Ovarian cancer is one of the most insidious malignancies in women. It is often diagnosed with widely disseminated disease and intraperitoneal ascites fluid. Angiogenesis and lymphangiogenesis, i.e. the development of new microvessels and lymphatic vessels in the tumor, are crucial in ovarian cancer development. In this thesis, promising antitumoral effects and safety of adenoviral antiangiogenic and antilymphangiogenic gene therapy in a human ovarian cancer xenograft model and in healthy rats are described. Furthermore, diffusion-weighted MRI (DW-MRI) was used to evaluate the early gene therapy responses in tumors.
Gene Therapy for Ovarian Cancer
LAURA TUPPURAINEN

Gene Therapy for Ovarian Cancer

Antiangiogenic and Imaging Study

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in the Auditorium 1, Kuopio University Hospital on Friday, March 31st 2017, at 12 noon

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Number 408

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Author's address: Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
E-mail: laura.tuppurainen@uef.fi

Supervisors: Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Eastern Finland
KUOPIO
FINLAND

Hanna Sallinen, M.D., Ph.D.
Department of Obstetrics and Gynecology
Kuopio University Hospital
KUOPIO
FINLAND

Reviewers: Docent Anna Kanerva, M.D., Ph.D.
Cancer Gene Therapy Group
University of Helsinki
Department of Obstetrics and Gynecology
Helsinki University Hospital
HELSINKI
FINLAND

Professor Oskari Heikinheimo, M.D., Ph.D.
Department of Obstetrics and Gynecology
Helsinki University Hospital
HELSINKI
FINLAND

Opponent: Docent Annika Auranen, M.D., Ph.D.
Department of Obstetrics and Gynecology
Tampere University Hospital
TAMPERE
FINLAND
Tuppurainen, Laura

Gene Therapy for Ovarian Cancer - Antiangiogenic and Imaging Study

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ABSTRACT:
Ovarian cancer is the most lethal of the malignant gynecological cancers. It is the second most common gynecological cancer, after cancer of the corpus uteri. Despite substantial research and the development of novel targeted therapies over the last decades, no significant improvement has been achieved in the treatment of ovarian cancer.

Angiogenesis and lymphangiogenesis, i.e. the development of new microvessels and lymphatic vessels in the tumor, are crucial for the development, malignancy, metastasis and accumulation of intraperitoneal ascites fluid in ovarian cancer. The objective of this thesis was to evaluate the role of the most relevant angiogenic and lymphangiogenic growth factors and their receptors in ovarian cancer and to develop a procedure to allow magnetic resonance imaging (MRI) in preclinical settings.

In the first study, the safety of adenoviral (Ad) soluble vascular endothelial growth factor receptors (VEGFR), AdsVEGFR2 and AdsVEGFR3, in combination with paclitaxel and carboplatin chemotherapy, was demonstrated in healthy rats. These results formed the foundation for further clinical studies with gene therapy in ovarian cancer using AdsVEGFR2 and AdsVEGFR3.

In the second study, adenoviral gene therapy was evaluated in intraperitoneal human ovarian cancer xenografts in mice by administering AdsVEGFR2, AdsVEGFR3 and soluble VEGFR coreceptors, neuropilins (NRP)-1 and -2. The survival of mice was prolonged and the growth of tumor microvessels was reduced with AdsVEGFR2 and AdsVEGFR3 gene therapy. We conclude that AdsNRP1 and AdsNRP2 gene therapy may exert antiangiogenic effects inhibiting the growth of ovarian tumors; however, their relevance in ovarian cancer is still unclear. We demonstrated that early changes in T2-relaxation time and diffusion-weighted MRI (DW-MRI) with apparent diffusion coefficient (ADC) values in tumor tissues after the gene therapy are potentially useful when evaluating the response of gene therapy targeting inside the tumors.

The third study examined the role of angiopoietins (Ang1 and Ang2) and their receptors (Tie1 and Tie2) in ovarian cancer development. In this experiment, AdsTie2 and AdsVEGFR2 restricted the growth of tumors and reduced the formation of intraperitoneal ascites in mice with ovarian cancer. Furthermore, we observed that chemotherapy has a relevant role in the treatment of ovarian cancer in our mouse model.

In conclusion, in the future, it is possible that patients with advanced ovarian cancer and malignant ascites may be treated with adenoviral antiangiogenic and antilymphangiogenic gene therapy combined with chemotherapy. These results are encouraging and represent a foundation for embarking on future clinical studies in ovarian cancer patients.
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TIIVISTELMÄ

Gynekologisista syövistä munasarjasyöpään liittyy suurin kuolleisuus. Se on naisten toiseksi yleisin gynekologinen syöpä kotonaan rungon syövän jälkeen. Vaikka munasarjasyöpää on tutkittu runsaasti ja hoitomenetelmät ovat kehittyneet viime vuosikymmenien aikana, merkittävää parannusta syövän hoidossa tai potilaiden ennusteessa ei ole saavutettu. Angiogeneesillä ja lymfangiogeneesillä, eli uusien mikroverisuonten kehittymisellä sekä imusuonten kehittymisellä kasvaimissa on keskeinen rooli munasarjasyövän kehittymisen, pahanlaatuistumisen, etäpesäkkeiden lähettämisen ja vatsaonteloon kertyvän askitesnesteen muodostumisessa. Tämän väitöskirjatutkimuksen tavoitteena oli arvioida keskeisimpien angiogeenisten ja lymfangiogeenisten kasvutekijöiden ja näiden reseptoreiden roolia munasarjasyövässä ja kehittää syövän magneettikuvantamista (MRI) preklinisissä tutkimuksissa.


Kolmannessa osatyössä tutkittiin angiopoietiinien (Ang1 ja Ang2) sekä niiden reseptoreiden (Tie1 ja Tie2) roolia munasarjasyövän kehittymässä. Tässä tutkimuksessa todettiin, että AdsTie2 ja AdsVEGFR2 vähensi vät kasvainten kasvua ja vähensi vät vatsaontelonsisäisen askitesnesteen muodostumista munasarjasyövän kokeeläinmallissa hiirillä. Lisäksi totesimme, että kemoterapialla on merkittävä rooli munasarjasyövän hoitossa kokeeläinmallissa.

Yhteenvetona voidaan todeta, että adenovirusvälitteinen antiangiogeeninen ja antilymfangiogeeninen geeniterapia yhdessä kemoterapian kanssa on mahdollinen uusi hoitomenetelmä pitkälle edenneen munasarjasyövän ja askitesnesteen kertymiseen hoidoon. Tutkimuksen tulokset ovat lupaavia ja puoltavat kliinisten tutkimusvaiheen aloitettuun munasarjasyövän hoitoon.
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Yleinen Suomalainen asiasanasto: adenovirukset; angiogeneesi; eläinkokeet; geeniterapia; kasvutekijät; munasarjasyöpä; reseptorit
We keep moving forward, opening new doors, and doing new things, because we're curious... and curiosity keeps leading us down new paths.

– Walt Disney
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List of the original publications

This dissertation is based on the following original publications:


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AAV  Adeno-associated virus
Ab  Antibody
ABL  Non-receptor tyrosine kinase protein
Ad  Adenovirus
Ad.mda-7  Adenoviral melanoma differentiation-associated gene-7
ADC  Apparent diffusion coefficient
ADP  Adenosine diphosphate
A  FOS  Alkaline phosphatase
ALAT  Alanine aminotransferase
Ang  Angiopoietin
B 0  External magnetic field
BRCA  Breast cancer-associated gene
CA12-5  Cancer antigen 12-5
CAR  Coxsackie adenovirus receptor
CD31  Cluster of differentiation 31-protein
CD34  Cluster of differentiation 34-protein
cDNA  Complementary deoxyribonucleic acid
CEPC  Circulating endothelial progenitor cells
CFDNA  Cell-free DNA
CMV  Cytomegalovirus
CRAd  Conditionally replicating adenovirus
CRISPR  Clustered regularly interspaced short palindromic repeats
CT  Computed tomography
DCE-MRI  Dynamic contrast-enhanced magnetic resonance imaging
DNA  Deoxyribonucleic acid
DNAseI  Deoxyribonuclease enzyme
DW-MRI  Diffusion weighted magnetic resonance imaging
E1A  Early region 1A
EGFR  Epidermal growth factor receptor
ELISA  Enzyme-linked immunosorbent assay
EMA  European Medicines Agency
FDA  US Food and Drug Administration
FDG  Fluorodeoxyglucose
FGF  Fibroblast growth factor
FIGO  International Federation of Gynecology and Obstetrics
Flk-1  Fetal liver kinase (VEGFR2)
Flt-1  Fms-like tyrosine kinase (VEGFR1)
Flt-4  Fms-like tyrosine kinase 4 (VEGFR3)
FOV  Field-of-view
FOXO1  Forkhead box O1
GMCSF  Granulocyte macrophage colony-stimulating factor
GMP  Good manufacturing practice
GT  Gene transfer
Hb  Hemoglobin
HCT  Hematocrit
HE4  Human epididymis secretory protein 4
HER  Human epidermal growth factor receptor
HIF  Hypoxia inducible factor
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<tr>
<td>AAV</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ABL</td>
<td>Non-receptor tyrosine kinase protein</td>
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<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>Ad.mda-7</td>
<td>Adenoviral melanoma differentiation-associated gene-7</td>
</tr>
<tr>
<td>ADC</td>
<td>Apparent diffusion coefficient</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>AFOS</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ALAT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>Ang</td>
<td>Angiopoietin</td>
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<tr>
<td>Bo</td>
<td>External magnetic field</td>
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<td>BRCA</td>
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HIV  Human immunodeficiency virus
HNPPCC  Hereditary non-polyposis colorectal cancer
HRD  Homologous recombination deficiency
HSPG  Heparin sulfate proteoglycan
HSV  Herpes simplex virus
i.m.  Intramuscular
i.p.  Intraperitoneal
i.v.  Intravenous
IFN  Interferon
Ig  Immunoglobulin
IGF-1  Insulin-like growth factor 1
IL  Interleukin
KDR  Kinase-insert domain receptor (VEGFR2)
Krea  Creatinine
LacZ  Betagalactosidase (marker gene)
LDH  Lactate dehydrogenase
LPL  Lipoprotein lipase
LV  Lentivirus
LYVE  Lymphatic vessel hyaluronan receptor
MAPK  Mitogen-activated protein kinase
MCH  Mean cell hemoglobin
MCHC  Mean cell hemoglobin concentration
MCV  Mean cell volume
MMR  Mismatch repair
MRI  Magnetic resonance imaging
mRNA  Messenger ribonucleic acid
MVD  Microvessel density
NMR  Nuclear magnetic resonance
NRP  Neuropilin
OS  Overall survival
OVCA  Ovarian cancer
PARP  Poly adenosine-diphosphate-ribose polymerase
PCR  Polymerase chain reaction
PD-1  Programmed cell death protein 1
PDGF  Platelet derived growth factor
PD-L1  Programmed cell death protein ligand
PET  Positron emission tomography
PFS  Progression-free survival
PI3K  Phosphatidylinositol 3-kinase
PLC-γ  Phospholipase C-γ
PLD  Pegylated liposomal doxorubicin
PIGF  Placental growth factor
PLT  Platelets
qRT-PCR  Quantitative reverse-transcriptase polymerase chain reaction
rAAV  Recombinant adeno-associated virus
RAFF  Relaxation along a fictitious field
rBC  Red blood cell
RF  Radiofrequency
RNA  Ribonucleic acid
ROI  Region of interest
SCID  Severe combined immunodeficiency
SEM  Standard error of the mean
SKOV  Human epithelial ovarian cancer cell line
SKOV-3m  Primary cell line derived from the SKOV3 cell line
SMA  Smooth muscle actin
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
</tr>
<tr>
<td>SRC-2</td>
<td>Steroid receptor coactivator-2</td>
</tr>
<tr>
<td>sTie</td>
<td>Soluble angiopoietin receptor</td>
</tr>
<tr>
<td>sVEGFR</td>
<td>Soluble vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>(T_{\text{ip}})</td>
<td>(T_1) relaxation in the rotating frame</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Tek</td>
<td>Angiopoietin receptor Tie2</td>
</tr>
<tr>
<td>Tie</td>
<td>Angiopoietin receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TVA</td>
<td>Total vascular area</td>
</tr>
<tr>
<td>T-VEC</td>
<td>Talimogene laherparepvec</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vp</td>
<td>Viral particle</td>
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<tr>
<td>WBC</td>
<td>White blood cell</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>(\alpha)-SMA</td>
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Introduction

Ovarian cancer is one of the most insidious malignancies since there is a low rate of symptoms in the early stage of the disease. The characteristics of the widely disseminated disease include the formation of intra-peritoneal ascites fluid, which is related to a poor prognosis and lethality.

The highest incidence of ovarian cancer occurs in the age group 60-64 years, although the incidence increases after the age of 45 years. The most important therapy for ovarian cancer is surgery and in most cases subsequent adjuvant therapy with chemotherapy (Ovarian cancer: Current Care Guidelines Abstract, 2012, Engholm et al., 2016).

The overall 5-year survival rate for ovarian cancer in Finland is 45% (2010-2014) (www.cancerregistry.fi).

Ovarian cancer is characterized as a highly disseminated peritoneal carcinosis. Cancer development, malignancy and metastasing are related to increased angiogenesis and lymphangiogenesis in tumors (Gavalas et al., 2013).

Angiogenesis, i.e. the formation of new blood vessels from existing vessels, is crucial in tumor development, since the growth of the tumor is dependent on oxygen and the presence of numerous growth factors. The role of vascular endothelial growth factors and angiopoietins is clearly related not only to increased angiogenesis in tumors but also with the aggressivity of the ovarian cancer (Liang et al., 2013; Monk et al., 2016).

In cancer development, the balance between angiogenic and antiangiogenic growth factors is disturbed and tilted in favor of the growth of new microvessels (Hanahan and Weinberg, 2011a).

The principle of gene therapy is to transfer modified nucleic acids encoding the therapeutic proteins into somatic cells of the targeted tissues. Gene therapy may be intended to restore a deficiency of certain nucleic acids and encoded proteins; it can be utilized in protein malfunction by replacing the faulty nucleic acids by functional ones (Ylä-Herttuala and Alitalo, 2003).

As the ovarian cancer is dependent on both angiogenesis and lymphangiogenesis, our aim was to target and inhibit the growth of new microvessels and lymphatic vessels by delivering soluble forms of vascular endothelial growth factor receptors (sVEGFR), their neuropilin-coreceptors (sNRP) and angiopoietin receptors (sTie).

The idea behind the soluble growth factor receptors was to inhibit the vascular sprouting angiogenesis and lymphangiogenesis in peritoneal ovarian tumors. Furthermore, as the role of vascular growth factors is related to the formation of peritoneal ascites fluid, which itself is linked with a poor prognosis in ovarian cancer, a secondary aim was to study the role of endothelial growth factors in ascites formation and also to prevent the amount of ascites in ovarian cancer xenografts (Sallinen et al., 2014; Smolle et al., 2014).

The imaging of peritoneally disseminated ovarian cancer with MRI is a routine method in clinical practice. Diffusion-weighted MRI is a more sensitive method for evaluating the nature of the disease and the clinical potential of this new technique for monitoring the therapeutic effects aimed at combating tumors has been under development (Rockall, 2014). As gene therapy focuses on changes inside of tumors and tumor microvessels, DW-MRI represents a potential innovative way of estimating the efficacy of tumor microvessel-targeted therapies at the molecular level.
1 Introduction

Ovarian cancer is one of the most insidious malignancies since there is a low rate of symptoms in early stage of the disease. The characteristics of the widely disseminated disease include the formation of intraperitoneal ascites fluid, which is related to a poor prognosis and lethality. The highest incidence of ovarian cancer occurs in the age group 60-64 years, although the incidence increases after the age of 45 years. The most important therapy for ovarian cancer is surgery and in most cases subsequent adjuvant therapy with chemotherapy (Ovarian cancer: Current Care Guidelines Abstract, 2012, Engholm et al., 2016). The overall 5-year survival rate for ovarian cancer in Finland is 45 % (2010-2014) (www.cancerregistry.fi). Ovarian cancer is characterized as a highly disseminated peritoneal carcinosis. Cancer development, malignancy and metastasing are related to increased angiogenesis and lymphangiogenesis in tumors (Gavalas et al., 2013). Angiogenesis, i.e. the formation of new blood vessels from existing vessels, is crucial in tumor development, since the growth of the tumor is dependent on oxygen and the presence of numerous growth factors. The roles of vascular endothelial growth factors and angiopoietins are clearly related not only to increased angiogenesis in tumors but also with the aggressivity of the ovarian cancer (Liang et al., 2013; Monk et al., 2016). In cancer development, the balance between angiogenic and antiangiogenic growth factors is disturbed and tilted in favor of the growth of new microvessels (Hanahan and Weinberg, 2011a).

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mon gynecological cancer unfortunately also young women with ovarian cancer. It has been dy of Finnish s type of further the age of carriers -.

- The risk of ovarian cancer in women at BRCA1 or BRCA2 positive cancer families are BRCA1 or BRCA2 positive HNPCC -.

- The most common predisposing gene mutations in hereditary ovarian cancer are childlessness, related to oophorectomy in BRCA1 or peritoneum other vaginal carcinogens upwards into cancer.

- Furthermore, the age of the risk of ovarian cancer is 40% and breast cancer, the prognosis mutation is 40% and systemic therapy was by estradiol and sequential estrogen and progesterone pills in women, postmenopausal (Gharwan et al., 2015; Gong et al., 2013).

2 Review of the literature

2.1 OVARIAN CANCER

2.1.1 Epidemiology and risk factors
Ovarian cancer is the second most common gynecological cancer after cancer of the corpus uteri, and the fifth most common malignancy in women in the developed countries with almost 100 000 new diagnosed cancers worldwide per year (Siegel et al., 2016; Torre et al., 2015). In the years 2010-2014, the incidence in Finland was 9.5 per 100 000 with 530 new diagnosed ovarian cancers and 372 deaths (Engholm et al., 2016; Engholm et al., 2010). Overall, the relative survival rate at one year after the cancer has been diagnosed in Finland is 78 %; the five year survival rate is even more dismal, only 45 %. The prevalence of ovarian cancer is at its highest in women in the age class 65 - 75 years. It is rarely diagnosed before the age of 40. Unfortunately, also young women with ovarian cancer are childlessness, (Rebbeck et al., 2009).

The known risk factors for ovarian cancer are childlessness, endometriosis, polycystic ovarian syndrome and overweight with a poor lifestyle (Al Bakir and Gabra, 2014; Dumesic and Lobo, 2013; Stewart et al., 2013). Furthermore the age of menarche is inversely related to the risk of ovarian cancer, relating to the higher amount of lifetime ovulations and the early presence of androgenic hormones in women who have menarche at an early age (Gharwan et al., 2015; Gong et al., 2013). According to a register data study of Finnish women, postmenopausal hormone replacement therapy with estrogen and sequential estradiol-progesterone therapy increased the total risk of ovarian cancer if this type of therapy was used for 5 years or more (Koskela-Niska et al., 2013). Furthermore, a large randomized trial with a 5.6 years’ follow-up in postmenopausal women without hysterectomy indicated that continuous combined therapy with estrogen and progestin elevated the risk of ovarian cancer (Hildebrand et al., 2010; Mørch et al., 2009).

A family history of ovarian cancer increases the risk by 3.6-fold if it is present in the first degree and by 2.9-fold in a second-degree relative. A hereditary reason for ovarian cancer is uncommon with only 5-10 % diagnosed new cancers being attributable to hereditary. The most common predisposing gene mutations in hereditary ovarian cancer are thought to be BRCA1, BRCA2 and Lynch syndrome (heritable non-polyposis colorectal cancer syndrome, HNPCC) (Al Bakir and Gabra, 2014; Burgess and Puhalla, 2014). In Finland, 26 % of ovarian cancer families are BRCA1 or BRCA2 positive (Sarantaus et al., 2001). BRCA1 mutation carriers have a 57 % lifetime risk and BRCA2 carriers carry a 49 % risk for suffering breast cancer. In women at the age of 70, the lifetime risk for ovarian cancer if she has the BRCA1 mutation is 40 % and lower, 18 %, if she has the BRCA2 counterpart (Chen and Parmigiani, 2007). Although these mutations are clearly related to high lifetime risk for ovarian or breast cancer, the prognosis is better in these patients than in patients without the inherited mutations (Boyd et al., 2000; Sun et al., 2014).

Tubal ligation has been shown to reduce the risk for ovarian cancer by 34- 40 %. Furthermore, the effect of hysterectomy and tubal ligation at the time of sterilization or cancer-prophylactic surgery may prevent the passage of uterine endometrial cells, and other vaginal carcinogens upwards into the fallopian tubes, fimbria, ovaries and peritoneum (Cibula et al., 2011; Dietl et al., 2011). It has been proposed that salpingooophorectomy in BRCA1 or BRCA2 mutation carriers can reduce the risk for breast cancer by 50 % and 80 % for ovarian or fallopian tube cancer (Rebeck et al., 2009).

