguez 2012). The majority of hereditary ovarian cancers are explained by specific mutations in the BRCA1/2 cancer susceptibility genes, and by the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (Hunn and Rodriguez 2012). Women with these conditions have an approximately 10% to 40% increased lifetime risk of ovarian cancer.
Hereditary ovarian cancer usually becomes manifest at an earlier age than sporadic ovarian cancer.

**Pathogenesis**

Owing to its heterogeneous nature, researchers have struggled to develop an unequivocal pathogenesis model for EOC. Traditionally, explanations of the pathogenesis of ovarian cancer have mainly been based on known risk factors such as ovulation, hormones or inflammation. Now, it has been accepted that such a heterogeneous disease cannot merely be explained by one or two factors but rather by synergy between all known factors. In addition, latest studies have seriously contested the traditional model where EOC originates from OSE (Piek *et al*. 2001, Shih and Kurman 2004, Prat 2012). This has led to the development of ovarian cancer models that strive to unite most of the known factors under one hypothesis.

One of the first decently working models was the Ovarian Surface Epithelium and Cortical Inclusion Cysts (OSE-CIC) model in which it was considered that all epithelial ovarian cancers arise from OSE (Karst and Drapkin 2010). Repeated ovulations inflict physical, inflammatory and oxidative damage to OSE cells, leading to malignant transformation (Karst and Drapkin 2010). Over time, sections of OSE can form invaginations into the cortical stroma which can finally turn into cortical inclusion cysts (CICs) (Karst and Drapkin 2010). The hormonal environment of the ovarian stroma can further induce proliferation in these OSE cell-based CICs.

However, the OSE-CIC model is unable to explain the wide genetic heterogeneity observed in the latest studies. The Two-Pathway model takes this into account and supplements the OSE-CIC model by dividing EOCs into type I and type II. Type I tumours usually originate from benign precursor lesions which first turn into borderline tumours and then advance slowly to low-grade carcinoma (Prat 2012). Endometriosis and endometriomas are suggested to be precursor lesions for type I endometrioid and type I clear cell carcinomas (Prat 2012). Mutations in cell-growth and proliferation-controlling genes such as *KRAS* and *BRAF* are characteristic of type I tumours. Type II tumours represent the aggressive phenotype of ovarian carcinomas. They are usually high-grade carcinomas, developing without clear precursor lesions, which spread rapidly and metastasize early (Prat 2012). Aggressiveness is partly explained by typical mutations in genes such as *TP53*, which are responsible for tumour suppression
and DNA repair (Karst and Drapkin 2010). The latest addition to the ovarian cancer model represents an attempt to clarify the origin of type II tumours. The results of most recent studies have suggested that high-grade type II serous ovarian tumours could originate from the fimbrial end of the fallopian tube (Piek et al. 2001, Karst and Drapkin 2010). Type I mucinous carcinoma has also been suggested to originate from the fallopian tube peritoneal junction (Seidman et al. 2011). It is now under a debate whether or not ovarian carcinoma originates from OSE at all.

2.3.3 Histology and subtypes

Most of the normal ovarian structure consists of stromal cells, collagen fibres and ground substance. The cortex of the ovarian stroma contains the developing and post-ovulatory follicles which are mainly responsible for the reproductive and hormonal functions of the ovary. The ovary is covered by a single layer of cuboidal or columnar epithelial cells of mesothelial origin (Young and Heath 2000).

Approximately 90% of ovarian malignancies are EOC and have been thought to originate from OSE cells. The remaining 10% arises from germ cells and stromal cells (Holschneider and Berek 2000, Rosen et al. 2009). There is great variety in the histological appearances of distinct subtypes and the wide heterogeneity between different subtypes of epithelial ovarian cancer has partly been explained by the mesothelial origin of OSE cells. Malignant ovarian tumours tend to resemble the various morphological features of the lower genital tract (Rosen et al. 2009).

Subtypes of epithelial ovarian neoplasms are serous, endometrioid, mucinous, clear-cell and transitional cell (Tavassoli and Devilee 2003). Epithelial tumours can also be classified as undifferentiated if the tissue shows no specific differentiation, or mixed type when the tumour has at least two histologically distinctive components (Tavassoli and Devilee 2003, Soslow 2008). Atypically proliferating tumours without stromal invasion are specified as borderline/low malignant potential tumours and they account for 10–20% of epithelial ovarian neoplasms (Holschneider and Berek 2000).

Serous ovarian carcinoma is the most common ovarian malignancy, representing over 80% of all epithelial ovarian carcinomas (Soslow 2008). Serous carcinomas have a polymorphic histological architecture and tend to mimic the morphology of other subtypes (Soslow 2008). Generally, the cell type of serous
carcinomas resembles the internal surface epithelium of the fallopian tube (Chen et al. 2003). Compared with the other major subtypes of epithelial ovarian cancer, serous carcinoma is associated with the worst overall 5-year survival rate (20–35%) (Rosen et al. 2009). This is mostly a result of the fact that serous carcinomas have often reached a more advanced stage (FIGO III–IV) at the time of diagnosis, whereas other subtypes are frequently found at stage I or II (Soslow 2008).

Other epithelial ovarian carcinoma subtypes are much rarer than serous carcinoma. Endometrioid carcinoma is the second most common epithelial ovarian carcinoma type, accounting for about 10% of cases. The endometrioid subtype resembles the cell type of the endometrium and is the most commonly found stage I ovarian malignancy (Soslow 2008). Mucinous and clear-cell carcinomas both represent under 10% of epithelial ovarian carcinomas. Mucinous carcinoma resembles either an intestinal or endocervical/Müllerian cell type (Chen et al. 2003). Endometrioid and clear-cell carcinomas are often associated with endometriosis. Transitional cell carcinoma resembling transitional epithelium/urothelium is the rarest subtype and it can often be hard to distinguish from serous carcinoma (Soslow 2008).

2.3.4 Prognostic factors

The prognosis of a patient with ovarian carcinoma can be evaluated with the use of several well characterized prognostic factors. By identifying a patient’s prognosis, it is possible to divide them into low-risk and high-risk categories and so optimize treatment and reduce unnecessary therapies. The best proven prognostic factors for ovarian cancer are stage, the amount of residual disease after initial cytoreductive surgery, age, performance status, histology and grade (Holschneider and Berek 2000, Akahira et al. 2001, Chi et al. 2001, Tingulstad et al. 2003, Winter et al. 2007, Chan et al. 2008).

Probably the most significant prognostic factor as regards ovarian cancer is the stage of the disease at the time of diagnosis. At present, clinicians utilize the staging system generated by the International Federation of Gynecology and Obstetrics (FIGO) Committee on Gynecologic Oncology (Table 4). The 5-year relative survival rate drops dramatically with more advanced stages: stage I (93%), stage II (70%), stage III (37%) and stage IV (25%) (Pecorelli et al. 1999). Along with stage, one of the most significant prognostic factors is the amount of residual
tumour after initial cytoreductive surgery. Optimal cytoreduction of the tumour is known to correlate directly to longer survival of the patient (Bristow et al. 2002).

A patient’s physical condition has a distinct effect on prognosis. Younger women and women with good performance status tend to endure heavy treatments better; they have fewer relapses and show longer overall survival (Holschneider and Berek 2000, Chi et al. 2001, Winter et al. 2007, Chan et al. 2008). Yong women, at the age of 30–40 years, are more often diagnosed with stage I–II disease than women older than 40 years (Winter et al. 2007). Tumour characteristics such as histology and grade also have prognostic significance. Clear-cell and mucinous tumours are generally associated with worse prognosis than other subtypes, mostly as a result of their inferior response to chemotherapy (Akahira et al. 2001, Winter et al. 2007).
Table 4. FIGO staging of ovarian cancer (FIGO Committee on Gynecologic Oncology 2009).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Growth limited to ovaries</td>
</tr>
<tr>
<td>Ia</td>
<td>Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact</td>
</tr>
<tr>
<td>Ib</td>
<td>Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact</td>
</tr>
<tr>
<td>Ic</td>
<td>Tumor either Stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings</td>
</tr>
<tr>
<td>Stage II</td>
<td>Growth involving one or both ovaries with pelvic extension</td>
</tr>
<tr>
<td>IIa</td>
<td>Extension and/or metastases to the uterus and/or tubes</td>
</tr>
<tr>
<td>IIb</td>
<td>Extension to other pelvic tissues</td>
</tr>
<tr>
<td>IIc</td>
<td>Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings</td>
</tr>
<tr>
<td>Stage III</td>
<td>Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive regional lymph nodes. Superficial liver metastases equals Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum</td>
</tr>
<tr>
<td>IIIa</td>
<td>Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery</td>
</tr>
<tr>
<td>IIIb</td>
<td>Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative</td>
</tr>
<tr>
<td>IIIc</td>
<td>Peritoneal metastasis beyond the pelvis &gt;2 cm in diameter and/or positive regional lymph nodes</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV</td>
</tr>
</tbody>
</table>

2.3.5 Treatment

Modern treatment of ovarian cancer is primarily based on initial maximal surgical cytoreductive removal of the malignant tissue, followed by adjuvant chemotherapy. No visible residual tumour after initial cytoreduction is considered optimal. Routine operation includes removal of the ovaries, fallopian tubes, uterus, appendix and ascites, and resection of the omentum. Pelvic and para-aortal lymph nodes are also removed. Bowel resection, peritonectomy and splenectomy are
performed if needed for optimal cytoreduction. Chemotherapy is based on a combination of paclitaxel and carboplatin combined with antiangiogenic therapy in high-risk patients.

