OXIDATIVE STRESS IN THE PATHOGENESIS AND PROGNOSIS OF OVARIAN CANCER

Marjo Pylväs-Eerola
MARJO PYLVÄS-EEROLA

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UNIVERSITY OF OULU, OULU 2015
Abstract

Ovarian cancer is the fifth leading cause of cancer-associated death in women in Finland. Although ovarian cancer is relatively common, the precise mechanism of its development is still unknown. Additionally, it appears that the modes of pathogenesis differ depending on histotype. Although the initial response to platinum-based chemotherapy is usually good, the majority of ovarian cancer patients relapse and develop platinum resistance. This is a major problem in the treatment of ovarian cancer.

Reactive oxygen species (ROS) are metabolites of oxygen. They are continuously formed in normal cells as a by-product of aerobic respiration and they play an important role in normal cell functions. Oxidative stress occurs when ROS formation overrides the antioxidative defence system. Oxidative stress is associated with carcinogenesis. A small proportion of cancer cells are stem cells that survive initial chemotherapy. These cells are suspected of being associated with the development of platinum resistance.

To evaluate the significance of oxidative stress in ovarian cancer we examined ROS-derived damage and antioxidant regulators in benign and borderline ovarian tumours and ovarian cancer samples by immunohistochemistry and analysis of serum samples. The existence of cancer stem cell markers was also assessed in ovarian cancer samples. The expression levels of various markers were compared with clinicopathological parameters.

Our results confirm that oxidative stress (8-hydroxydeoxyguanosine) exists in benign tumours and antioxidant enzymes, such as peroxiredoxins and thioredoxin are widely expressed in benign and borderline tumours. Oxidative stress was associated with poor survival, higher stage and platinum resistance in ovarian cancer. Oxidative stress markers were more strongly expressed in certain histotypes of ovarian cancer, such as serous and endometrioid type. Cancer stem cell markers were found in ovarian cancer and they were associated with the development of platinum resistance. These observations are beneficial in understanding the pathobiology of ovarian cancer and help in the design of new treatment options.

Keywords: antioxidant enzymes, cancer stem cell, immunohistochemistry, ovarian cancer, oxidative stress, platinum resistance, reactive oxygen species
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Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Oulun yliopistollinen sairaala
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


Tutkimuksessamme arvioimme oksidatiivisen stressin merkitystä munasarjakasvaimissa. Tutkimme vapaiden radikaalien aiheuttamia vaurioita ja antioksidatiivisia säätelyjärjestelmiä hyvänlaatuisissa, rajalaatuisissa sekä munasarjasyövässä kasvaimissa immunohistokemiallisesti ja seerumi-näytteistä.


Tulokset auttavat ymmärtämään munasarjasyövän syntymekanismia ja suunnittelemaan uusia hoitovaiheita erityissä kasvaimissa.

Asiakirjat:
- Antioksidatiiviset enzyymit, immunohistokemia, munasarjasyövä, oksidatiivinen stressi, platinareistenssi, syövän kantasolu, vapaa radikaalit
To my precious ones
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Oulu, September 2015

Marjo Pylväs-Eerola
Abbreviations

•OH  hydroxyl radical
8-OHdG  8-hydroxydeoxyguanosine
ALDH1  aldehyde dehydrogenase 1
AP-1  activating protein 1, collection of basic leucine zipper domain proteins
ARE  antioxidant response elements
ARID1A  AT rich interactive domain 1A (SWI-like)
BRAF  B-Raf proto-oncogene, serine/threonine kinase
BRCA1/2  breast cancer 1/2, early onset
BSO  bilateral salpingo-oophorectomy
c  cytoplasmic
CA12-5  cancer antigen 12-5
CD44  CD44 molecule (Indian blood group)
cNOS  constitutive nitric oxide synthase
CSC  cancer stem cell
CTNNB1  catenin (cadherin-associated protein), beta 1, 88kDa
Cu²⁺  copper ion
DFS  disease-free survival
DNA  deoxyribonucleic acid
EEC  endometrial endometrioid cancer
EGF  epidermal growth factor
ELISA  enzyme-linked immunosorbent assay
eNOS  endothelial nitric oxide synthase
EOC  epithelial ovarian cancer
ERBB2  erb-b2 receptor tyrosine kinase 2
ESC  endometrial serous cancer
FACS  fluorescence-activated cell sorting
Fe²⁺  ferrous ion
GPX  glutathione peroxidase
GSH  glutathione
GST  glutathione S-transferase
H₂O₂  hydrogen peroxide
HE4  human epididymis protein 4
HER2  human epidermal growth factor receptor 2
HGSC  high-grade serous cancer
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>HIF-1</td>
<td>hypoxia inducible factor 1</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary non-polyposis colorectal carcinoma</td>
</tr>
<tr>
<td>HMOX1</td>
<td>heme oxygenase 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemical/chemistry</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin 1 beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KEAP1</td>
<td>kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>KIT</td>
<td>v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LGSC</td>
<td>low-grade serous cancer</td>
</tr>
<tr>
<td>MAP3K5</td>
<td>mitogen-activated protein kinase kinase kinase 5</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAF</td>
<td>v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MLH1</td>
<td>mutL homolog 1</td>
</tr>
<tr>
<td>MSH2</td>
<td>mutS homolog 2</td>
</tr>
<tr>
<td>MSH3</td>
<td>mutS homolog 3</td>
</tr>
<tr>
<td>MSH6</td>
<td>mutS homolog 6</td>
</tr>
<tr>
<td>mtNOS</td>
<td>mitochondrial-specific nitric oxide synthase</td>
</tr>
<tr>
<td>MUTYH</td>
<td>mutY DNA glycosylase</td>
</tr>
<tr>
<td>MYH1</td>
<td>myosin, heavy chain 1, skeletal muscle, adult n</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEIL1</td>
<td>nei-like DNA glycosylase 1</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NRF2L2</td>
<td>nuclear factor, erythroid 2-like 2, NRF2 coding gene</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO'</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NQO1</td>
<td>NADPH dehydrogenase, quinone 1</td>
</tr>
<tr>
<td>NRF2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NT</td>
<td>nitrotyrosine</td>
</tr>
<tr>
<td>O2</td>
<td>oxygen molecule</td>
</tr>
<tr>
<td>O2^-</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>OGG1</td>
<td>8-oxoguanine DNA glycosylase</td>
</tr>
<tr>
<td>ONOO−</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribosyl) polymerase</td>
</tr>
<tr>
<td>PARK7</td>
<td>parkinson protein 7</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>p53</td>
<td>tumor protein p53</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PFS</td>
<td>progression-free survival</td>
</tr>
<tr>
<td>PMS1</td>
<td>PMS1 homolog 1, mismatch repair system component</td>
</tr>
<tr>
<td>PMS2</td>
<td>PMS1 homolog 2, mismatch repair system component</td>
</tr>
<tr>
<td>PRDX</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STIC</td>
<td>serous tubal intra-epithelial carcinoma</td>
</tr>
<tr>
<td>TATI</td>
<td>tumor-associated trypsin inhibitor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor protein p53 gene</td>
</tr>
<tr>
<td>TXN</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TXNRD</td>
<td>thioredoxin reductase (subtypes 1–3)</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
</tr>
</tbody>
</table>
List of original articles

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


Marjo Pylväs-Eerola (MPE) has collected clinical data and has been the main author in all publications. In Studies I, II and IV MPE has participated in assessing histological samples. In Studies II and III MPE has participated in ELISA-detection of the antigens. Ulla Puistola has conceived the studies and Peeter Karihtala has performed statistical analysis together with MPE. Saila Kauppila and Annikki Liakka have assessed histological samples. Ylermi Soini, Liisa Laatio and Jussi Koivunen have been specialists in their fields. All authors participated in writing the publications.
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1 Introduction

Ovarian cancer is the most lethal gynaecological malignancy, the five-year life expectancy rate being only 45% (Touboul et al. 2014). In Finland, the incidence of ovarian cancer is 8.0/100 000 per year (Finnish Cancer Registry, 2015). The early symptoms are usually few, which often leads to an advanced stage before diagnosis is made. Early diagnosis, a prerequisite for a favourable outcome, is hampered by the lack of good screening methods.

Known risk factors of ovarian cancer include smoking, infertility, late menopause, endometriosis, and obesity (Hunn & Rodríguez 2012). Protective factors include pregnancies, breastfeeding, contraceptive use, and sterilization. (Hunn & Rodríguez 2012, Modan et al. 2001, Tsilidis et al. 2011). The main cause and pathogenesis of ovarian cancer are still unclear. Over the last few years, reactive oxygen species (ROS) have been linked to the carcinogenesis of several cancer types (Thannickal & Fanburg 2000), but there are only a few studies concerning ROS in ovarian cancer.

Free radicals have an unpaired electron in their outer orbital, which makes them highly reactive. Free radicals and their metabolites (i.e. ROS) are formed, for example, as a result of ionizing and ultraviolet radiation, smoking, and alcohol, factors known as carcinogens. (Valko et al. 2004 & 2007). Small amount of ROS are physiological and essential in cell signalling systems, but when the production of ROS overrides antioxidant defence, they can cause mutations in deoxyribonucleic acid (DNA) and damage proteins and lipids. This process is called oxidative stress. ROS damage may accumulate in the genome over the years and it is associated with aging, degenerative diseases and cancer. (Thannickal & Fanburg 2000). Nitrogen-derived reactive molecules can also cause mutations, a process called nitrosative stress (Valko et al. 2006). The term is usually included under “ROS”.

To protect themselves from ROS, cells have several defence mechanisms, including action of the antioxidant enzymes peroxiredoxins (PRDXs) and thioredoxin (TXN) (Valko et al. 2006). Antioxidant enzymes can neutralize free radicals to water and other harmless compounds. The nuclear factor erythroid 2-related factor 2 (NRF2)-kelch-like ECH-associated protein 1 (KEAP1) complex and parkinson protein 7 (PARK7) are cell redox-state regulators that induce antioxidant mechanisms. (Gañán-Gómez et al. 2013, Junn et al. 2005).

Development of platinum resistance is a major problem in the treatment of ovarian cancer. The majority of ovarian cancer patients (~80%) relapse and nearly
all of them develop platinum resistance (Abubaker et al. 2013). In recent years so-called cancer stem cells (CSCs) have been suspected of causing platinum resistance. These cells have stem cell-like properties and it is suspected that some of them survive initial chemotherapy and become platinum-resistant partly via oxidative stress. (Foster et al. 2013, Garson & Vanderhyden 2015).

There are four major histological types of epithelial ovarian cancer (EOC), serous, endometrioid, clear cell, and mucinous. Until recently, they have not been separated in clinical practice. For example, the treatment has traditionally been similar for all types of EOC. However, according to recent observations, it seems that the pathogenesis and clinical course of the different histological types of cancer differ from each other. (Lengyel 2010, Gilks 2010).

Previous studies have indicated that antioxidant enzymes and ROS metabolites could be used as markers of cancer and for the evaluation of prognosis (Bur et al. 2014, Lehtonen et al. 2004, Ma et al. 2013, Pasanen et al. 2012). Further, it is possible that the levels of antioxidative markers are different in the various types of ovarian cancer (Koshiyama et al. 2015). The role of oxidative stress in ovarian tumours is unclear. There are no studies of CSCs and oxidative stress-regulating systems in neoadjuvant-treated ovarian cancer. The present work was undertaken to study oxidative stress in ovarian tumours.
2 Review of the literature

2.1 Epithelial ovarian carcinoma

2.1.1 Epidemiology

In Finland, ovarian cancer is the third most common gynaecological cancer after breast cancer and cancer of the uterine corpus and the eleventh most common of all cancers in women (Finnish Cancer Registry, 2015). Worldwide it is the seventh most common cancer in women (Ferlay et al. 2010). The prevalence increased in 1950–60 and has been approximately the same thereafter. In 2013, there were 434 new ovarian cancers in Finland, the incidence being 8.0/100 000. (Finnish Cancer Registry, 2015). The risk of ovarian cancer is highest at 60–64 years of age (Munasarjasyöpä, Käypä Hoito 2012) and lifetime risk of ovarian cancer is 1.4 % (Nakamura et al. 2014). In Finland, ovarian cancer is the fifth leading cause of cancer death in women and it is the most lethal malignancy of the female reproductive system (Finnish Cancer Registry, 2015, Luo et al. 2011).

2.1.2 Risk and protective factors

Risk factors

The main cause of ovarian cancer is still unclear because the intra-abdominal location of the ovaries makes it difficult to detect early changes for examination (Hunn & Rodriguez 2012). There are known risk factors of ovarian cancer, and together with hereditary gene mutations, these environmental factors are contributing to the development of ovarian cancer (Valko et al 2006).

Family history of ovarian cancer, with or without known hereditary gene mutations, is associated with a 2.9–3.6-fold increase in risk of ovarian cancer. Infertility and endometriosis are associated with a 2-fold increase in risk. Body mass index >30 kg/m² increases the risk by 30–83%. High age at menopause increases the risk by 46% (Tsilidis et al. 2011) and also smoking increases the risk. (Hunn & Rodriguez 2012). In a meta-analysis postmenopausal hormone therapy increased the risk of ovarian cancer, estrogen only up to 22% and combined estrogen-progestin treatment 10% (Pearce et al. 2009). The risk depends also on different cancer histotypes. Estrogen only increases the risk of serous ovarian
cancer 45% and decreases the risk of mucinous 35% in >5 years of use. Sequential estradiol-progestin therapy increases the overall risk of ovarian cancer 35%, in endometrioid subtype 88%, and in serous subtype 32% in >5 years of use. (Koskela-Niska et al. 2013).

Protective factors

Protective factors include reduced ovulation, such as with oral contraceptive use, pregnancies, and breastfeeding. One pregnancy reduces ovarian cancer risk by one third and minimum of three years of oral contraceptive use by 30–50%. (Hunn & Rodriguez 2012, Tsilidis et al. 2011). The protective effect lasts for 10-20 years (Hunn & Rodriguez 2012). For breast cancer 1/2, early onset gene (BRCA1/2) mutation carriers the risk of ovarian cancer is reduced cumulatively with each pregnancy but oral contraceptive use does not seem to reduce the risk (Modan et al 2001). Hysterectomy and tubal ligation reduce the ovarian cancer risk by 34% (Cibula et al. 2011, Whittemore et al. 1992).

Hereditary BRCA1/2 mutations

BRCA1 and BRCA2 genes are separate, localized in two different chromosomes (17q21 and 13q12.3, respectively), but disruption of these genes leads to a similar cancer type. Both proteins participate in repairing DNA and thus are considered to be tumour suppressor genes. (Yoshida & Miki 2004). Gene mutation carriers have an increased risk of breast and ovarian cancer. Deficiencies in BRCA1/2 genes lead to chromosome instability, such as aneuploidy and chromosome breaks and eventually accumulation of mutations. Platinum compounds induce the double-strand breaks of the DNA increasing spontaneous genetic aberrations. This is why ovarian cancer patients with BRCA1/2 mutation have better responses to platinum-based chemotherapy compared with sporadic ovarian cancer. (Girolimetti et al. 2014).