Pregnancy exerts a protective effect against ovarian cancer (Hinkula et al., 2006). In addition, the use of combined estrogen and progesterone contraceptive pills has been related to a lower risk for epithelial ovarian cancer with the relative risk of 0.73.
(Collaborative Group on Epidemiological Studies of Ovarian Cancer et al., 2008; Hankinson et al., 1992). Furthermore, hormone therapy with progesterone only, the so-called minipill, is believed to have a protective role in cancer development (Jeon et al., 2016). After four-years’ use of contraceptives, the decrease in the relative risk was estimated as 50% in postmenopausal women, furthermore the protective effect was assumed to remain for decades even after the termination of the medication (Grimbizis and Tarlatzis, 2010; Whittemore et al., 1992). It has been claimed that the long-time use of low-dose aspirin may reduce the risk for ovarian cancer by decreasing tissue inflammation (Baandrup et al., 2015; Trabert et al., 2014).

2.1.2 Etiology
The etiology and pathology behind the epithelial ovarian cancer are not fully understood. Several potential predisposing factors have been postulated i.e. frequent ovulation cycle, inflammation, changes in stromal tissue and influence of androgens or progestin or gonadotropins (Gharwan et al., 2015). Increased oxidative stress attributable to inefficient DNA repair mechanisms, as well as regular ovulation are prospective inflammatory factors and mechanisms behind the cancer development. Endometriosis is related to increased pelvic inflammation and increased production of prostaglandins, furthermore it has been associated with an increased risk of endometrial and clear cell ovarian cancer, but not for serous ovarian carcinoma (Babic et al., 2014; Merritt et al., 2013; Munksgaard and Blaakaer, 2012; Ness and Cottreau, 1999). It has been reported that the risk for ovarian cancer in patients with endometriosis is increased by 1.3 to 1.9 times as compared to women not suffering endometriosis (Munksgaard and Blaakaer, 2012). Although the development of ovarian cancer in women with endometriosis is relatively slow, a long-lasting disease with late diagnosis and childlessness are related to an increased risk of cancer (Lassus et al., 2015).

Histologically, the surface of the ovarian epithelium is identical to the pelvic and abdominal mesothelium, this has been related to behavior of ovarian cancer, especially its abdominal dissemination. The origin of high-grade serous ovarian carcinoma was recently related to serous carcinomas in the fimbria of the fallopian tubes and serous tubal intraepithelial carcinomas (Erickson et al., 2013; Reade et al., 2014; Swanson and Bakkum-Gamet, 2016; Tone et al., 2012). One theory about the origin of serous ovarian cancer originates from studies, where salpingo-oophorectomy was performed on BRCA1 or -2 mutation carriers as a prophylactic surgery and furthermore the neoplasia located in the fallopian tubes was monitored (Crum et al., 2007; Medeiros et al., 2006; Piek et al., 2001). According to this theory, the removal of the fallopian tubes and prophylactic salpingooophorectomy is recommended in women with low and high risk for ovarian cancer, especially in women with BRCA1 or -2 gene mutations (Oliver Perez et al., 2015). Salpingooophorectomy has been recommended for women with BRCA1 or -2 gene mutations after the age of 35-40 years; this procedure has been claimed to reduce the risk of ovarian cancer by 80-90% in mutation carriers (Domchek et al., 2010; Finch et al., 2014; Marchetti et al., 2014; Rebbeck et al., 2009). In addition to the cell-related pathway, there is another theory about the origin of ovarian cancer; in this case it is related to the development of the reproductive tract and Müllerian epithelium and Müllerian inclusions (Jarboe et al., 2008). During reproduction and ovulation, there is the formation of Müllerian inclusions and the invagination of the ovarian surface epithelium into the ovarian cortex is thought to be linked to the development of mucinous and low-grade serous neoplasms (Jarboe et al., 2008).

2.1.3 Genetics behind the development of ovarian cancer
The tumor cells in ovarian cancer display numerous mutations. Histological subtypes and the most well known mutations are presented in Table 1. Epithelial ovarian cancers may be classified according to their histological and pathological characteristics into either types I
or II. Type I includes mainly low-grade types of carcinoma with slow growth and histological types of serous, endometrioid, mucinous, clear cell and transitional (Table 1) (Banerjee and Kaye, 2013). High-grade serous, endometrioid, carcinosarcomas and undifferentiated carcinomas are considered as type II tumors; they have an aggressive phenotype and most importantly they seem to be linked with mutations in TP53 and BRCA genes (Kurman and Shih, 2011).

Mutation or methylation of BRCA1 or -2 genes leads to the inactivation of these genes, which in turn, favors cancer development. In Finland, inherited mutations in BRCA1 and -2 are related to the high regional presence of mutations in women living in the Oulu region (Huusko et al., 1998). The P53 tumor suppressor gene is one of the most frequently mutated genes in tumor oncogenesis and mutations in this gene can be detected in 73-96 % of high-grade serous ovarian carcinomas, in contrast, its prevalence is low in low-grade serous carcinomas (Ahmed et al., 2010; O’Neill et al., 2005). P53 mutations are also related to early-stage of lesions in fallopian-tubes in BRCA1 gene mutation carriers (Ahmed et al., 2010; Chmelarova et al., 2013; Lee et al., 2007).

HNPCC, Lynch syndrome has been associated with mutations in both germline and DNA mismatch repair (MMR) -genes (MLH1, MSH2, MSH6, PMS2) (Lynch and de la Chapelle, 2003). Mutation carriers have a 4 to 12 % life-time risk for developing ovarian cancer, and also elevated risks for other cancers i.e. 43-48 % risk for colorectal cancer and 40-62 % risk of developing endometrial cancer (Aarnio et al., 1999).

Table 1. Histological subtypes of epithelial ovarian cancer and the most relevant gene mutations.

<table>
<thead>
<tr>
<th>Type I ovarian cancer</th>
<th>Most relevant mutations</th>
</tr>
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<tbody>
<tr>
<td>serous</td>
<td>BRAF, KRAS, NRAS, ERBB2, PIK3CA, IGF</td>
</tr>
<tr>
<td>endometrioid</td>
<td>ARID1A, CTNNB1, PIK3CA, PTEN, PPP2R1a, MMR deficiency</td>
</tr>
<tr>
<td>mucinous</td>
<td>KRAS, HER2 amplification</td>
</tr>
<tr>
<td>clear cell</td>
<td>ARID1A, PIK3CA, PTEN, CTNNB1, PPP2R1a, ZNF217</td>
</tr>
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<table>
<thead>
<tr>
<th>Type II ovarian cancer</th>
<th>Most relevant mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>serous</td>
<td>TP53, germ-line and tumor BRCA1 and -2, NF1, RB1, CDK12, homologous recombination repair genes; MMR, CHK2, BARD1, BRI1, PALB2, RAD50, RAD51C, ATM, ATR, EMSY, Fanconi anemia genes</td>
</tr>
<tr>
<td>endometrioid</td>
<td>Pathway mutations; PI3K/KRAS/BRAF/NOTCH/FOXM1</td>
</tr>
<tr>
<td>carcinosarcoma</td>
<td>TP53, germ-line BRCA1, BRCA2</td>
</tr>
<tr>
<td>(malignant mixed mesodermal tumors)</td>
<td>TP53, BRCA1, BRCA2</td>
</tr>
<tr>
<td>undifferentiated</td>
<td>TP53, BRCA1, BRCA2</td>
</tr>
</tbody>
</table>

According to (Banerjee and Kaye, 2013; Kurman and Shih, 2011; Ledermann et al., 2016; Seo et al., 2016).

2.1.4 Clinical aspects of ovarian cancer
In ovarian cancer, the level of plasma glycoprotein biomarker, cancer antigen 12-5 (CA12-5), after the first chemotherapy can be used as a prognostic factor for overall survival and progression-free survival of ovarian cancer (Lee et al., 2016). Despite the good sensitivity and high specificity of CA12-5, 10-20 % of patients with advanced disease and 50 % with early stage ovarian cancer have normal CA12-5 plasma levels and its levels may be elevated as a result of other gynecological diseases, endometriosis and other cancers (Nowak et al.,
In clinical use, human epididymis protein 4 (HE4) is another biomarker proposed as being useful for evaluating the tumor malignancy (Hamed et al., 2013). HE4 is over-expressed in endometrioid and epithelial ovarian cancer cells, furthermore it has been utilized in cancer diagnosis in women with endometriosis and claimed to display higher accuracy than CA12-5 (Anastasi et al., 2013; McKinnon et al., 2015). It may be possible to enhance the accuracy of the ovarian cancer diagnosis and the evaluation of cancer malignancy in the clinic by combining both CA12-5 and HE4 biomarkers (Freydanck et al., 2012; Hamed et al., 2013).

One major challenge to diagnosing ovarian cancer is the asymptomatic nature of the early disease. By the time that the first symptoms are detected and a diagnosis can be made, widely disseminated carcinosis and the accumulation of ascites fluid are often present, complicating the response to therapy (Chen et al., 2003). The most common symptoms and clinical findings in ovarian cancer are presented in Table 2. In ovarian cancer, differential diagnosis predictive measurements, Risk of Malignancy Index (RMI) with analysed CA12-5 levels from plasma may be exploited in the differential diagnosis and when evaluating the malignancy in addition to menopause status and findings from an ultrasound scan (Tingulstad et al., 1996). The Risk of Malignancy Algorithm (ROMA) criteria are focused on evaluating the risk for ovarian cancer, although their relevance in clinical use is less than CA125 and HE4 (Montagnana et al., 2011; Moore et al., 2010, 2009).

Depending on the dissemination of ovarian cancer, based on surgical findings and radiological imaging, the staging of the disease is performed by the FIGO system, developed by the International Federation of Gynecology and Obstetrics (Heintz et al., 2006) (Table 3).

**Table 2.** Typical symptoms and clinical findings related to ovarian cancer and its diagnosis¹.

<table>
<thead>
<tr>
<th>Symptom or clinical finding</th>
<th>Early stage/Late stage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal tumor</td>
<td>40/ 75</td>
</tr>
<tr>
<td>Ascites accumulation</td>
<td>27/ 24-30</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>17/ 11</td>
</tr>
<tr>
<td>Intestinal and urinary disorders (nausea, constipation)</td>
<td>14/ 24</td>
</tr>
<tr>
<td>Gynecological bleeding</td>
<td>12/ 12</td>
</tr>
<tr>
<td>Fever</td>
<td>4/ 15</td>
</tr>
<tr>
<td>Dyspnea or backache</td>
<td>2/ 8</td>
</tr>
<tr>
<td>Weight loss</td>
<td>7/ ?</td>
</tr>
<tr>
<td>Fatigue</td>
<td>unknown</td>
</tr>
</tbody>
</table>

¹According to (Chan et al., 2003; Flam et al., 1988).
Table 3. FIGO staging of the ovarian carcinoma (Rio de Janeiro 1988) and relative 5-year survival of patients with ovarian cancer according to FIGO stage (Heintz et al., 2006; Prat, 2014; Ries LAG et al., 2007).

<table>
<thead>
<tr>
<th>FIGO stage</th>
<th>Distribution of tumor growth</th>
<th>Relative 5-year survival rate (%) according to FIGO stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>Limited to ovaries</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Growth in one ovary and the capsule of ovary is intact, no tumor growth on ovarian surface. No malignant cells in ascites or rinse water.</td>
<td>89</td>
</tr>
<tr>
<td>IB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Growth in both ovaries and the capsule of ovary is intact, no tumor growth on ovarian surface. No malignant cells in ascites or rinse water.</td>
<td>94</td>
</tr>
<tr>
<td>IC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor growth in both ovaries and the capsule is ruptured, tumor growth in ovarian surface or malignant cells present in ascites fluid or in rinse water.</td>
<td>91</td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>In one or both ovaries with pelvic tumors.</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Diffused growth in uterus and/or fallopian tubes. No malignant cells in ascites fluid or in rinse water.</td>
<td>76</td>
</tr>
<tr>
<td>IIB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diffused growth to other pelvic tissues. No malignant cells in ascites fluid or in rinse water.</td>
<td>67</td>
</tr>
<tr>
<td>IIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disseminated growth in other pelvic tissues and malignant cells present in ascites fluid or in rinse water.</td>
<td>57</td>
</tr>
<tr>
<td>Stage III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>In one or both ovaries with peritoneal dissemination outside the pelvic tumors and/or retropertitoneal or inguinal lymph nodes. Metastases on the surface of liver. Tumor limited to pelvis with histologically proven metastases to small bowel or omentum.</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Diffused growth in uterus and/or fallopian tubes. No malignant cells in ascites fluid or in rinse water.</td>
<td>45</td>
</tr>
<tr>
<td>IIIB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor growth in one or both ovaries, histologically confirmed implants, peritoneal metastasis, size of peritoneally disseminated metastases &lt; 2 cm. No metastases in lymph nodes.</td>
<td>39</td>
</tr>
<tr>
<td>IIIIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peritoneal metastases sized &gt; 2 cm or tumor in retroperitoneal or inguinal lymph nodes.</td>
<td>35</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Tumor in one or both ovaries. Distant metastases outside the peritoneal cavity. Malignant cells in the pleural fluid. Metastases in liver parenchyma.</td>
<td>18</td>
</tr>
</tbody>
</table>

2.1.5 Current therapies
The general guideline for advanced ovarian cancer is debulking surgery, which includes resection of the tumor, resection of ovaries, fallopian tubes, uterus, omentum and lymph nodes as well as taking tissue biopsies. In addition resection of appendix is often conducted in mucinous ovarian cancer (Chen and Lee, 1983; Pomel et al., 2007). When limited to ovaries, resection of adnexa or tumor with staging is sufficient, especially when trying to preserve the fertility of young women (Cadron et al., 2007; Palomba et al., 2010; Song et al., 2011). Most importantly, the amount of residual tumor after the surgery is related to ovarian cancer prognosis in addition to histological type, FIGO-staging, and performance and age of the patient (Brun et al., 2000; Friedlander, 1998; Voest et al., 1989). For patients
with high-grade epithelial ovarian cancer, the combination therapy of carboplatin and paclitaxel is the first-choice treatment (Bookman et al., 2009; du Bois et al., 2003; McGuire et al., 1996).

Even when surgery and paclitaxel-carboplatin chemotherapy have been conducted, there can be a relapse of ovarian cancer within 3 years in approximately 70 % of the patients (Ledermann et al., 2013a). The prognosis and response to second-line therapy in relapsed ovarian cancer depend on the progression-free time after the preceding chemotherapy. Platinum-refractory cancer progress during the therapy or within 4 weeks after the last chemotherapy, and similarly the growth of platinum-resistant cancer within 6 months of platinum-therapy are related to poor prognosis with an expected overall survival of less than 12 months. Partially platinum-sensitive cancer tends to progress between 6 to 12 months (Friedlander et al., 2011). In platinum-resistant disease, single-agent therapy with paclitaxel, topotecan, pegylated doxorubicin or gemcitabine has been related to an improved overall response rate and median progression-free survival of 3 to 4 months in phase III trials (Ledermann et al., 2013b; Pignata et al., 2011; Vasey et al., 2004). In patients with platinum-sensitive cancer but subsequently relapsed disease appearing between 6 to 12 months later, carboplatin and combined therapy with paclitaxel, gemcitabine or anthracycline has been associated with improved progression-free survival (Parmar et al., 2003).

The monoclonal antibody, bevacizumab (Avastin®) has been recommended for the treatment of patients with stage IIIC-IV epithelial ovarian, fallopian tube or peritoneal serose papillary cancer and suboptimal surgery and poor prognosis expected if they have been treated only with chemotherapy. Bevacizumab dosed with paclitaxel and carboplatin chemotherapy, and subsequent bevacizumab maintenance therapy has been shown to improve the progression-free survival from two to four months (Burger et al., 2011; Perren et al., 2011).

Patients with BRCA-mutations show a good response to chemotherapy and therefore their prognosis is more optimistic than in patients with other types of ovarian cancers. In their recent report, Kotsopoulos et al. stated that the most important survival-predicting factor was the absence of residual disease after the debulking surgery in both BRCA and non-mutation carriers i.e. this was more important than the presence of certain gene mutations (Kotsopoulos et al., 2016). Although a survival benefit was observed in BRCA-carriers in terms of short term survival, this was not achieved after 10 years’ follow-up i.e. the values were 30 % for non-carriers, 25 % for BRCA1 and 35 % for BRCA2 mutation carriers (Candido-dos-Reis et al., 2015; McLaughlin et al., 2013).

2.1.6 Targeted therapies for ovarian cancer

2.1.6.1 Angiogenesis and lymphangiogenesis in tumor formation of ovarian cancer

Angiogenesis refers to the formation of new blood vessels and branching or rearrangement of pre-existing ones. It is a process with a key role in the development and growth of tumors (Karamysheva, 2008; Risau, 1997). Lymphangiogenesis, i.e. the formation of new lymphatic vessels, has an essential role in tumor malignancy and metastasis via the lymphatic system (Alitalo et al., 2005; Kärpänen and Alitalo, 2008). Angio- and lymphangiogenesis are regulated by numerous growth factors and their receptors, most importantly vascular endothelial growth factor (VEGF). The breakthrough finding in angiogenesis and tumor-related studies was the discovery of VEGF-A, also called vascular permeability factor in the 1980’s (Leung et al., 1989; Senger et al., 1983). Angiogenesis takes place in normal physiological situations, such as embryogenesis, wound healing and the normal menstrual cycle of women in the uterus as well as in pathological conditions, such as the development of cancer, diabetic retinopathy, ischemia and inflammation (Byrne et al., 2005; Carmeliet, 2003; Conway et al., 2001). In angiogenesis, the newly formed
microvessels have loosened endothelial-cell junctions in their endothelium, leading to increased vascular permeability and the migration of endothelial cells into surrounding tissues and degradation of extracellular matrix (Conway et al., 2001). In sprouting angiogenesis, new blood vessels are formed from the stalk cells of existing vessels. Stalk cells further develop into endothelial tip cells in microvessel walls; this process is regulated by a high VEGF gradient and fibroblast growth factors (FGF) (Gerhardt et al., 2003). Newly formed microvessels are leaky with loose cell-junctions, although they later become stabilized by pericytes and smooth muscle cells, covering the endothelial cells. Endothelial cells produce the platelet-derived growth factor-B (PDGF-B), which increases the recruitment of pericytes to the microvessels. Furthermore, pericytes are able to stabilize the capillary diameter, regulate the proliferation of capillary endothelial cells and their cellular structure (Gerhardt and Betsholtz, 2003; Hellström et al., 2001). The relevance of VEGF-A and PDGF in promoting the growth of tumor microvessels has been demonstrated in several studies, while their inhibition with antiangiogenic inhibitors reduced the tumor vascularity and pericyte coverage (Sennino et al., 2009).

In malignant tumors, disorganized angiogenesis originates from the unbalanced production of pro- and antiangiogenic growth factors, and the insufficient presence of growth factor receptors in microvessel endothelium (Carmeliet and Jain, 2011; Maj et al., 2016; Zhao and Adjei, 2015). Liao et al. demonstrated that high levels of luteinizing hormone increased angiogenesis and promoted ovarian cancer development by activating the PI3K/AKT-mTOR-signaling pathway (Liao et al., 2012). A continuous source of oxygen and good energy supplies regulate tumor growth, driven by the presence of tumoral blood vessels.

In pathological situations, angiogenesis is promoted by hypoxia and increased production of hypoxia-inducible transcription factor HIF-1α (Pugh and Ratcliffe, 2003). HIF-1α is the main factor in oxygen homeostasis of tissues; this protein regulates the expression of genes coding for angiogenic, metabolic and erythropoietic factors. The production of VEGF-A is the main angiogenic growth factor regulated by HIF-1α, activated as a result of decreased tumor microvasculature and further by elevated tissue hypoxia (Forsythe et al., 1996; Hirota and Semenza, 2006). High levels of follicle stimulating hormone (FSH) have been related to increased HIF-1α and VEGF expression and further to increased angiogenesis in ovarian cancer female rats (Alam et al., 2004).

2.1.6.1.1 Vascular endothelial growth factors

The role of VEGF and VEGF-receptors in angiogenesis and lymphangiogenesis has been studied widely since they have a crucial role in the modulation of blood vessel permeability, remodeling of vessels, regulating endothelial cell survival, and the proliferation and migration of cells in the formation of new vessels (Ferrara et al., 2003; Hicklin and Ellis, 2005). VEGFs are dimeric glycoproteins of approximately 40 kDa. The VEGF-family consists of VEGF-A, -B, -C, -D and placental growth factors -1 and -2 (PIGF) (Figure 1). Structurally, VEGF proteins are related to VEGF in parapoxvirus (VEGF-E) and the snake venom VEGF-F–group of proteins (Suto et al., 2005; Takahashi and Shibuya, 2005).