2.4 Endometriosis

2.4.1 Epidemiology and clinical features

Endometriosis is a chronic, oestrogen-dependent, inflammatory disease characterized by the presence of endometrial-like tissue outside the uterine cavity (Giudice and Kao 2004, Bulun 2009). Endometriosis is one of the most common gynaecological disorders, affecting approximately 5–10% of all women of reproductive age and over 30% of all infertile women (Giudice and Kao 2004, Bulun 2009). Depending on the study population, there is great variation in the prevalence estimates of endometriosis. Among a group of women undergoing sterilization by tubal ligation, the prevalence ranged between 1% and 7% (Cramer and Missmer 2002). However, among women with pelvic pain or infertility, prevalence varies from 9% to 50% and 5% to 21%, respectively (Cramer and Missmer 2002). Adolescents suffering from severe pelvic pain or dysmenorrhoea have an approximately 50% chance of being diagnosed with endometriosis (Cramer and Missmer 2002).

Menstrual, environmental and hereditary factors affect the risk of endometriosis. Early menarche, short menstrual cycles, exposure to dioxins and proton irradiation, obesity, taller height, alcohol, caffeine and former use of oral contraceptives are linked to an increased risk of endometriosis (Viganò et al. 2004). First-degree relatives of a woman with endometriosis have an approximately seven-fold increased risk of developing endometriosis (Bulun 2009). In addition, endometriosis also seems to be associated with several autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, hypo- and hyperthyroidism, and multiple sclerosis (Viganò et al. 2004). Current use of oral contraceptives, smoking, regular exercise and higher parity have been suggested as protective factors against endometriosis (Viganò et al. 2004).

Clinical diagnosis of endometriosis can be challenging and is often delayed, since modern medicine does not include a reliable clinical examination, imaging method or blood test to diagnose endometriosis (Fassbender et al. 2013). As a
gold standard, diagnosis of endometriosis is verified by laparoscopic inspection accompanied by histological examination of endometriosis lesions (Fassbender et al. 2013). The severity of the disease can also be evaluated during laparoscopy, using the classification system of the American Society of Reproductive Medicine (American Society for Reproductive Medicine 1996). Abnormal growth of endometri-alike tissue in the pelvic peritoneum, ovaries or rectovaginal septum becomes manifest as a wide spectrum of different symptoms such as chronic pelvic pain, dyspareunia, dysmenorrhea, dysuria, dyschezia and infertility (Fassbender et al. 2013).

### 2.4.2 Pathogenesis

There are several promising theories concerning the pathogenesis of endometriosis. However, none of these theories have been able to explain reliably all aspects of endometriosis, in the light of the heterogeneity of the disease (Burney and Giudice 2012). The main debate between distinct theories is centred around the question of whether or not endometriosis lesions originate from the uterine endometrium.

A uterine origin of ectopic endometrial tissue is explained by retrograde menstruation through the fallopian tubes to the peritoneal cavity and lymphatic or haematogenous spread followed by transplantation and growth of endometriosis tissue (Baldi et al. 2008, Burney and Giudice 2012). The retrograde menstruation theory, proposed by Sampson in the 1920s, was one of the first and is one of the most studied theories addressing the pathogenesis of endometriosis and it has gained much supportive evidence over the years (Sampson 1927). It is known that the majority of healthy women have refluxed menstrual blood and endometrial tissue fragments in their peritoneal fluids and in women with endometriosis the volumes of these are increased (Baldi et al. 2008, Burney and Giudice 2012). In addition, obstruction of menstrual outflow in adolescents and in animal models results in increased retrograde menstruation and can lead to the development of endometriosis (Baldi et al. 2008, Burney and Giudice 2012). Further, endometriosis tissue detected in remote areas such as the lungs, brain, skin and lymph nodes could be a consequence of lymphatic or haematogenous spread of endometrial cells.

The latest thinking supporting a uterine origin is that mere physical displacement of endometrial cells cannot totally explain the pathogenesis of endometriosis, and some innate or acquired attribute or impairment of the
endometrium, peritoneal epithelium or immune response is required for the implantation, growth and survival of the ectopically spread endometrial cells (Burney and Giudice 2012). Several molecular abnormalities have been identified in ectopic endometriotic tissue and eutopic endometrial tissue in women with endometriosis compared with endometrial tissue from healthy women (Bulun 2009). Defective endometrial cell function may be a result of genomic alterations caused by oxidative stress-induced mutations or epigenetic dysregulation (Bulun 2009, Burney and Giudice 2012). Key factors are increased local production of estradiol and prostaglandin E2 accompanied by progesterone resistance arising from dysregulation of progesterone receptors in endometriotic tissue (Bulun 2009). These three factors maintain a positive-feedback loop which further promotes the activity of each member. Endometriotic tissue also overproduces several growth factors, cytokines and chemokines such as vascular endothelial growth factor, tumour necrosis factor-alpha, interleukins and matrix metalloproteinases (Burney and Giudice 2012). These molecular alterations promote the attachment, growth and survival of ectopic endometriotic tissue by damaging mesothelium, inducing angiogenesis, enhancing cell proliferation and decreasing apoptotic activity (Bulun 2009, Burney and Giudice 2012). In addition, a defective immune response may further support the implantation of ectopic endometrial tissue. Impaired function of natural killer cells and macrophages are possible explanations for the dampened immune response observed in connection with endometriosis (Burney and Giudice 2012).

Despite the wide scope of evidence, theories supporting a uterine origin of endometriosis struggle to explain the endometriosis cases observed in men, and in women without menstrual endometrium and why endometriosis affects only 5% to 10% of women when retrograde menstruation is observed in almost all healthy women. Therefore, theories suggesting a non-uterine origin of endometriotic lesions have emerged. The coelomic metaplasia theory proposes that endometriotic tissue could emerge from normal peritoneal tissue through metaplasia induced by either hormonal or environmental stimuli (Bulun 2009, Burney and Giudice 2012). The Müllerian or embryonic rest theory propounds that embryologic Müllerian cells in the peritoneal cavity could be activated by hormonal stimuli, initiating the formation of endometriotic tissue (Burney and Giudice 2012). In addition, one of the most recent theories suggests that bone marrow-derived stem/progenitor cells could differentiate into endometrial cells and possibly bring about formation of endometrial tissue outside the uterus (Sasson and Taylor 2008).
2.4.3 Treatment

The treatment of endometriosis is focused on the management of two major symptoms; pain and infertility. Treatment of endometriosis-related pain is based on either medical suppression or surgical removal of endometriotic implants. First-line medical pain treatment includes the use of combined oral contraceptives together with non-steroidal anti-inflammatory drugs (NSAIDs) (Giudice 2010). Progestins, GnRH agonists, aromatase inhibitors and androgens are used as second- and third-line medical treatments for pain (Giudice 2010). Since all of these agents (NSAIDs excluded) also suppress eutopic endometrium, medical treatment of endometriosis-related pain usually leads to amenorrhea and therefore medical therapies for pain are not useful for the treatment of infertility. First- and second-line surgical treatment for pain consists of lysis of adhesions, ablation, excision or fulguration of endometriotic implants, and ablation, excision or drainage of endometriomas (Giudice 2010). Hysterectomy accompanied by bilateral salpingo-oophorectomy can be used as a last-line treatment for women with severe endometriosis that is resistant to other treatments (Giudice 2010). In advanced-stage endometriosis, the effectiveness of operative treatment can be improved by means of postoperative medical treatment with GnRH agonists, oral contraceptives or androgens (Giudice 2010). Surgical treatment of endometriotic implants, endometriomas and adhesions also significantly improves the fertility of women with endometriosis (Giudice 2010). Other treatments for infertility caused by endometriosis include intrauterine insemination and in vitro fertilization (Giudice 2010). Preceding GnRH agonist therapy improves the success rates of fertility treatments (Giudice 2010).

2.4.4 Endometriosis and ovarian cancer

Currently, endometriosis is often considered to be a monoclonal, neoplastic disease and the literature shows strong evidence that women with endometriosis are at a significantly increased risk of developing epithelial ovarian cancer (EOC) (Mandai et al. 2009, Munksgaard and Blaakaer 2011). In particular, endometrioid and clear-cell subtypes of EOC are linked to endometriosis and it has even been suggested that endometriotic implants could work as precursor lesions of endometrioid and clear-cell EOC (Mandai et al. 2009, Munksgaard and Blaakaer 2011). In addition, there is some (but somewhat insufficient) evidence that endometriosis is also associated with several other malignancies such as breast
cancer, non-Hodgkin’s lymphoma and melanoma. Epithelial ovarian cancer arising from endometriosis is considered to have a unique aetiology, distinct from other ovarian malignancies, since it favours endometrioid and clear-cell subtypes, is found at earlier stages and has a relatively favourable prognosis (Mandai et al. 2009). Development of EOC from endometriotic lesions occurs probably through step-wise malignant transformation of endometriosis cells. Intermediary lesions between benign endometriosis and EOC have not yet been verified, but atypical endometriosis that often seems to be associated with EOC has been described (Worley et al. 2013).