It is estimated that BRCA1/2 mutations are involved in 65–75% of hereditary ovarian cancers (Bewtra et al. 1992). The prevalence of BRCA1/2 mutations varies according to different studies; in families with multiple cases of ovarian and breast cancer the prevalence is 9–46%, depending on selection criteria and ethnicity. In population-based cohorts, the prevalence of BRCA1/2 mutations among ovarian carcinoma patients is approximately 13–14%. In BRCA1/2-associated ovarian carcinomas, tumor protein gene (TP53) mutations have been
demonstrated as early events in high-grade serous cancer (HGSC) subtype. (Girolimetti et al. 2014). In the recent large Epidemiological Study of BRCA1 and BRCA2 mutation carriers (EMBRACE) study, it was concluded that the cumulative risk of breast cancer by age 70 would be 60% for BRCA1- and 55% for BRCA2-carriers. The risk of ovarian cancer by age 70 was 59% for BRCA1- and 16.5% for BRCA2-carriers. (Mavaddat et al. 2013). The mean age of patients with BRCA1/2-gene-associated cancer is lower than in those with sporadic ovarian cancer, 51.2 years for BRCA1 and 57.5 for BRCA2 (Rich et al. 2001).

Most BRCA1/2-associated ovarian cancers are high-grade and advanced-stage serous carcinomas. In the large Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) study, 67% of all BRCA1/2-associated cancers were serous and over 70% were grade 3 carcinomas. (Mavaddat et al. 2012). There were no significant differences in morphology or grade between BRCA1 and BRCA2 mutation carriers. BRCA1/2 mutation carriers have more favourable prognoses, overall survival (OS) and disease-free survival (DFS) (Zhong et al. 2015) than non-carriers (hazard ratio 0.73), and in BRCA2 carriers the survival is better than in BRCA1 carriers (hazard ratio 0.49) (Bolton et al. 2012).

**Hereditary Lynch syndrome**

Lynch syndrome is an autosomal hereditary dominant cancer syndrome that was previously called hereditary non-polyposis colorectal cancer syndrome (HNPCC). Patients with Lynch syndrome have a high risk of endometrial cancer, urinary tract cancer and small intestinal cancer. Additionally, it is related to 10–15% of hereditary ovarian cancers. (Bewtra et al. 1992). Lynch syndrome is caused by germline mutations in DNA mismatch repair genes. These genes include mutL homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 3 (MSH3), mutS homolog 6 (MSH6), PMS1 homolog 1, mismatch repair system component (PMS1), and PMS1 homolog 2, mismatch repair system component (PMS2), which are tumor-suppressor genes. Mutations in MLH1 and MSH2 account for 90% of cases in Lynch syndrome. (Nakamura et al. 2014).

The lifetime risk of ovarian cancer in Lynch syndrome is 8%, whereas in the general population it is 1.4%. In addition, the age at diagnosis is lower in patients with Lynch syndrome than in patients with sporadic cancer (42–49 years and 60–65 years, respectively). (Nakamura et al. 2014). Watson et al. (2001) and Ketabi et al. (2011) reported that the majority of ovarian cancers in Lynch syndrome are of epithelial non-serous type, well or moderately differentiated and 85% were
stage I–II at the time of the diagnosis. Watson et al. (2001) reported synchronous endometrial cancer in 21.5% of the cases. In Lynch syndrome, TP53 and Kirsten rat sarcoma viral oncogene homolog gene (KRAS) or B-Raf proto-oncogene, serine/threonine kinase (BRAF) mutations do not exist, while in sporadic ovarian cancer the incidences are 37% and 8%, respectively (Niskakoski et al. 2013). The prognosis is relatively good in Lynch syndrome-associated ovarian cancer. In one study, the 5-, 10-, 20- and 30-year survival rates were 83.7%, 80.6%, 78% and 71.5%, respectively. (Grindedal et al. 2010).

**Prophylactic aspects in hereditary gene mutations**

The use of oral contraceptives reduces the risk of ovarian and endometrial cancer by 50% and thus could be useful for risk reduction in patients with Lynch syndrome. Similar to sporadic ovarian cancer, there is no good screening method for ovarian cancer in either BRCA1/2 or Lynch syndrome mutation carriers. Several investigators support prophylactic hysterectomy with bilateral salpingo-oophorectomy (BSO) in cases of Lynch syndrome after the age of 35 or once childbearing is completed. (Nakamura et al. 2014). Prophylactic BSO is recommended at the age of 40 for BRCA1/2 mutation carriers, which reduces the risk of ovarian and Fallopian tube cancer by 75–96% (Girolimetti et al. 2014).

**2.1.3 Pathogenesis**

**The origin of ovarian cancer**

For a long time it was suggested that high-grade ovarian cancer originates from the surface epithelium of the ovary. Ovarian epithelium is a form of mesothelium and histologically identical to the peritoneal epithelium. They both originate from the mesonephros. The Fallopian tubes, endometrium, endocervix and upper vagina originate from the paramesonephros (Müllerian duct). (Zeppernick et al. 2015). It was assumed that ovulation causes continuous cellular disruption and damage, promoting wound healing and thus leading to DNA damage and eventually to malignancy (Foster et al. 2013, Koshiyama et al. 2014). Cortical inclusion cysts are thought to develop from invaginations of the surface epithelium and it has been hypothesized that ovarian cancer might originate from these cysts (Zeppernick et al. 2015). EOCs are of the Müllerian type and thus the
ovarian surface epithelium should go through transformation in the course of carcinogenesis. However, no evidence has been found to support this hypothesis. (Zeppernick et al. 2015).

Piek et al. (2003) proposed that the Fallopian tubes could be the origin of ovarian cancer. They found non-invasive and invasive tubal carcinoma in tissue specimens of prophylactic BSOs of \(BRCA1/2\) patients. The changes were similar to those in HGSC, but were found only in the tubes. In further studies of serous ovarian cancer patients, 52% had intra-epithelial carcinoma in the Fallopian tubes and exhibited strong tumor protein p53 staining indicative of \(TP53\) mutation. (Folkins et al. 2009, Kindelberger et al. 2007). Serous tubal intra-epithelial carcinoma (STIC) was found especially in the ends of the fimbriae (Medeiros et al. 2006). Cumulative evidence supports the Fallopian tube as the tissue of origin of HGSC (Callahan et al. 2007, Finch et al. 2006, Przybycin et al. 2010). The genetic profile of HGSC resembles that of the Fallopian tube, and HGSC stains for Müllerian marker but not for mesothelial marker (Zeppernick et al. 2015). Chene et al. (2014) found ovarian and tubal dysplasia and STIC significantly more commonly in prophylactic specimens from patients at genetic risk of ovarian cancer. In STICs p53 was over-expressed. Evidence suggests that tumour progression begins in the Fallopian tube with \(TP53\) mutations, evolves to serous intra-epithelial carcinoma and finally transforms to serous carcinoma and translocates to the ovary (Jones & Drapkin 2013). However, not all HGSCs clearly originate from the Fallopian tubes. On the other hand, precursor lesions in fimbriae have been found only in connection with the serous type of cancer and not with other histologies (Przybycin et al. 2010). It has been suggested that the pathogenetic mechanisms of low-grade serous cancer (LGSC) and HGSC differ from each other. Pearce et al. (2012) found an association between endometriosis and LGSC and they speculated endosalpingiosis might explain the result.

Oxidative stress in the pathogenesis of ovarian cancer

In recent years many studies have shown the role of oxidative stress in the development of ovarian and other cancers. Evidence suggests that ovulation increases the levels of inflammatory agents, which can further lead to mutations in DNA. (Murdoch & Martinchick 2004, Murdoch 2005, Ness & Cottreau 1999). Ovulation creates a void on the ovarian surface, which leads to wound-healing process, with increased levels of inflammatory mediators and ROS and could lead to borderline tumours and LGSC (Foster et al. 2013, Murdoch & Martinchick
2004, Murdoch 2005). It has been suggested that cancer cells from the Fallopian tubes, cervix or endometriosis migrate to the ovary and form tumours near the corpus luteum as a result of increased inflammation (Kessler et al. 2013, Yang-Hartwich et al. 2014). Studies carried out on murine tissues in vitro and in baboons in vivo showed increased oxidative stress in tubal epithelium and an increased number of macrophages close to the oocytes (King et al. 2011). Infections can also cause oxidative stress and thus chronic inflammation might induce carcinogenesis (Kessler et al. 2013). However, this topic is difficult to study in vivo.

In summary, it seems that HGSC mainly originates from the Fallopian tubes and in other histotypes the tumour cells might also translocate from other tissues to the ovary as a result of increased oxidative stress. In addition, some cancers develop in the ovary from benign precursors.

_Tumour spreading_

Ovarian carcinoma metastasizes directly from the ovaries or Fallopian tubes to neighbouring organs or by detachment of cells from the primary tumour. Tumour cells are transported by peritoneal fluid and disseminated within the abdominal cavity. The omentum is usually affected by cancer in advanced stage. (Lengyel 2010).

### 2.1.4 The grading and staging systems

Ovarian cancers can be classified as low- or high-grade according to pathological characterization. There is also the Federation Internationale de Gynecologie et d’Obstetrique (FIGO) three-tiered grading system (1997) based on the ratio of glandular or papillary structures to solid tumour in addition to the MD Anderson Cancer Center (MDACC) two-tiered grading system (Malpica et al. 2004). The latter is based on assessment of nuclear atypia and mitotic rate. The low-grade category includes tumours with mild to moderate nuclear atypia and up to 12 mitoses per 10 high-power fields (HPFs). The high-grade category includes tumours with marked nuclear atypia and more than 12 mitoses per 10 HPFs. (Malpica et al. 2004). The system seems to be easy to learn and reproducible (Gernshenson 2013), and is widely used.

The new FIGO staging system for ovarian cancer was introduced in January 2014 (Table 1). An update was needed, since increasing knowledge of the
heterogeneity of ovarian cancer indicated different morphology and biological behaviour between different histological types. (Prat 2014).

Table 1. FIGO staging of ovarian cancer (Prat 2014).

<table>
<thead>
<tr>
<th>Stages I-IV of ovarian cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I: Tumour confined to ovaries</td>
</tr>
<tr>
<td>IA Tumour limited to 1 ovary, capsule intact, no tumour on surface, negative washings</td>
</tr>
<tr>
<td>IB Tumour involves both ovaries, otherwise like IA</td>
</tr>
<tr>
<td>IC Tumour limited to 1 or both ovaries</td>
</tr>
<tr>
<td>IC1 Surgical spill</td>
</tr>
<tr>
<td>IC2 Capsule rupture before surgery or tumour on ovarian surface</td>
</tr>
<tr>
<td>IC3 Malignant cells in the ascites or peritoneal washings</td>
</tr>
<tr>
<td>Stage II: Tumour involves 1 or both ovaries with pelvic extension</td>
</tr>
<tr>
<td>IIA Extension and/or implant on uterus and/or Fallopian tubes</td>
</tr>
<tr>
<td>IIB Extension to other pelvic intraperitoneal tissues</td>
</tr>
<tr>
<td>Stage III: Tumour involves 1 or both ovaries with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes</td>
</tr>
<tr>
<td>IIIA Positive retroperitoneal lymph nodes and/or microscopic metastasis beyond the pelvis</td>
</tr>
<tr>
<td>IIIA1 Positive retroperitoneal lymph node only</td>
</tr>
<tr>
<td>IIIA1(i) Metastasis ≤ 10 mm</td>
</tr>
<tr>
<td>IIIA1(ii) Metastasis &gt; 10 mm</td>
</tr>
<tr>
<td>IIIA2 Microscopic, extrapelvic (above the brim) peritoneal involvement + positive retroperitoneal lymph nodes</td>
</tr>
<tr>
<td>IIIB Macroscopic, extrapelvic peritoneal metastasis ≤ 2 cm + positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen</td>
</tr>
<tr>
<td>IIIC Macroscopic, extrapelvic, peritoneal metastasis &gt; 2 cm + positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen</td>
</tr>
<tr>
<td>Stage IV: Distant metastasis excluding peritoneal metastasis</td>
</tr>
<tr>
<td>IVA Pleural effusion with positive cytology</td>
</tr>
<tr>
<td>IVB Hepatic and/or splenic parenchymal metastasis, metastasis to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)</td>
</tr>
</tbody>
</table>

2.1.5 Histological types

Ovarian tumours can arise from three different cell types; epithelial, germ and sex cord stromal cells. EOC is the most common, representing approximately 90% of
all ovarian cancers. (Foster et al. 2013, Jones & Drapkin 2013). This study is focused on EOC.

HGSC represents approximately 70%, endometrioid carcinoma 10%, clear-cell carcinoma 10%, LGSC <5%, and mucinous carcinoma 3% of EOCs (Prat 2014). Since HGSC is by far the most common type of ovarian cancer, it is challenging to examine other types of ovarian cancer and their behaviour (Gilks 2010).

**Serous cancer**

Serous EOC has glandular or papillary histology that resembles the surface epithelium of the Fallopian tube (Lengyel 2010). It is suggested that LGSC arises from the ovarian surface epithelium via tumours of low malignant potential (borderline) and HGSC from the Fallopian tube through rapid genetic changes, which could explain their different behaviours (Gilks 2010). The results of several studies suggest that significant differences exist in molecular and clinical characteristics of LGSC and HGSC types.

LGSC represents less than 10% of serous carcinomas (Gershenson 2013). Typically it is located in the ovary, behaves in an indolent manner and has mutations in \textit{KRAS} in 33% and in \textit{BRAF} in 30% of cases (Chen et al. 2014, Gilks 2010, Koshiyama et al. 2014). Mutations of \textit{TP53} are rare and expressed in < 10% of tumours (Koshiyama et al. 2014). LGSC expresses more estrogen and progesterone receptors than HGSC (Schlumbrecht et al. 2011, Wong et al. 2007) and the mitogen-activated protein kinase (MAPK) pathway also appears to play a role in its pathogenesis (Gernshe nson 2013). Unlike HGSC, LGSC was associated with endometriosis in a pooled analysis (Pearce et al. 2012). Because of the rarity of LGSC, the response to chemotherapy is difficult to estimate. In cases of serous borderline tumours progressing to carcinoma, response rates have been low. Low mitotic activity could be one reason for this finding. LGSC proceeds only rarely to HGSC. (Gilks 2010).

HGSC is a genetically diverse, fast growing, aggressive disease having only a small number of core somatic mutations (Kessler et al. 2013). Most have mutations in the \textit{TP53} gene and 40% have \textit{BRCA1/2} alterations (Chen et al. 2014). The median age of LGSC patients is lower than that of HGSC patients (Gernshenson et al. 2006, Schlumbrecht et al. 2011). In a study by Chen et al. (2014), LGSC patients were younger (46 vs. 56 years) and, unlike in HGSC, optimal cytoreductive surgery predicted progression-free survival (PFS). The
outcomes were similar between groups when the surgical result was not optimal. This supports the hypothesis that in LGSC the surgical result has an important effect on survival, since it is relatively chemoresistant. In one study, the prognosis of stage II–IV LGSC patients did not differ from that of HGSC (Ali et al. 2013). Malpica et al. (2004) showed significantly better OS in low-grade patients than in high-grade ones (4.2 vs. 1.7 years, respectively).

Endometrioid cancer

Endometrioid ovarian cancer is often associated with endometriosis and it has endometrioid-like glands (Kim et al. 2014, Koshiyama et al. 2014, Lengyel 2010, Pearce et al. 2012). The most common mutations are in the catenin (cadherin-associated protein), beta 1 (CTNNB1) gene in 38–50% of cases and in the phosphatase and tensin homologue (PTEN) gene in 20%. Endometrioid cancer is most commonly diagnosed at stage I or II and is low grade (1–2). (Gilks 2010). Endometriosis causes chronic inflammation with imbalance between cytokines and it also changes the hormonal balance and stimulates the production of growth factors (Koshiyama et al. 2014). It seems that the hormonal environment plays a role in the development of endometrioid ovarian cancer; virtually all cases express estrogen receptor. Endometriotic cysts include old blood with a high concentration of iron. (Yamaguchi et al. 2008). Iron is associated with carcinogenesis, since it can cause persistent oxidative stress and sensitise cells to DNA damage (Koshiyama et al. 2014, Yamaguchi et al. 2008). Up to 20% of patients with endometrioid ovarian cancer have synchronous atypical hyperplasia or endometrioid adenocarcinoma of the endometrium (Gilks 2010).