VEGF-A (also called VEGF) is the best known member of the VEGF family, being the first of the VEGF-family to be cloned in 1989 (Ferrara and Henzel, 1989; Leung et al., 1989). VEGF-A binds to two receptors, VEGFR1 and VEGFR2. VEGF-A has also been called vascular permeability factor (VPF) since it increases vascular permeability, and it further induces endothelial cell migration and promotes endothelial cell survival and lymphangiogenesis (Shibuya and Claesson-Welsh, 2006). VEGF is also produced by the foetus and is crucial in embryonic development (Ferrara et al., 2003). In ovarian cancer as well as in many other cancers, the expression of VEGF-A in tumor cells is elevated and the extent of this up-regulation has been associated with increased angiogenesis (Gutman et al.,
The expression of VEGF-A can be induced by many stressors e.g. induced by hypoxia, tissue damage and inflammation (Takahashi and Shibuya, 2005). Several isoforms of VEGFs with variable numbers of amino acids have been detected: VEGF121, VEGF145, VEGF162, VEGF165, VEGF183, VEGF189 and VEGF206 (Ferrara et al., 2003; Houck et al., 1991). The most important and the forms that are mostly secreted are VEGF121, VEGF165 and VEGF189 (Robinson and Stringer, 2001) VEGF165 is the most important isoform with the highest expression level and greatest biological activity in comparison to the other forms. VEGF165 over-expression has been detected in various human tumors with the extent being correlated with cancer progression, invasion and metastasis (Kim et al., 1992).

VEGF-B is expressed in muscle, myocardium and brown fat and is involved in inflammatory angiogenesis (Mould et al., 2003). It is produced in mouse and humans as two isoforms, VEGF-B162 and VEGF-B186. VEGF-B binds to VEGFR1 and NRPI and furthermore, it may form heterodimers with VEGF-A (Olofsson et al., 1999). The highest expression of VEGF-B is present in heart in adult mice, but it is also present in skeletal muscles and diaphragm. VEGF-B expression has been linked with various tumors, such as adenocarcinomas, breast cancer, lymphoma and melanoma; its expression is also thought to be upregulated in ovarian cancer (Nash et al., 2006; Sowter et al., 1997).

VEGF-C and VEGF-D are produced by proteolytic processing from precursors, which allows their binding to VEGFR2, albeit with rather low affinity. VEGF-C induces endothelial cell mitogenesis, migration and endothelial cell survival and is crucial in embryonic lymphangiogenesis (Karkkainen et al., 2004; Lymboussaki et al., 1999). VEGF-C binds to the VEGFR3 receptor and regulates lymphangiogenesis (Veikkola et al., 2001). Angiogenesis is regulated by VEGF-C through VEGFR2 signaling. In clinical cancer studies, an elevated VEGF-C expression has been related to lymphatic invasion, metastasis and poor survival of patients (He et al., 2002).

VEGF-D activates two receptors, VEGFR2 and VEGFR3, and has a role in endothelial cells as an angiogenic and lymphangiogenic growth factor. In mice, VEGF-D binds only to VEGFR3 which indicates that it possesses a different role in mice (Baldwin et al., 2001). During embryogenesis, VEGF-D is expressed in humans in lungs and skin. In cancer, VEGF-D has been linked to increased growth of lymphatic vessels and metastases through lymphatic system (He et al., 2004).

Placental growth factor (PlGF), a homolog to VEGF is mainly expressed in placenta, during the embryogenesis in the heart and lungs and is mainly upregulated in pathological conditions (De Falco, 2012). In cancer, increased PlGF levels have been related to cancer malignancy and poor prognosis in colorectal and breast cancer and although there are dissenting voices, PlGF has been claimed to be a proangiogenic factor (Carmeliet and Jain, 2011; Maae et al., 2012; Wei et al., 2005). PlGF stimulates the revascularization of ischemic tissue and was demonstrated to enhance the VEGF-related angiogenesis after ischemia (Autiero et al., 2003; Carmeliet et al., 2001a). In ischemic heart and limb, adenoviral-mediated PlGF achieved a strong angiogenic effect and increased the number of vessels including larger vessels (Luttmun et al., 2002). PlGFs bind to cell surface heparin sulfate proteoglycan (HSPG) and act as a ligand for VEGFR1. PlGFs have been shown to stimulate the angiogenesis, and maturation and survival of endothelial cells by increasing the proliferation of smooth-muscle cells and fibroblasts (Carmeliet et al., 2001b; Kim et al., 2012).

2.1.6.1.2 VEGF-receptors
Vascular endothelial growth factor receptors (VEGFR) as well as receptors for platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) belong to the same subgroup of endothelial growth factors. VEGFs can be thought of as ligands that bind to their VEGF-receptors, leading to receptor dimerization and activation of intracellular signaling pathways, for example in endothelial cells of newly formed microvessels during
angiogenesis and lymphangiogenesis (Olsson et al., 2006) (Figure 1). The receptors’ intrinsic signaling pathways are distinct, despite their similar intrinsic tyrosine-kinase domains. Signaling through VEGFR1 in microvessel endothelium is related to activation of the phospholipase C-γ (PLC-γ), Src homology phospholipase-2 (SRC-2) and growth-factor-bound protein (Grb2) signaling proteins. In contrast, VEGFR2 signaling acts through activated intrinsic PLC-γ and mitogen-activated protein kinase (MAPK) signaling, which leads to increased DNA synthesis in endothelial cells. In addition, an activation of phosphatidylinositol 3-kinase (PI3K) has been related to increased cell migration in microvessels (Holmqvist et al., 2004; Shibuya and Claesson-Welsh, 2006).

VEGFR1 (Flt-1) is a tyrosine-kinase receptor with seven extracellular immunoglobulin (Ig) domains; it has a transmembrane region and an intracellular tyrosine-kinase domain (Shibuya et al., 1990). VEGFR1 binds VEGF, VEGF-B and PIGF and has a role in angiogenesis in vessel endothelium, its expression is increased as a result of tissue hypoxia (Ferrara et al., 2003). VEGFR1 is also expressed in osteoblasts, monocytes and macrophages, pericytes, placental trophoblasts and in hematopoietic stem cells and it is involved in the recruitment of bone-marrow derived progenitor cells (Zachary and Gliki, 2001). On its own, VEGFR1 has only a weak mitogenic activity in endothelial cells, but it can form heterodimers with VEGFR2, leading to higher signaling efficacy than either receptor alone (Huang et al., 2001).

VEGFR2 (KDR/Flk-1) is a tyrosine-kinase receptor with a rather similar structure to VEGFR1 (Terman et al., 1992). VEGFR2 binds to VEGF-A, VEGF-C and VEGF-D and has a major role in VEGF-A signaling in endothelial cells in angiogenesis, although it has lower binding affinity to VEGF-A in comparison to VEGFR1 (Gille et al., 2001). VEGFR2 regulates microvascular permeability as well as controlling the proliferation of endothelial cells, migration of endothelial cells in new microvessels and invasion of angiogenesis. Furthermore, it is expressed in neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells (Matsumoto and Claesson-Welsh, 2001). In normal, quiescent blood vessel endothelial cells, VEGFR2 expression is downregulated but it becomes activated in angiogenic vessels. The soluble form of VEGFR2 is also believed to occur naturally in both mice and humans (Ebos et al., 2004). It is highly expressed in early embryogenesis and vascular endothelial progenitor cells. VEGFR2 deficient mice fail to develop blood vessels, leading to embryonic fatality, highlighting its crucial role in the development of the blood-circulatory system (Shalaby et al., 1995).

Increased VEGFR2 expression in the ovaries has been related to ovarian cancer, whereas VEGFR2 is not expressed in normal ovaries (Klasa-Mazurkiewicz et al., 2011; Spannuth et al., 2009). Additionally, VEGFR2 levels have been shown to be relatively high in ovarian malignancies, evidence of the pathogenetic role of this receptor in the formation of ovarian tumors. Increased VEGFR1 expression has not been related to the progression of ovarian tumors (Spannuth et al. 2009). In anti-cancer therapies, VEGF-A and VEGFR2 have proved to be the most important targets, as their expression is highly related to cancer progression and they are known to take part in physiological and pathological events in newly formed vascular endothelial cells (Takahashi, 2011).

VEGFR3 (Flt4) consists of six extracellular Ig-domains with the fifth Ig-domain being proteolytically cleaved, leaving a disulfide bond between polypeptide chains (Pajusola et al., 1994). VEGFR3 binds both VEGF-C and VEGF-D and it is present during development. In adults, its expression is restricted to lymphatic endothelial cells and fenestrated blood vascular endothelial cells (Achen et al., 1998; Joukov et al., 1996). VEGFR3 is expressed in angiogenic sprouts and tumor microvessels and furthermore, it regulates the vascular density, blood vessel branching, and proliferation of endothelial cells. During embryonal development, VEGFR3 is present in blood vessel endothelium cells, and later in the lymphatic endothelium in adults (Tammela et al., 2008). Under pathological conditions, VEGFR3 is up regulated in blood vascular endothelial cells, in solid and vascular tumors and is related to increased lymphatic metastasis in cancers (Stacker et al., 2001).
2.1.6.1.3 Neuropilins

Neuropilins (NRP) -1 and -2 are 130-140 kDa transmembrane non-tyrosine kinase glycoproteins, first identified as being specific in the developing nervous system (Chen et al., 1997; Takagi et al., 1987). NRPs are VEGF-coreceptors, which are known to increase the ligand-binding affinity of VEGFs to VEGF-receptors (Soker et al., 2002, 1998). NRP1 is related to arterial angiogenesis, while NRP2 is mainly expressed in endothelial cells of lymphatic vessels and venules (Kitsukawa et al., 1995; Yuan et al., 2002). NRP1 binds to the VEGF165 isoform, placental growth factor-2 (PIGF-2) (Migdal et al., 1998), VEGF-B (Mäkinen et al., 1999), VEGF-C, VEGF-D (Kärpänen et al., 2006) and VEGF-E. NRP1 is considered to function as a VEGFR2 coreceptor. Blockade of NRP1 signaling has been related to inhibited VEGFR signaling and may be useful in anti-VEGF therapies (Pan et al., 2007). Recently, NRP1 has been shown to promote angiogenesis through the NRP1-ABL and PDGF(R) -pathway in addition to the VEGF-VEGFR2 pathway. This newly discovered signaling pathway activates RAD51 protein, which has been associated with radio- and chemoresistance of cells, although it was recently reported that the benefits of NRP1 inhibition in cancer therapy may be limited (Hu and Jiang, 2016).

A high expression of NRP1 has been related to a poor prognosis of human malignant glioma and over-expression of NRP1 was also detected in human colon adenocarcinoma (Hu et al., 2007; Osada et al., 2004; Parikh et al., 2004). In ovarian cancer, an increased expression of NRP1 has been related to cancer development and malignancy, as well as curtailed survival of patients (Baba et al., 2007; Jiang et al., 2015). The role of NRP2 in ovarian cancer is poorly understood. Caunt et al. stated that an inhibition of NRP2 reduced tumoral lymphangiogenesis and metastasis via lymphatic vessels in a mouse model (Caunt et al., 2008). Increased expression of NRP1 and NRP2 was linked to increased vascularity and development of cancer in patients with small cell lung carcinoma (Kawakami et al., 2002). The soluble form of neuropilins lack their transmembrane and intracellular domains, although they do possess the extracellular domains that bind to ligands and c-domains. It has been postulated that the soluble forms of neuropilins act as antagonists in VEGF-signaling and may increase apoptosis of tumor cells (Gagnon et al., 2000).
Figure 1. Description of VEGFs, VEGF-receptors and their NRP-coreceptors and Tie-receptors. VEGFRs consist of seven immunoglobulin-like (Ig) domains and an intracellular tyrosine-kinase component. In VEGFR3, between the fourth and sixth Ig-domain, there is a disulfide-bond (S-S) attached. NRPs consist of four extracellular domains and a transmembrane protein. In the angiopoietin/Tie-signaling pathway, the extracellular domains of Tie-receptors consist of two Ig-like domains and followed by three epidermal growth factor (EGF)-like repeats and three fibronectin-like repeats (Huang et al., 2010; Nakamura and Goshima, 2002; Shibuya and Claesson-Welsh, 2006).

2.1.6.1.4 Anti-VEGF trials in ovarian cancer

Bevacizumab (Avastin®) is a humanized anti-VEGF-A monoclonal antibody binding to all isoforms of VEGFs, and it has exerted a beneficial effect in treatment of ovarian cancer patients with primary and recurrent disease (Ferrara et al., 2005). Bevacizumab has been approved by U.S. Food and Drug Administration (FDA) for the treatment of platinum-resistant ovarian carcinoma (Robert A Burger et al., 2011; Perren et al., 2011) and recurrent or advanced cervical cancer (Tewari et al., 2014). Furthermore, bevacizumab has been approved in combination with chemotherapy for the treatment of metastatic colorectal cancer (Hurwitz et al., 2004) and also for recurrent or advanced non-small cell lung cancer (Sandler et al., 2006), glioblastoma (Cohen et al., 2009) and metastatic renal cell carcinoma (Summers et al., 2010). In Europe, bevacizumab has been approved by European Medicines Agency (EMA) for the first-line treatment of ovarian cancer (Burger et al., 2011, Oza et al., 2015; Perren et al., 2011), platinum-sensitive ovarian cancer (Aghajanian et al., 2015, 2012) and platinum-resistant recurrent ovarian cancer (Pujade-Lauraine et al., 2014; Stockler et al., 2014). Furthermore it has received approval by EMA for the treatment of recurrent or advanced cervical cancer (Penson et al., 2015).

The main results from phase III trials in ovarian cancer are presented in Table 4. Overall survival was not improved by bevacizumab, but the progression-free survival was significantly improved from 2 to 4 months. There were some important adverse events
related to bevacizumab i.e. hypertension, gastrointestinal-wall disruption, bleeding, thromboembolic events, thrombocytopenia and proteinuria (Aghajanian et al., 2015a; Burger et al., 2011; Oza et al., 2015a; Perren et al., 2011; Pujade-Lauraine et al., 2014a).

Advanced ovarian cancer is characterized by the formation of malignant intraperitoneal ascites (Shen-Gunther and Mannel, 2002). Ascites is defined as an accumulation of fluid in the abdominal cavity, containing also cancer cells (Barni et al., 2011). In the treatment of malignant ascites, traditional methods such as restricted use of salt, diuretics, radioactive isotopes, paracentesis and shunt placement are used, although their efficacy is limited (Eskander and Tewari, 2012). The tri-functional antibody to epithelial cell adhesion molecule and cluster of differentiation 3, catumaxomab, reduced significantly the accumulation of ascites and is being assessed in Europe as therapy for malignant ascites (Burges et al., 2007; Heiss et al., 2010). Since VEGF has been related to increased vascular permeability, it also has a role in ascites formation. Blockade of VEGF-signaling has been related to an inhibition of ascites formation and further, intraperitoneally administered bevacizumab has been claimed to reduce the formation of peritoneal ascites and its efficacy has been reported in single patients with ovarian cancer (El-Shami et al., 2007; Kobold et al., 2009; Numnum et al., 2006). Aflibercept (VEGF Trap), which targets VEGFR1 and VEGFR2 and inhibits the signaling of VEGF and PI GF, has also been evaluated in the treatment of malignant ascites. In preclinical studies conducted in ovarian cancer mouse models, the i.p. administration of aflibercept and paclitaxel significantly inhibited the formation of ascites (Hu et al., 2005) as well as only single-dosed aflibercept (Byrne et al., 2003). In phase II trials, aflibercept has also been studied in malignant ascites in ovarian cancer patients. Colombo et al. examined the efficacy of i.v. aflibercept as a treatment of malignant ascites every two weeks with 16 patients, resulting in a 4.5 times longer duration between the time needed for paracentesis than at baseline (Colombo et al., 2012). In another phase II clinical trial in 29 ovarian cancer patients with malignant ascites, the i.v. administration of aflibercept dosed every two weeks achieved a significantly longer period between repeated paracentesis as compared to placebo treated patients. It was noted that there were severe adverse events with aflibercept, i.e. severe hypertension and weight loss and furthermore, three patients of 29 patients suffered a gastrointestinal perforation (Gotlieb et al., 2012). At present, no larger clinical studies for the treatment of malignant ascites have been performed in patients with ovarian cancer.
Table 4. Examples from recent Phase III antiangiogenic trials for ovarian cancer. CP=carboplatin, P=paclitaxel, Bev=bevacizumab

<table>
<thead>
<tr>
<th>Study</th>
<th>Cancer type</th>
<th>Therapy agents</th>
<th>Target</th>
<th>Main results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOG218 (n=1873)</td>
<td>Newly diagnosed OVCA, stage III/IV,</td>
<td>CP, P + placebo vs. CP, P + Bev vs.</td>
<td>VEGF</td>
<td>Improved PFS (10.3 vs. 11.2 vs. 14.1</td>
<td>Burger et al., 2011</td>
</tr>
<tr>
<td></td>
<td>first-line therapy</td>
<td>CP, P + Bev + Bev-maintenance</td>
<td></td>
<td>months)</td>
<td></td>
</tr>
<tr>
<td>ICON7 (n=1528)</td>
<td>High-risk early stage OVCA or advanced</td>
<td>CP, P vs. CP, P + Bev + Bev-maintenance</td>
<td>VEGF</td>
<td>Improved PFS (17.4 vs. 19.8</td>
<td>Oza et al., 2015; Perren</td>
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<td></td>
<td>cancer, first-line therapy</td>
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<td>et al., 2011</td>
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<td>OCEANS (n=484)</td>
<td>Recurrent, platinum-sensitive OVCA,</td>
<td>Gemcitabine, CP+ P or Bev</td>
<td>VEGF</td>
<td>Improved PFS (8.4 vs. 12.4</td>
<td>Aghajanian et al., 2015,</td>
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<td>second-line therapy</td>
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<td>AURELIA (n=361)</td>
<td>Platinum-resistant recurrent OVCA,</td>
<td>Chemotherapy (Pegylated liposomal</td>
<td>VEGF</td>
<td>Improved PFS (3.4 vs. 6.7</td>
<td>Pujade-Lauraine et al.,</td>
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<td>second-line therapy</td>
<td>doxorubicine, paclitaxel or topotecan)</td>
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<td>vs. chemotherapy+ Bev</td>
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<td>TRINOV1 (n=919)</td>
<td>Recurrent OVCA</td>
<td>P + placebo or trebananib</td>
<td>Ang1, Ang2, Tie2</td>
<td>Improved PFS (5.4 vs. 7.2</td>
<td>Monk et al., 2016, 2014a</td>
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<td>ICON 6 (n=486)</td>
<td>Recurrent platinum-sensitive OVCA</td>
<td>Placebo, P + placebo-maintenance vs.</td>
<td>VEGFR1-3</td>
<td>Improved PFS (8.7 vs. 9.9</td>
<td>Jonathan A Ledermann et</td>
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<td>Cediranib, P + placebo-maintenance vs.</td>
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<td>vs. 11.0 months)</td>
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<td>Cediranib, P + cediranib-maintenance</td>
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<td>AGO-OVAR 16 (n=940)</td>
<td>Newly diagnosed advanced OVCA, adjuvant therapy</td>
<td>Pazopanib or placebo up to 24 months</td>
<td>VEGFR1-3, PDGFR, c-Kit</td>
<td>Improved PFS (17.9 vs. 12.3</td>
<td>Du Bois et al., 2014; Floquet et al., 2015</td>
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<td>AGO-OVAR 12 (n=1366)</td>
<td>Newly diagnosed advanced OVCA</td>
<td>C, P + Nintedanib or placebo</td>
<td>VEGFR, PDGFR, FGF-receptor</td>
<td>Improved PFS (17.2 vs. 16.6</td>
<td>Du Bois et al., 2016</td>
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2.1.6.1.5 Angiopoietins and their receptors

Angiopoietins and their Tie-receptors participate in many physiological and pathological conditions, they also have a role in the formation of ovarian cancer, cancer angiogenesis and further in tumor progression and malignancy (Hata et al., 2004). The angiopoietin-family consists of Ang1, Ang2, Ang3, Ang4 and their Tie2 (also called Tek) and Tie1 tyrosine-kinase receptors. The roles of Ang3 or Ang4 have not been identified other than that Ang3 seems to be the mouse orthologue of human Ang4 (Lee et al., 2004). Ang4 is upregulated in human glioblastoma multiforme and is thought to play a role in glioma progression (Brunckhorst et al., 2010).

Ang1 is expressed in quiescent normal blood vessels, in mural cells, fibroblasts, non-vascular cells and in tumor cells. Ang1 activates Tie2 and takes part in vascular maturation as well as stabilization of vessel endothelium by Akt signaling cascade, and phosphorylates the transcription factor of forkhead box O1 (FOXO1) (Brunet et al., 1999). phosphorylation
of FOXO1 is known to inhibit DNA binding and transcription in endothelial cells of microvessels, and further to induce the expression of genes needed for vessel stabilization and inhibition of Ang2 (Daly et al., 2004; Zhang et al., 2002).