Mechanisms behind the malignant transformation of endometriosis are still largely unknown, but some similar genetic defects in benign endometriosis and endometrioid and clear-cell EOC have been found. The findings suggest that malignant transformation of benign endometriosis cells could be caused by mutations in oncogenes such as KRAS and PI3K or in tumour-suppressor genes such as PTEN and ARID1A (Worley et al. 2013). A high frequency of these genetic mutations and therefore an increased risk of EOC associated with endometriosis could be a consequence of a harsh microenvironment around endometriotic lesions, with increased oxidative stress, chronic inflammation and hyperestrogenism (Worley et al. 2013). Constant haemorrhage induces release of haem and free iron in endometriotic tissue, which sustains increased production of reactive oxygen species, causing persistent oxidative stress that has the ability to damage DNA and lead to genetic mutations (Worley et al. 2013). In addition, endometriotic tissue overproduces several inflammatory cytokines and chemokines that are also linked to the development and progression of EOC (Worley et al. 2013). Finally, enhanced oestrogen production and activity in endometriotic tissue promotes excessive cell proliferation and production of harmful cytokines, hence increasing the chance of genetic mutations (Worley et al. 2013).

2.5 Polycystic ovary syndrome

2.5.1 Epidemiology

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age (Goodarzi et al. 2011). In addition, it represents the most frequent hormonal disruption and it can cause anovulatory infertility,
hyperandrogenism, menstrual irregularity, hirsutism, acne, seborrhoea and pattern alopecia (Normal et al. 2007, Rosenfield 2008). Globally, the prevalence of PCOS is approximately 6–15% depending on the classification criteria (Nicandri and Hoeger 2012). General risk factors of PCOS include obesity, menstrual dysfunction, hirsutism, polycystic ovaries and a family history of PCOS (Norman et al. 2007, Goodarzi et al. 2011).

2.5.2 Pathogenesis

The underlying aetiology of PCOS is still relatively poorly understood. Current opinion is that the cause of PCOS is probably a complex combination of environmental and genetic factors centred around defective interaction between the ovary, the hypothalamic-pituitary axis and insulin activity (Norman et al. 2007). It appears that a key factor of PCOS is hyperandrogenism. Most of the other aspects of the syndrome either contribute to or are a consequence of abnormal androgen activity and production in one way or another.

Compensatory hyperinsulinemia, caused by peripheral insulin resistance, is often observed in women with PCOS (Goodarzi et al. 2011). Hyperinsulinemia in cases of PCOS appears to be present in women of normal weight but is naturally aggravated by obesity (Norman et al. 2007, Goodarzi et al. 2011). High circulating insulin levels are known to contribute to the pathogenesis of PCOS via several specific mechanisms that mostly result in elevated androgen activity and production. Insulin suppresses the hepatic production of sex hormone-binding globulin (SHBG) and insulin-like growth factor-binding protein, resulting in elevated bioactivity of testosterone and insulin-like growth factor 1 (IGF-1) (Goodarzi et al. 2011, Nicandri and Hoeger 2012). IGF-1 and also insulin itself stimulate androgen production directly in ovarian theca cells (Goodarzi et al. 2011, Nicandri and Hoeger 2012). In addition, insulin might promote gonadotrophin releasing-hormone (GnRH) -dependent LH secretion in the pituitary gland (Norman et al. 2007). One reason for increased insulin resistance connected to PCOS might be dysfunction of adipose tissue, resulting in abnormal secretion of several metabolically active adipocytokines from adipocytes (Nicandri and Hoeger 2012). Obesity is known to aggravate PCOS symptoms by increasing insulin resistance, hyperandrogenism and menstrual dysfunction (Norman et al. 2007).

In PCOS, abnormal gonadotrophin secretion is also one of the main contributors to the hyperandrogenism observed. Probably as a result of elevated
insulin activity and deficiency of steroid hormone negative feedback, the hypothalamic pulse frequency of GnRH is increased (Goodarzi et al. 2011, Nicandri and Hoeger 2012). High GnRH activity leads to enhanced secretion of LH, whereas FSH secretion remains normal or is decreased (Norman et al. 2007, Nicandri and Hoeger 2012). An abnormally high LH-FSH ratio leads to hyperandrogenism by increasing androgen production in theca cells, while aromatase activity in granulosa cells is dampened (Goodarzi et al. 2011, Nicandri and Hoeger 2012). In addition, recent studies have revealed elevated anti-Müllerian hormone (AMH) levels in women with PCOS. AMH is secreted from granulosa cells and it acts as an inhibitor of FSH-stimulated aromatase activity in these cells (Goodarzi et al. 2011, Nicandri and Hoeger 2012).

In addition to the above aspects, the structure and function of the ovary is significantly altered in PCOS. Polycystic ovaries typically have an excess number of early developing follicles and increased volume. Normal folliculogenesis is disrupted by cooperative action of hyperandrogenism, hyperinsulinemia and elevated LH, AMH and IGF-1 stimulation, leading to a state called follicular arrest (Norman et al. 2007, Goodarzi et al. 2011). Follicular maturation stagnates at the antral stage, resulting in anovulation, menstrual irregularity and polycystic morphology of the ovary (Goodarzi et al. 2011). Furthermore, an increased amount of developing follicles results in high numbers of androgen-secreting theca cells (Norman et al. 2007).

2.5.3 Clinical features and diagnostic criteria

Polycystic ovary syndrome was originally recognized as a combination of amenorrhoea, hirsutism and obesity and was named Stein–Leventhal syndrome (Nicandri and Hoeger 2012). However, modern research has enabled redefinition of PCOS in much more detail. At present, the diagnosis of PCOS is centred around three main clinical features; hyperandrogenism, chronic anovulation and polycystic ovaries (Norman et al. 2007). There are three slightly different views on how these main characteristics should be emphasized when diagnosing PCOS.

The first up-to-date diagnostic criteria were proposed in 1990 by the US National Institutes of Health (NIH) and PCOS was defined as a combination of hyperandrogenism and chronic anovulation (Norman et al. 2007). In 2003, the Rotterdam European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine consensus workshop group included polycystic ovaries in ultrasonography in the list of criteria and required
the presence of at least two of the three main characteristics for the diagnosis of PCOS (Table 5) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004, Norman et al. 2007). This diagnostic criteria is in clinical use in Finland and was also used in this thesis. Most recently, in 2009, the Androgen Excess and PCOS Society suggested that PCOS should be primarily defined by hyperandrogenism associated with either chronic anovulation or polycystic ovaries (Goodarzi et al. 2011). Some other conditions, such as Cushing’s syndrome, androgen-secreting tumours, congenital adrenal hyperplasia, elevated prolactin levels and lack of luteinising hormone, can cause similar clinical manifestations as PCOS and therefore all of these diagnostic definitions require exclusion of these conditions before deciding on a diagnosis of PCOS (Norman et al. 2007).

**Table 5. The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group revised 2003 criteria for PCOS.**

<table>
<thead>
<tr>
<th>PCOS diagnosis requires 2 out of 3</th>
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<tbody>
<tr>
<td>1. Oligo- and/or anovulation</td>
</tr>
<tr>
<td>2. Clinical and/or biochemical signs of hyperandrogenism</td>
</tr>
<tr>
<td>3. Polycystic ovaries</td>
</tr>
<tr>
<td>Exclusion of other aetiologies required (congenital adrenal hyperplasias, androgen-secreting tumours, Cushing’s syndrome).</td>
</tr>
</tbody>
</table>

Hyperandrogenism is thought to be the most important aspect of PCOS and it can be diagnosed either clinically or biochemically. However, there is no 100% reliable diagnostic method for the detection of hyperandrogenism, which makes the diagnosis of PCOS challenging. Clinical diagnosis of hyperandrogenism is based on symptoms caused by adverse androgen activity such as hirsutism, acne and female-pattern alopecia (Norman et al. 2007). Biochemically the most reliable method to diagnose hyperandrogenism is to define bioavailable testosterone by measuring the concentrations of serum testosterone and SHBG and using the mass action equation for calculation (Norman et al. 2007). A diagnosis of chronic anovulation can be made when a woman has either oligomenorrhoea (<8–10 menses a year) or amenorrhoea (>3 months with absence of menses) (Nicandri and Hoeger 2012). Polycystic ovaries are diagnosed in ultrasonography when there are 12 or more developing follicles present in one ovary, or ovarian volume is increased by more than 10 mL (Nicandri and Hoeger 2012).
Women with PCOS have a higher prevalence of metabolic syndrome and cardiovascular disease risk factors, such as increased insulin resistance-derived type 2 diabetes mellitus, obesity, dyslipidaemia, hypertension and elevated levels of inflammation markers (Norman et al. 2007, Goodarzi et al. 2011).

2.5.4 Treatment

The treatment of PCOS is focused mainly on symptoms but also on the prevention of long-term risks.

Treatment of menstrual irregularity, hirsutism and acne is largely based on the use of combined oral contraceptives, which stabilize endometrial proliferation via progestins, increase SHGB production and decrease the production and activity of androgens (Norman et al. 2007). Alternatively, stabilization of menstruation can also be achieved by administration of cyclic/continuous progestins or use of a progestin-releasing intrauterine device (Goodarzi et al. 2011). Hirsutism treatment can be enhanced by combining an insulin sensitizer such as metformin, or a peripheral androgen blocker such as spironolactone with oral contraceptives (Goodarzi et al. 2011). Lifestyle changes in the form of caloric restriction and exercise are also crucial for all PCOS patients with excessive weight. A minor weight loss of as little as 2–5% significantly decreases insulin resistance, menstrual irregularity, testosterone and SHGB levels and the incidence of miscarriage and it can even normalize ovulation (Norman et al. 2007). Lifestyle changes, insulin sensitizers and statins are used to prevent type 2 diabetes mellitus and cardiovascular diseases related to PCOS (Goodarzi et al. 2011).