Clear-cell cancer

Clear-cell cancer is also associated with endometriosis and it has histological features of serous and endometrioid ovarian carcinoma (Kim et al. 2014, Lengyel 2010, Pearce et al. 2012). All clear-cell carcinomas are high-grade and they usually present at an early stage. The clear-cell type of ovarian cancer is aggressive; there is a significant risk of relapse and response rates to chemotherapy vary between 15% and 45%. It has a low mitotic rate, which may explain the poor response to chemotherapy. It lacks expression of hormone receptors and is not associated with germline mutations and typically does not have chromosomal instability. (Gilks 2010). High incidences of
phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) and the AT rich interactive domain 1A (SWI-like) (ARID1A) gene mutations have been found (Kessler et al. 2013).

Mucinous cancer

Mucinous cancers have a texture resembling endocervical or gastrointestinal epithelium (Lengyel 2010). The origin of mucinous cancer is not clear, but an association with Brenner tumour has been recognized and some can develop in teratomas (Koshiyama et al. 2014). Most of these cancers are confined to the ovary at diagnosis. If the disease relapses, there are no effective treatments. Most common mutations are in the KRAS gene, which can be seen in benign-appearing areas of the tumours, suggesting an early event during oncogenesis from adenoma to cancer. (Gilks 2010). Human epidermal growth factor receptor 2 (HER2) overexpression and amplification can be seen in 15–20% of mucinous cancers (Gilks 2010, McAlpine et al 2009). HER2 is coded by erb-b2 receptor tyrosine kinase 2 (ERBB2) gene and its amplification is also present in mucinous ovarian cancers in 14–18% of the cases (Chao et al 2014).

2.1.6 Type I and II ovarian cancer

EOC can be divided in two subtypes (Fig. 1) according to the existence of precursor lesions (Type I) or de novo formation of the tumour (Type II). Type I is composed of low-grade serous, endometrioid, clear-cell and mucinous tumours. They have low malignant potential and represent 30% of ovarian cancers. They usually present at stage I–II (tumour confined to the ovary or pelvis) and develop from well-established precursors starting from cystadenoma and developing to non-invasive borderline tumours. (Bützow 2014, Koshiyama et al. 2014, Kurman 2013, Zeppernick et al. 2015). The tumours are characterized by specific mutations, including KRAS, BRAF, PTEN and ERBB2 and they are relatively stable genetically. Type I cancer patients are young, have a favourable DFS and are relative resistant to carboplatin and taxane chemotherapy. (Lengyel 2010).

In type II cancers, no clear precursor lesions have been found and tumours may develop de novo from tubal or ovarian surface epithelium (Koshiyama et al. 2014). HGSC represents 60–70% of Type II ovarian cancers. Patients also present with undifferentiated cancer, carcinosarcoma and endometrioid cancer. Type II cancers are aggressive and present typically at an advanced stage. (Lengyel 2010).
It seems that malignant cells spread easily to the peritoneal cavity from the Fallopian tubes, explaining the rapid progression (Nezhat et al. 2015). In general, there are mutations of TP53 in 50–80% of type II cancers. The frequency is even higher in serous cancers, 90% or more. (Kohler et al. 1993, Singer et al. 2005). At the time of diagnosis, they demonstrate marked chromosomal aberrations, but over the course of the disease these changes remain relatively stable (Bützow 2014, Kurman 2013). Type II cancers are usually found in postmenopausal women; they are initially chemosensitive but DFS is shorter than in Type I cancer (Lengyel 2010).

Fig. 1. Two types of ovarian cancer. Reprinted with permission from Ronny Drapkin; Jones & Drapkin, Front Oncol 2013;3:217.

Dividing EOC to Type I and II is a quite new model and has not yet reached clinical practise. It is suggested that in the future genetic profiling would assist in individual therapy designs. (Koshiyama et al. 2014).

2.1.7 Diagnosis of ovarian cancer

EOC is usually diagnosed because of increased pain or swallowing in the stomach area (Chan et al. 2003). Other symptoms include constipation, nausea, bleeding, urinary urgency, and loss of weight. None of the symptoms are specific for EOC.
Ultrasound usually reveals ascites and a cystic-solid mass of tumour. Computer tomography or magnetic resonance imaging is used to find out metastases. Serum level of ovarian cancer marker, cancer antigen 12-5 (CA12-5), is usually increased at the time of the diagnosis. Tumour-associated trypsin inhibitor (TATI) can be used, especially in mucinous cancer its serum levels increase. Also human epididymis protein 4 (HE4) is a potential marker of EOC (Granato et al 2015). Risk of Malignancy Index can be used as a tool in differential diagnostic. It is calculated by multiplying pre-or postmenopausal status (1 or 4 points) by ultrasound imaging result (simplex cyst 1 point, ascites, solid or multicystic areas, and tumour in both ovaries 4 points), and by serum CA12-5. Score above 200 indicates malignant tumour. Risk of Malignancy Algorithm is a predictive index, which calculates the risk of malignancy by using CA12-5 and HE4 values and postmenopausal status. It classifies patients as low or high risk for malignancy. However, none of these markers are specific for EOC and no good screening method exists. (Munasarjasyöpä, Käypä Hoito 2012).

2.1.8 Treatment of ovarian cancer

The standard treatment of EOC is maximal cytoreductive surgery including hysterectomy, BSO, and omentectomy. Lymphadenectomy and also peritoneectomy is recommended, to achieve optimal (residual tumour <1 cm) surgical result. After surgery, adjuvant chemotherapy is usually administered. (Della Pepa et al. 2015, Munasarjasyöpä, Käypä Hoito 2012). McGuire et al. (1996) and Piccart et al. (2000) compared cisplatin and cyclophosphamide versus cisplatin and paclitaxel in the treatment of ovarian cancer and found significantly improved OS and DFS with the latter combination. Du Bois et al. (2003) compared paclitaxel and cisplatin versus paclitaxel and carboplatin, and found significantly reduced side-effects in the carboplatin group. Since then, paclitaxel and carboplatin have been the standard first-line chemotherapy. Initial treatment is effective in approximately 70% of patients, resulting in a partial or complete response, which is seen as a reduction in tumour volume and normalisation of serum CA12-5 levels (Foster et al. 2013).

Other chemotherapy options, such as gemcitabine, liposomal pegylated doxorubicin, topotecan, and epirubicin are also available. Despite different combinations of chemotherapy agents and doses, significant improvement in survival has not been achieved (Della Pepa et al. 2015). New biological
molecules have been under investigation and bevacizumab, a monoclonal antibody for vascular endothelial growth factor A (VEGFA), seems to increase PFS combined with certain chemotherapy agents in primary and recurrent EOC. (Aghajanian et al 2012, Perren et al. 2011, Pujade-Lauraine et al 2014). Poly(ADP-ribose) polymerase (PARP) proteins are involved in DNA repair, their function is related to homologous recombination pathway. This pathway may be defective, like in BRCA1/2 mutations. In these cancers, PARP inhibitors cause accumulation of mutations and cell death and increase PFS in EOC. (Della Pepa et al 2015). PARP inhibitor, Olaparib, is recently approved in the treatment of BRCA1/2 associated ovarian cancer in Finland.

**Platinum resistance**

Despite effective first line treatment, approximately 80% of EOC patients experience recurrence within 6–20 months after chemotherapy (Abubaker et al. 2013). Recurrent disease is less sensitive to chemotherapy. Patients who relapse 12 months or later after platinum-based chemotherapy are classified as platinum-sensitive. The response rate to further platinum treatment in these patients varies between 30% and 90% and median survival is two years. (Davis et al. 2014). Patients who relapse within six months after platinum-based chemotherapy are platinum-resistant. Their response rate to any form of chemotherapy is less than 15% and median survival less than one year. (Davis et al. 2014). Patients who relapse between six and 12 months are classified as partially sensitive; of those with HGSC, 27% will respond to platinum treatment (Cooke et al. 2011). New platinum analogues have been developed to overcome cisplatin resistance, for example oxaliplatin and enloplatin (Galluzzi et al. 2014). However, once platinum resistance has developed, it usually affects all forms of platinum-based chemotherapy. Clear-cell, mucinous and LGSC types usually show resistance to platinum-based compounds. The response rate to platinum therapy is higher in BRCA1/2 mutation carriers than in patients with sporadic cancer. (Davis et al. 2014).

The mechanisms behind platinum resistance are complex and not fully understood, since there are several different pharmacological pathways. As being the first platinum drug that was developed, cisplatin is the most studied one. Cisplatin resistance can develop via alterations in transporters that move cisplatin in and out of the cell (Fig. 2). For example, copper transporter mediates the uptake of cisplatin and it has been confirmed that cisplatin triggers its degradation.
(Dasari & Tchounwou 2014). Hydroxylated cisplatin molecule can react with any nucleotide in a cell and cause DNA or mitochondrial damage. DNA repair systems patch a large proportion of cisplatin-induced DNA lesions. (Galluzzi et al. 2014). Cisplatin affects a variety of cell signalling pathways and changes in the proteins involved can either sensitise, or usually, cause resistance in cells as regards cisplatin-induced cell death (Dasari & Tchounwou 2014). Malignant cells are usually more resistant to environmental changes than normal cells and they are capable of adapting by acquiring additional mutations easily (Galluzzi et al. 2014). Additionally, platinum resistance seems to be influenced by mesenchymal cells, extracellular matrix proteins, and immunosuppressive cells (Galluzzi et al. 2014).
Fig. 2. Mechanisms of cisplatin resistance. A variety of genetic and epigenetic alterations can cause cisplatin (CDDP) resistance. Pre-target processes can cause resistance before binding of cisplatin to the target, on-target processes can repair the damage caused by cisplatin and post-target mechanisms impair the signals leading to cell apoptosis. Off-target mechanisms are usually not elicited by cisplatin and they stimulate the pro-survival signals that antagonize cisplatin cytotoxicity. ATP7B = ATPase, Cu²⁺ transporting, β polypeptide, CTR1 = copper transporter, MRP2 = multidrug resistance-associated protein, UPR = unfolded protein response. Reprinted with permission from Guido Kroemer; Galluzzi et al., Cell Death Dis 2014;5:e1257.


2.1.9 Prognosis

Stage at the time of the diagnosis, maximum cytoreductive surgery, and the performance status of the patient are the major factors defining prognosis. If complete surgical reduction is not possible, the size of the residual tumour should be less than 1 cm. (Elattar et al. 2011). Five-year survival is 89% in stage I, 66% in stage II, 34% in stage III, and 18% in stage IV (American Cancer Society, 2014). Usually EOC is diagnosed at an advanced stage, since early symptoms are few and no good screening methods exist. Approximately 75% of patients have stage III–IV disease at diagnosis. (Chen et al. 2014). Mortality rates are high; 60% of patients relapse within five years (Touboul et al. 2014) and ultimately develop platinum resistance. Despite progress in surgery and the broad range of chemotherapy, only a minor improvement in prognosis has been achieved during the past 10 years (Touboul et al. 2014). Five-year survival is 45% worldwide (Touboul et al. 2014) and 41% in Finland according to the latest data from the Finnish Cancer Registry (2015).

2.2 Reactive oxygen species

2.2.1 Background

ROS are molecules, which are produced in aerobic organisms. Under the term ROS includes several types of reactive oxygen metabolites such as free radicals, which are defined as molecules with one or more unpaired electrons in atomic or molecular orbitals. (Karihtala & Soini 2007). Because of the unpaired electrons, ROS are unstable and react easily with other compounds to form new compounds. ROS are products of normal cellular metabolism, formed mainly as a consequence of aerobic respiration in mitochondria. Small amounts of ROS can also be formed in phagocytes, lymphocytes and peroxisomes. ROS formation is induced by exogenous sources, including ionising radiation, ultraviolet light, smoking, ozone, chlorinated compounds, barbiturates, and metal ions. (Klaunig et al. 2010 & 1997). It is estimated that an average person has 100 000–200 000 free radicals attacking each cell every day, and an athlete as much as 50% more. ROS-induced oxidative damage accumulates during the lifespan and ROS seem to play a key role in aging and the development of cancer, arteriosclerosis, arthritis, and neurodegenerative diseases, for example. (Thannickal & Fanburg 2000, Valko et al. 2004 & 2007).
2.2.2 Formation of ROS

Superoxide radical ($O_2^-$)

Mitochondria are responsible for energy production and cellular respiration. Electrons are passed between molecules, with each pass producing energy. When electrons leak from the respiratory chain, they may react with oxygen ($O_2$) to produce superoxide radicals: $O_2 + e^- \rightarrow O_2^-$. About 1–3% of the $O_2$ in the mitochondria are converted to $O_2^-$. (Valko et al 2004). $O_2^-$ are incapable of penetrating through lipid membranes and are mostly present in mitochondria. If $O_2^-$ is not destroyed properly, it can cause accumulation of damage, inhibit mitochondrial function, and finally shut down mitochondria. (Karihtala & Soini 2007).

Hydrogen peroxide ($H_2O_2$)

Superoxide dismutase (SOD) enzymes can disseminate $O_2^-$, which then forms hydrogen peroxide: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. A small amount of $H_2O_2$ can also be formed in peroxisomes directly from $O_2$. $H_2O_2$ can diffuse throughout mitochondria and cross cell membranes, thus causing cellular injury, for example to the nucleus. (Karihtala & Soini 2007). It is reduced by catalase, glutathione peroxidases (GPXs) or PRDXs to $O_2$ and water. $H_2O_2$ is also involved in the actions of epidermal growth factor (EGF), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), platelet-derived growth factor (PDGF) and angiotensin II. (Karihtala & Soini 2007).

Hydroxyl radical ($\cdot OH$)

The main injuries brought about by ROS are mediated by hydroxyl radicals (Karihtala & Soini 2007). They oxidize all parts of the cell – lipids, proteins and DNA (Schieber & Chandel 2014). Hydroxyl radical can be generated via the metal-catalyzed Haber–Weiss reaction ($O_2^- + H_2O_2 \rightarrow Fe^{2+} + O_2 + \cdot OH + OH^-$). It seems that this mechanism proceeds through the catalytic activity of metal ions such as iron ($Fe^{2+}$) or copper ($Cu^{2+}$). $\cdot OH$ has a short half-life, less than 1 ns. This explains its high reactivity close to its site of formation. It appears that the majority of $\cdot OH$s are formed via the Fenton reaction in the presence of $Fe^{2+}$:
Cu+/Fe2+ + H2O2 $\rightarrow$ Cu2+/Fe3+ + $\cdot$OH + OH−. (Schieber & Chandel 2014, Valko et al 2004 & 2006).

2.3 Reactive nitrogen species

Reactive nitrogen species (RNS) are nitric oxide (NO$^\cdot$)-derived radicals. Overproduction of RNS is called nitrosative stress. (Valko et al. 2006). In many cases the term ROS also covers RNS (Karihtala & Soini).