Ang2 is produced by endothelial cells in the presence of an inflammatory stimulus from TNFα or other cytokines, such as VEGFA, IGF-1 and PDGF-B (Fiedler et al., 2006; Phelps et al., 2006). Ang2 inhibits competitively the signaling of Ang1 via the Tie2-receptor and suppresses the Tie2 signaling, leading to enhanced vascular remodeling (Benest et al., 2013; Maisonpierre et al., 1997). Ang2 has some relevance as an angiogenic factor as its production is controlled by hypoxia and upregulation of TNFα (Kim et al., 2000; Koga et al., 2001). Ang2 regulates the growth of new blood vessels in pathological situations, paradoxically it may either over-activate or inactivate Tie2 in vessel remodeling (Kim et al., 2016). In addition to angiogenic signaling, Ang2 has been implicated in the development of lymphatic vessels and clinical symptoms in sepsis (Ziegler et al., 2013). Ang2 is also claimed to prevent vascular leakage of vessels by preserving the organization of the endothelial cells (Daly et al., 2006). In their study, Daly et al. claimed that the Ang2-Tie2 interaction and its protective role in endothelial cells could inhibit the antivascular effect of anti-VEGF therapies (Daly et al., 2013). Serous human ovarian cancers express Ang2 and Ang4 and their receptor Tie2, furthermore they are related to the promoted growth of intraperitoneal ovarian cancer and shortened survival in preclinical and clinical studies (Brunckhorst et al., 2014).

Tie1 is an orphan receptor without natural identified ligands (Jeltsch et al., 2013). It has a role in tumor angiogenesis, as Tie1 deletion inhibited angiogenesis and tumor growth (D’Amico et al., 2014). Tie1 is also expressed in endothelial cells of adult organs such as heart, kidneys and lungs (Korhonen et al., 1992). Tie1 regulates the Ang2-Tie2 signaling; furthermore, it regulates the presence of Tie2 receptors in endothelial cells of microvessels. In addition, binding of Ang1 or Ang2 to Tie2-receptor increases the interaction between Tie1 and Tie2 receptors (Korhonen et al., 2016; Savant et al., 2015). It has been stated that vascular remodeling and angiogenesis via angiopoietins is regulated by both Tie2 overactivation and inactivation (Boscolo et al., 2015; Bureau et al., 2006; Vikkula et al., 1996). Tie2 plays an important role in gestational angiogenesis and cardiovascular development (Puri et al., 1999).

### 2.1.6.1.6 Trials targeting angiopoietin inhibition

The angiopoietin-related neovascular pathways have been mostly studied in cancer research and the angiopoietin/Tie2-pathway also has a role in tumor neovascularization (Huang et al., 2010). High Ang2 levels in serum have been related to poor survival and high malignancy in ovarian cancer patients (Sallinen et al., 2014, 2010), also the high expression of VEGF and Ang1/Ang2 has been related to ovarian cancer and malignancy in humans (K Hata et al., 2004; Yamamoto et al., 1997). Elevated Ang2 levels have been linked with a more rapid progression of ovarian cancer and invasion and they promote the cancer-related fibroblast proliferation in the tumor microenvironment (Brunckhorst et al. 2014).

The antiangiogenic molecule AMG 386 (Trebananib), targeting Ang1/Ang2 and Tie2-receptors, is currently undergoing clinical phase III studies in patients with ovarian cancer. In TRINOVA-1 which was a trial in patients with recurrent ovarian cancer receiving Trebananib, the drug prolonged progression-free survival of ovarian cancer patients when combined with weekly paclitaxel (Monk et al., 2014b) (Table 2). An overall survival benefit was reached in the TRINOVA-1 trial with trebananib in patients with ascites at baseline (Monk et al., 2016). The adverse events associated with trebananib were a higher incidence of oedema, ascites, neutropenia and abdominal pain. In the trebananib treated group, 34 % of patients and in placebo group 28 % of patients experienced serious adverse events (Monk et al., 2014b). At the moment, two phase III clinical trials with trebananib are ongoing; in the TRINOVA-2 study, Trebananib and pegylated liposomal doxorubicin (PLD) are being
administered to women with recurrent, partially platinum sensitive or resistant epithelial ovarian, primary peritoneal or fallopian tube cancer (identifier in clinical trials.gov NCT01281254). The other currently ongoing phase III study is TRINOVA-3 with Trebananib and carboplatin and paclitaxel in patients with recently diagnosed epithelial ovarian, primary peritoneal or fallopian tube cancer (identifier in clinical trials.gov NCT01493505) (Marchetti et al., 2015).

Currently, no clinical trials targeting both VEGF and Ang/Tie2 pathways in ovarian cancer have been published. A few preclinical cancer studies have been reported targeting both the VEGF/VEGFR and Ang/Tie signaling pathways. The interaction between VEGFR1 and Tie2 signaling in tumor growth was first evaluated in a human melanoma xenograft where it was found that these represented two independent angiogenic pathways. It was observed that these receptors were essential for tumor growth and angiogenesis. In addition, the relevance of the interaction between VEGF and Tie1 or VEGFR3 signaling was assumed to be nonsignificant with respect to the growth of melanoma (Siemeister et al., 1999). In a non-small cell lung cancer xenograft, an anti-tumoral agent, tubeimoside-1, stimulated the proteosomal degradation of VEGFR2 and Tie2 in endothelial cells and inhibited angiogenic signaling (Gu et al., 2015). Altiratinib, which inhibits the signaling of MET, Tie2 and VEGFR2, was mainly designed for the treatment of brain cancers and brain metastases as a way of inhibiting tumor initiation, progression and drug resistance (Smith et al., 2015). The role of Ang2-VEGFR2 signaling in pancreatic neuroendocrine tumors and mammary adenocarcinomas and the importance of Ang2-Tie signaling in tumor resistance to anti-VEGFA therapy have also been studied (Rigamonti et al., 2014). Tumor growth and angiogenesis were inhibited in a human melanoma xenograft model with subcutaneous tumors when VEGFR2 and Tie2 signaling were inhibited (Jendreyko et al., 2005).

2.1.6.2 Biomarkers in antiangiogenic therapy for ovarian cancer

Cancer antigen 12-5 (CA12-5) is a biomarker which has proved to be useful not only in epithelial ovarian cancer diagnosis but also for monitoring the efficacy of therapy in the clinic (Lee et al., 2016; Sölétormos et al., 2016). Unfortunately, the link between CA12-5 and tumor size is not sufficiently reliable to allow the evaluation of all therapy responses in ovarian cancer patients. Although many biomarkers for monitoring ovarian cancer progression, survival and malignancy have been postulated, it does not seem as if they are beneficial for assessing the effects of antiangiogenic therapies (Secord et al., 2014; Trachana et al., 2016). The relevance of circulating biomarkers for predicting anti-vascular therapy effects has been poor, as the expression of biomarkers varies extensively between patients and the biomarkers also exhibit low sensitivity (Duncan et al., 2008).

It has been proposed that the amount of malignant ascites could be used as an independent prognostic factor and clinical biomarker for antiangiogenic therapy, i.e. it could also serve as a surrogate factor in evaluating which patients would benefit from antiangiogenic therapy (Ferriss et al., 2015). Hypertension is related to VEGF, as when this VEGF binds to VEGFR-2, this upregulates the production of nitric oxide and leads to local vasodilatation and reduced blood pressure (Facemire et al., 2009). In studies with bevacizumab, one of the main side-effects has been severe hypertension, reflecting the blockade of these cardiovascular effects of VEGF, and thus hypertension may offer a useful way of monitoring the effects of antiangiogenic therapy in the clinic (Maitland et al., 2010; Mir et al., 2011; Mourad et al., 2008).

Levels of VEGF, FGF, PIGF, Ang2 and VEGFR2 in plasma have been claimed as being the most relevant biomarkers when predicting the patient outcomes (Hepler et al., 2006; Li et al., 2004; Sallinen et al., 2010; Wei et al., 2005). Plasma VEGF levels have been shown to reflect the response to anti-VEGF therapy, as high VEGF-levels seem to correlate with shorter survival (Han et al., 2010a; Lambrechts et al., 2013; Smerdel et al., 2010). In addition, the circulating levels of Ang1 and Tie2 have been evaluated as potential predictive
biomarkers for anti-VEGF therapies in ovarian cancer (Backen et al., 2014). Recently, the potential of plasma Tie2 and CA12-5 levels to function as biomarkers for evaluating the efficacy of anti-VEGF therapy was proposed in conjunction with bevacizumab therapy (Zhou et al., 2016).

Endothelial biomarkers have been studied as potential predictive factors with which to evaluate the antiangiogenic therapy effects in tumor microvessels. In a phase II trial with epithelial ovarian cancer, a high CD31-microvessel density in tumor biopsies was associated with a poor response to bevacizumab therapy and shortened median survival (Han et al., 2010b). In addition, other endothelial markers such as CD105 which was used to evaluate the numbers of proliferating endothelial cells and neoangiogenesis in tumors may be potentially useful in antiangiogenic trials (Rubatt et al., 2009). It has been claimed that the expression levels of the cellular adhesion molecule, thrombospondin-1, in ovarian cancer cells may be an independent prognostic factor of poor progression-free and overall survival in clinical trials and may reflect the response to anti-VEGF therapy, although the results are controversial and need to be studied further (Garcia et al., 2008; Secord et al., 2007).

In preclinical studies in ovarian cancer, changes in the levels of tumor-derived cell-free DNA (cfDNA) in the blood have been postulated to reflect the response of antivascular therapy (Kamat et al., 2006b). High cfDNA levels have been related to advanced ovarian cancer reflecting its potential as a surrogate marker for clinical use (Kamat et al., 2006a). High levels of circulating endothelial progenitor cells (CEPCs) have also been related to ovarian cancer; in a preclinical study, these levels correlated with efficacy of antivascular therapy (Kamat et al., 2007). Recently in a clinical ovarian cancer phase II trial examining the efficacy of the PARP-inhibitor olaparib (Lynparza®) combined with the VEGFR1-3 inhibitor cediranib, the levels of circulating endothelial cells (CECs) and IL-8 were found to predict the therapy response and prognosis (Lee et al., 2015).

Microarray studies have been performed to profile the ovarian tumor gene expression, and also angiogenesis-related genes have been studied (Beck et al., 2013). In high-grade ovarian cancer, two different subtypes with different survival outcomes have been identified. Longer survival was related to high expressions of AKT1 and CD44 genes, and furthermore a high expression of EPHB2, ERBB2, FLT1, PF4, NRPI, COL4A3 and ANGPTL3 associated with longer survival (Siamakpour-Rehani et al., 2015). A microarray study with specimens from patients with high-grade serous ovarian cancer showed that angiogenic gene upregulation and immune gene upregulation was related to the response to bevacizumab therapy (Gourley et al., 2014).

The predictive value of tumor imaging methods has also been assessed; dynamic-contrast enhanced-MRI (DCE-MRI) was claimed to be a potential biomarker for predicting the altered vascular permeability and the changed blood flow as well as the blood volume in tumors. DCE-MRI has been evaluated in the assessment of antiangiogenic therapy responses and changes in tumor perfusion. In a phase II study with epithelial ovarian cancer, DCE-MRI showed potential for revealing the relative blood flow in tumors and progression-free survival after bevacizumab therapy (Chase et al., 2012a). Tumor T1 relaxation time has been recently evaluated as a potential predictive marker for detecting the therapeutic response to bevacizumab in a mouse model of ovarian cancer. In that study, bevacizumab inhibited the tumor growth and an increased T1 relaxation time was observed at both 2 days and 2 weeks after the therapy (Ravoori et al., 2015). Positron-emission tomography (PET) imaging has been also used to assess the changes in tumor metabolic activity and hypoxia as a result of antiangiogenic therapy. In preclinical studies with ovarian cancer, radiolabeled bevacizumab has been developed as a means of imaging VEGF in tumors (Nagengast et al., 2007; Nayak et al., 2011). In an in vivo trial in animals with an ovarian cancer xenograft, the biodistribution of monoclonal antibodies against EGFR and VEGFR3 in the tumors was revealed by single-photon emission computed
tomography and computer tomography (SPECT/CT) and it has been speculated that this approach could be utilized in future imaging trials (Huhtala et al., 2010).

2.1.7 Novel therapies for ovarian cancer

2.1.7.1 PARP-inhibitors

In human serous ovarian cancer, aberrations in homologous recombination repair (HRR) have been detected. HRR is critical in the repair of damaged DNA in normal situations (Helleday et al., 2008). Genetical mutations, for example in BRCA1 and BRCA2 or ATM, CHEK2, RAD51 and PALB2 genes in high-grade ovarian cancer, result a homologous repair deficiency (HRD) and thus in an inadequate DNA repair, and this property has been exploited in the development of PARP inhibitors (Pennington et al., 2014). PARP is a DNA repair enzyme which is involved in base-excision repair, and therefore drugs which inhibit its action (PARPis) disrupt the DNA damage repair and therefore can be beneficial as cancer therapies (Schreiber et al., 2006). The mechanism of the PARP-inhibitor, olaparib, is to trap the PARP at sites of damaged DNA but to block the base-excision repair, leading to impaired DNA replication forks and the accumulation of DNA double-strand breaks (Murai et al., 2012). This leads to lethality in tumors with deficient homologous recombination repair mechanisms, such as in BRCA-mutated tumors (Farmer et al., 2005).

Olaparib (Lynparza®) is the first oral PARP-inhibitor that has conferred clinical benefits in ovarian cancer patients with BRCA1 and BRCA2 mutations. In phase II trials, the efficacy of olaparib was so clear that it could be recommended as a maintenance monotherapy in patients with platinum-sensitive recurrent ovarian cancer (Kaufman et al., 2015; Ledermann et al., 2014, 2012). Accordingly, olaparib was approved by FDA for the treatment of relapsed germline-BRCA-mutated ovarian cancer, after at least three prior chemotherapies. It has been approved by EMA for the maintenance therapy in BRCA-mutated ovarian cancer with platinum-sensitive recurrence, in patients who show a response to platinum. In a recent phase II study conducted by Ledermann et al. the maintenance monotherapy with olaparib improved the PFS in patients with platinum-sensitive recurrent serous ovarian cancer and BRCA mutations (Ledermann et al., 2016b).

Recently, results from a phase III trial with another PARP 1 and 2 inhibitor, niraparib, were published. Niraparib is a PARP inhibitor which has been claimed to display minimal toxicity in clinical studies, although some dose-related myelotoxicity has been reported (Sandhu et al., 2013). In the trial, niraparib was compared to placebo treatment in patients with platinum-sensitive, recurrent ovarian cancer and a possible germline BRCA mutation. PFS was significantly improved in the women treated with niraparib as compared to placebo (21.0 vs. 5.5 months) in germline-BRCA mutation carriers. Correspondingly, in non-germline BRCA mutated patients, the PFS was also improved with niraparib (9.3 vs. 3.9 months) as well as in non-germline BRCA mutated patients with homologous recombination deficiency (HRD) status (12.9 vs. 3.8 months) (Mirza et al., 2016).

2.1.7.2 Ovarian cancer immunotherapies

Development of immunotherapies in ovarian cancer stemmed from the hypothesis that immunogenic pathways and the immune system are activated during cancer development (Drerup et al., 2015; Lin Zhang et al., 2003). The principle of immunotherapy is to target immunogenic antigens expressed on the surface of tumor cells. Immunotherapies may be categorized into four main categories; antibodies, immune checkpoint inhibitors, vaccines and adoptive cell therapy. The fundamental principle of immunotherapy is to trigger the activation of tumor-specific helper T-cells (CD4) and cytotoxic T-lymphocytes (CD8). The administration of tumor-infiltrating lymphocytes (TIL) alone and combined with cisplatin chemotherapy was studied in seven patients with ovarian cancer. The results were
encouraging as there was a regression cancer and even complete response achieved in some of the patients (Aoki et al., 1991). There has been a dramatic development of new immunotherapeutic drugs during the last decades, although currently there is no approved immunotherapy for ovarian cancer.

The main advances in immunotherapies have been achieved with anti-CTLA-4 and anti-PD-1 in the treatment of malignant and advanced melanoma, as well as in the development of personalized CAR therapy for leukemia (Couzin-Frankel, 2013). One novel form of immunotherapy in ovarian cancer is to deliver monoclonal antibodies targeted to the programmed cell death-1 (PD-1) receptor expressed on T cells, and its signaling pathway. Programmed cell death ligands (PD-L1 and PD-L2) are present on the tumor cell surface, for example in ovarian cancer cells and binding of PD-1 and PD-L1 inhibits T-cell proliferation (Freeman et al., 2000). In recurrent ovarian cancer, the administration of an anti-PD-1 antibody, nivolumab, to block PD-1 signaling, has been studied in a phase II trial where it demonstrated tolerable safety and clinical efficacy. In that trial, the overall response rate was 15 % (Hamanishi et al., 2015). The other anti-PD-1-antibody for advanced recurrent ovarian cancer, pembrolizumab, has been recently studied in a phase Ib trial and resulted in a complete response in one out of 26 patients, with an overall response rate of 11.5 % (KEYNOTE-028, NCT02054806) (Varga et al., 2015). Avelumab, a fully humanized anti-PD-L1 IgG1 antibody has also been evaluated in women with recurrent or refractory epithelial ovarian cancer; the overall response rate was 9.7 %, but also 44.4 % experienced a stabilization of their disease. Currently, a phase I trial with avelumab is ongoing (NCT01772004)(Disis et al., 2016).

Our knowledge has increased about the connection between ovarian cancer and immune system and this resulted in the development of chimeric antigen receptor (CAR) or T cell receptor (TCR) modified T cells. CAR-T cell therapy has enjoyed a remarkable progress in preclinical and clinical trials. There have been encouraging results, for example in treatment of B-cell hematologic malignancies and in the treatment of patients with acute lymphoblastic leukemia, chronic lymphocytic leukemia and B-cell non-Hodgkin lymphoma (Lee et al., 2015; Maus et al., 2014; Singh et al., 2016; Wang et al., 2016). In clinical oncological trials with CAR-T cell therapy, the tumor antigen recognition is achieved with genetically modified T cells that are highly expressing chimeric antigen receptors (CAR). Genetical constructs are introduced into autologous peripheral T cells collected from the patient either by lentiviral transduction or by electroporation. Furthermore, CAR redirects the T-cells and cytotoxic immunoreactivity to target cells, which express the targeted antigen (Boulassel and Galal, 2012; Jena et al., 2010). In the treatment of solid tumors, the efficacy of CAR-T cell immunotherapy has been limited as a result of the immunosuppressive environment and also the lack of ideal targets for the therapy. Only one clinical phase I study with metastatic ovarian cancer and its specific folate receptor-α (FRα) has been published. This study indicated that CAR-T cell therapy was safe but resulted only a transient effect on therapeutic cells and poor results without any therapeutic tumor responses (Kershaw et al., 2006). FRα is highly expressed in certain cancers, for example in ovarian cancer cells and it can be considered as an appropriate target for CAR-T therapy (Weitman et al., 1992). Currently, a phase I study with CAR-T cell therapy is ongoing in 15 patients with recurrent ovarian cancer to study the feasibility, safety and preliminary activity of FRα-CAR-T cells, administered after lympho-depletion (Kandalaf et al., 2012). Attempts are being made to improve the efficacy of CAR-T cell therapy in the treatment of solid tumors. For example, numerous clinical phase I and II trials targeted to different antigens are ongoing, such as HER2 in colon cancer and malignant gliomas, CEA in stomach cancer, metastatic adenocarcinoma and metastatic breast cancer and mesothelin FRα in mesothelioma, lung, breast and ovarian cancer (Zhang et al., 2016). In preclinical studies, the CAR-T cell therapy has been targeted against the following proteins; mesothelin, MUC-16 and L1CAM (Chekmasova et al., 2010; Hong et al., 2016; Lanitis et al., 2013).
2.2 OVARIAN CANCER IMAGING

2.2.1 MRI in ovarian cancer
Magnetic resonance imaging (MRI) is a method to obtain anatomical images without subjecting the patient to ionizing radiation. Nuclear magnetic resonance (NMR) forms the basis for MRI; this is a phenomenon for instance occurring in spin ½ nuclei. Certain nuclei, such as 1H, exhibit split energy states in an external magnetic field (B0), which enables the excitation of the spin system with external radiofrequency (RF) pulses and the detection of nuclear magnetic resonance (NMR). RF energy is often applied as short pulses, leading to the transition of the nucleus from higher to lower energy levels and subsequently the return of the spin system to the equilibrium state, a property called relaxation. The released energy is detected with MRI sensors. There are two types of relaxation times, longitudinal T1 and transverse T2-relaxation times (McRobbie et al., 2007; Moser et al., 2009). In T1-weighted images, dark areas are detected with tissues with long T1-relaxation times, such as water and cerebrospinal fluid and other areas i.e. fat, with short T1 times are bright areas (Grover et al., 2015). In MRI with T1 relaxation in the rotating frame (T1ρ), the spin magnetization is flipped into transverse plane (in the rotating frame) (Rommel and Kimmich, 1989). The advantage with T1ρ measurement compared to T1 relaxation is its higher sensitivity to detect the low-frequency fluctuations and slow molecular motions in tissues as well as capturing the death of tumor cells during the early phases of the tumor cell related therapy (Hakumäki et al., 2002; Kettunen et al., 2007). In order to overcome the high specific absorption rate in T1ρ, a novel method relaxation along a fictitious field (TRAFT) was developed (Limatainen et al., 2010, 2012). TRAFT measurements have shown potential when evaluating the molecular dynamics and contrast between white and gray matter in human and rat brain, and also in prostate cancer (Jambor et al., 2016a, 2016b). These studies are encouraging the wider use of TRAFT for imaging other cancers including ovarian cancer.