Infertility in women with PCOS is caused by anovulation and thus the treatment is based on ovulation-induction agents. The oestrogen-receptor modulator clomiphene citrate and the aromatase inhibitor letrozole can be used to induce ovulation by balancing the abnormal LH/FSH ratio (Norman et al. 2007). The use of metformin, especially in combination with weight loss in obese women, can also restore ovulation (Goodarzi et al. 2011). Second- and third-line infertility treatments for women with PCOS are gonadotrophin administration and in vitro fertilization, respectively (Goodarzi et al. 2011).

2.5.5 PCOS and gynaecological malignancies

There is firm evidence that the risk of endometrial cancer is increased among women with PCOS (Fauser et al. 2012). In addition, there are few studies
suggesting that also epithelial ovarian cancer might be associated with PCOS, whereas the relationship between PCOS and breast cancer seems to be either relatively weak or nonexistent (Gadducci 2005, Chittenden et al. 2009). The link between PCOS and gynaecological malignancies seems logical, since many key features of PCOS can be considered to be risk factors of endometrial and ovarian cancer.

In women with PCOS the risk of developing endometrial cancer is increased by approximately three-fold compared with healthy women (Chittenden et al. 2009). Risk factors linking endometrial cancer to PCOS are obesity, insulin resistance, type II diabetes mellitus, nulliparity, infertility, chronic anovulation, and tamoxifen and exogenous oestrogen treatment (Gadducci 2005, Chittenden et al. 2009, Shafiee et al. 2013). Most of these features lead to increased oestrogen and androgen activity without sufficient progesterone opposition, promoting mitotic activity and thus increasing the possibility of DNA replication errors in endometrial cells (Gadducci 2005).

Evidence connecting PCOS with epithelial ovarian cancer is based around a handful of studies with relatively low numbers of patients (Gadducci 2005, Chittenden et al. 2009, Shafiee et al. 2013). On the other hand, there is no incontrovertible proof suggesting otherwise. Theoretically, PCOS and epithelial ovarian cancer share several common pathogenic features, which suggests that there could be a connection between these diseases. Increased activity of oestrogens, androgens and gonadotrophins, nulliparity and infertility, chronic inflammation and obesity are all factors that are linked in the pathogenesis of epithelial ovarian cancer and are also important in PCOS (Gadducci 2005, Goodarzi et al. 2011, Hunn and Rodriguez 2012). Polycystic ovaries also seem to be associated with a higher rate of hyperplastic and metaplastic changes in the surface epithelium or in the inclusion cysts compared with healthy ovaries, indicating possible involvement of PCOS in the development of precursor lesions of epithelial ovarian cancer (Gadducci 2005).
3 Aims of the study

The aims of this work were:

1. To evaluate the prognostic significance of 8-OHdG in breast cancer.
2. To investigate the role of ROS-derived DNA damage in the pathogenesis of breast cancer and endometriosis-associated ovarian cancer.
3. To evaluate the significance of 8-OHdG, Prx II and Prx VI in the transformation of benign endometriosis to endometriosis-associated ovarian cancer.
4. To investigate oxidative stress in patients with PCOS compared with healthy controls
5. To evaluate the effect of metformin treatment on serum 8-OHdG levels in patients with PCOS.
4 Materials and methods

4.1 Study material

The material for study I consisted of 173 pre-operative venous blood samples from breast carcinoma patients and 150 tumour blocks from the same patients. The samples were taken in 2003–2005 and comprised 140 ductal carcinomas, 25 lobular carcinomas and 8 other types of breast carcinoma. Histopathological typing was carried out according to WHO classification (Tavassoli and Devilee, 2003). The mean follow-up time for survival analysis was 40.5 months.

Study II included 22 tissue samples from patients with benign endometriosis (BE) and 33 tissue samples from patients with endometriosis-associated ovarian cancer (EAC) operated upon in 1999–2009. Ovarian cancer blocks were selected to represent both sample types; endometriosis-associated ovarian cancer and ovarian cancer-associated endometriosis (CAE). Representative cancerous and endometriotic tissues were obtained in 32 out of 33 cases. Ovarian cancer cases comprised 17 endometrioid and 16 clear-cell subtypes, determined according to WHO classification (Tavassoli and Devilee, 2003). Tumor characteristics from study I and II are shown in table 6.

In study III, the material consisted of 50 venous blood samples from women with PCOS and 20 venous blood samples from age- and BMI-matched healthy women as controls. The women were recruited in 2006–2008 and PCOS was diagnosed according to the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine consensus definition. The women were selected to form two equally sized BMI groups; obese (BMI \(\geq 27\) kg/m\(^2\)) and non-obese (BMI < 27 kg/m\(^2\)).

In study IV, the material consisted of venous blood samples from 110 women with PCOS, selected from a cohort of a prospective multicentre, randomized, placebo-controlled study on the effects of metformin on miscarriage, pregnancy and miscarriage rates (Morin-Papunen et al. 2012). The women were recruited to the large cohort in 2003–2009 and PCOS was diagnosed according to the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine consensus definition. One hundred and ten women were selected according to their age and BMI to form two distinct BMI groups in the same manner as in study II. The patients were randomized to receive metformin (Diformin, Leiras) or placebo for 3 months and blood samples were
taken before and after the treatment. Obese women received metformin at 1000 mg × 2 daily and non-obese women received metformin at 500 mg + 1000 mg daily. Fifty-three women (23 obese, 30 non-obese) received metformin and 57 women (27 obese, 30 non-obese) received placebo. Patient characteristics in studies III and IV are shown in table 7.

All serum samples were acquired from the Department of Oncology and from the Department of Obstetrics and Gynaecology, Oulu University Hospital. All tissue samples were acquired from the archives of the Department of Pathology, Oulu University Hospital. Clinical data was obtained from the files of Oulu University Hospital. All serum samples were stored in polypropylene or polystyrene tubes at -20 °C and all tissue samples were fixed in neutral formalin and embedded in paraffin.

Table 6. Tumor characteristics in study I and II.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study I ductal carcinomas</th>
<th>Study I other carcinomas</th>
<th>Study II endometrioid carcinomas</th>
<th>Study II clear cell carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>140</td>
<td>33</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>18</td>
<td>9</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>64</td>
<td>20</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>58</td>
<td>4</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>T (breast carcinoma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>89</td>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>44</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3+4</td>
<td>7</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N (breast carcinoma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>75</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Figo stage (ovarian carcinoma)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>8</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>III + IV</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 7. Clinical parameters in study III and in study IV before metformin treatment. Hirsutism score according to Ferriman-Gallwey criteria.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study III PCOS</th>
<th>Study III Control</th>
<th>Study IV PCOS metformin</th>
<th>Study IV PCOS placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obese</td>
<td>Nonobese</td>
<td>Obese</td>
<td>Nonobese</td>
</tr>
<tr>
<td>Number of cases</td>
<td>25</td>
<td>25</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age (y)</td>
<td>27.8 ± 4.7</td>
<td>26.6 ± 3.8</td>
<td>34.8 ± 8.3</td>
<td>26.3 ± 5.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.8 ± 4.7</td>
<td>22.4 ± 4.2</td>
<td>31.5 ± 3.3</td>
<td>22.1 ± 2.2</td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>7.9 ± 4.0</td>
<td>4.3 ± 2.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serum testosterone (nmol/L)</td>
<td>1.7 ± 0.8</td>
<td>1.6 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serum SHBG (nmol/L)</td>
<td>35.7 ± 14.0</td>
<td>59.1 ± 21.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fasting insulin (μIU/mL)</td>
<td>16.7 ± 14.0</td>
<td>5.1 ± 2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>5.3 ± 0.3</td>
<td>5.0 ± 0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oligomenorrhea or amenorrhea</td>
<td>25/25</td>
<td>25/25</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Hirsutism score according to Ferriman-Gallwey criteria.
4.2 Methods

4.2.1 ELISA

In studies I, III and IV, serum levels of 8-OHdG were measured by using enzyme-linked immunosorbent assay (ELISA) kits from the Japan Institute for the Control of Aging, Fukuroi, Japan. The assay is named “Highly Sensitive 8-OHdG Check ELISA” and it utilizes anti 8-OHdG monoclonal antibody (clone N45.1). According to the manufacturer, the antibody should not cross-react with RNA oxidation products such as 8-hydroxy-guanine and 8-hydroxy-guanosine. A few modifications were made to the manufacturer’s assay instructions to fit more samples in one microtitre plate. Serum samples were filtered by using Millipore Microcon filters to separate interfering substances. Before use, the filters were moistened by centrifuging 100 mL of distilled water through them. Leftover water was removed from the filters by centrifuging them upside down for 5 minutes. Serum samples were then filtered through the moistened Millipore Microcon filters by centrifuging them for 30 minutes at 14,000 × g. Primary antibody and primary antibody solution were mixed together until the antibody was dissolved completely. Filtered serum samples, and standards (both 50 mL) were added to the wells of a microtitre plate. Forty-one samples in duplicate were placed in one plate by utilizing all the wells. Reconstituted primary antibody (50 mL) was then added to each well. The plate was shaken, covered with an adhesive strip and incubated overnight at +4 °C. After incubation, the wells were emptied and washed three times with 250 mL of washing solution. Secondary antibody was mixed with secondary antibody solution and dissolved completely. Reconstituted secondary antibody (100 mL) was added to each well. The plate was shaken, covered with an adhesive strip and incubated at room temperature for one hour. After incubation the wells were again emptied and washed three times with 250 mL of washing solution. Enzyme substrate solution was then reconstituted and 100 mL added to each well. The plate was shaken, covered with aluminium foil and incubated in the dark, at room temperature, for 15 minutes. After incubation, 100 mL of reaction termination solution was added to each well. Absorbance values were read at 450 nm and the amount of 8-OHdG in the samples was determined by utilizing a standard curve. If 8-OHdG concentrations in duplicate samples differed by more than 10%, the sample was re-assayed.
4.2.2 Immunohistochemistry