2.3.1 Nitric oxide (NO$^\cdot$)

NO$^\cdot$ is formed from L-arginine by three NO$^\cdot$ synthases (NOS). The reaction also requires O$_2$ and cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH) and citrulline. The majority of NO$^\cdot$ is synthesized by inducible NOS (iNOS, NOS-2). It exists in a variety of cell types including hepatocytes, vascular smooth muscle cells, fibroblasts, macrophages, and epithelial cells and is induced by inflammation. NO$^\cdot$ can be formed in the cells of the immune system during the oxidative burst triggered by inflammatory processes (Valko et al 2006). Other forms of NOS include mitochondrial-specific NOS (mtNOS), endothelial NOS (eNOS, NOS-3), and neuronal NOS (nNOS, NOS-1). mtNOS eNOS, and nNOS are grouped as constitutive NOS (cNOS) and are regulated by intracellular calcium concentrations. (Davis et al. 2001, De Sanctis et al. 2014).

NO$^\cdot$ is a short-lived free radical, its half-life is less than 30 s. It has greater stability at a low O$_2$ concentration. (Jomova & Valko 2013). It can diffuse through cytoplasm and plasma membranes, since it is soluble in aqueous and lipid media (Valko et al. 2006). NO$^\cdot$ has direct effects, which are usually interactions with metals, and indirect effects, such as DNA oxidation, nitration, and strand breaks. Direct effects are mediated by NO$^\cdot$ molecule and indirect effects by RNS, produced after interaction of NO$^\cdot$ with O$_2$ or O$_2^\cdot$. The effects depend on NO$^\cdot$ concentration, location and timing. Concentration is thought to be the principal determinant of diverse actions, at low concentrations direct effects dominate, whereas at higher concentrations indirect effects prevail. NO$^\cdot$ is second messenger that has effects far from its site of production. (Davis et al. 2001).

NO$^\cdot$ has several physiological functions, for example in cellular adhesion, neurotransmission, bronchodilatation, vascular tone and permeability, defence mechanisms, and smooth muscle relaxation (Davis et al. 2001, Jomova & Valko 2013). On the other hand it has been implicated in the pathology of arthritis,
myocarditis, colitis, diabetes, neurodegenerative diseases and cancer (Jomova & Valko 2013).

2.3.2 Peroxynitrite (ONOO⁻) and formation of nitrotyrosine

Under pathological conditions (sustained inflammation), ROS reach high concentrations and affect cellular metabolism by direct oxidation of macromolecules or after combining with NO'. In addition, since in oxidative bursts both ROS and RNS are formed, NO' can react with $O_2^{•−}$, a powerful oxidant, which leads to peroxynitrite formation ($NO' + O_2^{•−} \rightarrow ONOO^{-}$). (Valko et al. 2006). The ONOO⁻-producing reaction is extremely rapid and depends on the redox environment and NO' concentrations, and produces two different types of post-translational modifications. In equal concentrations, the reaction leads to ONOO⁻ formation (nitration), and a two- to threefold excess of NO' leads to dinitrogen trioxide ($N_2O_3$) formation, which is an $S$-nitrosylating agent in the intracellular microenvironment. $S$-nitrosylation is a process in which RNS attach to cysteine residues and modify proteins, for example p53. $S$-nitrosylated proteins have been associated with cancer. (De Sanctis et al. 2014). ONOO⁻ chemistry is dependent on pH, so that nitration is maximal at physiological pH (7.4). ONOO⁻ acts as a nitrating agent and a powerful oxidant, which can modify proteins, lipids and nucleic acids. SODs are important regulators of ONOO⁻ formation by regulating $O_2^{•−}$ levels. ONOO⁻ can react with DNA, causing strand breakage. Additionally, it is involved in nitration of tyrosine residues of proteins and disturbs the mitochondrial respiratory chain. Nitrotyrosine (NT) is a stable end-product from the conversion of tyrosine residues. (De Sanctis et al. 2014).

2.4 Effects of ROS and RNS

2.4.1 DNA damage and formation of 8-OHdG

ROS can create various types of DNA damage; modification of all bases, deletions, frame shifts, strand breaks, DNA-protein cross-links, and deoxyribose backbone and chromosomal rearrangements. $O'H$ and ONOO⁻ in particular can react with all components of DNA and form several new compounds. One of these products is 8-hydroxydeoxyguanosine (8-OHdG), which is formed quite easily and is a good biomarker of oxidative stress. DNA single nucleotide repair
mechanisms repair approximately 90% of DNA damage and the remaining 10% is fixed by a long-patch excision repair mechanism. (Valko et al. 2006). Base excision repair enzymes such as 8-oxoguanine DNA glycosylase (OGG1) and nei-like DNA glycosylase 1 (NEIL1) repair most 8-OHdG lesions. In addition, nucleotide excision repair (NER) can be involved in removing the lesion. (Klaunig et al. 2010). Myosin, heavy chain 1, skeletal muscle, adult (MYH1) and mutY DNA glucosylase (MUTYH) genes code defence mechanisms against accumulating 8-OHdG (Nakabepu 2014). Despite repair mechanisms, ROS-derived damage is known to accumulate in DNA and have an effect on aging, for example (Valko et al 2006).

Besides causing direct genetic DNA mutations, ROS can cause epigenetic alterations such as DNA methylation, histone modification and production of small ribonucleic acids (RNAs). Hypomethylation and regional hypermethylation can be found in early neoplasia. Oncogene transcription is activated by hypomethylation, whereas hypermethylation can cause silencing of tumour suppressor genes. (Wu & Ni 2015).

p53

The protein p53 guards the cell cycle (as a tumour suppressor) and can initiate apoptosis. During cell proliferation, p53 checks the integrity of DNA and triggers apoptosis if the cell is damaged. Inactivation of p53 leads to uncontrolled cell division. (Valko et al. 2007). TP53 gene disruption is associated with more than 50% of all human cancers (Hofseth et al. 2004). TP53 is activated by ultraviolet radiation, hypoxia, gamma-radiation, and nucleotide deprivation (Valko et al. 2007). Under normal/low cellular stress, low concentrations of p53 induce the expression of antioxidant genes, but under severe cellular stress, high concentrations of p53 promote genes that induce ROS formation and p53-mediated apoptosis (Sablina et al. 2005).

2.4.2 Lipid damage

Unsaturated phospholipids of cell membrane are sensitive to oxidation. The most common fatty acid in cells is linoleic acid. Fatty acid oxidation products are short-lived hydroperoxides and their reaction with metals creates, for example, aldehydes and epoxides. Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are mutagenic products of lipid oxidation and can further react with DNA.
(Klaunig et al. 2010). HNE is weakly mutagenic but seems to be the major toxic product of lipid peroxidation and it has effects on signal transduction pathways. Nucleotide excision repair pathways and mismatch repair can fix mutations. (Valko et al. 2007 & 2004).

2.4.3 Protein damage

ROS can damage proteins directly or indirectly through lipid peroxidation. Protein damage influences various cell functions, such as enzyme activity, transport across membranes and interaction with receptors. (Karihtala & Soini 2007). RNS can also affect the structure of individual proteins and modify protein–protein interaction and function. Post-translational modifications can result from direct interaction with NO$^+$ or after exposure to RNS (ONOO$^-$) and other products that arise when NO$^+$ reacts with ROS such as O$_2$$^+$-. (De Sanctis et al. 2014).

2.4.4 Beneficial effects of ROS and RNS

ROS and RNS in immune system

At low/moderate concentrations ROS have physiological roles in cellular responses against infectious agents (Valko et al. 2007 & 2006). Both oxygen radicals and NO$^+$ function are part of the immune system. Macrophages and neutrophils use oxidative bursts as a defence mechanism against environmental pathogens. NADPH oxidase is a component of this system. It is normally disassembled in a cell, but upon stimulation, by e.g. interferon gamma (IFN-$\gamma$), its components assemble and begin to work. (DeCoursey & Ligeti 2005, Thannickal & Fanburg 2000). NADPH oxidase transfers electrons from NADPH to oxygen, generating both O$_2$$^+$ and NO$^+$ (DeCoursey & Ligeti 2005, Hampton et al. 1998). Macrophages and neutrophils increase oxygen consumption up to 100-fold during oxidative burst, which leads to increased ROS formation during this inflammatory process (DeCoursey & Ligeti 2005).
Cells communicate with each other, a process called cell signalling or signal transduction. Extracellular agents, such as hormones, growth factors, and cytokines can trigger cell signalling. Transcription factors transmit gene expression signals to the nucleus, thus inducing various biological activities, such as cell growth, muscle contraction and gene expression. ROS have a major role in the intracellular signalling and the cells are capable of generating ROS. (Thannickal & Fanburg 2000, Valko et al. 2007).

At low concentrations ROS induce a mitogenic response, stimulating proliferation and enhanced survival. At high concentrations ROS are mediators of damage to cell structures, causing cell death or necrosis. (Valko et al. 2006). The type and local concentration of ROS determine whether reduction-oxidation, i.e redox signalling, or oxidative damage occurs. For example, $O_2^{\cdot-}$ accumulation has different effects in mitochondria and cytosol since the content of iron is high in the mitochondria. (Schieber & Chandel 2014). It seems that the $H_2O_2$ level and compartmentalization has an effect on the redox system, whereas $O_2^{\cdot-}$ and $\cdot OH$ are involved in oxidative stress (Schieber & Chandel 2014).

Several cytokine receptors such as those for tumor necrosis factor (TNF), interleukin-1 beta (IL-1β), and IFN-γ can generate ROS and have an effect on several cell signalling pathways (Valko et al. 2006, Fig. 3). Activated growth factors (tyrosine kinases) EGF, PDGF and VEGFA play an important role in the information transmission from outside the cell in to the cytoplasm and nucleus (Valko et al. 2006). Activated growth factor receptor signalling leads to increased ROS production via NADPH oxidases (Thannickal & Fanburg 2000, Schieber & Chandel 2014). ROS can inactivate protein tyrosine phosphatases, which has a function in redox control and cell signalling. The MAPK family is regulated by oxidants and activated by metals. (Thannickal & Fanburg 2000, Valko et al. 2006). MAPK pathways activate nuclear transcription factors, which control the expression of damaged DNA-protective genes and induce apoptosis. These transcription factors include NF-κB, which plays a role in cell growth and immune and inflammatory responses, activating protein 1 (AP-1), which is a collection of basic leucine zipper domain proteins inducing cell growth, and hypoxia inducible factor (HIF-1), which regulates the expression of several cancer-related genes such as $VEGF$. $H_2O_2$ is the major ROS responsible for the induction of HIF-1. The nuclear factor of activated T-cells (NFAT) family regulates cytokine formation and muscle growth. High calcium levels leads to the
activation of NFAT and ROS are known to increase intracellular calcium. (Valko et al. 2006 & 2007).

2.5 ROS and RNS in carcinogenesis

Oxidative stress occurs, when the ROS production exceeds the capacity of antioxidative mechanisms. Chronic inflammation causes chronic oxidative stress and is associated with carcinogenesis, for example in colitis ulcerosa and chronic gastritis. (Klaunig et al. 2010). ROS cause accumulation of DNA damage and it is suggested that mutations of certain genes in proliferating cells can initiate the development of cancer. ROS may stimulate the proliferation of transformed cell clones by modulating genes related to apoptosis or proliferation. They also affect intracellular calcium concentration, which leads to activation of proto-oncogenes.
Chronic inflammation induced by carcinomas further increases the generation of ROS, causing more genetic instability. (Karihtala & Soini 2007, Valko et al. 2006).

Cancer cells constantly activate growth factor pathways to sustain cell growth. In addition, hypermetabolism causes increased generation of ROS and stimulates NADPH oxidases. The antioxidant system in cancer cells is also high and balances the high ROS production. This prevents cells from undergoing apoptosis. (Schieber & Chandel 2014).

Cancer is a process of multiple events starting from a mutation in a single cell. Carcinogenesis can be divided into three stages: initiation, promotion and progression. (Fig. 4; Valko et al. 2006). ROS act at all three stages of carcinogenesis. At the initiation stage there are non-lethal mutations in DNA, which lead to altered cell behaviour. At the promotion stage the number of initiated cells increases by means of increased cell division or decreased apoptosis. (Klaunig et al. 2010 & 1997). Both of these stages are reversible. The progression stage is irreversible and characterised by accumulation of genetic damage, which finally alters the cell from benign to malignant. (Valko et al. 2006).
2.5.1 8-OHdG in cancer

8-OHdG is a stable end product of •OH attack. It is the most studied product of ROS-induced damage, because it is easily detectable and can be reliably measured by IHC and enzyme-linked immunosorbent assay (ELISA). (Davis et al. 2001, Valko et al. 2004). Systemic 8-OHdG levels reflect oxidative stress in the whole organism (Klaunig et al. 2010). 8-OHdG is also mutagenic, it can induce G:C→T:A transversion, which leads to altered proteins and tumour suppressor genes. Hard physical labour, shift work, and smoking increase 8-OHdG levels, whereas moderate physical exercise reduces them. (Valko et al. 2006). 8-OHdG expression is increased in several cancers and usually associated with worse prognosis (Table 2).

There are only few studies of 8-OHdG in ovarian cancer. High 8-OHdG IHC expression has been correlated with poor survival, higher stage and non-optimal surgical outcome in ovarian cancer (Karihtala et al. 2009). In endometriosis-associated ovarian cancer, serum 8-OHdG was down-regulated compared with
benign endometriosis, suggesting that endometriosis is a premalignant lesion. It was speculated that in invasive cancer 8-OHdG leaks from tumour to serum (Sova et al. 2012).

2.5.2 Nitrotyrosine in cancer

NT is a stable end product of ONOO⁻ metabolism and can be used as an indicator of RNS (Davis et al. 2001, Karihtala & Soini 2007). Increased NT expression has been detected in various cancers (Table 2). To our knowledge, there are no studies of NT in EOC.
Table 2. Oxidative stress marker expression in various cancers.

<table>
<thead>
<tr>
<th>Oxidative stress marker</th>
<th>Lymphoma</th>
<th>Breast cancer</th>
<th>Urinary tract and prostate cancers</th>
<th>Hepatic and pancreatic cancers</th>
<th>Gastrointestinal (oesophageal, gastric, colorectal) cancers</th>
<th>Head and neck squamous cell cancer</th>
<th>Lung cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG</td>
<td>▲▼</td>
<td>▼▼</td>
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<tr>
<td>NT</td>
<td>▲▼</td>
<td>▲▼</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td>▼▼</td>
<td>▲</td>
</tr>
</tbody>
</table>

▲=increased expression in cancer tissue, ▼=decreased expression in cancer tissue, ▶=better prognosis, ◀=worse prognosis (including platinum resistance, higher stage or higher grade)
2.6 Redox-regulating mechanisms

DNA repair enzymes prevent the accumulation of DNA damage. Antioxidants protect cells from free radicals and maintain cellular redox state. They are substances such as selenium, carotenoids and vitamins A, C and E. Antioxidants deactivate free radicals before they cause damage. However, it is unclear whether taking antioxidant supplements provides any benefit. Excessive doses of antioxidants can decrease ROS levels and stimulate the survival of damaged cells, thus promoting carcinogenesis. (Jomova & Valko 2013, Valko et al. 2006 & 2007).

There are several antioxidant enzymes, such as SOD, catalase, GPXs, PRDXs, and TXN. Mitochondria are the major site of ROS formation and thus they have high concentrations of antioxidants such as glutathione (GSH), SOD and GPXs in the membranes. (Valko et al. 2006). NRF2 is a cytoprotective transcription factor, which regulates cell response to oxidative, physical or chemical insults. NRF2 regulates the formation of several antioxidant enzymes, such as PRDX1, TXN, GPX, and aldehyde oxygenase. (Gañán-Gómez et al. 2013). Also PARK7 protects cells from oxidative stress, partly together with NRF2 (Raninga et al. 2014).