CT has been the first line method for the preoperative assessment of ovarian cancer, cancer diagnosis and when evaluating the indications for surgery (Forstner et al., 2016a). Nonetheless, compared to CT, MRI and functional-MRI techniques may represent more accurate methods to characterize the primary tumor, tumor metastasis, possible limitations to surgery and to evaluate preoperatively the indications for neoadjuvant therapy (Forstner et al., 2016b; Low et al., 2015; Vargas et al., 2013). MRI without contrast agents has been preferred in patients with contraindications to CT, such as pregnant women, individuals with renal dysfunction or contrast agent allergies (Forstner et al., 2010). When diagnosing the peritoneal ovarian cancer, the sensitivity of MRI was 98 % and specificity 84 %. In addition to characterizing the incidental ovarian masses, MRI improves the positive predictive value of imaging if combined with CT and positron emission tomography (PET)/CT (Schmidt et al., 2015).

Positron-emission tomography (PET) with fluorodeoxyglucose (FDG) is preferred in the detection of ovarian cancer and recurrent disease, as malignant tumors usually exhibit high FDG metabolism. 18F-FDG-PET has been used in the diagnosis of ovarian cancer since it offers a potential way of identifying distant metastases in lymph nodes and bone, and in suspected recurrence (Lee et al., 2015). 18F-FDG-PET may be superior to CT when evaluating the spread of ovarian cancer in extra-abdominal areas, in bowel mesentery or supradiaphragmatically. Furthermore, 18F-FDG-PET has shown potential in the evaluation of the likely response to neoadjuvant chemotherapy (Hynninen et al., 2013, 2012; Vallius et al., 2016). When compared to PET-imaging, DW-MRI has gained some popularity in gynecological cancers as a non-imaging method not involving radiation. It has been stated that no significant additional information has been acquired when FDG-PET was performed in addition to MRI to patients with ovarian cancer (Kawahara et al., 2004). Furthermore, the high rate of false-positive results with PET/CT has been related to the diagnosis of primary ovarian cancer, therefore it is not recommended for the primary diagnosis and favors the use of MRI or CT in early cancer detection as well as in diagnosis
and planning the optimal therapy (Iyer and Lee, 2010). When CT, MRI and PET/CT were compared, MRI was estimated to possess the highest sensitivity of these methods (98 %) whereas PET/CT exhibited the highest specificity (96 %) (Schmidt et al., 2015). Although there did not seem to be any major significant differences between these methods, FDG/PET was assessed as the most accurate method when diagnosing the supradiaphragmatic lesions of peritoneal ovarian cancer and carcinomatosis (Schmidt et al., 2015). In a study with multiple imaging methods, the whole-body MRI and DW-MRI were preferred to CT and PET/CT in ovarian cancer when imaging the bowel and assessing the mesenteric spread of cancer (Michielsen et al., 2014).

2.2.2 DW-MRI in ovarian cancer and other gynecological cancers
Diffusion weighted-MRI (DW-MRI) is based on visualizing the microscopic random movement of water molecules (Brownian motion) in tissues, and offers improved contrast as compared to traditional MRI with T1 and T2 relaxation times (Koyama and Togashi, 2007; Low et al., 2009; Padhani et al., 2009). In DW-MRI, the different gradient amplitudes, durations and time intervals (b-value measured as sec/mm²) between diffusion gradients are all factors that influence the intensity of the detected signal due to the movement of water during the time interval between the gradient pulses (Koh and Collins, 2007). With different b-values, the quantitative analysis of apparent diffusion coefficient (ADC)-values are calculated and parametric ADC maps are reconstructed (Koh and Collins, 2007). High ADC values reflect fast diffusion i.e. motion of water molecules within the tissue and also capillary perfusion in tissues (Le Bihan et al., 1988). In highly cellular areas such as malignant tumors, the water diffusion is restricted leading to lower ADC values as compared to areas with low cellularity, which give rise to higher ADC values (Koh and Collins, 2007). Low ADC values with restrained water diffusion have been detected in tumors, and also in metastatic lymph nodes with cervical cancer studies (Bollineni et al., 2015; Miccò et al., 2014).

The higher contrast between normal and malignant tissues as well as the contrast inside the tissue are the two major advantages of DW-MRI as compared to CT or MRI. Furthermore, DW-MRI is completely noninvasive and performed without ionizing radiation. Since the ADC value provides a quantitative measure, it has been used as a biomarker for evaluating the therapy response and tumor malignancy in breast, rectal and intracranial tumors as well as in gynecological cancers of epithelial ovarian cancer and uterine cancers (Chen et al., 2010; Oh et al., 2015b; Padhani et al., 2009; Pickles et al., 2006). The limitations of DW-MRI relate to artifacts from bowel, low resolution, poor image quality caused by the sensitivity of imaging method to tissue movement and difficulties in localization of peritoneal tumors as well as false-positives attributable to inflammation (Low et al., 2009).

In the clinic, DW-MRI has been used in the diagnosis of ovarian cancer, cancer staging before the surgery and when evaluating the therapy protocols (Fan et al., 2015; Fujii et al., 2008; Medeiros et al., 2011) (Table 5). Benign and borderline ovarian tumors may be differentiated from malignant tumors by their low intensity in DW-MRI and correspondingly higher ADC-values in benign tumors, although on the basis of only DW-MRI, the differentiation of tumor malignancy is difficult (Takeuchi et al., 2010; Thomassin-Naggara et al., 2008). DW-MRI has been utilized in the diagnosis of endometrial cancer, cervical cancer, squamous cell carcinoma of the uterine cervix and ovarian cancer, as well as in the identification of liver metastases (Bollineni et al., 2015; Chenevert et al., 2000a; Koyama and Togashi, 2007; Liu et al., 2009; Schmid-Tannwald et al., 2014). In addition, it may be possible to diagnose the status of pelvic lymph nodes and potential cancer metastases with DW-MRI and altered ADC-values as compared to normal lymph nodes (Kim et al., 2008; Winfield et al., 2015). In clinical studies with gynecological cancers, DW-MRI and DCE-MRI have been used for evaluating the early therapy response after
chemotherapy and to differentiate tumor recurrence from radiation fibrosis in cervical cancer (Sala et al., 2010). In ovarian cancer patients with metastatic disease, increased ADC-values in tumors were observed when the patient responded to effective chemotherapy with a loss of tumoral cells (Kyriazi et al., 2011; Sala et al., 2012). Recently a clinical study with DW-MRI was performed, in which the clinical outcome of ovarian cancer patients was related to lower ADC-values in malignant tumors, poorly differentiated ovarian cancer and reduced 3-year overall survival (Lindgren et al., 2017). Also, in metastases higher VEGF-A and -C and VEGFR1-3 mRNA expression were observed as compared to primary tumors. Furthermore, low ADC-values correlated with low expression of VEGFR mRNA in metastases. These results imply the potential role of DW-MRI and reduced ADC-values when assessing the outcome of ovarian cancer patients and histological severity of the cancer (Lindgren et al., 2017). Currently no clinical trials with DW-MRI have been performed to evaluate the efficacy of antiangiogenic therapies.

2.2.3 DCE-MRI in ovarian cancer and other gynecological cancers

With dynamic contrast-enhanced MRI (DCE-MRI), the measurement has been performed after i.v. injection of contrast agent at multiple time points reflecting the blood flow in tumor blood vessels. The contrast agent leaks from tumor capillaries into the tumor interstitial space and this allows the volume of tumor vascularity and capillary permeability to be measured (Chase et al., 2012b). DCE-MRI offers the potential to evaluate the perfusion and therefore oxygenation of tumor tissue, and this may be exploited when assessing the tumor’s response to radiation therapy (Zahra et al., 2007). When imaging the female pelvis, its main applications are in staging of endometrial and ovarian cancer (Sala et al., 2010). DCE-MRI is a potential technique for evaluating the response of antivascular therapies in ovarian cancer and to differentiate between benign and malignant tumors (Li et al., 2015). The effects of antivascular therapies and tumor angiogenesis may be evaluated with DCE-MRI, as the leakage of contrast agent into extravascular space reflects the capillary permeability and possible changes in tumor microvessels (Padhani and Leach, 2005).

DW-MRI and DCE-MRI are sensitive imaging modalities with which to characterize the indeterminate tumor masses detected with ultrasound. Furthermore, these methods are more accurate for ovarian cancer staging than CT or PET (Forstner et al., 2016a). A recent study achieved 94 % accuracy with whole body DW-MRI when detecting the recurrent ovarian cancer, while the accuracy with CT was lower, 78 % (Michielsen et al., 2016). DW-MRI and DCE-MRI have better accuracy than CT for detecting the peritoneal dissemination of the cancer (Low and Barone, 2012). Recently Fehniger et al. assessed the potential of DW-MRI to diagnose metastatic gynecological cancer in a retrospective clinical study with patients of stage IIIC/IV disease. DW-MRI was estimated to be more accurate than CT when evaluating the presence of cancer in the diaphragm and disseminated disease (Fehniger et al., 2016).
Table 5. Summary of clinical studies with diffusion-weighted-MRI (DW-MRI) in ovarian tumors. Abbreviations: ADC; water apparent diffusion coefficient, CT; computed tomography, DCE-MRI; dynamic contrast enhanced-MRI

<table>
<thead>
<tr>
<th>Study</th>
<th>MRI</th>
<th>N</th>
<th>Therapy</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujii et al., 2008</td>
<td>DW-MRI</td>
<td>N=123</td>
<td>No</td>
<td>No significant difference between ADC in malignant and benign lesions. Sensitivity of 81.0 %, specificity of 57.1 %</td>
</tr>
<tr>
<td>Kyriazi et al., 2011</td>
<td>DW-MRI</td>
<td>N=42</td>
<td>Three cycles of platinum-chemotherapy</td>
<td>Chemotherapy response reflects early increase in ADC-values</td>
</tr>
<tr>
<td>E. Sala et al., 2012</td>
<td>DW-MRI</td>
<td>N=22</td>
<td>Neoadjuvant chemotherapy, three cycles of carboplatin and paclitaxel or carboplatin alone</td>
<td>Baseline ADC-values differ between primary ovca, omental cake and peritoneal tumors. Elevated ADC-values reflects the therapy response</td>
</tr>
<tr>
<td>Zhang et al., 2012</td>
<td>MRI vs. DW-MRI</td>
<td>N=43 benign, N=42 malignant tumors</td>
<td>No</td>
<td>ADC values are lowest in malignant tumors and highest in benign tumors. Sensitivity of 97.0 %, specificity of 92.2 %, accuracy of 96.4 %</td>
</tr>
<tr>
<td>Li et al., 2012</td>
<td>DW-MRI</td>
<td>N=127</td>
<td>No</td>
<td>Mean ADC values associated with malignant tumors were lower than in benign tumors. Sensitivity of 90.1 %, specificity of 89.9 %</td>
</tr>
<tr>
<td>Zhao et al., 2014</td>
<td>DW-MRI</td>
<td>N=102</td>
<td>No</td>
<td>ADC values are lower in malignant than in borderline epithelial tumors</td>
</tr>
<tr>
<td>Michielsen et al., 2014</td>
<td>DW-MRI</td>
<td>N=32</td>
<td>No</td>
<td>DW-MRI showed the highest accuracy in characterizing primary tumors and peritoneal and distant staging compared to CT or FDG-PET/CT</td>
</tr>
<tr>
<td>Fan et al., 2015</td>
<td>DW-MRI</td>
<td>N=64</td>
<td>No</td>
<td>DW-MRI is superior to CT in differentiating benign and malignant tumors. DW-MRI has higher accuracy, sensitivity and specificity</td>
</tr>
<tr>
<td>Meng et al., 2016</td>
<td>DW-MRI</td>
<td>N=559 malignant, N=600 benign tumors</td>
<td>No</td>
<td>DW-MRI is excellent in discriminating benign and malignant ovarian neoplasms, and predicts the surgical outcome. Pooled sensitivity 93.0 %, specificity 89.0 %</td>
</tr>
<tr>
<td>Kim et al., 2016</td>
<td>DW-MRI</td>
<td>N=731 malignant, N=918 benign lesions</td>
<td>No</td>
<td>There is no significant difference in ADC between benign and malignant lesions, furthermore it is not reliable in differential diagnosis of ovca. Sensitivity 76.0 %, specificity of 84.0 %</td>
</tr>
<tr>
<td>Lindgren et al., 2017</td>
<td>DW-MRI</td>
<td>N=40</td>
<td>Therapy was dosed after diagnostic DW-MRI, paclitaxel-carboplatin adjuvant therapy (one patient with only carboplatin). Bevacizumab in primary setting (n=8) or in recurrent disease (n=4)</td>
<td>Reduced ADC correlates to poorly differentiated tumors, high tumor cell proliferation and reduced OS. Expression of VEGF, VEGF-C and VEGFR1-3 mRNA is higher in tumor metastases than in primary tumors</td>
</tr>
</tbody>
</table>
2.2.4 MRI applications in preclinical cancer studies

In preclinical studies, the use of MRI when detecting the transplanted ovarian tumors has proved challenging because of its low resolution when imaging the intraperitoneal tumors (Klostergaard et al., 2006). DW-MRI has been exploited in preclinical animal studies as a noninvasive method to detect the cellular effects of tumor-targeted therapy and as a way to reveal functional alterations in tissue even before there have been morphological alterations in tumors (Chenevert et al., 2000b, 1997; Hundt et al., 2015; Lyng et al., 2000; Ross et al., 2003).

Decreased tumor cellularity in central parts of tumors has been detected with DW-MRI in preclinical cancer studies (Morse et al., 2007). Elevated ADC-values as a result of cellular lost and higher water diffusion have been observed in the necrotic areas of tumors, furthermore, it has been speculated that the higher ADC values may be related to increased tumor cell apoptosis (Hakumäki et al., 2002; Lyng et al., 2000; Papaevangelou et al., 2015). In animal models of breast cancer (Whisenant et al., 2014), glioma (Chenevert et al., 2000a), liver tumors (Youn et al., 2008), prostate cancer (Jennings et al., 2002) and colon and small cell lung cancers (Jordan et al., 2005), an altered ADC value has also been observed as a response to effective therapy within hours to days. By monitoring the ADC values after tumor-targeted therapies, it may be possible to evaluate and thus optimise the dosing of therapeutic drugs, such as cytotoxins (Ross et al., 2003). The potential of DW-MRI for characterizing the tumor microenvironment and stroma has been studied in cervical cancer xenografts (Hompland et al., 2014). In a mouse model of breast cancer, the elevated ADC-values have been related to necrotic areas of tumors after the chemotherapy. Early elevation in ADC-values was observed at 2-4 days after taxane administration and furthermore there were higher ADC-values present before there was any detectable reduction in tumor size (Morse et al., 2007). In addition, in a preclinical study in a mouse model of breast cancer, increased ADC values were detected four days after the paclitaxel therapy in drug sensitive but not in resistant tumors, and it was concluded that DCE-MRI could be a viable way to evaluate the achievable therapeutic effects before therapy (Galons et al., 1999).

In a mouse model of breast cancer investigating antiangiogenic therapy with bevacizumab, the early change as reflected as an increase in the ADC value of the tumors. In that study, the increased uptake of contrast agent was also observed in the central parts of tumors with DCE-MRI (Moestue et al., 2013). Recently, Gaustad et al. examined human melanoma xenografts and observed that although the early effects of bevacizumab therapy were highly heterogenic between the tumors, nonetheless the antiangiogenic effects and vascular normalization could be detected with DW-MRI and DCE-MRI (Gaustad et al., 2015).

So far, only a couple of studies related to preclinical in vivo DW-MRI experiments with ovarian cancer have been published (presented in Table 6). There is only one antiangiogenic therapy-related study with MRI, i.e. recently Ravoori et al. reported that bevacizumab resulted in increased T1-relaxation time in SKOV3 i.p. tumors in nude mice after the therapy (Ravoori et al., 2015). DW-MRI studies in ovarian cancer described reduced ADC-values after the chemotherapy with cisplatin (Pisanu et al., 2014a) and increased ADC values after inhibited PI3K/mTOR pathway signaling with BEZ235 inhibitor (Cebulla et al., 2015). Interestingly Pisanu et al. utilized an intraperitoneal SKOV3 ovarian cancer xenograft in mice and observed lowered ADC-values after cisplatin chemotherapy (Pisanu et al., 2014b).

As a result of tumor cell targeted therapies and cellular loss, ADC values are normally elevated, although in that study, the early reduction in the ADC value may have been related to transient drug-induced swelling of tumor cells before necrosis, and this property will need to be clarified in trials with DW-MRI in cancer patients (Pisanu et al., 2014a).
Table 6. Summary of preclinical MRI, DW-MRI and DCE-MRI trials with animal models of ovarian cancer.

<table>
<thead>
<tr>
<th>Study</th>
<th>MRI</th>
<th>Therapy</th>
<th>Model</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pisanu et al., 2014b</td>
<td>MRS DW-MRI</td>
<td>Cisplatin N=12, saline-controls N=14</td>
<td>i.p. SKOV3 SCID mice</td>
<td>ADC reduction was noticed at 24-48 h after chemotherapy as therapy response.</td>
</tr>
<tr>
<td>Ravoori et al., 2015</td>
<td>MRI</td>
<td>Bevacizumab N=6, PBS-controls N=6</td>
<td>i.p. SKOV3 nude mice</td>
<td>T_1-relaxation increased in bevacizumab treated mice 2 days and 2 weeks after therapy.</td>
</tr>
<tr>
<td>Gossmann et al., 2000</td>
<td>DCE-MRI</td>
<td>VEGFαb N=6, saline-controls N=6</td>
<td>i.p. SKOV3 athymic rats</td>
<td>DCE-MRI can be used to measure a reduction in tumor microvascular permeability after VEGFαb treatment.</td>
</tr>
<tr>
<td>Cebulla et al., 2015</td>
<td>DW-MRI DCE-MRI</td>
<td>PI3K/mTOR inhibitor BEZ235 N=8, controls without therapy N=4</td>
<td>s.c. clear cell and endometrioid OVCA, BalbC/nu mice</td>
<td>ADC increased as a response to therapy until day 3 and induced tumoral cell death.</td>
</tr>
<tr>
<td>Lee et al., 2013</td>
<td>DW-MRI FDG-microPET</td>
<td>No N=5</td>
<td>i.p. ID8 murine OVCA cells, syngenic C57BL/6 mice</td>
<td>DW-MRI offers potential in evaluating peritoneal OVCA dissemination</td>
</tr>
</tbody>
</table>

2.3 GENE THERAPY FOR OVARIAN CANCER

2.3.1 Strategies for gene therapy

Gene therapy studies are focusing on diseases lacking effective therapy with a poor prognosis. Moreover, a more ambitious aim is to develop gene therapy for diseases considered as untreatable (Kumar et al., 2016). Gene therapy is based on the delivery of nucleic acids into somatic cells of individuals, resulting in the production of endogenous therapeutic proteins (Ylä-Herttuala and Martin, 2000). The gene encoding the production of the therapeutic protein is transduced into target cells by gene delivery vectors, such as viral vectors. As gene delivery is achieved with modified and selectively tissue-targeted vectors, it represents a specific therapy method. The delivery of therapeutic gene products can be performed “in vivo”, by transferring the transgene within the vector into the patient to allow the vector to seek out the affected cells. The other method is “ex vivo” gene therapy, when the therapeutic gene is transferred in the laboratory and the transduced cells are returned to the patient’s body. The therapeutic effect of the gene construct may be coded by protein, RNA or by initiating RNA interference. Furthermore, the newest gene constructs contain specific 3D-structures and possess enzymatic activity. In addition, they are able to change their conformation, for example, they can further activate microRNAs which can initiate RNA interference. Currently the discovery of the CRISPR-Cas system has been investigated in preclinical studies, it allows the targeted changing of genome and is optimized to a certain organ and its cells (Savitskaya et al., 2016).