Immunohistochemistry was used in studies I and II to assess expression of 8-OHdG, Prx II and Prx VI in tissue samples. Before use, tissue samples were fixed in 10% phosphate-buffered neutral formalin and embedded in paraffin. Four-μm thick sections were cut from paraffin-embedded samples and mounted on SuperFrostPlus glass (Menzel–Gläser, Germany). The sections were then deparaffinized in xylene and re-hydrated through a series of graded alcohol solutions. They were pre-digested by placing them in 10 mM citric acid monohydrate and heating them in a microwave oven for 10 minutes. They were then cooled at room temperature. To neutralize endogenous peroxides, the sections were immersed in 3% hydrogen peroxide in methanol for 15 minutes. Primary antibodies were added and the sections incubated overnight at +4 °C. After that, they were incubated with secondary antibody for 30 minutes. Aminoethyl carbazole (Zymed Laboratories Inc., South San Francisco, CA, USA) was used as a chromogen in both immunohistochemistry studies. After the addition of chromogen, the sections were counterstained with haematoxylin and mounted with Immu-Mount (Shandon, Pittsburgh, PA, USA).

In Study I, mouse monoclonal 8-OHdG antibody (clone N45.1) (Gentaur, Belgium) was used as primary antibody with biotinylated secondary antibody (Dakopatts, Glostrup, Denmark), and an avidin-biotin-peroxidase complex (Dakopatts, Glostrup, Denmark) was used for 8-OHdG immunostaining. In study II, mouse monoclonal 8-OHdG antibody (clone N45.1) (Japan Institute for the Control of Aging, Fukuroi, Japan), rabbit polyclonal Prx II antibody (Ab Frontier, Seoul, Korea) and rabbit polyclonal Prx VI antibody (Ab Frontier, Seoul, Korea) were used as primary antibodies, together with Novocastra Novolink Polymer Detection Systems Kits (Leica Microsystems, Wezlar, Germany) for 8-OHdG, Prx II and Prx VI immunostaining (Table 8). The outcomes of the staining reactions were divided into four (study I) and three (study III) groups according to the percentage of cells showing positive immunostaining. In study I, the groups were – (<5%), + (5–20%), ++ (21–80%) and +++ (81–100%). In study III, the groups were – (<10%), + (10–70%) and ++ (>70%). Expression of 8-OHdG in the nuclei of cancer cells was evaluated in study I. In study II, expression of nuclear and cytoplasmic 8-OHdG, Prx II and Prx VI was evaluated in ovarian cancer cells, epithelial cells in ovarian cancer-associated endometriosis and in epithelial cells in cases of benign endometriosis. In study II, Prx II and Prx VI were stained in...
only 13 out of 22 benign endometriosis samples owing to exhaustion of representative paraffin blocks

Table 8. Antigens, antibodies and immunohistochemistry methods used in studies I and II.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary antibody</th>
<th>Source of primary antibody</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG (study I)</td>
<td>Mouse monoclonal 8-OHdG antibody (clone N45.1)</td>
<td>Centaur, Belgium</td>
<td>A biotinylated secondary antibody, avidin-biotin-peroxidase complex (Dakopatts, Denmark)</td>
</tr>
<tr>
<td>8-OHdG (study II)</td>
<td>Mouse monoclonal 8-OHdG antibody (clone N45.1)</td>
<td>Japan Institute for the Control of Aging, Japan</td>
<td>Novocastra Novolink Polymer Detection Systems Kits (Leica Microsystems, Germany)</td>
</tr>
<tr>
<td>Prx II (study II)</td>
<td>Rabbit polyclonal Prx II antibody</td>
<td>Ab Frontier, Seoul, Korea</td>
<td>Novocastra Novolink Polymer Detection Systems Kits (Leica Microsystems, Germany)</td>
</tr>
<tr>
<td>Prx VI (study II)</td>
<td>Rabbit polyclonal Prx VI antibody</td>
<td>Ab Frontier, Seoul, Korea</td>
<td>Novocastra Novolink Polymer Detection Systems Kits (Leica Microsystems, Germany)</td>
</tr>
</tbody>
</table>

4.2.3 Statistical methods

Statistical analysis was carried out by using SPSS versions 15.0 (I) and 16.0 (II, III, IV) for Windows, and R-language (I). The Chi-square test (II), Fisher’s exact test (I, II, III), Student’s t-test (IV), the paired samples t-test (IV), the Mann–Whitney U test (I, III), Spearman’s test (I), Pearson’s test (I, IV), the general linear model (IV) and Cox’s multivariate regression analysis (I) were used to determine the significance of associations. Kaplan–Meier curves with log-rank, Tarone –Ware and Breslow tests were used for survival analysis (I). Receiver operating characteristic curves were used to assess an optimal cut-off point in study III. A value of p of < 0.05 was considered statistically significant.

4.2.4 Ethics

These studies were approved by the National Supervisory Authority for Welfare and Health (D1339/ 05.01.00.06/2009) and the Ethics Committee of the Northern Ostrobothnia Hospital District (1396/2004).
5 Results

5.1 8-OHdG in breast cancer (study I)

Fifteen per cent of all cancer tissue samples showed negative (<5% of cells showing nuclear positivity) 8-OHdG immunostaining and the patients concerned had a significantly higher risk of breast cancer-specific death when compared with patients with positive 8-OHdG immunostaining (Figure 2 and Figure 3). According to the results of (Cox’s) multivariate regression analysis, negative 8-OHdG expression in breast cancer cells was an independent prognostic factor as regards poor survival. Negative 8-OHdG immunostaining also correlated with negative HER-2 and negative p53 expression in all patients and with positive nodal status among ductal carcinoma patients. In line with immunohistochemical findings, low 8-OHdG levels in serum correlated with aggressive features: lymphatic vessel invasion and positive lymph node status in all patients, and higher grade, positive lymph node status, lymphatic vessel invasion and blood vessel invasion in patients with ductal carcinoma. The expression of 8-OHdG in cancer tissue correlated positively with 8-OHdG levels observed in serum.

Fig. 2. An example of highly positive (A) and negative (B) 8-OHdG immunostaining in breast carcinoma tissue. Magnification x 210.
Fig. 3. Kaplan – Meier curves showing breast cancer-specific survival among all breast carcinomas (A) and among ductal carcinomas only (B). Comparison is made between positive and negative 8-OHdG immunostaining.

5.2 8-OHdG, Prx II and Prx VI in endometriosis-associated ovarian cancer (study II)

In analysis of three groups of patients (EAC, CAE and BE), cancer cells showed significantly weaker nuclear and cytoplasmic 8-OHdG expression and nuclear Prx II expression than epithelial cells in endometriotic tissue (Figure 4). This trend remained when the groups were compared in pairs. Epithelial cells in ovarian cancer-associated endometriosis showed stronger expression of nuclear and cytoplasmic 8-OHdG and Prx VI and stronger cytoplasmic Prx II expression compared with ovarian cancer cells. Epithelial cells in benign endometriosis showed stronger cytoplasmic 8-OHdG and nuclear Prx II expression but weaker cytoplasmic Prx VI expression compared with ovarian cancer cells. Ovarian cancer cells with poor differentiation (grade 2–3) showed significantly low cytoplasmic 8-OHdG expression in comparison with grade 1 cancer cells. In addition, low-grade cancer cells showed significantly elevated nuclear Prx VI expression. In histological subtype analysis, the endometrioid subtype showed stronger cytoplasmic 8-OHdG expression compared with the clear-cell subtype.
Fig. 4. Pillar diagrams showing the difference in positive and negative immunostainings and p-values according to Chi-square test between all three patient groups.

Abbreviations: 8-OHdG = 8-hydroxydeoxyguanosine; BE = Benign endometriosis; EAC = Endometriosis-associated ovarian cancer; PRX = Peroxiredoxin.

5.3 8-OHdG in PCOS (study III and IV)

Serum 8-OHdG levels in studies III and IV are shown in Table 9. Women with PCOS had significantly lower serum levels of 8-OHdG among all subjects and also separately among obese and non-obese groups when compared with healthy age- and BMI-matched women (Study III). According to the receiver operating characteristic curve, the optimal 8-OHdG cut-off value to distinguish women with PCOS from healthy women was 184.69 pg/mL (study III). With 85% sensitivity and 90% specificity, 90% of women with PCOS and only 15% of healthy women had serum levels of 8-OHdG below this cut-off value.