2.6.1 NRF2 & KEAP1

NRF2 activates transcription of a variety of genes whose protein products protect against oxidative stress and restoration of homeostasis. Under basal conditions it is tethered to its cytoplasmic inhibitor, KEAP1-Cullin 3-Roc1-dependent mechanism. Oxidative stress triggers the disassociation of NRF2 from KEAP1, leading to NRF2 translocation to the nucleus. (Gañán-Gómez et al. 2013, Konstantinopoulos et al. 2011, Shibata et al. 2008, van der Wijst et al. 2014). In the nucleus it binds with the v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF) proteins and antioxidant response elements (AREs) located in its target genes. NRF2 regulates genes such as heme oxygenase 1 (HMOX1), NADPH dehydrogenase, quinone 1 (NQO1), glutathione S-transferase (GST), thioredoxin reductase 1 (TXNRI1) and PRDXI. NRF2 activates also some of the multidrug resistance genes. In physiological conditions NRF2 is quickly deactivated once the insult is withdrawn, but under pathological conditions it is altered. (Gañán-Gómez et al. 2013, Shibata et al. 2008). Sun et al. (2011) showed that under basal conditions KEAP1 shuttles between cytoplasm and nucleus independently of NRF2. Thus KEAP1 can translocate to the nucleus and modulate NRF2 signalling by transporting NRF2 back to the cytoplasm for
degradation (Fig. 5). Low levels of NRF2 increase ROS production and DNA damage, exposing tissues to tumorigenesis, but elevated levels also seem to increase the risk (Klaunig et al. 2010). Platinum-based chemotherapy generates electrophilic molecules that can damage DNA. Etoposide and doxorubicin can also induce production of ROS and/or RNS, which interact with DNA and activate NRF2 (Gañán-Gómez et al. 2013).

Besides its role as an antioxidant, NRF2 seems to have a role in cell growth as well, it partially regulates EGF receptors. It appears that p53-dependent apoptosis requires the inhibition of NRF2-regulated antioxidant genes. (Gañán-Gómez et al. 2013).

Fig. 5. NRF2 regulation by KEAP1. (1) Oxidative stress brings about a change in KEAP1-Cul3-E3 ubiquitin ligase by acting on cysteine residues. (2) NRF2 translocates to the nucleus and activates a variety of genes. (3) KEAP1 translocates to the nucleus with the assistance of importin β. (4) The KEAP1–NRF2 complex is translocated out of the nucleus. (5) In the cytosol NRF2 is degraded. Reprinted with permission from American Society for Microbiology; Sun et al. 2011;31:1800-1811.
Recent findings suggest that NRF2 contributes to the development of platinum resistance. Increased NRF2 activity and mutations in NRF2 or its gene, nuclear factor, erythroid 2-like 2 (NFE2L2) has been detected in several malignancies, Table 3. KEAP1 mutations lead to increased NRF2 expression and also KEAP1 or KEAP1 abnormalities are associated with several cancers (Table 3). In endometrial serous cancer (ESC) NRF2 expression was detected, while no expression was found in complex hyperplasia or the endometrial type of endometrioid cancer (EEC) (Jiang et al. 2010). The ESC cell line showed highest NRF2 expression and also resistance to cisplatin and paclitaxel. Silencing of NRF2 cells led to increased sensitivity to chemotherapy.

Konstantinopoulos et al. (2011) showed that the NRF2 pathway is associated with platinum resistance and OS in EOC. The NRF2 pathway seems to have a key role in ovarian carcinogenesis and cisplatin resistance in Western blotting, polymerase chain reaction (PCR) and IHC studies (Bao et al. 2014, Konstantinopoulos et al. 2011, Liao et al. 2012, Martinez et al. 2014). Gorrini et al. (2014) showed that estrogen regulates NRF2 activation in BRCA1-deficient cells, resulting in the induction of antioxidant genes that protect cells from ROS-induced death. This may lead to cumulative mutations and eventually cancer. In vitro studies have shown, that depressing NRF2 leads to cell growth arrest and increased sensitivity to docetaxel (Manandhar et al. 2012), doxorubin (Mandanhar et al. 2010), and cisplatin (Cho et al. 2008) in ovarian cancer cells.
Table 3. NRF2 and KEAP1 expression in various cancers.

<table>
<thead>
<tr>
<th>Studied marker</th>
<th>Breast cancer</th>
<th>Urinary tract and prostate cancers</th>
<th>Hepatic and pancreatic cancers</th>
<th>Gastrointestinal (oesophageal, gastric, colorectal) cancers</th>
<th>Head and neck squamous cell cancer</th>
<th>Lung cancer</th>
<th>Endometrial cancer</th>
</tr>
</thead>
</table>

↑=increased expression of protein or mutations  ➤better prognosis,  ➤=worse prognosis (including platinum resistance)
2.6.2 Peroxiredoxins

The PRDX family (PRDX1–6) is considered to be the most important group of cell redox state-regulating enzymes. They degrade hydroperoxides to water or corresponding alcohol. These enzymes are distributed subcellularly, in contrast to most other antioxidant enzymes and are found in various tissue fluids. PRDX1 and PRDX2 are located both in the cytoplasm and nucleus and interact with transcription factors. (Ishii et al. 2012). PRDX3 is located in the mitochondrial matrix, PRDX4 in the endoplasmic reticulum, PRDX5 in mitochondria, peroxisomes and cytosol, and PRDX6 in the cytosol (Poynton & Hampton 2014). The production of PRDXs increases when oxidative stress occurs (Rabilloud et al. 2002). In degrading process, PRDXs use their cysteine site, which is oxidized to sulphenic acid by hydroperoxides (Poynton & Hampton 2014). Under oxidatives stress, NRF2 increases PRDX expression (Gañán-Gómez et al. 2013).

PRDXs are highly homologous, but they seem to have different roles. Besides their antioxidative role, they seem to modulate inflammation, tissue repair after damage, and tumour progression. (Ishii et al. 2012). They protect cells from oxidative damage through their peroxidase activity. PRDX1 also suppresses oxidative stress-induced cell death by interacting with kinases and enzymes that regulate cell death and apoptosis (Ishii et al. 2012). PRDX2 is most abundant in neurons and levels are elevated in Parkinson’s disease (Ishii et al. 2012). Expression of PRDX5 has been found in osteoarthritic cartilage, suggesting a protective role against oxidative stress in human cartilage (Wang et al. 2002). PRDX6 plays an important role in lung surfactant degradation and synthesis (Fisher et al. 2005). Several studies have also shown increased expression of PRDXs in various cancers (Table 4). In ovarian cancer, strong IHC PRDX4 expression was associated with better prognosis, and PRDX5 and 6 with higher stage (Karihtala et al. 2009).
Table 4. Expressions of PRDXs, TXN, and PARK7 in various cancers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Lymphoma</th>
<th>Breast cancer</th>
<th>Urinary tract and prostate cancers</th>
<th>Hepatic and pancreatic cancers</th>
<th>Gastrointestinal (oesophageal, gastric, colorectal) cancers</th>
<th>Head and neck squamous cell cancer</th>
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<tr>
<td>PRDX2</td>
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<td>(Lee et al. 2007)</td>
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<td>(Chung et al. 2001)</td>
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<td>PRDX5</td>
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<td>(Karihtala et al. 2003)</td>
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<tr>
<td>PRDX6</td>
<td>↑↑</td>
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<td>(Karihtala et al. 2003)</td>
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<tr>
<td>Marker</td>
<td>Lymphoma</td>
<td>Breast cancer</td>
<td>Urinary tract and prostate cancers</td>
<td>Hepatic and pancreatic cancers</td>
<td>Gastrointestinal (oesophageal, gastric, colorectal) cancers</td>
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<td>TXN</td>
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<td>PARK7</td>
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</table>

↑=increased expression in cancer tissue, ↓=decreased expression in cancer tissue, →better prognosis, ↔worse prognosis (including higher stage or grade)
2.6.3 Thioredoxin

The thioredoxin system includes TXN, NADPH, and TXNRD. There are three different TXNRD proteins; TXNRD1 is in the extracellular space, nucleus, cytoplasm, and plasma membranes. TXNRD2 and TXNRD3 are restricted to mitochondria. (Karihtala & Soini 2007). TXNRDs have a celenocysteine-containing active site and together with NADPH they reduce back oxidized TXN (Arné & Holmgren 2006). Under oxidative stress, NRF2 induces TXN expression (Raninga et al. 2014). TXN also acts together with PRDXs in supplying electrons and together they form the thioredoxin peroxidase system to reduce peroxides with NADPH. TXN has anti-apoptotic effects by suppressing mitogen-activated protein kinase kinase kinase 5 (MAP3K5) activation, and anti-inflammatory effects. Under oxidative stress, TXN dissociates from MAP3K5, which leads to increased apoptosis. PARK7 has been shown to prevent this dissociation under oxidative stress. (Raninga et al. 2014). Inside the cell, TXN reduces oxidative stress and regulates a variety of signalling pathways (NF-κB, p53) and ROS-independent genes. Outside the cell it acts as a growth factor or cytokine and promotes cellular responses. (An & Kang 2014, Park et al. 2014b). TXN is a proto-oncogene and is seen in many cancers. It promotes tumour angiogenesis and inhibits spontaneous apoptosis and drug-induced apoptosis, thus stimulating cancer cell survival. (An & Kang 2014, Karihtala & Soini 2007). TXN is essential for mammals; deficient cells succumb to apoptosis and TXN knockout mice die embryonically (Nakamura et al. 2006).

Park et al. (2014b) showed that serum TXN levels combined with CA12-5 were useful for the early diagnosis of ovarian cancer. Expression of TXN in other cancers is seen in Table 4.

2.6.4 PARK7

PARK7, also known as DJ-1, is a protein that is involved in diverse cell processes such as chemotaxis, cell migration, cell adhesion, angiogenesis, apoptosis, cell–extracellular matrix interactions, and immune regulation. PARK7 is located mainly in cytoplasm, but under oxidative stress, some of the protein translocates to the mitochondria and to the nucleus. (Junn et al. 2009). PARK7 protects cells from oxidative stress by quenching ROS, apparently by several different mechanisms. For example, PARK7 increases the expression of glutamate cysteine ligase, which is needed in GSH synthesis. Additionally, PARK7 down-regulation
decreases extracellular SOD expression. (Junn et al. 2005, Raninga et al. 2014). PARK7 also stabilizes NRF2 by preventing its association with KEAP1 (Clements et al. 2006, Raninga et al. 2014). However, it does not disturb the NRF2-ARE pathway and transcription and the precise role of PARK7 in NRF2 regulation is unclear (Raninga et al. 2014). Under oxidative stress PARK7 inhibits PTEN, leading to activated cell growth. PARK7 also regulates p53 transcriptional activity, but it is unclear whether the effect is positive or negative. PARK7 is a positive regulator of NFKB transcription, leading to enhanced cell survival and proliferation and it also regulates MAP3K5-TXN function. (Raninga et al. 2014).

PARK7 is associated with early onset Parkinsonism (Bonifati et al. 2003), hence its name. PARK7 overexpression increases the resistance of neoplastic cells to apoptosis by inhibiting apoptotic pathways (Junn et al. 2005, Kim et al. 2005) and it is overexpressed in several malignant tumours (Table 4). Chen et al. (2015) showed increased serum PARK7 levels with metastatic uveal melanoma. Increased PARK7 expression in EEC compared with healthy controls and even higher expression in ESC compared with EEC was found (Morelli et al. 2014). In ovarian cancer, PARK7 has been associated with advanced-stage disease (Davidson et al. 2007).

2.7 Cancer stem cell theory

Previously it was assumed that any tumour cell is capable of forming new tumours. Increasing knowledge of the heterogeneity of malignant cells and clinical course of ovarian cancer suggests that there are distinct populations of cells in a tumour. The identification and isolation of CSCs was carried out in connection with acute myeloid leukaemia by Lapidot et al. (1994) and Bonnet & Dick (1997). Most ovarian cancer cells are initially chemosensitive and it is suspected that there is a population of cells that survive and become chemo resistant (Shah & Landen 2014). CSC theory could explain the behaviour of ovarian cancer; a small proportion of cancer cells have stem cell-like properties. These cells have unlimited proliferative potential through self-renewal, but they can also differentiate to generate cells with limited proliferative potential that form the bulk of the tumour. (Foster et al. 2013, Garson & Vanderhyden 2015). CSCs are mostly in a dormant state and can survive chemotherapy, which could explain recurrence after an initial total response (Bonneau et al. 2015). Physiologically, stem cells play a role in immunotolerance and other functions
such as inhibition of apoptosis and fibrosis, stimulation of angiogenesis, and reduction of oxidative stress (Touboul et al. 2014).

CSCs have been found in several malignancies and it appears that every cancer has different types of CSCs. Bapat et al. (2005) first isolated tumorigenic clones from the ascites fluid of advanced ovarian cancer. These cells had stem cell-like characteristics and could form tumours. Moserle et al. (2008) found a population of ovarian stem cells in mice that were highly tumorigenic compared with other cells. In ovarian cancer, the presence of CSCs is associated with poor survival and chemoresistance (Abubaker et al. 2013, Chau et al. 2013). Identification of CSCs is based on surface expression of proteins, such as CD44 molecule (Indian blond group) (CD44), aldehyde dehydrogenase 1 (ALDH1), and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT). CSCs can be detected, for example, by immunohistochemistry (IHC), flow cytometry, fluorescence-activated cell sorting (FACS), and side population and aldefluor assays. At present, there is no standard method for CSC detection and the methods in use are not specific. The degree of surface expression of CSC markers depend on the in vitro or in vivo environment, including the cell line that is used, and also the enzymatic or mechanical disaggregation method. (Tirino et al. 2013). Two- or three-dimensional cell culture systems are widely used for CSC detection, the main issues of concern include dilution, temperature, and possible toxicity of agar. In sphere-formation assays, it may be difficult to distinguish spheres with high cell densities and to detect quiescent CSCs. In tumour xenotransplantation, tumour cell manipulation may affect cell behaviour and viability. (Garson & Vanderhyden 2015, Tirino et al. 2013).

2.7.1 CD44

CD44 is a single-chain transmembrane glycoprotein of the hyaluronate receptor family. It is a major adhesion molecule of the extracellular matrix that binds primarily to glycosaminoglycans. (Hiraga et al. 2013). It has functions not only in cell adhesion, but also in extracellular matrix interactions. Acting as a ligand receptor and co-receptor, it can modulate the function of other membrane tyrosine receptors. (Dan et al. 2014). CD44 works in several physiological processes such as leukocyte homing and activation, wound healing, and cell migration (Hiraga et al. 2013).

Al-Hajj et al. (2003) showed CD44 to be a marker of tumour stem cells. In vitro studies have shown that CD44+ cells have self-renewal properties; they
show extensive proliferation and differentiation and have the ability to form metastases (Hiraga et al. 2013, Ponti et al. 2005). However, the findings have been conflicting in clinical studies of cancers (Table 5).