The first gene transfer in humans with a retrovirus was performed in 1989 in patients with advanced melanoma (Rosenberg et al., 1990). The first gene therapy in humans in Finland was performed in Kuopio at 1995 to a patient with malignant glioma (Puimalainen et al., 1998b; Ylä-Herttuala et al., 1996). The first “ex vivo” trials were performed in a four-year-old girl with ADA disease (Blaese et al., 1995). Currently about 2000 clinical trials have been concluded or are ongoing with gene therapy. Gene therapy products approved for the human therapy and their indications are presented in Table 7.

In the treatment of malignant brain tumors, adenovirus-mediated herpes simplex virus-thymidine kinase gene Cerepro® produced in Kuopio is currently being evaluated in EMA (Westphal et al., 2013). The AAV-based vector alipogene, tiparvovec (Glybera®) was approved by EMA in 2012 for the treatment of familial lipoprotein lipase (LPL) deficiency
with severe hypertriglyceridemia, metabolic complications, chylomicronemia and development of pancreatitis. Gene therapy with Glybera® significantly decreased the incidence of life-threatening pancreatitis in LPL-patients (Carpentier et al., 2012; Gaudet et al., 2013; Ylä-Herttuala, 2012). Approval of Glybera® represents the first approved gene therapy in Western countries (Kastelein et al., 2013). Later, in clinical gene therapy trials, breakthroughs have been made in the treatment of monogenic diseases such as primary immune deficiencies, hemoglobinopathies, hemophilia B, neurological diseases, ocular diseases and cancer immunotherapies (Kumar et al., 2016). The oncolytic herpes simplex 1 virus-based immunotherapy with Imlygic® (talimogene laherparepvec/T-VEC) was the second viral-based therapy to be approved for clinical use in the treatment of cutaneous and subcutaneous metastatic melanoma (Johnson et al., 2015; Liu et al., 2003; Ross et al., 2014). In addition, Imlygic® was the first approved oncolytic virotherapy to receive global recognition; it represents a milestone in the development of clinical gene therapies.

The challenges with gene therapy have focused on its side-effects, especially in i.v. delivery of gene therapy (Ylä-Herttuala and Alitalo, 2003). One of the main challenges encountered in the development of viral therapies has been the production of neutralizing antibodies, which limits the use of medication, i.e. the therapy can only be administered on one single occasion. One option to avoid the formation of antibodies would be to select an alternative delivery route to intravenous injection for the administration of the viral therapy. Since the neutralizing antibodies to adenoviruses may be present also in ascites fluid, the i.p. dosing of viral therapy may lead to reduced therapy response (Stallwood et al., 2000). Furthermore, this phenomenon may be related to the poor response in preclinical viral gene therapies after i.p. gene transfers. In future trials, the potential of activated immunoresponse and neutralizing antibodies as a result of targeted therapies should be considered.

Table 7. Approved indications and gene therapy products for human gene therapy.

<table>
<thead>
<tr>
<th>Product trade name</th>
<th>Indication</th>
<th>Viral vector</th>
<th>Approved by/ year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glybera® (Alipogene tiparvovec)</td>
<td>LPL-deficiency with severe or multiple attacks of pancreatitis</td>
<td>AAV</td>
<td>EMA/ 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st approved gene therapy in Western countries</td>
</tr>
<tr>
<td>Imlygic® (Talimogene laherparepvec/T-VEC)</td>
<td>Metastatic melanoma</td>
<td>Oncolytic herpes simplex 1 virus</td>
<td>EMA and FDA/ 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st approved oncolytic virotherapy</td>
</tr>
<tr>
<td>Strimvelis®</td>
<td>ADA-SCID immunodeficiency</td>
<td>Hematopoietic stem cells from patient are transduced to express ADA</td>
<td>EMA/ 2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1st approved ex vivo gene therapy</td>
</tr>
<tr>
<td>Gendicine®</td>
<td>Head- and neck squamous cell carcinoma</td>
<td>Recombinant adenvirus-p53</td>
<td>China’s State Food and Drug Administration (SFDA)/ 2003</td>
</tr>
<tr>
<td>Oncorine®</td>
<td>Nasopharyngeal cancer</td>
<td>Adenovirus</td>
<td>China’s State Food and Drug Administration (SFDA)/ 2005</td>
</tr>
</tbody>
</table>

2.3.2 Vectors

Nucleic acids RNA or DNA are not stable when delivered into the biological environment and furthermore, they are not able to to enter the cytoplasm or nucleus. Viral vectors are categorized into DNA and RNA viruses, integrated and non-integrated vectors (Chira et al., 2015a). As in gene therapy, since the production of therapeutic nucleic acids and proteins is
encoded in RNA or DNA, it is essential to deliver the modified gene into the target cells. At present, viral vectors are the most promising constructs for gene delivery and targeting the genetic material into the host tissue (Chira et al., 2015a). When compared to non-viral vectors, such as plasmids, the superior transfection efficacy with viral vectors is the reason for their use in preclinical and clinical trials. In gene therapy, the development of effective gene delivery vectors has proved to be one of the main challenges. Although, the adenovirus has been one of the most commonly and widely studied viral vectors utilized in gene therapy trials, during the last ten years the possibility of exploiting long-expressing gene delivery vectors with retroviruses, lentiviruses and adeno-associated viruses (AAV) as the viral vector has attracted considerable attention (Kotterman and Schaffer, 2014).

2.3.2.1 Adenoviruses
Adenovirus is known as a ‘flu-virus causing respiratory infections, conjunctivitis and gastro-intestinal infections in humans. In gene therapy, these viruses have been one of the most widely studied and extensively used viral vectors. Currently, adenoviruses are the most studied viral vectors in gene therapy clinical trials i.e. 21.4 % of all vectors (n=532) (www.wiley.co.uk/genmed/clinical). The adenovirus is a DNA-virus with an icosahedral capsid and 36 kbp linear double-stranded DNA. At least 57 serotypes of human adenoviruses have been characterized; in gene therapy, the most widely used adenoviral vectors are produced from Ad5 and Ad2 serotypes (Connelly, 2001; Parks et al., 1999).

Adenoviruses are popular in gene therapy trials, since the production of these vectors with high titters is straightforward and furthermore, they are non-integrating vectors, which means that adenoviruses are safer for therapeutical use. Human AdV5 serotype has been the most commonly used in gene therapy trials, as there are advantages in its manufacturing, titters up to 10^13 viral particles per ml (vp/ml) may be produced (Alonso-Padilla et al., 2015). Transgene expression time with adenoviral vectors is only weeks with the highest expression occurring at 1-2 weeks after the i.v. transfer (Bouard et al., 2009).

Adenoviruses and their vectors activate the innate immune system, with antibodies directed against viral capsid proteins, which is relevant in the development and safety of viral vectors.

If one wishes to generate a therapeutic gene delivery vector, the normal lysogenic cycle must be bypassed to avoid the lysis of vector. In replication defective vectors, essential deletions in E1A and E1B regions of adenoviral genome are generated and replaced with a high activity promoter such as the cytomegalovirus (CMV) promoter in order to drive the expression of foreign transgene (Figure 2). The early E1A proteins are crucial for the replication of the virus since they induce the expression of early genes in the E1B, E2, E3 and E4 transcription units. The E3 gene is usually deleted in adenoviral vectors to inhibit the elimination of vector-infected cells by the activated immune system. Since the genome adenoviruses has a relatively large size, the modification of their genome is possible, i.e. it provides a high capacity of 7-8 kb for inserted genes (Lukashev and Zamyatnin, 2016; Tong et al., 1998; Wold and Toth, 2013; Ylä-Herttuala and Martin, 2000).

The transduction of adenoviruses is usually initiated by binding of the virus to the cellular coxsackievirus and adenovirus receptors (CAR). As a result of adenovirus binding to fiber protein components in the CAR, there is an interaction of Ad penton base with cellular αβ integrins and this allows the virus to become fused into the target cell by receptor-mediated endocytosis and subsequently the viral genome is translocated into the cell’s nucleus (Meier and Greber, 2003). Adenoviral administration via the i.v. route has demonstrated a major transduction efficacy in liver via kupffer cell uptake, and further in spleen, heart, lung and kidneys in mice, although this is not correlated with the highest CAR levels in tissues (Tao et al., 2001; Wood et al., 1999). Indeed, the signs of liver toxicity as a result of high transduction efficacy in liver cells have been observed after i.v. adenoviral transfers (Bergelson, 1999; Bergelson et al., 1997). One of the problems
encountered with adenoviral vectors in gene therapy trials, especially with their systemic administration, has been the binding of viral particles to blood components (proteins, complements, erythrocytes and platelets), and their possible inactivation before they reach their target cells (Lyons et al., 2006). It is also possible that there will be an activation of the inflammatory response after their systemic administration, and this may even lead to lethal complications in the worst case scenario (Hendrickx et al., 2014; Raper et al., 2003). In addition to Ad5-vector binding to liver CARs, the vector binds to blood coagulation factor X mediating the hepatocyte cell infection by heparin sulfate proteoglycans on hepatocytes and a role for the hexon in viral infectivity of hepatocytes has been demonstrated (Kalyuzhniy et al., 2008; Piccolo et al., 2013; Shayakhmetov et al., 2005).

One of the challenges with adenoviral oncolytic therapies in cancers, including ovarian cancer, is the variability and low levels of CAR present in tumors. In studies with modified adenoviral vectors, the virus selectivity into cancer cells has been enhanced by modifying the viral binding proteins (Kanerva et al., 2002a; Kelly et al., 2000). CARs are also expressed in normal epithelial tissues, which support the development of targeted viral vectors, as there is the possibility of toxicity if the vectors become transported into normal cells (Tomko et al., 1997).

![Diagram](image)

*Figure 2.* Schematic figure of adenoviral genome. E1 and E3 transcription units are deleted and a transgene for example encoding the soluble VEGF-receptors or Tie-receptors, with a CMV promoter is transduced.

### 2.3.2.2 Other viral vectors and non-viral vectors

Retroviruses are RNA viruses, which integrate into the host genome resulting in a life-long transgene expression when used in gene transfers. These types of vectors were used to modify the genome before the discovery of the CRISPR-Cas system and they were used in trials with monogenic disorders and malfunctions in single genes. The potential of retroviral vectors in gene therapy has focused on hematopoietic cells. The problem with retroviral gene therapy is the possibility of harmful integration and dysregulated expression of oncogenes, and further the early adverse events encountered with γ-retroviral vectors meant that lentiviral vectors became the preferred gene delivery vectors (Hacein-Bey-Abina et al., 2008). For example, γ-retroviral vectors were used in the treatment of SCID-X1 immunodeficiency in early gene therapy trials, and resulted in a major setback when *ex vivo* gene transfer led to development of leukemia in five out of twenty treated patients (Hacein-Bey-Abina et al., 2003, 2008).

Lentiviral vectors are a group of retroviruses including HIV, which have become the most popular vectors in gene therapy trials. Lentiviruses integrate into the host genome
resulting in a life-long transgene expression, but they are thought to activate oncogenesis less frequently than retroviral vectors since their integration site preference is further away from the start of transcription units (Montini et al., 2006; Naldini et al., 1996). Lentiviral vectors have been most often used in the treatment of inherited genetical disorders, being mainly targeted to the hematopoietic systems (Montini et al., 2006).

Adeno-associated viruses (AAV) are small non-enveloped, single-stranded DNA-viruses with a 4.7 kbp genome. AAVs allow the incorporation of transgenes with sizes up to 4 kb. AAVs do not integrate into the host genome and further they are not genotoxic vectors. The achievable time of transgene expression is months to years, probably life-long, it has been reported that these vectors exhibit low immunogenicity and good safety. The immunogenicity of AAVs is lower than with adenoviruses. The transgene expression with AAVs decreases and leads to decreased transgene levels, which means that AAVs tend to be used in slowly dividing cells (Kotterman and Schaffer, 2014).

Non-viral vectors are manufactured biological vectors, in which the therapeutic gene is usually attached to plasmid DNA. Non-viral vectors are easier to produce than viral vectors and they also possess a lower risk for evoking immunoreactions. They have been less commonly applied in gene therapies since their transduction efficacy is low, as a result of the limited stability of the plasmids (Thierry et al., 1995). DNA and RNA molecules and autologous or allogenic cells have also been used in non-viral delivery of gene constructs. The main challenge with non-viral vectors is poor targeting of vectors and inadequate delivery of material into the cells of the target organ. DNA-plasmids are the simplest non-viral vectors, expressing a gene that induces protein synthesis or interferes with cellular RNA. They are easy to produce, but their stability is relatively low and their therapeutic effect is transient. DNA-constructs have been used in laboratory studies and in the development of veterinary vaccines (Chira et al., 2015a; Nayerossadat et al., 2012).

2.3.2.3 Oncolytic viruses

Oncolytic viruses promote an anti-tumoral response by two distinct mechanisms; by activated selective tumor cell killing and by inducing systemic anti-tumor immunity. The selective replication of oncolytic viruses in neoplastic cells results in the lysis of the tumor cells and the further release of new infectious viral particles (Kaufman et al., 2015). The antiviral response mounted by the host cell influences the activity of oncolytic viruses. Oncolytic viruses are able to enter not only cancer cells but also normal cells, but cancer cells are particularly sensitive to be lysed by the viruses because of their different cell signaling, disturbed homeostasis and distinct responses to stress (Hanahan and Weinberg, 2011b). Oncolytic viruses are able to promote the immune response against the tumor cells and themselves, leading to tumor cell lysis. Unfortunately, the neutralizing antiviral response may inhibit the viral replication and thus reduce the antitumoral efficacy of viruses (Kaufman et al., 2015). After the infection of neoplastic cells by oncolytic vectors, in addition to direct viral oncolysis, apoptosis, necrosis and autophagic cell lysis may also occur (Atherton and Lichty, 2013).

Engineered oncolytic viruses have been developed to improve the penetration of therapeutic viruses in the treatment of solid tumors, for example, adenovirus, herpes-simplex virus, vaccinia reovirus, measles virus and vesicular stomatitis virus have been investigated. In ovarian cancer studies, conditionally replicating adenoviruses (CRAds) have been the most widely studied oncolytic viruses (Kanerva et al., 2004). Type I CRAds maintain the characteristics as tumor specific-replication, which is achieved by deletions in genes critical to normal viral replication in normal cells, but not in tumor cells (Alemany et al., 2000).

Preclinical studies with ovarian cancer have revealed the efficacy of other CRAds, by targeting ovarian cancer cells with deficient retinoblastoma/p16 signaling pathway (Hakkarainen et al., 2006; Rein et al., 2005). Type II CRAds are engineered by adding
heterologous promoters into the adenovirus genome, and further by controlling expression of early genes, which are essential for virus replication. In studies of ovarian cancer, it has been shown that promoters of IAI.3B, cox-2 and SLPI seem to be the crucial sites (Hamada et al., 2003; Rein et al., 2005). The most widely studied CRAAd is the E1B gene-deleted Ad5 ONYX-015 which targets the tumor cells and reduces the binding of the P53 tumor suppressor gene, leading to p53-induced apoptosis in tumor cells (Bischoff et al., 1996).

2.3.3 Animal models for ovarian cancer

Depending on the desired applications, several animal models are available for use in preclinical studies of ovarian cancer. Mouse models are categorized into three class, xenografts, syngeneic meaning genetically identical and genetically engineered models (House et al., 2014; Lengyel et al., 2014). Epithelial ovarian cancer has also been studied in a strain of chickens with a high rate of spontaneous epithelial ovarian cancer (Johnson and Giles, 2013; Lim and Song, 2013) and also in rats (Fan et al., 2014).

Mouse s.c. xenografts derived from human ovarian cancer have been exploited in many ovarian cancer models (Kullander et al., 1978). In ovarian cancer, since the malignant cells have a propensity towards peritoneal dissemination and ascites formation, subcutaneous preclinical models are not optimal; this has led to the development of peritoneal disease models (Astoul et al., 1993; Fu and Hoffman, 1993; Hu et al., 2002; Klostergaard et al., 2006). Patient-derived ovarian cancer xenografts from ascites have been used, for example in gene therapy studies with AAV9, since this model resembles more closely human ovarian cancer (Pépin et al., 2015). A recent study defined the different characteristics of high-grade serous ovarian carcinoma models, which would be most suitable for development of ovarian cancer therapies (Mitra et al., 2015).

Transplantation of xenografts, reflecting the human origin of the tumor, has been the most generally used mouse model. In xenografts, the human tumor cells are transplanted into a host lacking a normal immune system. Intraperitoneal xenografts resemble the disseminated disease and therefore they have been favoured in many studies investigating ovarian cancer. Models utilizing subcutaneous xenografts with ovarian cancer have been described, but their limitation is the regional nature of the tumor formation (Molthoff et al., 1991). The limitation with cancer xenografts is the lack of an immune response in these models. In some ovarian cancer studies, severe combined immunodeficient mice (SCID) are utilized, which lack both T and B-cell related immune responses (Elkas et al., 2002). As the potential of activating an effective immune response is involved in most anti-tumor therapies, xenografts are not totally suitable for this type of research. In addition, immunocompetent Syrian hamsters have been used in ovarian cancer studies with oncolytic adenoviruses, as the immunotolerant animal models are not suitable for these studies (Thomas et al., 2006).

Many commercial ovarian cancer lines are available, although their characteristics may vary according to their genetics and the cell lines may possess different replication efficacies. As the culturing of cell lines is known to be related to increased aggressivity of cell line and further to genetical alteration, cell-culturing needs to be optimized in preclinical cancer studies. The challenge with utilizing cell lines derived from human primary tumors is to establish a high-quality and permanent cell line with limited diversity of tumors when used in different models, for example in mouse xenografts. Therefore also new ovarian cancer cell lines with an improved molecular profile, drug responses and more permanent quality features have been established (Ince et al., 2015).

In syngeneic mouse models, the tumors are produced in immunocompetent mice with cells from the same strain. Syngeneic mice are have also been utilized in preclinical ovarian cancer trials and VEGF-related therapies (Roby et al., 2000; Toyoshima et al., 2009). The advantage of these models is their high histopathological convergence with human tumors. In these models, the mice can mount a normal immune response, permitting
studies with anti-tumor immune mediated therapies. The disadvantage with syngeneic models is the production of cancer from animal cells, therefore they are not optimal for studying human cancers (House et al., 2014).

Genetically modified mouse models have been produced to clarify the genetical alterations behind the cancer development. In human ovarian cancer, the genetic alterations which have been studied are related to TP53, c-MYC, K-RAS, AKT, BRCA1 and BRCA2 (Sharpless and DePinho, 2006; Xing and Orsulic, 2006). As the origin of serous ovarian cancer was recently related to fallopian tube epithelium, new models with fallopian tube epithelium-related cancer have been recently produced with genetically modified mice (Morin and Weeraratna, 2016). The challenge with these models and studying the ovarian cancer is the complexity of cancer and variations in genes between different mouse strains (House et al., 2014).

2.3.4 Preclinical gene therapy studies in ovarian cancer
The safety of adenoviral vectors has been examined in in vivo studies, as the i.p. administration of Ad5 resulted in bowel adhesion, perforation and obstruction in a phase I/II clinical trial (Vasey et al., 2002a). In that preclinical study, repeated i.p. administration of the Ad11 serotype was related to a reduced inflammatory response and less liver toxicity than encountered with the Ad5 serotype (Thoma et al., 2013).

High EGFR expression is related to a poor prognosis of ovarian cancer. In a preclinical in vivo study conducted with combined retrovirus and inhibited EGFR signaling, the growth of ovarian tumors was inhibited and furthermore, the tumor cell sensitivity to cisplatin chemotherapy was enhanced (Chan et al., 2005). In addition, the potential of adenoviral p53 therapy was demonstrated in vivo with inhibited growth of ovarian cancer cells (Santoso et al., 1995). Preclinical virotherapy trials with ovarian cancer have focused on improving the adenoviral oncolytic viruses, such as Ad5-Δ24RGD (CRAd) (Bauerschmitz et al., 2002; Kanerva et al., 2004; Lam et al., 2003) and Ad5/3-Δ24 (Kanerva et al., 2003). The potential of these viruses has been revealed in preclinical studies as they displayed enhanced viral infectivity and higher efficacy in tumor cell killing. In a preclinical study with cisplatin resistant ovarian cancer, the p53-mediated apoptotic response to cisplatin was promoted by administering the Ad12 E1B oncoprotein (Wang et al., 2015). Adenoviral melanoma differentiation-associated gene-7 (Ad.mda-7) therapy has been shown to induce apoptosis and to suppress the growth of the tumor cells. In further in vitro studies with infectivity-enhanced Ad.RGD.mda-7, tumor apoptosis was significantly induced (Leath et al., 2004; Mahasreshti et al., 2006).