Among the whole study population in study IV, women with PCOS treated with metformin for 3 months showed a significant decrease in serum 8-OHdG levels, while there was a slight increase in 8-OHdG serum levels in the placebo group. Baseline levels of 8-OHdG were comparable in the metformin and placebo groups. The effect of metformin treatment on 8-OHdG serum levels was most significant among obese women. The body weight of obese women fell significantly during metformin treatment. The decrease in 8-OHdG serum levels solely among non-obese women with PCOS did not reach statistical significance, even though a clear drop from baseline levels was observed.
Table 9. Serum 8-OHdG levels in studies III and IV. Study III p values between PCOS and control groups according to Mann – Whitney U tests. Study IV p values between 8-OHdG levels before and after the treatment according to paired-samples t-tests.

<table>
<thead>
<tr>
<th>Study</th>
<th>8-OHdG in study III and before treatment in study IV (pg/mL)</th>
<th>8-OHdG after treatment in study IV (pg/mL)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All PCOS (n=50)</td>
<td>137.8 ± 44.4</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>All Control (n=20)</td>
<td>219.7 ± 57.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese PCOS (n=25)</td>
<td>149.3 ± 42.0</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Obese Control (n=10)</td>
<td>229.0 ± 51.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonobese PCOS (n=25)</td>
<td>126.3 ± 44.5</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nonobese Control (n=10)</td>
<td>210.3 ± 65.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study IV Metformin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients (n=53)</td>
<td>291.8 ± 94.0</td>
<td>263.2 ± 71.2</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Obese (n=23)</td>
<td>314.1 ± 88.6</td>
<td>271.2 ± 76.9</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Nonobese (n=30)</td>
<td>274.7 ± 95.9</td>
<td>257.1 ± 67.2</td>
<td>NS</td>
</tr>
<tr>
<td>Study IV Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All patients (n=57)</td>
<td>273.4 ± 68.4</td>
<td>274.1 ± 100.6</td>
<td>NS</td>
</tr>
<tr>
<td>Obese (n=27)</td>
<td>292.4 ± 49.4</td>
<td>328.7 ± 95.2</td>
<td>NS</td>
</tr>
<tr>
<td>Nonobese (n=30)</td>
<td>256.2 ± 78.9</td>
<td>225.0 ± 78.4</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>
6 Discussion

6.1 ROS in breast cancer

Exposure to oestrogen is one of the key risk factors of breast cancer. Interestingly, oestrogen and its hydroxylated metabolites seem to increase ROS production by regulating mitochondrial function and bringing about lipid peroxidation (Vera-Ramirez et al. 2011). It has been suggested that the carcinogenic effects of oestrogen exposure may be partly explained by ROS-derived genomic instability and activation of tumourigenic signalling pathways (involving NF-κB, nrf-2, AP-1) (Okoh et al. 2011). Higher levels of 8-OHdG and the hydroxylated oestrogen metabolite 4-OH-E2 have been observed in breast cancer tissue compared with benign breast tissue (Roy et al. 2007). In addition, 8-OHdG is more abundant in ER+ tumours compared with ER- tumours (Roy et al. 2007, Karihtala et al. 2011). This might also indicate that less aggressive and early-stage breast carcinomas express higher ROS levels to initiate the carcinogenic process, but the most aggressive tumours and tumours at later stages prefer ROS at physiological levels for the stimulation of tumour growth and survival-supporting signalling pathways. Reactive oxygen species can also bring about phosphorylation and proteolysis of oestrogen receptors and this is thought to be one of the mechanisms turning ER+ tumours into ER- tumours, finally leading to endocrine therapy resistance (Vera-Ramirez et al. 2011).

In general, levels of oxidative stress markers, including the DNA damage marker 8-OHdG and the lipid peroxidation markers MDA and 4-HNE, are elevated in breast cancer tissue and in the serum and urine of breast cancer patients when compared with healthy controls (Karihtala et al. 2006, Karihtala and Puistola 2011, Karihtala et al. 2011, Pande et al. 2011, Vera-Ramirez et al. 2011, Pande et al. 2012). Urinary levels of 8-OHdG have been observed to drop significantly after surgical removal of breast cancer tissue, which indicates that a malignant process can be a major source of the total oxidative DNA damage in the human body (Cho et al. 2009). This idea is also supported by our finding that 8-OHdG expression in breast cancer tissue is associated with 8-OHdG serum levels in breast cancer patients. Also in line with increased oxidative damage, most antioxidant enzymes, such as Prxs, Trx, SOD and GPx are up-regulated in breast cancer patients compared with healthy controls (Karihtala et al. 2003, Turunen et al. 2004, Cha et al. 2009, Karihtala et al. 2011, Karihtala and Puistola
Among antioxidant enzymes, catalase is the only exception to this trend, since its expression has been consistently shown to be decreased in breast cancer (reviewed in Karihtala and Puistola 2011). Down-regulation of catalase might have a major role in the oxidative stress-derived initiation of breast carcinogenesis, since acatalatic mice are known to develop significantly more breast cancer (Ishii et al. 1996).

Even though 8-OHdG is generally increased in breast cancer patients compared with healthy controls, lower 8-OHdG levels often correlate with more aggressive carcinoma phenotypes in several types of carcinoma (Matsui et al. 2000, Kuo et al. 2007, Karihtala et al. 2011). These findings are supported by our results, where low 8-OHdG expression in breast cancer tissue and low 8-OHdG serum levels were found to be associated with poor survival and aggressive features. Our results strengthen the overall hypothesis that low expression of 8-OHdG observed with more advanced breast cancer could indicate that the levels of H2O2 are maintained at near physiological levels in the cells. Under these circumstances, ROS would merely promote growth- and survival-signalling pathways, while there would not be excess H2O2 available for the initiation of apoptosis or for the production of damaging OH via the Haber–Weiss reaction. Low OH production would naturally lead to minimal DNA damage and low immunohistochemical expression of oxidatively damaged DNA adducts. In addition, low 8-OHdG levels observed in the serum of our breast cancer patients is also a natural consequence of minimal DNA damage in cancer cells, since after damaged guanosine is cleaved from DNA, it is secreted to the urine via the circulation. Furthermore, similar to low 8-OHdG expression, a recent study revealed low expression of hOGG1, the repair enzyme removing 8-OHdG from DNA, to be associated with aggressive breast cancer phenotypes (Karihtala et al. 2012). This is probably a result of the low demand for 8-OHdG repair in the most aggressive tumours.

### 6.2 ROS in endometriosis and endometriosis-associated ovarian cancer

Oxidative stress is considered to be one of the main factors contributing to malignant transformation of benign endometriosis into epithelial ovarian cancer (Worley et al. 2013). Interestingly, other suggested mechanisms behind malignant transformation of endometriosis, i.e. inflammation and hyperestrogenism, are also closely connected to oxidative stress (Reuter et al. 2010, Vera-Ramirez et al. 2011,
Persistent high-level oxidative stress is probably potent in initiating the carcinogenic process by causing mutations in crucial oncogenes and tumour suppressor genes linked to the development of endometriosis-associated ovarian cancer such as *PTEN, TP53, NF-kB, ARID1, KRAS* and *PI3K* (Shigetomi *et al.* 2012, Worley *et al.* 2013). As noted before, most of these EOC-associated gene mutations are known to be closely related to ROS. Recent studies also indicate that over-expression of HNF-1β by endometriotic cells could promote their survival in a highly oxidative environment (Shigetomi *et al.* 2012). HNF-1β also seems to be up-regulated in ovarian clear-cell carcinoma, further suggesting the significance of oxidative stress in the carcinogenesis of endometriosis-associated ovarian cancer (Worley *et al.* 2013). In addition to being linked to the malignant transformation of endometriosis, oxidative stress is also considered to have a significant impact on the pathogenesis and progression of endometriosis itself (Carvalho *et al.* 2012). Oxidative stress has been shown to activate ERK1/2 and PI3K/mTOR/AKT pathways in benign endometriotic tissue and lead to disease progression (Ngô *et al.* 2009, Leconte *et al.* 2011). Activation of these signalling pathways is also closely linked to carcinogenesis.

In the majority of recent studies, oxidative stress markers, including 8-OHdG, have been reported to be increased in women with endometriosis when compared with healthy controls (Murphy *et al.* 1998, Szczepanska *et al.* 2003, Jackson *et al.* 2005, Kao *et al.* 2005, Verit *et al.* 2008, Lambrinoudaki *et al.* 2009, Carvalho *et al.* 2012). The main source of increased oxidative stress in endometriotic tissue is suggested to be repetitive production of haemorrhage-derived free iron, which increases the production of •OH via the Haber–Weiss reaction. Increased free iron levels in endometriotic cysts have been proved to correlate with increased 8-OHdG levels and in addition, the ovarian cortex around endometriotic cysts expresses elevated 8-OHdG levels (Yamaguchi *et al.* 2008, Matsuzaki and Schubert 2010). Only a few studies have been concerned with antioxidant activity or antioxidant enzyme expression in endometriosis. Reduced expression of SOD, GPx and total thiol has been observed in the peritoneal fluids of endometriosis patients (Szczepanska *et al.* 2003, Jackson *et al.* 2005). Antioxidant-poor diets have been connected to endometriosis and antioxidant supplementation seems to reduce oxidative stress and relieve symptoms in endometriosis (Agarwal *et al.* 2012, Santanam *et al.* 2013). In contrast, SOD expression seems to be up-regulated at tissue level in endometriosis, which could reflect a local response to high-level oxidative stress in endometriosis cells (Ota *et al.* 1999). Constant high
consumption of antioxidant enzymes in cells would naturally lead to low extracellular levels of antioxidants.