Several studies have been carried out on CD44 in EOC, but no consensus of opinion regarding its clinical significance has been reached. Zhang et al. (2008) isolated a CD44- and KIT-positive cell population from primary ovarian carcinoma that could form self-renewing spheres in vitro and re-establish the original tumour hierarchy in mice. Alvero et al. (2009) and Shi et al. (2010) demonstrated CD44 expression in vitro in ovarian cancer and found that the cells have a high capacity for repair, tumour formation and chemoresistance. Steffensen et al. (2011) found 17.1% of the ovarian cancer samples to have CD44+ cells. The number of CD44+ cells was significantly higher in early-stage ovarian cancer and was associated with shorter PFS. In ovarian cancer, the percentage of CD44+ cells may vary from 0.5% to 85% (Kryczek et al. 2012). Most methods of detection do not discriminate between different isoforms of CD44, which may affect the results (Garson & Vanderhyden 2015).

Cannistra et al. (1995) found CD44 variant expression in ovarian cancer by PCR analysis, but there was no correlation with prognosis. Kayastha et al. (1999) showed IHC expression of CD44 to correlate with poor survival, whereas Sillanpää et al. (2003) found IHC expression of CD44 more frequently in well-differentiated, early-stage tumours and in patients with longer survival. In a study by Meng et al. (2012), CD44 associated with an increased risk of recurrence and shorter PFS. In a study by Bonneau et al. (2015), a decrease of CD44 expression after chemotherapy was associated with poor OS in EOC. They also found chemoresistance in CD44+ cells in vitro. They hypothesized that microscopic residual tumour containing dormant CSCs and a large proportion of rapidly dividing bulk cells explained early recurrence (Bonneau et al. 2015).
Table 5. Expression of CSC markers in various cancers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Gastric and colorectal cancer</th>
<th>Head and neck squamous cell cancer</th>
<th>Hepatic and pancreatic cancers</th>
<th>Melanoma</th>
<th>Urinary tract cancers</th>
<th>Breast cancer</th>
<th>Lung cancer</th>
<th>Endometrial and cervical cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑leftrightarrow</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td></td>
<td>↑</td>
<td>↑leftrightarrow</td>
<td></td>
</tr>
<tr>
<td>KIT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑leftrightarrow</td>
<td>↑leftrightarrow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑leftrightarrow</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td>↑leftrightarrow</td>
<td>↑leftrightarrow</td>
<td>(Yasuda et al. 2006)</td>
</tr>
<tr>
<td>ALDH1</td>
<td>↑leftrightarrow</td>
<td>↑leftrightarrow</td>
<td>↑</td>
<td></td>
<td></td>
<td>↑leftrightarrow</td>
<td>↑leftrightarrow</td>
<td></td>
</tr>
</tbody>
</table>

↑=increased expression, ↓=decreased expression, ↑leftrightarrow=better prognosis, ↔worse prognosis in vivo or in humans, including platinum resistance
2.7.2 KIT

KIT, also known as CD117, is a surface marker of embryonic stem cells, haematopoietic stem cells, and mesenchymal cells. It maintains cells in their undifferentiated state and confers the ability to self-renew. (Luo et al. 2011, Palmqvist et al. 2005). KIT is encoded by the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog gene (KIT). KIT is a tyrosine kinase receptor for stem cell factor (SCF), it activates for example PIK3CA, and initiates a broad programme of signal transduction, which mediates cell survival, migration, and proliferation. (Garson & Vanderhyden 2015). The SCF-KIT signalling system supports KIT-expressing cells, such as melanocytes and germ cells (Yasuda et al. 2006).

KIT mutations have been found in gastrointestinal stromal tumours (Duensing et al. 2004) and ovarian dysgerminomas (Hoei-Hansen et al. 2007). Increased KIT expression exists in various cancers (Table 5). Zhang et al. (2008) described KIT-positive cells with self-renewing growth in ovarian cancer. Stewart et al. (2011) detected KIT expression in 45% of EOCs by FACS, but Kryczek et al. (2012) only 0% to 10% by immunofluorescence technique. In vitro, KIT+ ovarian cancer cells formed tumours and reproduced the original tumour. KIT expression was also associated with resistance to platinum-paclitaxel chemotherapy and short DFS. (Luo et al. 2011). Cisplatin chemotherapy in vitro has led to enrichment and enhanced stem cell-like characteristics of dormant ovarian cancer cells, which expressed high levels of KIT (Zhou et al. 2014).

Zeng et al. (2014) showed increased IHC expression of KIT in recurrent serous ovarian cancer. Moreover, Huang et al. (2014) found high expression of KIT in fibroblast-like stromal cells and correlation with an advanced FIGO stage, poor grade, and short OS and PFS in ovarian cancer. KIT expression was in 9% of tumour cells, with no correlation with clinical parameters. The authors concluded that the microenvironment is important for tumour growth, and fibroblast cells act synergistically with carcinoma cells. (Zeng et al. 2014).

2.7.3 ALDH1

ALDH1 is a cytosolic enzyme that catalyzes the oxidation of aldehydes to carboxylic acids. It protects organisms from harmful aldehydes and cytotoxic drugs. It also plays a role in signalling and other cellular processes, such as oxidation of retinol to retinoic acid. ALDH1 is widely expressed in normal tissue,
but its expression is altered in carcinomas. (Garson & Vanderhyden 2015, Penumatsa et al. 2010, Wang et al. 2012). ALDH1 expression in various cancers is shown in Table 5.

Landen et al. (2010) first described an association between ALDH1 and poor outcome in patients with ovarian cancer. Subsequently, other investigators confirmed this finding. ALDH1 expression was low in non-dysplastic epithelium and STIC found in specimens of prophylactic BSO, but high in dysplasia (Chene et al. 2014). Expression of ALDH1 in epithelial cells isolated from fresh ovarian tumours was 0.3–7.1% (Kryczek et al. 2012). ALDH1+ cells have been found to be more chemoresistant and tumorigenic (Silva et al. 2011). Kryczek et al. (2012) showed ALDH1 expression of CSCs to be highly variable, with overlap between patients in immunofluorescence staining. They also showed that ALDH1+ cells could generate heterogeneous tumour populations in vivo.

Primary ovarian cancer samples had low densities of ALDH1, but after chemotherapy stronger expression was seen Zhang et al. (2012). Steg et al. (2012) also found an increase of ALDH1 expression after primary chemotherapy from 23.4% to 29.2%. In persistent tumour, the proportion of ALDH1-positive cells increased from 29.7% to 54.9% (Steg et al. 2012). High ALDH1 expression has been found to correlate with poor clinical outcome (Kuroda et al. 2013, Liebscher et al. 2013), chemoresistance (Landen et al. 2010), and CD44 expression (Wang et al. 2012) in ovarian cancer. ALDH1 associated with chemoresistance and tumour progression in ovarian cancer (Deng et al. 2010)

On the other hand, ALDH1 has been found to be a favourable prognostic factor in ovarian carcinoma (Chang et al. 2009). Penumatsa et al. (2010) found a weaker expression of ALDH1 in ovarian cancer compared with normal ovarian tissue and benign tumours. The results were similar with PCR, Western blot and IHC methods. In addition, IHC expression of ALDH1 did not correlate with that of CD44 and KIT. Bonneau et al. (2015) found, in post-chemotherapy samples, decreased ALDH1 expression to be associated with shorter DFS in patients with EOC.
3 Aims of the study

The present work was undertaken

1. To investigate the roles of oxidative stress and major antioxidant enzymes in ovarian tumours.
2. To study the roles of serum and IHC 8-OHdG as prognostic factors in ovarian cancer.
3. To evaluate the roles of serum 8-OHdG and PARK7 in the outcome of disease in different EOC histotypes.
4. To study the CSC theory, NRF2, KEAP1, and PARK7 and their correlation to platinum resistance, clinicopathological parameters and survival in neoadjuvant-treated ovarian cancer.
4 Materials and methods

4.1 Ethical aspects

This study was approved by The Regional Ethics Committee of the Northern Ostrobothnia Hospital District (53/2008) and the National Supervisory Authority for Welfare and Health (1339/05.01.00.06/2009).

4.2 Study population

Study I included 71 patients with benign and borderline ovarian tumours diagnosed during 2000–2007. Study II included 84 patients with ovarian cancer diagnosed during 1997–2005. Serum samples were available from all patients and tumour samples from 78. In Study III, there were 112 EOC patients diagnosed during 1996–2009, 48 samples were available for PARK7 measurements. Serum samples in Studies II and III were collected on the day before surgery and tumour samples during primary surgery. For Study III we included 35 patients (32 serous, 2 endometrioid, 1 clear cell) from Study II. Study IV included 38 ovarian cancer patients whose diagnoses were made during 2004–2014. Tumour samples were collected during primary surgery before chemotherapy and at debulking surgery after chemotherapy. The total number of patients in Studies I–IV was 270, of whom 20 had a benign, 51 a borderline and 199 a malignant tumour. Pertinent characteristics of patients in Studies I–IV are found in Table 6. All patients were diagnosed and treated at Oulu University Hospital.

Tumour samples were fixed in neutral formalin, embedded in paraffin blocks and stored at the Department of Pathology. One sample in Study IV was fixed in alcohol. Serum samples were stored at −70 °C until analysed. Histological diagnoses of the tumours were determined according to the criteria of the most recent WHO classification of ovarian cancer. In Study IV, the samples were regraded according to the Malpica grading system to low and high grade (Malpica et al. 2004).

Clinicopathologic data were collected from hospital records and included patient age at diagnosis, tumour stage, grade, histology, disease recurrences, development of platinum resistance, and time of death.
Table 6. Clinical characteristics of the patients, studied markers, and used methods in Studies I–IV.

<table>
<thead>
<tr>
<th>Study</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technique</td>
<td>IHC</td>
<td>IHC, ELISA</td>
<td>ELISA</td>
<td>IHC</td>
</tr>
<tr>
<td>Studied markers</td>
<td>8-OHdG, NT, PRDX1–6, TXN</td>
<td>8-OHdG</td>
<td>8-OHdG, PARK7</td>
<td>CD44, KIT, ALDH1, PARK7, NRF2, KEAP1</td>
</tr>
<tr>
<td>Number of patients</td>
<td>71</td>
<td>84</td>
<td>112</td>
<td>38</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>49 (19–80)</td>
<td>58 (32–81)</td>
<td>59 (22–85)</td>
<td>62 (28–79)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>10 benign</td>
<td>63</td>
<td>38</td>
<td>36</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>0</td>
<td>11</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>10 benign</td>
<td>7</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Clear-cell</td>
<td>0</td>
<td>2</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>11</td>
<td>22</td>
<td>1 (Low)</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>25</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>47</td>
<td>71</td>
<td>37 (High)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>13</td>
<td></td>
<td>I+II 50</td>
<td>0</td>
</tr>
<tr>
<td>Stage II</td>
<td>1</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Stage III</td>
<td>51</td>
<td></td>
<td>III+IV 60</td>
<td>25</td>
</tr>
<tr>
<td>Stage IV</td>
<td>17</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal</td>
<td>28</td>
<td>55</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>56</td>
<td>56</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>1</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

1 No macroscopic residual tumour
4.3 Methods

4.3.1 Detection of DNA damage, antioxidant enzymes and CSC markers

ELISAs

ELISA was used to measure serum levels of 8-OHdG (Highly Sensitive 8-OHdG Check ELISA kit; Gentaur Europe, Brussels, Belgium in Study II; JaICA, Fukuroi, Japan in Study III) and PARK7 (Nordic Biosite, Täby, Sweden). In Study II, serum 8-OHdG concentrations were measured as follows:

Serum samples were pre-processed using Millipore Microcon filters (Millipore, Billerica, MA, USA). The filters were dampened with 100 µL of distilled water and centrifuged at 14,000 × g for five min. To remove the remaining water, filters were turned over and centrifuged for a further five minutes. The filters were then moved to new tubes, 200 µL of serum was added, and the tubes were centrifuged at 14,000 × g for 30 min. The primary antibody was reconstituted with primary antibody solution. After this, 50 µL of standard sample was added in duplicate to the wells of an assay plate before adding 50 µL of reconstituted primary antibody to each well. The plate was shaken, covered with an adhesive strip and incubated at 4 °C overnight. The contents of the wells were poured off and each well was washed three times with 250 µL of washing solution. Then 100 µL of reconstituted secondary antibody was added to each well. The plate was shaken, covered with adhesive strip and incubated for one hour at room temperature. At the end of the incubation period, washing was repeated. Substrate solution was then prepared and 100 µL was added to each well. The plate was shaken and incubated in the dark for 15 min at room temperature. Reaction terminating solution (100 µL) was added to each well and the plate was shaken. Absorbance was measured at 450 nm in a plate reader and a standard curve was used to determine the levels of 8-OHdG in the samples. In one plate, forty samples were assayed as a duplicate. The outermost wells of the microtitre plates were also used. The assay was repeated if the measured values in duplicate samples differed by more than 10%.

In Study III, serum 8-OHdG concentrations were measured as described above, except that filtering was omitted from the procedure. Levels of serum PARK7 were measured with a similar method. The volume of standard solution...
and diluted sample was 100 μL. After incubation at room temperature for one hour, each well was washed four times with 350 μL of wash buffer. Then 100 μL of detection antibody was added, and samples were incubated again at room temperature for one hour. The wells were then washed four times with 350 μL of wash buffer, 100 μL substrate reagent was added, and incubation was continued for 10–20 minutes at room temperature. Finally, 100 μL of stop solution was added.

In Study II, we used the ROC curve and in Study III, the median concentration of 8-OHdG and PARK7 as the cut-off point for survival analysis.