Only a few antiangiogenic preclinical gene therapy trials with VEGFR-targeted therapies have been published so far. As far as is known, no preclinical studies in ovarian cancer with neuropilin-targeted therapies have been published. The preclinical trials testing antiangiogenic gene therapy in ovarian cancer are presented in Table 8. The adenoviral soluble VEGFR1 was studied by Mahasreshti et al. in ovarian cancer. It was noted that i.p. xenografts, and i.p. delivered gene therapy inhibited the growth of tumors and increased the survival of mice (Mahasreshti et al., 2001). In their subsequent study, the safety of i.v. adenoviral gene therapy was examined; it was found that a high expression of sVEGFR1 in liver was associated with significant hepatotoxicity (Mahasreshti et al., 2003). Recombinant AAV-2 mediated sVEGFR1 has been tested in vivo in ovarian cancer by Mahendra et al.; these workers detected significantly inhibited growth of intraperitoneal tumors as well as increased the disease-free survival when the gene therapy was delivered intraperitoneally (Mahendra et al., 2005). In preclinical ovarian cancer studies with adenovirus, the efficacy of combined soluble VEGFR1, VEGFR2 and VEGFR3 i.v. -gene therapy in ovarian cancer has been revealed i.e. significantly reduced growth of tumors, antiangiogenic therapy effect and reduced ascites fluid formation (Sallinen et al., 2009). Furthermore, in another mouse study with i.p. ovarian cancer xenografts, there was evidence of prolonged survival,
reduced growth of tumors and reduced tumor vascularity (Sopo et al., 2012). In the trial conducted by Sopo et al., the anti-tumoral efficacy was potentiated when adenoviral gene therapy was combined with paclitaxel chemotherapy; in that study the efficacy of bevacizumab in ovarian cancer therapy was poorer than achieved with gene therapy (Sopo et al., 2012). The role of angiopoietin/Tie-signaling in ovarian cancer has been evaluated in \textit{i.p.} ovarian cancer xenografts, in which the combined \textit{i.v.} AdsVEGFR1, AdsVEGFR2 and AdsTie2 -gene therapy reduced the growth of tumors and had a significant antiangiogenic effect in tumors (Sallinen et al., 2011). In that study, the \textit{i.v.} AdsTie1 and AdsTie2 gene therapy shortened the survival of mice with ovarian cancer and there were signs of liver toxicity attributable to the therapy (Sallinen et al., 2011). In an \textit{in vivo} study of ovarian cancer with either baculovirus expressing soluble Tie2 -therapy on its own or combined with sVEGFR1, the peritoneal angiogenesis and ascites formation were reduced, as were the number of tumor cells and invasive tumor cells (D’Souza et al., 2010).

Some experiments have been conducted with adeno-associated viral vector (AAV). A recombinant AAV-P125A-endo construct targeting human endostatin, a hormone with a putative angiogenic activity, reduced the growth of ovarian cancer cells and prevented angiogenesis (Subramanian et al., 2005). In another study with AAV-P125A-end, a prolongation in tumor-free survival was achieved in a orthotopic mouse model of ovarian cancer and there was also improved tumor inhibition to treatment with carboplatin (Subramanian et al., 2006). In addition, the potential of \textit{i.m.} recombinant AAV-gene therapy encoding both endostatin and angiostatin was shown in mice as improved tumor-free survival and lower ascites volume. Furthermore the beneficial effects were improved when combined to chemotherapy with paclitaxel (Isayeva et al., 2006, 2005; Ponnazhagan et al., 2004). Gene therapy with lentiviruses and IL-21-secreting mesenchymal cells was examined in ovarian cancer xenografts; there was a significantly reduced tumor growth, although only a couple of studies with lentiviral vectors in ovarian cancer gene therapy have been published (Indraccolo et al., 2005; Zhang et al., 2014).
Table 8. Preclinical trials of ovarian cancer in which the gene therapy has targeted angiogenesis.
LV=lentivirus, ovca=ovarian cancer

<table>
<thead>
<tr>
<th>Viral vector</th>
<th>GT delivery</th>
<th>Animal model</th>
<th>Main results</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdsVEGFR1</td>
<td>i.p. 1 and 14 days after tumor cell inoculation</td>
<td>i.p. ovca in mice</td>
<td>Increased survival</td>
<td>Mahasreshti et al., 2001</td>
</tr>
<tr>
<td>AdsVEGFR1</td>
<td>i.v. or i.p.</td>
<td>i.p. ovca in mice</td>
<td>i.v. GT is hepatotoxic</td>
<td>Mahasreshti et al., 2003</td>
</tr>
<tr>
<td>AAV-sVEGFR1</td>
<td>Cancer cells were transduced with AAV-sVEGFR1 cDNA before inoculation</td>
<td>i.p. ovca in mice</td>
<td>Reduced ascites and cancer cells, prolonged survival</td>
<td>Hasumi et al., 2002</td>
</tr>
<tr>
<td>AAV-sVEGFR1</td>
<td>i.m. 3 weeks before tumor cell inoculation</td>
<td>i.p. ovca in mice</td>
<td>Increased disease-free survival and reduced tumor growth</td>
<td>Mahendra et al., 2005</td>
</tr>
<tr>
<td>AAV-sVEGFR1</td>
<td>i.m. 9 days before tumor cells</td>
<td>i.p. ovca in mice</td>
<td>Growth of tumors and dissemination were suppressed by inhibited angiogenesis</td>
<td>Takei et al., 2007</td>
</tr>
<tr>
<td>AdsVEGFR2</td>
<td>i.v. or combined with i.p. cis-diaminedichloroplatinum 8 days after tumor cells</td>
<td>s.c. ovca in mice</td>
<td>Inhibited tumor growth, suppressed angiogenesis and induced apoptosis</td>
<td>Wu et al., 2006</td>
</tr>
<tr>
<td>AdsVEGFR1, AdsVEGFR2, AdsVEGFR3</td>
<td>i.v. 1 day after the first visible tumors in MRI</td>
<td>i.p. SKOV3m ovca in mice</td>
<td>Reduced tumor growth, tumor vascularity and ascites</td>
<td>Salilnen et al., 2009</td>
</tr>
<tr>
<td>AdsVEGFR1, AdsVEGFR3, AdsTie2 or AdsTie1, AdsTie2</td>
<td>i.v. 1 day after the first visible tumors in MRI</td>
<td>i.p. SKOV3m ovca in mice</td>
<td>Reduced tumor growth, tumor vascularity. Liver toxicity and shortened survival with AdsTie1 and AdsTie2</td>
<td>Salilnen et al., 2011</td>
</tr>
<tr>
<td>Ad-sVEGFR1, AdsVEGFR2, AdsVEGFR3 and chemotherapy and bevacizumab</td>
<td>i.v. 1 day after the first visible tumors in MRI</td>
<td>i.p. SKOV3m ovca in mice</td>
<td>GT with paclitaxel prolonged the survival. GT reduced tumor growth and tumor vasculature. Bevacizumab did not improve the survival</td>
<td>Sopo et al., 2012</td>
</tr>
<tr>
<td>Ad-vector encoding antiangiogenic angiostatin, endostatin, PIGF4, angiotatin and endostatin fusion protein</td>
<td>i.p.</td>
<td>i.p. ovca in mice</td>
<td>Reduced ascites formation, tumor growth, vascularity and prolonged survival</td>
<td>Hampl et al., 2001</td>
</tr>
<tr>
<td>AAV9-LRMIS</td>
<td>Ad-vector</td>
<td>i.m.</td>
<td>i.p. ovca in mice</td>
<td>Improved tumor-free survival, reduced ascites formation</td>
</tr>
<tr>
<td>(modified multien or inhibiting substance)</td>
<td>i.v. 1 day after the first visible tumors in MRI</td>
<td>i.p. SKOV3m ovca in mice</td>
<td>Ovarian cancer xenografts derived from ascites</td>
<td>Pépin et al., 2015</td>
</tr>
<tr>
<td>AAV-mediated bevacizumab, AAVrh10.BevMab</td>
<td>i.p. single administration</td>
<td>i.p. ovca in mice</td>
<td>High local expression of bevacizumab in peritoneal cavity and suppressed carcinomatosis, prolonged survival</td>
<td>Xie et al., 2014</td>
</tr>
<tr>
<td>LV-IFNo</td>
<td>i.p.</td>
<td>IGROV-1 or OC316 ovca in SCID mice</td>
<td>Reduced hemorrhagic ascites, increased tumor necrosis, reduced microvessel density</td>
<td>Indraccolo et al., 2005</td>
</tr>
<tr>
<td>Baculovirus-sTie2 and sVEGFR1</td>
<td>i.p.</td>
<td>i.p. ovca mice</td>
<td>Reduced angiogenesis and ascites formation</td>
<td>D’Souza et al., 2010</td>
</tr>
</tbody>
</table>
2.3.5 Clinical trials of gene therapy in ovarian cancer

Gene therapy trials may be divided into suicidal gene therapy, molecular therapy, replacement of tumor suppressor gene therapy and inhibition of growth factors and their regulators. Clinical trials are subdivided into four phase studies (I-IV). In phase I trials, the safety of drugs is evaluated in terms of optimal dosing and potential side effects. In phase II trials, the drug is given to a larger number of patients and the efficacy of drugs along with a safety evaluation is performed. In phase III trials, a large group of patients is medicated and the efficacy of drug is evaluated, also therapy is compared to the common therapy of the disease and side effects and safety data are monitored. Phase IV trials are done after the marketing of drug or therapy to gather information about the effects from various populations as well as identifying possibly rarer side effects after the long-term use.

The most relevant clinical trials of gene therapy in combatting ovarian cancer performed with adenoviruses are presented in Table 9. Adenoviral p53 gene therapy has been developed to induce the tumoral p53, which is also the most common molecular abnormality encountered in the development of ovarian tumors (Von Grunigen et al., 1999). There have been promising results emerging from preclinical and clinical trials in patients with recurrent ovarian cancer receiving disease Adp53 gene therapy. Subsequently, in an international randomized phase II/III trial with Adp53 gene therapy in ovarian cancer, no significant therapeutic effect was reached and increased morbidity was shown. After these poor results, the trials with Adp53 were suspended (Zeimet et al., 2003). Afterwards the safety of therapy has been studied in more detail; it was reported that multiple dosing had been well tolerated and six out of eleven patients had a stable disease (Wolf et al., 2004). Furthermore, a phase II study was performed with wild-type p53 vaccine and s.c. administration in stage II, IV or recurrent ovarian cancer patients, no serious systemic side effects were encountered and a survival benefit was obtained with specific immune response against p53 (Rahma et al., 2012).

As ovarian cancer cells have undergone widespread genetical alterations, many gene therapy trials targeting these genes have been performed. Ovarian cancer suicidal gene therapy targeting BRCA1 has been studied in phase I and II trials, but significant toxical side effects were encountered without beneficial therapeutic effects (Tait et al., 1999). Over-expression of the ErbB-2 gene (HER2/neu) may occur in human ovarian cancers and this has been related to cancer aggressivity and metastasis (Felip et al., 1995; Meden et al., 1994). A phase I trial with ErbB-2 in ovarian cancer has also been performed (Table 9). Phase I/II trials with IL-12 plasmids and carboplatin and docetaxel chemotherapy have been conducted in platinum-sensitive patients as well as in subjects with resistant/recurrent ovarian cancer. Although there were few side-effects, no significant improvement in antitumoral response or progression-free survival was reached (Alvarez et al., 2014; Anwer et al., 2013). The safety of i.p. retroviral LXSN-BRCA1sv gene therapy was studied in patients with recurrent or persistent epithelial ovarian cancer. This trial was terminated after the treatment of six patients because of vector instability and viral antibody development, and furthermore no clinical response to therapy was obtained (Tait et al., 1999).

In a clinical phase I trial with recurrent ovarian cancer the administration of oncolytic measles virus expressing the carcinoembryonic antigen (MV-CEA) improved overall survival, and decreased CA-125 levels in patients with heavily pretreated recurrent ovarian cancer (Galanis et al., 2010). The efficacy of oncolytic therapy with vaccinia virus (JX-594) has been examined in a phase I study with two ovarian cancer patients (Breitbach et al., 2011). Two experimental studies with a granulocyte macrophage colony-stimulating factor (GMCSF)-coding adenovirus have been performed in the treatment of different types of cancers (four patients with ovarian cancer in both studies) to examine the possible induction of antitumor immunity with Ad5-Δ24 and Ad5/3-Δ24 oncolytic vectors (Cerullo et al., 2010; Pesonen et al., 2012). These studies revealed the good tolerability of viral therapies and the induction of antitumoral and antiviral immune responses (Kanerva et al., 2002; Hasumi et al., 2005; Mahasreshti et al., 2009; Wu et al., 2006; Sallinen et al., 2009; Tait et al., 2010; Pesonen et al., 2012).
2013; Koski et al., 2010). A phase I study with GMCSF-expressing oncolytic virus (ONCOS-102) and cyclophosphamide was performed in patients with solid tumors (number of X ovarian cancer patients) to assess the safety of ONCOS-102 and the potential of oncolytic virus as a immunosensitizing agent (Ranki et al., 2016).

No clinical trials in ovarian cancer with viral antiangiogenic gene therapies targeting the tumor vasculature have been published. Therefore, there is a clear need for ovarian cancer gene therapy studies with optimal dosing of vectors and improved delivery methods. Furthermore, these studies should focus on improving the efficacy i.e. better targeted therapies with higher transduction properties as well as optimizing the quality of tumors established by the xenografts.
A phase I study with GMCSF-expressing oncolytic virus (ONCOS-102) and cyclophosphamide was performed in patients with solid tumors (number of ovarian cancer patients) to assess the safety of ONCOS-102 and the potential of oncolytic virus as a immunosensitizing agent (Ranki et al., 2016). No clinical trials in ovarian cancer with viral antiangiogenic gene therapies targeting the tumor vasculature have been published. Therefore, there is a clear need for ovarian cancer gene therapy studies with optimal dosing of vectors and improved delivery methods. Furthermore, these studies should focus on improving the efficacy i.e. better targeted therapies with higher transduction properties as well as optimizing the quality of tumors established by the xenografts.

Table 9. Clinical adenoviral gene therapy studies with ovarian cancer patients administered to more than five patients. (Ad-RSV-tk; recombinant adenovirus containing the HSV-tk gene, GT; gene therapy, ovca; ovarian cancer)

<table>
<thead>
<tr>
<th>Stage of the disease (n)</th>
<th>Phase</th>
<th>Therapy</th>
<th>Main results</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrent ovca stage IIIc after debulking surgery, n=10</td>
<td>I</td>
<td>i.p. Ad-RSV-tk followed by i.v. acyclovir and topotecan 24 h after vector delivery</td>
<td>At second-look surgery, 2/5 of patients were free of tumor. Improved OS when pretreated with chemotherapy</td>
<td>Hasenburg et al., 2001</td>
</tr>
<tr>
<td>Recurrent ovca stage IIIc after debulking surgery, n=10</td>
<td>I</td>
<td>i.p. Ad-RSV-tk followed by i.v. acyclovir and topotecan 24 h after vector delivery</td>
<td>No serious complications or toxicity related to GT</td>
<td>Hasenburg et al., 2000</td>
</tr>
<tr>
<td>Recurrent peritoneal or fallopian tube cancer with aberrant or mutant p53, n=41</td>
<td>I/II</td>
<td>i.p. rAd/p53 alone and with platinum-chemotherapy</td>
<td>No dose-limiting toxicity and GT was well tolerated. GT+platinum can be related to reduced CA12-5</td>
<td>Buller et al., 2002b</td>
</tr>
<tr>
<td>Recurrent ovca, n=15</td>
<td>I</td>
<td>i.p. Adp53 dosed multiple times (safety study)</td>
<td>One patient had prolonged decrease in CA12-5 and stable disease. No severe toxicities were found.</td>
<td>Wolf et al., 2004</td>
</tr>
<tr>
<td>Recurrent ovca, with erbB2 overexpression, n=15</td>
<td>I</td>
<td>i.p. adenoviral anti-erbB-2 antibody (ad21)</td>
<td>No dose-limiting vector-related toxicity. 5/6 patients displayed increases in anti-adenoviral ab titer</td>
<td>Alvarez et al., 2000a</td>
</tr>
<tr>
<td>Recurrent ovca, n=14</td>
<td>I</td>
<td>i.p. AdHSV-tk followed by 14 days ganciclovir starting 2 days from GT</td>
<td>Survival benefit, 5 (38%) in patients with stable disease and in 8 (62%) with progressive. 10/11 had increased anti-adenoviral ab titers. GT was well tolerated</td>
<td>Alvarez et al., 2000b</td>
</tr>
<tr>
<td>Ovca with HER-2/neu overexpression, n=15</td>
<td>I</td>
<td>i.p. adenoviral E1A-lipid complex, dosed weekly for 3 to 4 weeks (max. 6 cycles)</td>
<td>GT is safe and feasible with limited toxicity</td>
<td>Madhusudan et al., 2004</td>
</tr>
<tr>
<td>Recurrent ovarian cancer, n=16</td>
<td>I</td>
<td>i.p. adenoviral oncolytic virus dl1520 (ONYX-015) with E1B deletion and targeted to lysis of p53-deficient tumor cells</td>
<td>Safety of oncolytic virus was shown, 1st oncolytic adenoviral therapy in clinics</td>
<td>Vasey et al., 2002b</td>
</tr>
<tr>
<td>Ovca, n=21</td>
<td>I</td>
<td>i.p. adenoviral oncolytic virus Ad-Δ24-RGD</td>
<td>No vector related toxicity, potential antitumoral response. After 1 month 15 (71%) had stable disease, 6 (29%) progressive disease. No partial or complete responses</td>
<td>Kimball et al., 2010</td>
</tr>
</tbody>
</table>
2.4 SAFETY AND ETHICAL ASPECTS OF GENE THERAPY

In development of gene therapy, the first setback in clinical trials was encountered when a 18-year old boy with ornithine transcarbamylase (OTC) deficiency died as a result of a severe immune response after he had received adenoviral gene therapy (Raper et al., 2003). Following the next study, ten children with X-linked severe combined immunodeficiency (SCID-X1), the so-called "bubble boy" syndrome, were treated with retroviral vectors and treatment gene was delivered into their white blood cells; in that study, two of the children developed leukemia almost three years after the gene therapy (Hacein-Bey-Abina et al., 2003). After these studies, a wide range of trials for ADA-SCID patients has been performed and vector production has been substantially improved. As a result, the third viral-based therapy was approved by EMA in the year 2016 for the treatment of patients with ADA-SCID-immunodeficiency without suitable bone-marrow donors (Agency, 2016; Hoggatt, 2016). This product, with the product name of Strimvelis® consists of a retroviral vector encoding the human ADA cDNA sequence (Roy et al., 2005). In studies relating to this gene product, there have been significant prolongations in patient survival, on average seven years. Normally ADA-SCID is fatal within 1-2 years when untreated. The gene therapy showed an adequate safety profile (Aiuti et al., 2009).

The safety of gene therapy often involves the question of whether the mutated genes can be incorporated into the germ line. According to the current legislation of gene therapy, only gene therapy targeting somatic cells is approved. The safety of gene therapies has been widely studied but nonetheless the use of integrating viral vectors is one of the main concerns in clinical applications (Li et al., 2011). There have also been worries about the safety of adenoviral vectors in gene therapy; this issue has been studied widely in preclinical and in clinical studies. It is now apparent that the activation of an immune response as a result of viral-based therapy is a possible side effect in clinical settings. As a result of the activated immune response, the efficacy of viral gene therapy may become reduced and this is a phenomenon needing to be taken into account in future trials of novel viral vectors (Hedman et al., 2009; Immonen et al., 2004; Muona et al., 2012).
3 Aims of the study

The aim of this thesis was to develop antiangiogenic and antilymphangiogenic adenoviral gene therapy in ovarian cancer xenografts with soluble VEGF-, angiopoietin- and neuropilin-receptors. In addition, the efficacy of combined gene- and chemotherapy in ovarian cancer was studied and the safety of these therapies was evaluated. To detect the early responses of gene therapy in epithelial ovarian tumors at the molecular level, a secondary aim was to improve the ovarian cancer imaging with DW-MRI in our animal model.

The specific aims of individual studies were as follows:

I To evaluate the safety and biodistribution of i.v. AdsVEGFR2 and AdsVEGFR3 gene therapy and chemotherapy with carboplatin and paclitaxel in healthy female rats. The study was performed as a preclinical evaluation for forthcoming clinical trials with ovarian cancer gene therapy.

II To study the ovarian cancer gene therapy with AdsVEGFR2, AdsVEGFR3, chemotherapy, AdsNRP1 and AdsNRP2 in mice xenografts. Furthermore, MRI and relaxation time measurements $T_{1p}$, $T_2$, $T_{RAFF2}$ and $T_{RAFF4}$ with DW-MRI were performed to evaluate the early treatment responses of adenoviral gene therapy and chemotherapy in ovarian tumors.