Studies on oxidative stress markers and antioxidants in ovarian cancer have mostly been focused on serous ovarian carcinomas, but endometriosis-associated ovarian cancer is usually known to become manifest in the form of endometrioid or clear-cell subtypes (Munksgaard and Blaakaer 2011). Studies concerning mostly serous and mucinous subtypes have generally revealed increased levels of oxidative stress markers in ovarian cancer compared with healthy controls (Senthil et al. 2004, Sanchez et al. 2006). In addition, high serum levels and strong tissue expression of 8-OHdG has been associated with poor survival and aggressive clinicopathological features in ovarian cancer (Karihtala et al. 2009, Pylväs et al. 2011). Antioxidant activity in ovarian cancer seems to have some enzyme-specific variation, since SOD and CAT show decreased expression, while GSH-dependent enzymes such as GPx seem to be up-regulated (Senthil et al. 2004, Sanchez et al. 2006, Karihtala and Puistola 2011). Borderline ovarian tumours show increased expression of Prx II and Prx VI compared with benign tumours and the expression Prx V and Prx VI is increased along with ovarian cancer stage (Karihtala et al. 2009, Pylväs et al. 2010).

We focused on endometrioid and clear-cell cancers. Our results suggest that 8-OHdG and Prx II expression decrease at tissue level along with the carcinogenic progress of endometriosis-associated ovarian cancer. Strong expression of the oxidative stress marker 8-OHdG and the antioxidant Prx II in endometriosis tissue seems to be in line with the results of previous studies (Murphy et al. 1998, Szczepanska et al. 2003, Jackson et al. 2005, Kao et al. 2005, Verit et al. 2008, Lambrinoudaki et al. 2009, Carvalho et al. 2012). High Prx II levels are likely to be a result of the induction of local antioxidant defence in response to high ROS production in endometriosis tissue. However, high 8-OHdG expression shows that antioxidant activity is still deficient in endometriosis tissue, since there is an abundance of damaged DNA adducts in endometriosis cells. This indicates that high-level oxidative stress could have a significant role in the early stages of the malignant transformation of endometriosis. Decreased expression of 8-OHdG and Prx II in endometriosis-associated ovarian cancer seems to be directly opposite the trend that has usually been observed in previous studies mostly concerning serous ovarian carcinomas (Senthil et al. 2004, Sanchez et al. 2006, Karihtala et al. 2009, Pylväs et al. 2010, Pylväs et al. 2011). This is probably a result of totally different aetiologies of these ovarian cancer subtypes and our results strengthen this idea even further.
Endometriosis-associated ovarian cancer is known to bear a unique molecular signature, to develop at a younger age and is found at earlier stages compared with other ovarian cancer subtypes (Banz et al. 2010, Munksgaard and Blaakaer 2011). At advanced stages, these cancer types behave more aggressively and are more resistant to platinum-based chemotherapy. According to our results, the contribution of ROS to the carcinogenic process could be similar in breast cancer and endometriosis-associated ovarian cancer. High-level oxidative stress is crucial for the initiation of carcinogenesis, while lower-level ROS production is more favourable in later stages, promoting growth, survival and resistance to chemotherapy. Decreased Prx II expression in endometriosis-associated ovarian cancer might simply be a result of a reduced need of this antioxidant enzyme, either because of decreased ROS production, increased activity of other antioxidants or enhanced repair of ROS-mediated damage in cancer cells.

### 6.3 ROS in PCOS

Oxidative stress is known to be closely related to some of the main characteristics of PCOS, such as insulin resistance, obesity, inflammation and oestrogen and androgen activity. In addition, the latest evidence suggests that there could be a close connection between hyperandrogenism, insulin resistance, inflammation and oxidative stress in the pathogenesis of PCOS (González 2012). The development of insulin resistance in type 2 diabetes is suggested to be partly explained by the high oxidative stress observed in these patients (Henriksen et al. 2011). Additionally, obese people show aberrantly high levels of lipid peroxidation, protein oxidation and oxidized DNA adducts compared with people of normal weight and this phenomenon can partially be explained by hyperglycaemia, chronic inflammation and defective antioxidant activity, among other things (Vincent et al. 2007). Increased levels of serum 8-OHdG correlate with obesity and type 2 diabetes and 8-OHdG levels are known to be connected to body weight and the inflammation marker (high-sensitivity) CRP in healthy women (Sakano et al. 2009, Al-Aubaidy and Jelinek 2011). The effect of androgens as regards oxidative stress is obvious in prostate cancer, where they are known to increase the levels of oxidized DNA adducts, including 8-OHdG, in prostate cancer cells (Miyake et al. 2004, Pathak et al. 2008, Gupta-Elera et al. 2012). Finally, patients with PCOS might have a slightly increased risk of developing ovarian cancer, the pathogenesis of which is nowadays thought to be closely linked to ROS (Karihtala and Puistola 2011). Since ROS seem to be
connected to the major characteristics of PCOS on such a wide scale, interest in research concerning the role of oxidative stress in the pathogenesis of PCOS and PCOS-related cancers has increased substantially in recent years.

Various markers of oxidative stress, and antioxidants, have been studied widely in connection with PCOS, but there are only a few studies concerning oxidative DNA damage in women with this condition. There is good evidence that patients with PCOS have significantly increased serum and urinary levels of lipid peroxidation products (in most studies measured as MDA), increased protein oxidation and an elevated total oxidative state when compared with healthy age- and BMI-matched controls (Sabuncu et al. 2001, Fenkci et al. 2003, Yilmaz et al. 2005, Kaya et al. 2008, Verit and Erel 2008, Kuxsxcu and Var 2009, Rajendran et al. 2009, Kurodoglu et al. 2012, Blair et al. 2013, Gao et al. 2013, Hilali et al. 2013, Kuxsxcu and Var 2009, Rajendran et al. 2009, Kurodoglu et al. 2012, Blair et al. 2013, Enli et al. 2013, Hilali et al. 2013, Meng et al. 2013, Murri et al. 2013). Among antioxidants, SOD has been consistently reported to be up-regulated, whereas GSH, Prx IV and thiol-based total antioxidant status are down-regulated in women with PCOS, which could suggest generally defective H2O2 scavenging in cases of PCOS (Sabuncu et al. 2001, Fenkci et al. 2003, Dinger et al. 2005, Yilmaz et al. 2005, Kaya et al. 2008, Kuxsxcu and Var 2009, Rajendran et al. 2009, Kurodoglu et al. 2012, Blair et al. 2013, Enli et al. 2013, Hilali et al. 2013, Meng et al. 2013, Murri et al. 2013). In three recent studies concerning 8-OHdG in cases of PCOS, two revealed elevated plasma/serum levels of 8-OHdG and one showed no significant difference in urinary 8-OHdG levels in women with PCOS compared with healthy controls (Hamurcu et al. 2010, Gao et al. 2013, Shreeve et al. 2013). These results indicate that oxidative stress is increased among PCOS patients, inflicting damage to proteins and lipids at least. Whether or not PCOS patients have significantly increased ROS-mediated DNA damage is still controversial as a result of the low number of studies carried out.

A number of investigators have linked increased oxidative stress to the insulin resistance, inflammation and hyperandrogenism observed in PCOS. Increased oxidative stress and decreased antioxidant levels correlate with serum testosterone levels, high insulin levels and insulin resistance in women with PCOS (Fenkci et al. 2003, Dinger et al. 2005, Yilmaz et al. 2005, Kaya et al. 2008, Macut et al. 2011, González 2012). Physiological hyperglycaemia seems to increase ROS production and the inflammatory response of mononuclear cells in cases of PCOS and this phenomenon is independent of obesity (González et al. 2006). Interestingly, hyperandrogenism activates mononuclear cells and the amount of mononuclear cells is increased in PCOS (González et al. 2006).
Increased ROS production further promotes the expression of TNF-α and IL-6 via activation of the transcription factor NF-κB (González et al. 2006, González 2012). Increased expression of proinflammatory cytokines is related to the development of insulin resistance and hyperandrogenism (González et al. 2006, González 2012). It has been shown in vitro that ovarian steroidogenic CYP17 enzyme activity and androgen production from theca cells can be promoted by proinflammatory stimuli (Spazynsky et al. 1999, Piotrowski et al. 2005). Victor et al. (2011) observed defective mitochondrial function (i.e. decreased oxygen consumption), increased ROS production and decreased GSH levels in the polymorphonuclear cells of patients with PCOS. It has also been suggested that inflammation may directly induce hyperandrogenism in PCOS, without the development and contribution of hyperinsulinaemia (González 2012).