**Immunohistochemistry**

Sections with a thickness of 3.5–4 μm were cut from paraffin blocks and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). They were de-paraffinised in xylene and re-hydrated in a descending series of ethanol solutions, incubated in 10 μM of citrate buffer (pH 6.0, but pH 9.0 in Study IV for NRF2, KEAP1 and KIT), boiled in a microwave oven for 10 minutes in Studies I and II and for 17 minutes in Study IV and thoroughly cooled to room temperature before adding the primary antibody. In negative controls, the primary antibodies were replaced with phosphate-buffered saline and serum isotype controls (Zymed Laboratories Inc, California, United States). A detailed list of the used antibodies and antigens is shown in Table 7. In Studies I and II, immunoreactivity was evaluated by categorising the staining into four groups: 0 = no staining, 1 = weak staining (20–49% of the cells stained), 2 = moderate staining (50–89%) and 3 = strong staining (≥ 90%). In Study IV, the samples were considered negative if less than 5% of the cells stained, ± if 5–50% and ++ if more 50% of the cells stained. Nuclei were categorised as positive if over 5% expressed positivity.
Table 7. Details of antigens and antibodies used in the IHC studies.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Immunostaining method</th>
<th>Source of primary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRDX1</td>
<td>Rabbit polyclonal PRDX1 antibody</td>
<td>1:1500</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>PRDX2</td>
<td>Rabbit polyclonal PRDX2 antibody</td>
<td>1:1000</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>PRDX3</td>
<td>Rabbit polyclonal PRDX3 antibody</td>
<td>1:750</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>PRDX4</td>
<td>Rabbit polyclonal PRDX4 antibody</td>
<td>1:750</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>PRDX5</td>
<td>Rabbit polyclonal PRDX5 antibody</td>
<td>1:2000</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>PRDX6</td>
<td>Rabbit polyclonal PRDX6 antibody</td>
<td>1:2000</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Labfrontier, York, United Kingdom</td>
</tr>
<tr>
<td>TXN</td>
<td>Goat polyclonal anti-human TXN antibody</td>
<td>1:200</td>
<td>Biotinylated secondary anti-goat antibody; avidin-biotin-peroxidase complex</td>
<td>American Diagnostica, Greenwich, CT</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>Mouse monoclonal 8-OHdG antibody</td>
<td>1:100 ¹</td>
<td>Dako Envision Kit</td>
<td>Gentaur, Brussels, Belgium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:125 ²</td>
<td>Dako Envision Kit</td>
<td>Gentaur, Brussels, Belgium</td>
</tr>
<tr>
<td>NT</td>
<td>Rabbit polyclonal NT antibody</td>
<td>1:100</td>
<td>Histostain-Plus Bulk Kit</td>
<td>Upstate, NY, USA</td>
</tr>
<tr>
<td>CD44</td>
<td>Mouse monoclonal CD44 antibody</td>
<td>1:400</td>
<td>Dako REAL antibody diluent</td>
<td>Cell Signaling Technology, Danvers, USA</td>
</tr>
<tr>
<td>KIT</td>
<td>Rabbit polyclonal KIT antibody</td>
<td>1:200</td>
<td>Dako Envision Kit</td>
<td>Dako Denmark, Agilent Technologies, Glostrup, Denmark</td>
</tr>
<tr>
<td>ALDH1</td>
<td>Mouse monoclonal ALDH1 antibody</td>
<td>1:300</td>
<td>Dako Envision Kit</td>
<td>BD Transduction laboratories, New Jersey, USA</td>
</tr>
<tr>
<td>NRF2</td>
<td>Rabbit monoclonal NRF2 antibody</td>
<td>1:300</td>
<td>Dako Envision Kit</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>KEAP1</td>
<td>Rabbit polyclonal KEAP1 antibody</td>
<td>1:500</td>
<td>Dako Real antibody diluent</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>PARK7</td>
<td>Rabbit polyclonal PARK7 antibody</td>
<td>1:5000</td>
<td>Dako Envision Kit</td>
<td>Abcam, Cambridge, UK</td>
</tr>
</tbody>
</table>

¹ Study I, ² Study II
4.3.2 Statistical analysis

IBM SPSS 16.0 (Study I), 17.0 (Study II) and 21.0.0.0 (Studies III and IV) for Windows (Chicago, IL, USA) software were used for statistical analyses. The significance of associations between the expressions of 8-OHdG, PRDXs, TXN and NT in benign and borderline tumours was determined by using two-sided Fisher’s exact probability test in Study I. Ovarian cancer-specific survival and DFS were analysed with Kaplan–Meier curves with log-rank test in Studies II, III, and IV and also with Tarone–Ware and Breslow tests in Study II. Cox multivariate regression analysis was used to evaluate prognostic factors in Studies II and III. Association between 8-OHdG and clinicopathological features was analysed with Mann–Whitney U test and Pearson’s Chi-square test in Study II, and with Mann-Whitney U test and Kruskal–Wallis test in Study III. In Study IV, Pearson’s Chi-square test and 2-sided McNemar’s tests were used to analyze associations between CSC markers and clinicopathological features. Probability values of < 0.05 were considered significant.
5 Results

5.1 Expression of antioxidant enzyme and oxidative stress (Studies I-III)

In the benign and borderline tumours, 20% of cells expressed 8-OHdG. The staining was mostly weak. NT expression was seen in 45% of the benign and 41.1% of the borderline tumours; the difference was not statistically significant (Table 8). PRDX expression generally was stronger in borderline than in benign tumours. The differences in the cytoplasmic and nuclear expression patterns of the PRDX subtypes in benign and borderline tumours are found in Table 8. Cytoplasmic (c) and nuclear (n) staining of TXN was strong both in benign and borderline tumours. Expression of nTXN was stronger in benign tumours ($p = 0.003$, Table 8). In ovarian cancer, 8-OHdG immunostaining was located mainly in the nuclei. Of these cancer samples, 41% were negative for 8-OHdG, whereas 24.4% stained weakly, 16.7% moderately, and 17.9% strongly.

In Study II, the median concentration of serum 8-OHdG was 176.4 pg/mL (mean 198.6 pg/mL, range 61.7 – 502.8 pg/mL) in cancer samples. A serum 8-OHdG level of 140 pg/mL was recognized as an optimal cut-off level for survival analysis. In Study III, the mean 8-OHdG concentration was 3 220 pg/mL (95% CI 2 730 – 3 710 pg/mL) in cancer samples. The median 8-OHdG concentration was 2 530 pg/mL and was used as an optimal cut-off level for survival analysis. The mean concentration of PARK7 was 15 990 pg/mL (95% CI 12 200 – 19 800 pg/mL). The median PARK7 concentration was 10 500 pg/mL and was used as an optimal cut off level.
Table 8. Oxidative stress markers in benign and borderline ovarian tumours. NS= not significant

<table>
<thead>
<tr>
<th>Studied marker</th>
<th>p-value</th>
<th>Higher expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>cPRDX1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>nPRDX1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>cPRDX2</td>
<td>0.000004</td>
<td>Borderline</td>
</tr>
<tr>
<td>nPRDX2</td>
<td>0.000002</td>
<td>Borderline</td>
</tr>
<tr>
<td>cPRDX3</td>
<td>0.02</td>
<td>Borderline</td>
</tr>
<tr>
<td>cPRDX4</td>
<td>0.02</td>
<td>Borderline</td>
</tr>
<tr>
<td>cPRDX5</td>
<td>0.0006</td>
<td>Borderline</td>
</tr>
<tr>
<td>nPRDX5</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>cPRDX6</td>
<td>0.0003</td>
<td>Borderline</td>
</tr>
<tr>
<td>nPRDX6</td>
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<td>Borderline</td>
</tr>
<tr>
<td>cTXN</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>nTXN</td>
<td>0.003</td>
<td>Benign</td>
</tr>
</tbody>
</table>

5.2 Association between oxidative stress and antioxidant enzymes (Study I)

Significant correlations between various markers of oxidative stress were found both in benign and borderline tumours. 8-OHdG was significantly associated with cPRDX3 (p = 0.02), nPRDX5 (p = 0.01), and nPRDX6 (p = 0.006) expression in benign tumours. In addition, NT expression was associated with cPRDX3 (p = 0.06), cPRDX4 (p = 0.01), cPRDX5 (p = 0.007), nPRDX5 (p = 0.01) and nPRDX6 (p = 0.003) expression. A highly significant correlation between 8-OHdG and NT (p = 0.0002) was seen in benign tumours. In borderline tumours, 8-OHdG was associated with increased expression of cPRDX1 (p = 0.002) and cPRDX3 (p = 0.002). NT was associated with higher cPRDX1 (p = 0.01).

5.3 Oxidative stress and antioxidative enzyme expression in different histologies (Studies I-III)

Expressions of cPRDX3 (p = 0.01), cPRDX4 (p = 0.03), cPRDX5 (p = 0.03), cPRDX6 (p = 0.01), and cTXN (p = 0.03) were stronger in serous benign tumours than in mucinous benign tumours. Similarly, expressions of cPRDX1 (p = 0.04), cPRDX2 (p = 0.002), cPRDX4 (p = 0.008), cPRDX5 (p = 0.005), cPRDX6
(p = 0.00004), and cTXN (p = 0.04) were significantly stronger in serous than in mucinous borderline tumours. Serum 8-OHdG expression was significantly stronger in serous cancer compared with other histologies, p = 0.03 (Study II) and p = 0.033 (Study III).

5.4 Correlation of 8-OHdG expression with survival (Studies II and III)

Serum concentration of 8-OHdG above 140 pg/mL correlated to poor survival (log-rank test, p = 0.049; Breslow test, p = 0.038; Tarone–Ware test, p = 0.041, Fig. 6A) in EOC. Mean survival was 64.5 months for patients with serum 8-OHdG concentration above 140 pg/mL, whereas patients with a lower 8-OHdG level showed mean survival of 95.9 months. The association was stronger in the grade 1–2 diseases (log-rank test, p = 0.032; Breslow test, p = 0.040; Tarone–Ware test, p = 0.034, Fig. 6B). Highly positive 8-OHdG IHC expression was associated with poor survival (log-rank test, p = 0.053; Breslow test, p = 0.045; Tarone–Ware test, p = 0.043, Fig. 6C).

Higher serum 8-OHdG concentration (with concentration 2530 pg/mL as cut-off) was associated with poor DFS (p = 0.020) as well as with poor OS (p = 0.019) in the whole EOC study group (Figs. 7A & B). In patients with endometrioid tumour, serum 8-OHdG levels were associated significantly with DFS (p = 0.005, Fig. 7C). A high 8-OHdG concentration in the patients with endometrioid histology was a more significant predictor of poor DFS in Cox regression analysis (OR 1.387; 95% CI 1.095–1.758; p = 0.007) than suboptimal surgery (OR 2.898; 95% CI 0.925–9.076; p = 0.068), high stage (OR 1.221; 95% CI 0.215–6.915, p = 0.822) or high grade (OR 1.566; 95% CI 0.259–9.457, p = 0.625).
Fig. 6. Kaplan–Meier curves demonstrating ovarian cancer survival in relation to A) serum 8-OHdG, all patients B) serum 8-OHdG, patients with grade 1–2 disease and C) IHC 8-OHdG staining, all patients.
5.5 Correlation of 8-OHdG with prognosis (Studies II and III)

Residual disease after surgery was the only predictor of survival in multivariate Cox regression analysis in Study II. A high serum 8-OHdG concentration (with concentration 140 pg/mL as cut-off) was associated with an advanced stage \((p = 0.021)\), serous histology \((p = 0.002)\), and suboptimal surgery \((p = 0.007)\) according to the Mann–Whitney test. Strong IHC staining of 8-OHdG correlated with higher grade \((p = 0.041)\) and showed a trend to correlate with suboptimal surgery \((p = 0.094)\). No correlation between serum 8-OHdG levels and tissue 8-OHdG expression was found.

A high serum 8-OHdG concentration (with concentration 2530 pg/mL as cut-off) was associated with serous histology, platinum resistance, stage III–IV, and a
suboptimal surgical result, (Table 9). Among the patients with endometrioid ovarian cancer, high serum 8-OHdG concentration was associated with platinum resistance and a suboptimal surgical result. High PARK7 levels were associated with high grade and non-significantly with serous histology (Table 9).

Table 9. Associations of serum 8-OHdG and PARK7 with prognostic factors in ovarian cancer. Data on 8-OHdG are shown separately for the whole Study III population and for the subgroup with endometrioid ovarian cancer.

<table>
<thead>
<tr>
<th>Studied marker</th>
<th>Serous histology</th>
<th>Platinum resistance</th>
<th>Stage III–IV</th>
<th>Inoperability</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 8-OHdG</td>
<td>All patients</td>
<td>0.033</td>
<td>0.002</td>
<td>0.009</td>
<td>0.012</td>
</tr>
<tr>
<td>High 8-OHdG</td>
<td>Endometrioid histology</td>
<td>NS</td>
<td>0.007</td>
<td>NS</td>
<td>0.025</td>
</tr>
<tr>
<td>High PARK7</td>
<td>All patients</td>
<td>0.089</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are p-values, NS=no statistical significance

5.6 Expression of CSC markers and antioxidative defence mechanisms in neoadjuvant-treated cancer (Study IV)

Positive immunostaining of KIT was rare (10%) in pre-treatment ovarian cancer samples. Of the same samples, 67% stained positively for CD44, 39% for cALDH1, 11% for nALDH1, 86% for cNRF2, 42% for nNRF2, 100% for cKEAP1, 86% for nKEAP1, 97% for cPARK7, and 94% for nPARK7. Changes in the expression of the markers after neoadjuvant chemotherapy are shown in Table 10. All post-treatment samples were positive for nKEAP1 ($p=0.025$), whereas cNRF2 expression decreased ($p = 0.016$). An inverse correlation between pre-treatment nALDH1 and cNRF2 expression was observed ($p = 0.034$).

Pre-treatment nKEAP1 expression was higher in stage II–III disease compared with stage IV ($p = 0.0003$). nKEAP1 was increased in post-treatment samples and correlated with higher stage ($p = 0.0001$). In post-treatment samples, cPARK7 expression was increased and correlated with stage IV ($p = 0.035$).
Table 10. Changes in the expression of oxidative stress markers and CSC markers after neoadjuvant treatment.

<table>
<thead>
<tr>
<th>Studied marker</th>
<th>Decreased (%)</th>
<th>Stable (%)</th>
<th>Increased (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>32</td>
<td>50</td>
<td>18</td>
</tr>
<tr>
<td>KIT</td>
<td>6</td>
<td>91</td>
<td>3</td>
</tr>
<tr>
<td>cALDH1</td>
<td>12</td>
<td>62</td>
<td>27</td>
</tr>
<tr>
<td>nALDH1</td>
<td>6</td>
<td>88</td>
<td>6</td>
</tr>
<tr>
<td>cNRF2</td>
<td>44</td>
<td>41</td>
<td>15</td>
</tr>
<tr>
<td>nNRF2</td>
<td>21</td>
<td>62</td>
<td>18</td>
</tr>
<tr>
<td>cKEAP1</td>
<td>3</td>
<td>94</td>
<td>3</td>
</tr>
<tr>
<td>nKEAP1</td>
<td>0</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>cPARK7</td>
<td>6</td>
<td>82</td>
<td>12</td>
</tr>
<tr>
<td>nPARK7</td>
<td>0</td>
<td>94</td>
<td>6</td>
</tr>
</tbody>
</table>

5.7 Association between CSC markers and platinum resistance (Study IV)

There was a correlation between negative pre-treatment CD44 expression and increased platinum sensitivity \( (p = 0.013) \). In addition, increased CD44 expression after platinum treatment correlated with platinum resistance \( (p = 0.001, \text{Fig. 8A}) \). Positive pre-treatment cALDH1 expression correlated with platinum resistance \( (p = 0.017, \text{Fig. 8B}) \). The combination of negative pre-treatment CD44 and positive cALDH1 expression predicted platinum resistance \( (p = 0.047) \).
5.8 Summary of the main results

Study I revealed that oxidative stress exists in benign and borderline ovarian tumours. In particular, PRDX2 and PRDX6 expressions were significantly higher in the borderline tumours and most of the PRDX isoenzymes correlated with the...
oxidative stress markers 8-OHdG or NT. The expression of nearly all studied markers was stronger in serous benign and borderline tumours than in the mucinous ones, Table 11.

In Study II, high serum 8-OHdG levels were associated with poor survival in patients with EOC, especially in grade 1–2 disease. Serum 8-OHdG levels were higher in patients with serous ovarian carcinoma than in those with other histological types, and in patients with stage III–IV cancers compared those with less advanced cancers. A high preoperative serum 8-OHdG level predicted suboptimal surgical result. A correlation between strong IHC 8-OHdG expression and poor prognosis was observed.

Study III revealed that high preoperative serum 8-OHdG levels were significantly associated with serous histology, platinum resistance, higher stage, and suboptimal surgical results and with poor OS and DFS. In the endometrioid cancers, 8-OHdG was a powerful predictor of decreased DFS and platinum resistance. The results support the hypothesis of the role of oxidative stress in the development of platinum resistance in ovarian cancer. Results also confirm the different carcinogenetic backgrounds of ovarian cancer histotypes.

In Study IV, negative pre-treatment CD44 expression predicted platinum sensitivity, and positive cALDH1 expression predicted platinum resistance. In addition, we found that the NRF2-KEAP1 system is highly active and even further stimulated after neoadjuvant treatment, especially in advanced stages of cancer. Together, CD44 and cALDH1 could be potential predictors of platinum sensitivity and might be of value in designing chemotherapy for individual patients.
Table 11. Summary of the main results in Studies I–IV.