In study III, we reported significantly reduced 8-OHdG levels in the serum of women with PCOS when compared with healthy controls. However, in study IV mean 8-OHdG serum levels of PCOS women are higher than in control group or in PCOS women group in study III. At first glance this may seem paradoxical, since the results of previous studies have convincingly shown that oxidative stress is increased in women with PCOS, at least according to the observed oxidative damage to proteins and lipids. The variation observed in 8-OHdG levels could be explained by enhanced antioxidant defence in response to increased oxidative stress. It could be expected that during oxidative stress, the integrity of DNA is the number one priority as regards antioxidant protection and this could lead to low oxidative DNA damage during mild oxidative stress. Furthermore, growing oxidative stress is likely to be reflected first as protein oxidation and lipid peroxidation, as seen in PCOS, since proteins and lipids are far more abundant components than DNA in our cells and do not have specific repair mechanisms (Davies et al. 2005). Therefore, only high-level oxidative stress may be capable of overwhelming antioxidant defence, leading to interaction with DNA. Aberrant 8-OHdG levels in PCOS could merely be a sign of constantly varying oxidative stress in ovaries where antioxidant activity is only momentarily able to maintain balance resulting into the fluctuation of oxidative DNA damage but rather constant lipid and protein damage. Also insulin resistance, testosterone and SHBG levels have notable diurnal intraindividual variation in PCOS women which should, in the light of previous observations, be reflect as a variation in oxidative stress rate (Jayagopal et al. 2002, Jayagopal et al. 2003). Another possible explanation for low 8-OHdG serum levels could be enfeebled repair of oxidized DNA adducts, where they would not be secreted to the circulation. However, this
would not explain the high 8-OHdG levels. Differences in 8-OHdG levels between study III and IV could also result from methodological issues like deviance in ELISA kit shipments which would make the comparison of the absolut 8-OHdG levels between these two studies unreliable. Overall, it would seem that the most harmful effect of oxidative stress in PCOS is not the oxidative DNA damage caused by high-level oxidative stress, as in endometriosis, but rather the contribution of ROS as signalling molecules as regards increased androgen activity and development of insulin resistance, together with mild inflammation. This resembles the function of ROS also observed in the later stages of breast cancer and endometriosis-associated ovarian cancer. Furthermore, low oxidative DNA damage observed among PCOS women could partly explain the weak connection between ovarian cancer and PCOS.

### 6.4 Effect of metformin on oxidative stress in PCOS patients

Metformin administration is the first-line treatment for type II diabetes because of its great antihyperglycaemic properties and also because of its preventive effects on secondary complications (Rojas and Gomes 2013). The most important effects of metformin are inhibition of hepatic gluconeogenesis and increase of glucose uptake in peripheral tissues, thus reducing insulin resistance and glucose levels in plasma (Mahmood et al. 2013). Metformin also decreases intestinal glucose uptake and circulating levels of free fatty acids (Mathur et al. 2008). These effects of metformin are mostly mediated by activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway, which has also been shown to be associated with reduction of vascular inflammation and promotion of antioxidants (Mathur et al. 2008, Hou et al. 2010). Oxidative stress is thought to contribute significantly to the development of insulin resistance in type 2 diabetes and, interestingly, several investigators have reported that metformin treatment decreases oxidative stress in type 2 diabetes patients (Henriksen et al. 2011, Rojas and Gomes 2013). Metformin has been shown to decrease levels of oxidative stress markers and increase antioxidant activity in vivo and in vitro (Pavlović et al. 2000, Bonnefont-Rousselot et al. 2003, Formoso et al. 2008, Esteghamati et al. 2013). The effect of metformin on oxidative stress has also been studied in several animal models, with intriguing results. Metformin treatment decreased oxidative stress and NF-kB activation, up-regulated the expression of several antioxidant genes and inhibited TNF-alpha and IL-6 genes in diabetic rats (Alhaider et al. 2011, Zheng et al. 2012). In addition, metformin increases the life
span of *C. elegans* by promoting antioxidant defence via the transcription factor SKN-1/Nrf2 (Onken and Driscoll 2010).

Recent studies have also shown beneficial effects of metformin in PCOS and encouraged its wider use in this complex disease (Lebinger 2007, Mathur *et al.* 2008). Use of metformin in women with PCOS has been shown to regularize menstruation, improve ovulation, decrease body weight, increase insulin sensitivity, lower circulating androgen levels and reduce long-term metabolic complications (Mathur *et al.* 2008). The advantageous effects of metformin in PCOS are likely to originate mostly from its antihyperglycaemic properties. However, in the light of the results of previous studies, metformin should also have an impact on the imbalanced redox status observed in PCOS patients. In study IV, we observed a significant reduction in serum 8-OHdG levels in PCOS patients after three months of metformin treatment in comparison with the placebo group. The decrease was evident among obese women with PCOS but did not reach statistical significance among lean PCOS patients alone. Only one study has previously concerned the effect of metformin treatment on oxidative stress in women with PCOS. In the study, metformin treatment did not have a significant effect on total antioxidant status or MDA levels (Yilmaz *et al.* 2005). The study included only lean women with PCOS and the results are therefore in line with our findings. In addition to decreases in 8-OHdG, testosterone and glucose levels, and body weight of obese patients, testosterone levels in lean patients were significantly reduced during metformin treatment, which is also in line with the results of previous studies, where hyperandrogenism, insulin resistance and oxidative stress have been closely connected in cases of PCOS. The decrease in 8-OHdG levels is therefore probably explainable by the combination of reduced body weight, increased insulin sensitivity and decreased androgen excess. In addition, the beneficial effects of metformin in PCOS might also be connected to its ability to reduce oxidative stress. Reactive oxygen species and antioxidant enzymes also have a central role in the regulation of follicle maturation, ovulation and corpus luteum activity, indicating that maintenance of a strict redox balance in the ovary is crucial for normal female reproduction (Agarwal *et al.* 2012). Anovulation and infertility related to PCOS could therefore at least partly be explained by the aberrant ROS and antioxidant balance observed in women with PCOS. Metformin has been proven to improve pregnancy and live-birth rates in cases of PCOS, which might also partly be explained by the normalized redox balance associated with metformin use in PCOS patients (Morin-Papunen *et al.* 2012).
6.5 Challenges in oxidative stress research

There seems to be many mixed results and unexpected associations in the field of oxidative stress research and often results leave a lot of room for speculation. This emphasizes the major problem of studies in which attempts are made to evaluate the significance of oxidative stress in the pathogenesis of cancer and other diseases. There is such a huge number of different sources of ROS and antioxidant enzymes contributing to the overall redox status of a tissue that measuring a single metabolic product or antioxidant is able to give answers only to specific questions. ROS, antioxidants and oxidative damage markers are constantly produced and catabolized in our cells and therefore one of the biggest problems in the study considering oxidative stress is that we don’t know yet what are the normal levels of these molecules in our cells (Halliwell 2000, Kryston et al. 2011). Furthermore, ROS and many enzymatic antioxidants are so highly reactive that direct measurement of them by current means is challenging (Tarpey et al. 2004, Valko et al. 2006). In addition, measurement of oxidative DNA damage markers from directly from DNA, like 8-OHdG, are vulnerable for artificial DNA oxidation during DNA extraction or analysis whereas indirect methods (enzymatic assays) give much lower values of 8-OHdG (Valavanidis et al. 2009, Kryston et al. 2011).

After all, what matters most is the total cellular balance of reducing and oxidizing agents that finally determines whether or not the tissue is suffering from oxidative stress. It would seem that high levels of ROS and oxidative stress are important in the initiation and early stages of cancer, but at later stages tumours may profit more from physiological ROS levels. Furthermore, the significance of ROS in benign diseases seems to be highly dependent on the magnitude of oxidative stress, as in endometriosis a high level of oxidative stress might be strongly related to the initiation of carcinomas, whereas low to moderate levels of oxidative stress in PCOS might not be likely to induce carcinogenesis but rather promote ROS signalling to aggravate the disease itself. The problem is that even these kinds of hypothesis do not completely indicate what levels of antioxidants or 8-OHdG should be expected. 8-OHdG is a marker of ROS-derived DNA damage and ultimately its expression can be considered to increase upon oxidative stress. However, high levels of 8-OHdG do not reveal how efficiently this DNA damage is repaired, whereas low levels of 8-OHdG do not tell us if they are a consequence of low ROS production, effective DNA repair or a high level of antioxidant activity. Furthermore, the expression of a few antioxidant enzymes
does not reveal much about the current redox status of a cell, since if one antioxidant system is down-regulated, other antioxidant enzymes compensate for this, keeping the total redox status close to normal. It would also seem that ROS production and cellular redox status might be in a state of constant change even in benign tissue because of proliferation or temporary external sources of oxidative stress, for example. This makes it even harder to evaluate the overall oxidative stress in a tissue, especially if a study is carried out at only one point of time. Keeping these problems in mind, future studies should aim to address oxidative stress as a whole, by measuring several members of antioxidant systems and oxidative damage markers at the same time and repeating the measurements several times in the same patients to see if observed trends are persistent through time.
7 Conclusions

- Low-level oxidative DNA damage is associated with aggressive breast cancer phenotypes, suggesting that these tumours maintain a cellular redox balance near physiological levels to promote ROS-mediated signalling for enhanced growth and survival and to avoid ROS-mediated apoptosis.
- There seems to be a decreasing trend in oxidative DNA damage and Prx II expression along with carcinogenic development of endometriosis-associated ovarian cancer. High-level oxidative DNA damage observed in endometriotic tissue is probably potent in initiating malignant transformation, while later stages of carcinogenesis may benefit more from lower levels of oxidative stress.
- PCOS patients show increased oxidative protein and lipid damage but decreased oxidative DNA damage (i.e. lower serum levels of 8-OHdG) compared with healthy controls. This might indicate either only mild oxidative stress and enhanced antioxidant defence or enfeebled repair of oxidized DNA lesions. Harmful effects of ROS in PCOS might therefore be focused more around abnormal ROS signalling leading to increased androgen activity and the development of insulin resistance rather than pure DNA damage.
- Metformin treatment decreases the oxidative DNA damage observed in obese PCOS patients. This phenomenon is probably explainable by the combination of reduced body weight, increased insulin sensitivity and decreased androgen excess.
References


Brown GC & Borutaite V (2012) There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. Mitochondrion 12: 1–4.


Sampson JA (1927) Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obst Gynecol 14: 442–469.


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