<table>
<thead>
<tr>
<th>Significant change in expression of markers in neoadjuvant-treated ovarian cancer</th>
<th>Significant change in the expression of markers in neoadjuvant-treated ovarian cancer</th>
<th>Correlation of 8-OHdG with clinicopathological features</th>
<th>Correlation of 8-OHdG with survival in ovarian cancer</th>
<th>Significantly stronger expression of markers in serous tumours</th>
<th>Significantly stronger PRDX expression in borderline tumours compared with benign ones</th>
</tr>
</thead>
<tbody>
<tr>
<td>cPRDX2, nPRDX2, cPRDX3, cPRDX4, cPRDX5, cPRDX6, nPRDX6</td>
<td>In benign tumours: cPRDX3, cPRDX4, cPRDX5, cPRDX6, cTXN</td>
<td>Decreased OS and DFS in the whole study population.</td>
<td>Higher stage, higher grade, serous histology, suboptimal surgery, platinum resistance</td>
<td>Increased nKEAP1, decreased cNRF2</td>
<td>Increased post-treatment CD44 expression with platinum resistance. Positive pre-treatment cALDH1 expression with platinum resistance</td>
</tr>
</tbody>
</table>
6 Discussion

6.1 Antioxidant enzymes in ovarian tumours

Our research group has previously shown that increased expression of cPRDX4 associates with a better prognosis, and cPRDX5 and cPRDX6 are associated with a higher stage in ovarian cancer (Karihtala et al. 2009). No studies on benign and borderline ovarian tumours have been published. In Study I, increased expression of PRDX2 and PRXD4, in particular, was seen in borderline tumours compared with benign tumours. These results support the hypothesis that PRDXs have different roles in ovarian tumours. Possibly they could be used in differential diagnostic between tumours.

In Study I, TXN expression was significantly stronger in the nuclei of benign tumours compared with borderline tumours. TXN translocates to the nucleus as a result of oxidative stress or chemotherapy, where it can activate transcription factors (Woolston et al. 2010). TXN can also become pro-oxidant at high levels and it has been shown to enhance apoptosis (Ravi et al. 2005). Low pre-treatment cTXN expression has been associated with better DFS in ovarian cancer, whereas the combination of low cTXN and high nTXN expression was associated with better DFS and OS (Woolston et al. 2010).

The possible association of PRDXs and TXN with the development of platinum resistance has been raised in ovarian and other cancers in other studies. High PRDX3 expression associated with platinum resistance and in vitro, the role of PRDX1, PRDX2, PRDX3, and PRDX6 in cisplatin resistance was suggested in ovarian cancer (Kalinina et al. 2012, Wang et al. 2013). Association between high TXN expression and paclitaxel resistance in vitro was found in ovarian cancer (Wang et al. 2015).

Antioxidant enzymes, such as PRDX1–6 and TXN, are essential in normal tissues. However, these enzymes may also protect cancer cells from ROS, induced by the tumour itself or chemotherapy, which may lead to increased drug resistance and cancer cell survival. The precise role of PRDXs and TXN in ovarian cancer is still unclear and further studies of ovarian carcinogenesis are needed. Several studies investigate antioxidant enzymes as targets of therapeutic intervention in cancer. For example, inhibition of GSH and TXN pathways led to mammary cancer cell death in vitro and in vivo (Harris et al. 2015).
6.2 Significance of oxidative stress in ovarian tumours

Oxidative stress was seen in benign ovarian tumours. ROS production is necessary for normal cell signalling cascades and thus low levels of oxidative stress are physiological in normal human tissue (Valavanidis et al. 2009). However, not all samples expressed oxidative stress markers or antioxidant enzymes suggesting variability in ROS formation in benign and borderline tumours.

8-OHdG and NT expression were seen both in benign and borderline tumours. Our group has previously shown that 8-OHdG is associated with poor differentiation, non-optimal surgical outcome and higher stage in ovarian carcinomas. However, NT expression was not associated significantly with clinicopathological parameters. (Karihtala et al. 2009). In the present study, 8-OHdG also associated with poor survival, higher stage, and suboptimal surgical result. 8-OHdG clearly associates with worse prognosis in EOC, but the role of NT does not seem so obvious.

6.3 Significance of oxidative stress in different histologies

Previously, histologies have not necessarily been separated in clinical studies of EOC. According to recent findings, it is supposed that there are differences in the development and clinical courses of different histologies in EOC (Berns & Bowtell 2012, Romero & Bast 2012). An association between 8-OHdG and poor prognosis in HGSC has been confirmed (Xu et al. 2013) but there are no published studies of associations between other histologies and 8-OHdG. In our ovarian cancer study population, strong 8-OHdG expression was also associated with serous histology. Additionally, already benign serous tumours expressed more strongly almost all studied PRDXs and TXN than mucinous tumours. It appears that serous tumours are under increased oxidative stress.

In Study III, we found significantly reduced DFS especially in patients with endometrioid cancer, and also correlation between 8-OHdG expression and platinum resistance. Most endometrioid ovarian cancers arise from endometriosis and in theory the increased oxidative stress in endometriosis could result in a more aggressive cancer. However, a recent study revealed no effect on survival when compared endometriosis-associated endometrioid or clear-cell cancers with non-endometriosis-associated ones (Kim et al. 2014). In that study, endometriosis-associated cancers were more often stage I–II and grade 1. Sova et
al. (2012) found that, compared with endometriosis, the expression of 8-OHdG and PRDX is lower in endometriosis-associated endometrioid and clear-cell ovarian cancers. It was hypothesized that, in contrast to endometriosis, oxidative stress during carcinogenesis would induce the DNA repair enzymes, leading to decreased levels of antioxidants and 8-OHdG.

Earlier studies and the findings in this study support the idea that the level of oxidative stress determines the outcome of patients with serous and endometrioid types of ovarian cancers. Increased oxidative stress associates with platinum resistance in endometrioid cancer. So far, chemotherapy protocols have been similar in all tumour histologies. Further studies are needed to evaluate oxidative stress in other ovarian cancer histotypes for a more precise categorization of the tumours into different prognostic groups.

6.4 Association of 8-OHdG and antioxidant defence mechanisms with platinum resistance

In Study III, we found an association between preoperative serum 8-OHdG levels and the development of platinum resistance in EOC. The mechanism of platinum-based treatment is partly based on ROS formation as chemotherapy agents create molecules that damage DNA (Gañán-Gómez et al. 2013). It is possible that oxidative stress induces antioxidant enzyme formation in the tumour, which manifests clinically as chemoresistance. However, preoperative 8-OHdG expression could be a potential predictor of platinum resistance and assist in choosing the optimal chemotherapy agent. To our knowledge, this is the first study on 8-OHdG and platinum resistance in ovarian cancer.

The NRF2-KEAP1 system and PARK7 are activated as a result of oxidative stress. In previous studies, high expression of NRF2 has usually been associated with increased platinum resistance in ovarian cancer (Bao et al. 2014, Konstantinopulos et al. 2011, Liao et al. 2012, Martinez et al. 2014). Increased expression of PARK7 has been shown to be associated with poor prognosis in lung cancer (Merikallio et al. 2012). However, there are no studies on PARK7 in connection with platinum resistance and only few studies on PARK7 in ovarian cancer. In Study III, there was no association between serum PARK7 and platinum resistance. Only 48 of the initial samples were available for PARK7 measurements, which may have affected the result. In Study IV, IHC expression of PARK7 increased after platinum-based treatment, especially in patients with stage IV disease, which may reflect enhanced antioxidant defence after an
oxidative insult. However, there was no correlation between IHC PARK7 expression and platinum resistance. Additionally, there was no association between the NRF2-KEAP1 system and platinum resistance, even though there were changes in their expression after platinum-based treatment. Statistical significance was found for the increase of nKEAP1 expression and decrease of cNRF2 expression. The study population in Study IV was quite small and prognostically homogeneous, which makes it challenging to investigate the effect of these markers on OS. There are also other effective treatment options for platinum-resistant patients, which may have an effect on OS. There are several ongoing studies to evaluate the possibility that NRF2 blocking increases platinum sensitivity. Encouraging results have already been published (van der Wijst et al. 2015).

6.5 Expression of CSC markers

The expression of ovarian CSC markers has varied considerably in previous studies, especially for CD44 (range 0.5%–85%) (Kryczek et al. 2012). CSC marker expression after chemotherapy has been either increased or decreased, depending on the study and the investigated markers. After primary therapy for ovarian cancer, an increase in CD44 expression from 6.2% to 11% has been observed (Steg et al. 2012, Zhang et al. 2012). In persistent tumours, an increase in CD44+ cells from 8.3% to 21.2% has been reported (Steg et al. 2012). All pre-treatment samples that were assessed for CSC markers in Study IV showed some expression; the strongest positivity was for CD44 (67%). Statistically significant alterations in the expression of the markers were not observed. In addition, no correlation between KIT, CD44, and ALDH1 was seen in our study. Similarly, in previous studies, the associations between CSC markers have been variable or nonexistent in ovarian and other cancers (Bonneau et al. 2015, Pitule et al. 2014). These findings are not completely surprising because CSCs have different functional mechanisms.

6.6 CSC theory and platinum resistance

Several in vitro studies have revealed an association between CSCs and more aggressive tumour growth, development of metastasis and platinum resistance in ovarian cancer. However, the findings have not been unequivocal in clinical studies. Mostly the results have indicated poor prognosis in patients with high
CSC marker expression (Landen et al. 2010, Luo et al. 2011, Park et al. 2013), but a favourable association between CSCs and survival has also been reported (Bonneau et al. 2015, Dan et al. 2014, Sillanpää et al. 2003). Most studies have included tumours of different histologies and stages, which may have affected the results. CSCs have different isoforms. Although their role may be variable in cancer, the type of CSC isoforms has not been specified in most of the studies. (Garson & Vanderhyden 2015).

In Study IV, negative CD44 pre-treatment expression was a powerful predictor of platinum sensitivity, and pre-treatment cALDH1 positivity signalled platinum resistance. Recent results reported by Gao et al. (2015) support our findings regarding CD44, both metastatic and recurrent ovarian cancers, which are more likely to be platinum resistant, expressed CD44 more strongly than primary tumours. Strong expression also correlated with poor prognosis. In vitro and in vivo models showed decreased proliferation and improved drug sensitivity after CD44 knockdown. Also in vitro, cisplatin chemotherapy led to CSC enrichment and enhanced stem cell-like characteristics of dormant ovarian cancer cells, which expressed high levels of CD44 (Zhou et al. 2014).

In our study, positive pre-treatment nALDH1 expression was associated with decreased pre-treatment cNRF2 expression ($p = 0.034$), which is in accordance with the results published by Mizuno et al (2014). In their study of clear-cell ovarian cancer, strong ALDH1 expression correlated to advanced stage and poor PFS but not with CD44 expression. Cells with strong ALDH1 expression had a much lower intensity of ROS and a higher intensity of NRF2. This finding could be associated with the poor response to platinum-based chemotherapy in the clear-cell type of ovarian cancer. ALDH1 expression was high in 10–20% of clear ovarian cancer cells, whereas in serous cancer only 0.1–5% of the cells were positive. (Mizuno et al. 2014). Earlier studies have shown that ALDH1 expression is increased in post-chemotherapy groups and that strong ALDH1 expression is associated with poor prognosis and platinum resistance (Deng et al. 2010, Landen et al. 2010, Park et al. 2013). Unfortunately, the majority of these studies have been carried out in vitro. In our study, the increase in ALDH1 in the post-treatment group was not statistically significant. However, our study is the first to show an association between pre-treatment cALDH1 expression and chemoresistance. The results from Study IV could be highly valuable in clinical therapeutic treatment designs, as currently there is no method to evaluate whether or not a patient will benefit from platinum-based neoadjuvant chemotherapy.
6.7 Challenges in assessing oxidative stress and CSCs \textit{in vivo}

Free radicals are short-lived and react quickly with other components. Thus, it is difficult to measure their concentrations directly. Certain end products of cell metabolism, e.g. 8-OHdG and NT, are stable and seem to be reliable IHC markers for the indirect measurement of oxidative stress. (Hwang & Bowen 2007). However, the preparation and examination of both histology and paraffin blocks requires experience and skill to find the most representative parts of the tumour without false negativity and necrosis. In addition, the level of oxidative stress may vary within the tumour. Thus, a small sample of tumour may not always represent the level of oxidative stress in the whole tumour. Investigation of cancer CSCs may also be problematic, because a tumour may contain different clones of CSCs (Shah & Landen 2014). Further, as found in the current study, tumour cell populations may vary within one histological plate. These findings indicate that one tumour sample may not represent the whole tumour. On the other hand, IHC assessment allows examination of the localization of oxidative stress and CSC markers (nuclei, cytoplasm, surrounding stroma).

Since oxidative stress is a physiological phenomenon, it is difficult to assess pathological levels of 8-OHdG in serum samples (Hwang & Bowen 2007). In Study II, we used the ROC curve and in Study III, the median concentration of 8-OHdG as the cut-off point for survival analysis. This is a common methodological tool that has been frequently used in other studies as well. There have also been problems in the sensitivity of 8-OHdG assays in other studies. Compared with chromatographic methods, ELISA seems to overestimate 8-OHdG levels because of cross-reaction, which also depends on the used antibody. (Peoples & Karnes 2005, Rossner & Sram 2012). At present, several commercial antibodies are available for 8-OHdG measurements. Of these, we used the one that is highly specific and has been widely used in earlier studies. Study III included cases from Study II and for some of these cases ELISA was repeated with consistent results.

In Study II, 8-OHdG serum concentration and IHC staining intensity were associated with poor prognosis. However, a correlation between serum and tissue 8-OHdG levels was not found. In advanced stage of cancer, the tumour vasculature may leak 8-OHdG from cells as a result of enhanced repair enzyme functions, which leads to increased serum 8-OHdG levels. Oxidative bursts, other inflammatory factors, tumour necrosis, and renal elimination (Rossner & Sram 2012) may also affect serum 8-OHdG levels. Additionally, smoking, obesity,
diabetes, cardiovascular diseases and aging, for example, increase serum 8-OHdG levels. (Hwang & Bowen 2007, Rossner & Sram 2012).
7 Conclusions

Oxidative stress markers and antioxidant enzymes exist in benign and borderline ovarian tumours. Some antioxidant enzymes are more strongly expressed in borderline tumours. Further studies are needed to evaluate their role in the pathogenesis and differential diagnostics of ovarian tumours.

8-OHdG expression is associated with serous histology, poor prognosis, platinum resistance, higher stage, and suboptimal surgical result in EOC. In the endometrioid subtype, 8-OHdG associates with poor prognosis, platinum resistance, and suboptimal surgical result.

CSC markers exist in ovarian cancer and they are associated with the development of platinum resistance. Further studies are needed to evaluate their expression in different histotypes and stages of ovarian cancer.
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Li Xs, Xu Q, Fu XY, Luo WS (2014) ALDH1A1 overexpression is associated with the progression and prognosis in gastric cancer. BMC Cancer 14:705.


**Original publications**


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Original publications and not included in the electronic version of the dissertation.
1308. Myllymäki, Satu-Marja (2015) Specific roles of epithelial integrins in chemical and physical sensing of the extracellular matrix to regulate cell shape and polarity

1309. Antonoglou, Georgios (2015) Vitamin D and periodontal infection

1310. Valtokari, Maria (2015) Hoitoon pääsyn moniulotteisuus erikoissairaanhoidossa


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OXIDATIVE STRESS IN THE PATHOGENESIS AND PROGNOSIS OF OVARIAN CANCER

Marjo Pylväs-Eerola