III To study the antiangiogenic and antilymphangiogenic gene therapy in ovarian cancer xenografts by targeting two essential signaling pathways in ovarian cancer development with AdsVEGFR2 and AdsTie2. The role of VEGFR2 and Tie2 in the formation of malignant ascites was evaluated. In addition, the DW-MRI and relaxation time measurements in tumor imaging were studied further.
Intraperitoneal ovarian xenografts, all tumors were generated by inoculating $1 \times 10^6$ cells into the peritoneal cavity of a nude mouse. Further, after development of tumor, tumor pieces were suspended in 1 ml Optimem and counted.

Before infection, the cells were trypsinized, centrifuged and suspended in McCoy's 5A medium (Gibco, Invitrogen, Life Technologies, USA). The highly aggressive SKOV3m cells were cultured in McCoy's 5A medium (McCoy's 5A medium (Gibco, Invitrogen, Life Technologies, USA)). Cells were trypsinized, centrifuged and suspended in 1 ml Optimem and counted.

In the study with ovarian cancer xenografts, animals were followed for four weeks after the gene therapy. In addition, ascites fluid was collected and was measured in study I to evaluate the biodistribution and biodistribution of adenovirus. In studies II and III, SKOV3m cells were injected i.v., the final volume of virus was $200 \mu l$ (studies II, III) and $200 \mu l$ (study I).

Before induction 4.5 % isoflurane and 450 ml air, maintenance; 1.7 % oxygen, 4% carbon dioxide in air, and 40 ml per kg of body weight per minute, the animals were anesthetized with 3.5 ml/kg anesthetic solution containing solution (studies II, III) were weighed and weighed. At the time of death, all animals were dissected and samples from muscle, ovary, uterus, pancreas and heart were collected and weighed. From liver, spleen, kidney, lung, bowel and peritoneum, additional samples were collected and weighed. Blood was collected and its hematological parameters were evaluated by weekly DW measurements.

Cells were inoculated intraperitoneally. The growth characteristics of SKOV3m cells were evaluated by weekly DW measurements (studies II, III). The development of tumors was followed by weekly MRI and the effect of therapies. The tumor pieces were stained with hematoxylin and eosin and were evaluated by light microscopy.

The safety of the adenovirus was evaluated by monitoring the appearance of symptoms or weight loss demanding the sacrifice. At the time of death, the rats were followed for four weeks after the gene therapy. The animals were followed until the death or appearance of symptoms demanding the sacrifice. At the time of death, the animals were dissected and samples from muscle, ovary, uterus, pancreas and heart were collected and weighed. From liver, spleen, kidney, lung, bowel and peritoneum, additional samples were collected and weighed. Blood was collected and its hematological parameters were evaluated by weekly DW measurements.

The epithelial cell line was obtained from the American Type Culture Collection (HTB176, ATCC, Manassas, USA). The highly aggressive SKOV3m cell line has an epithelial phenotype. The epithelial SKOV3m cell line has been developed previously (Sallinen et al., 2006). It has demonstrated the capacity for metastatic spread and has an epithelial phenotype.

Materials and methods

Animals used in studies

In the study with ovarian cancer xenografts, female Balb/cA (Taconic, Danisco, USA) immunocompetent nude mice were used. The development of tumors was followed by weekly MRI and the effect of therapies. The tumor pieces were stained with hematoxylin and eosin and were evaluated by light microscopy.

The growth characteristics of SKOV3m cells were evaluated by weekly DW measurements (studies II, III). The development of tumors was followed by weekly MRI and the effect of therapies. The tumor pieces were stained with hematoxylin and eosin and were evaluated by light microscopy.

All animals were kept in a pathogen-free isolated unit at the National Experimental Animal Center of the University of Eastern Finland, Kuopio.

Before infection, the cells were trypsinized, centrifuged and suspended in 1 ml Optimem and counted.

Before induction 4.5 % isoflurane and 450 ml air, maintenance; 1.7 % oxygen, 4% carbon dioxide in air, and 40 ml per kg of body weight per minute, the animals were anesthetized with 3.5 ml/kg anesthetic solution containing solution (studies II, III) were weighed and weighed. At the time of death, all animals were dissected and samples from muscle, ovary, uterus, pancreas and heart were collected and weighed. From liver, spleen, kidney, lung, bowel and peritoneum, additional samples were collected and weighed. Blood was collected and its hematological parameters were evaluated by weekly DW measurements.

Cells were inoculated intraperitoneally. The growth characteristics of SKOV3m cells were evaluated by weekly DW measurements (studies II, III). The development of tumors was followed by weekly MRI and the effect of therapies. The tumor pieces were stained with hematoxylin and eosin and were evaluated by light microscopy.

The safety of the adenovirus was evaluated by monitoring the appearance of symptoms or weight loss demanding the sacrifice. At the time of death, the rats were followed for four weeks after the gene therapy. The animals were followed until the death or appearance of symptoms demanding the sacrifice. At the time of death, the animals were dissected and samples from muscle, ovary, uterus, pancreas and heart were collected and weighed. From liver, spleen, kidney, lung, bowel and peritoneum, additional samples were collected and weighed. Blood was collected and its hematological parameters were evaluated by weekly DW measurements.

The epithelial cell line was obtained from the American Type Culture Collection (HTB176, ATCC, Manassas, USA). The highly aggressive SKOV3m cell line has an epithelial phenotype. The epithelial SKOV3m cell line has been developed previously (Sallinen et al., 2006). It has demonstrated the capacity for metastatic spread and has an epithelial phenotype.
4 Materials and methods

4.1 CELL LINES (STUDIES II, III)

The highly aggressive SKOV-3m cell line has been developed previously from the SKOV-3 cell line (Sallinen et al., 2006). The epithelial human ovarian adenocarcinoma cell line was obtained from the American Type Culture Collection (HTB-77, ATCC, Manassas, USA). McCoy’s 5A medium (Gibco, Invitrogen, Life Technologies) was used to culture the cells. Before the inoculation, cells were trypsinised. After centrifugation, cell pellets were suspended in 1 ml Optimem and counted. A total of 2 x 10⁶ cells were inoculated s.c. into the flank of a nude mouse and further, after development of tumor, tumor pieces were transplanted into the peritoneal cavity of a nude mouse. The growth characteristics of SKOV-3m cell line have an epithelial phenotype and the cells resemble the original cell line. Chromosome analysis using G-banding has demonstrated that SKOV-3m cells were of human origin (Sallinen et al., 2006).

In studies II and III, SKOV-3m cells were cultured in McCoy’s 5A medium (M8403, Sigma-Aldrich, Zwijndrecht, NL) with fetal bovine serum (Sigma-Aldrich, Zwijndrecht, NL) and 1 % penicillin-streptomycin in Opti™-MEM (Sigma-Aldrich, Zwijndrecht, NL). Cells were trypsinized, centrifuged and suspended in Opti-MEM®-GlutaMAX™ (GIBCO™ Life Technologies, USA). The cells were counted and 1x10⁷ SKOV-3m cells were injected intraperitoneally into nude mice (n=30 in study II and n=44 in study III).

4.2 ANIMALS USED IN STUDIES (STUDIES I-III)

The safety and biodistribution of adenoviral gene therapy were evaluated in healthy, immunocompetent HsdHan:Wist (Harlan Laboratories Inc., Füllinsdorf, Switzerland) female rats (n=90, Study I).

In the gene therapy study with ovarian cancer xenografts, seven to 12-week (n=30, study II) or eight week (n=44, study III) old immunodeficient female Balb/cA-nu (Taconic Biosciences) mice were used. The study groups are presented in Tables 10 and 11. All animals were kept in a pathogen-free isolated unit at the National Experimental Animal Center of the University of Eastern Finland, Kuopio. Fodder, water and beddings were autoclaved and animals received fodder and water ad libitum. Intraperitoneal ovarian tumors were generated by inoculating 1x10⁷ SKOV-3m cells (studies II, III) i.p. via a 22 G needle. The development of the tumors was followed by weekly MRI and the effect of therapies on tumor growth and the numbers of tumor cells was evaluated by weekly DW-MRI and relaxation time measurements (studies II, III).

Gene transfers were performed intravenously via the tail vein, the final volume of virus-containing solution was 500 µl (study I) or 200 µl (studies II, III) in 0.9 % saline. In the safety-study, the rats were followed for four weeks after the gene therapy with individual rats being sacrificed on days 3, 14 and 28 (study I). In studies with ovarian cancer xenografts (studies II, III), the mice were followed until their death or the appearance of significant symptoms or weight loss demanding their sacrifice. At the time of death, samples were taken from liver, spleen, kidney, lung, bowel and peritoneum, all tumors were dissected and weighed; ascites fluid was collected and its volume was measured (studies II, III). In addition, samples from muscle, ovary, uterus, pancreas and heart were collected in study I to evaluate the biodistribution and safety of the gene therapies. In the MRI study, during administration of i.v. gene therapy and in the collection of blood samples, all animals were anesthetized with isoflurane (Isoflurane, Baxter Medical AB; induction 4.5 % isoflurane and 450 ml air, maintenance; 1.7-2 % isoflurane and 250 ml air).
All studies were approved by the Experimental Animal Committee of the University of Eastern Finland.

Table 10. Summary of the gene therapies in study groups in study I with rats.

<table>
<thead>
<tr>
<th>Adenoviral vector</th>
<th>N</th>
<th>Titer of each vector per animal (vp)</th>
<th>Total amount of vectors (vp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sR2, sR3</td>
<td>25</td>
<td>5x10^{10} (low-dose)</td>
<td>1x10^{10} / 1 ml</td>
</tr>
<tr>
<td>sR2, sR3</td>
<td>25</td>
<td>5x10^{11} (high-dose)</td>
<td>1x10^{11} / 1 ml</td>
</tr>
<tr>
<td>LacZ</td>
<td>35</td>
<td>1x10^{11}</td>
<td>1x10^{11} / 0.5 ml</td>
</tr>
</tbody>
</table>

Table 11. Summary of the gene therapies in study groups in studies II and III conducted in mice.

<table>
<thead>
<tr>
<th>Adenoviral vector</th>
<th>N</th>
<th>Study</th>
<th>Titer of each vector per animal (vp)</th>
<th>Total amount of vectors (vp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sR2, sR3</td>
<td>13</td>
<td>II</td>
<td>5x10^{10}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
<tr>
<td>sTie1, sTie2</td>
<td>6  + 6 healthy mice</td>
<td>III</td>
<td>5x10^{10}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
<tr>
<td>sR2, sTie2</td>
<td>24</td>
<td>III</td>
<td>5x10^{10}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
<tr>
<td>2x sR2, sTie2</td>
<td>12</td>
<td>III</td>
<td>2 x (5x10^{10})</td>
<td>2x10^{11} / 0.4 ml</td>
</tr>
<tr>
<td>sNRP1, sNRP2</td>
<td>6</td>
<td>II</td>
<td>5x10^{10}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
<tr>
<td>LacZ</td>
<td>11</td>
<td>II</td>
<td>1x10^{11}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
<tr>
<td>CMV</td>
<td>25</td>
<td>III</td>
<td>1x10^{11}</td>
<td>1x10^{11} / 0.2 ml</td>
</tr>
</tbody>
</table>

4.3 VIRAL VECTORS AND GENE THERAPY (STUDIES I-III)

Serotype 5 adenoviral vectors were used in all studies. The vectors were replication-deficient (E1-E3 deleted) and produced in 293 cells under clinical GMP-grade conditions (Himadri Roy et al., 2005). All sVEGFR constructs contain an immunoglobulin Fc domain to ensure the dimerization of the soluble decoy receptors (Jauhiainen et al., 2011; Lin et al., 1998; Mäkinen et al., 2001; H Roy et al., 2005). As a control vector AdLacZ (study I) and adenovirus vector with a cytomegalovirus (CMV) promoter without an insert (AdCMV) were used (Hedman et al., 2003; Puimalainen et al., 1998a). The viruses were tested to be free from lipopolysaccharides, replication competent viruses and bacteriological contaminants. All study groups are presented in Tables 10 and 11, and protocol of the study is presented in Figure 3. The principle of these preclinical studies with ovarian cancer gene therapy in mice is presented in Figure 4.
All studies were approved by the Experimental Animal Committee of the University of Eastern Finland.

Table 10. Summary of the gene therapies in study groups in study I with rats.

<table>
<thead>
<tr>
<th>Adenoviral vector</th>
<th>NTiter of each vector per animal (vp)</th>
<th>Total amount of vectors (vp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sR2, sR3</td>
<td>25 x10^10</td>
<td>1 x10^10/1 ml</td>
</tr>
<tr>
<td>sR2, sR3</td>
<td>25 x10^11</td>
<td>1 x10^11/1 ml</td>
</tr>
<tr>
<td>LacZ</td>
<td>35 x10^11</td>
<td>1 x10^11/0.5 ml</td>
</tr>
</tbody>
</table>

Table 11. Summary of the gene therapies in study groups in studies II and III conducted in mice.

<table>
<thead>
<tr>
<th>Adenoviral vector</th>
<th>Study</th>
<th>Titer of each vector per animal (vp)</th>
<th>Total amount of vectors (vp/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sR2, sR3</td>
<td>II</td>
<td>5 x10^10</td>
<td>1 x10^11/0.2 ml</td>
</tr>
<tr>
<td>sTie1, sTie2</td>
<td>III</td>
<td>6 + 6 health y mice</td>
<td>5 x10^10/0.2 ml</td>
</tr>
<tr>
<td>sR2, sTie2</td>
<td>III</td>
<td>5 x10^10</td>
<td>2 x10^11/0.4 ml</td>
</tr>
<tr>
<td>sNRP1, sNRP2</td>
<td>II</td>
<td>5 x10^10</td>
<td>1 x10^11/0.2 ml</td>
</tr>
<tr>
<td>LacZ</td>
<td>II</td>
<td>1 x10^11</td>
<td>1 x10^11/0.2 ml</td>
</tr>
<tr>
<td>CMV</td>
<td>III</td>
<td>1 x10^11</td>
<td>1 x10^11/0.2 ml</td>
</tr>
</tbody>
</table>

4.3 VIRAL VECTORS AND GENE THERAPY (STUDIES I-III)

Serotype 5 adenoviral vectors were used in all studies. The vectors were replication-deficient (E1-E3 deleted) and produced in 293 cells under clinical GMP-grade conditions (Himadri Roy et al., 2005). All sVEGFR constructs contain an immunoglobulin Fc domain to ensure the dimerization of the soluble decoy receptors (Jauhiainen et al., 2011; Lin et al., 1998; Mäkinen et al., 2001; H Roy et al., 2005).

As a control vector AdLacZ (study I) and adenovirus vector with a cytomegalovirus (CMV) promoter without an insert (AdCMV) were used (Hedman et al., 2003; Puumalainen et al., 1998a).

The viruses were tested to be free from lipopolysaccharides, replication competent viruses and bacteriological contaminants.

All study groups are presented in Table 10 and 11, and protocol of the study is presented in Figure 3.

The principle of these preclinical studies with ovarian cancer gene therapy in mice is presented in Figure 4.

Figure 3. Protocol of the studies II and III. Development of intraperitoneal tumors was followed by weekly MRI, the first visible tumors were observed on day 10 after the SKOV3m cell injections. Gene transfers (GT) were performed one day after the first visible tumors in MRI and the second GT or chemotherapy was performed one week later on day 7. The first MRI and DW-MRI was performed 4 days after the GT and then performed weekly until the death of the mice to evaluate the growth of tumors and molecular changes taking place in the tumors. Plasma samples were collected before the study and on days 6, 13, 21 and 28 (red arrows).

Figure 4. Simplified principles of ovarian cancer gene therapy in mouse xenografts. The human ovarian SKOV3-cancer cells, collected and developed from ascites from a patient with ovarian cancer, were cultured in laboratory for the cell transplantation into a nude mouse. Further, an intraperitoneal ovarian cancer mouse model with the developed SKOV3m-ovarian cancer cells was produced. After the first visible tumors were detected in MRI, the i.v. adenoviral gene therapy with sVEGFR, sNRP or sTie was administered to the mice. At the endpoint, all visible tumors were collected for testing e.g. histological analyses.
4.4 CHEMOTHERAPY (STUDIES I-III)

The chemotherapy was paclitaxel infusion concentrate (Paclitaxel Hospira 6 mg/ml, Hospira UK Limited, England) and carboplatin concentrate (Carboplatin Hospira 10 mg/ml, Hospira UK Limited, England). In the safety and toxicology study I, the chemotherapy was dosed i.v. via a 24 G needle at one and two weeks after the i.v. gene therapy. Chemotherapy was dosed once seven days after the i.v. gene therapy via a 30 G needle i.p. into the mice with ovarian cancer (studies II, III). The dose of paclitaxel for mice was 20 mg/kg i.p. and 6 mg/kg for rats i.v. The dose of carboplatin for mice was 80 mg/kg i.p. and 40 mg/kg for rats i.v. The doses of chemotherapy were optimized for the animals according to previous preclinical studies. The study groups treated with chemotherapy are presented in Table 12.

Table 12. Study groups treated with chemotherapy and gene therapy.

<table>
<thead>
<tr>
<th>Adenoviral vector</th>
<th>sVEGFR2</th>
<th>sVEGFR3</th>
<th>sTie2</th>
<th>LacZ (control)</th>
<th>CMV (control)</th>
<th>- (only chemotherapy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study (I-III) I, II</td>
<td>III</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>1</td>
</tr>
<tr>
<td>N (chemotherapy) I: 20 II: 7</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (total)</td>
<td>27</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel (mg/kg)</td>
<td>6 (I)</td>
<td>20 (II)</td>
<td>20</td>
<td>6</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Carboplatin (mg/kg)</td>
<td>40 (I)</td>
<td>80 (II)</td>
<td>80</td>
<td>40</td>
<td>80</td>
<td>40</td>
</tr>
</tbody>
</table>

4.5 MRI, DW-MRI AND RELAXATION TIME MEASUREMENTS (STUDIES II, III)

In studies II and III, all MRI experiments were performed with a horizontal 7 T magnet (Bruker PharmaScan, Bruker BioSpin MRI GmbH, Ettlingen, Germany) using a volume quadrature coil with 40 mm diameter for signal transmission and reception. In the MRI protocols, the animals were anesthetized with isoflurane inhalation and respiration was monitored during imaging with a pneumatic pillow under the mouse abdomen (SA Instruments, Stony Brook, NY, USA). All imaging was triggered with respiration and respiration was adjusted to 30-40 breaths/min to avoid the substantial movement of mice during the MRI. The temperature of the mice was maintained near to physiological level with a warm water pad.

Multislice fast spin echo T₂-weighted axial images were taken (repetition time (TR) = 3 s, effective echo time (TE) = 44 ms, field of view (FOV) = 40 x 40 mm², matrix size = 256 x 256, slice thickness 1 mm, 30 slices). The slice stack covered the mouse body from bladder to kidneys. For the relaxation time measurements and diffusion-weighted imaging, the largest tumor in the abdomen was selected (study I). Tumor volumes (mm³) and area of the tumor (mm²) were calculated from T₂-weighted anatomical images by hand drawn regions of interest (ROIs). Tumors were detected from surrounding healthy soft tissue due to their deviant intensity and location. All analyses were performed with Aedes software package (aedes.uef.fi) on Matlab platform (MathWorks, Natick, MA).

We assessed the following relaxation time parameters; transversal relaxation time T₂ (adiabatic Hahn double echo preparation with TE = 8 - 22 ms), longitudinal relaxation time in the rotating frame T₁p (spin-lock time = 0 - 45.4 ms, RF amplitude (γB1/(2π)) =1250 Hz) [36], and two novel rotating field frame relaxation time; relaxation along a fictitious field in the 2nd (TRAFF2) and 4th frame (TRAFF4) (study I) (TRAFF or 4 -pulses [37,45], pulse train length of 0 - 36 ms, γB1/(2π) = 1250 Hz for RAFF2 and 648 Hz for RAFF4). The radio frequency field B1 was measured by incrementing the length of a hard pulse (0 - 1.6 ms, γB1/(2π) = 1250 Hz) placed in front of the readout sequence. In all of the relaxation time and B1 measurements,
a fast spin echo sequence was used as readout (TR = 1 s, effective TE = 7.13 ms, FOV = 40 × 40 mm², matrix size = 256 × 256 (study I) and TR = 3 s, TE = 8.5 ms, FOV = 40 × 40 mm², matrix size = 128 × 128, 4 shots (study II). The apparent diffusion coefficient (ADC) of water was determined by diffusion weighted imaging using a spin echo sequence with diffusion weighting in three orthogonal directions (TR = 1 s, TE = 27 ms, b-value = 0, 500 s/mm², FOV = 40 × 40 mm², matrix size = 128 × 64). All relaxation time maps, water ADC-maps and B1 maps were reconstructed on a pixel-by-pixel basis from the signal intensities. Additionally, T₁, T₂, TRAFF2 and TRAFF3 relaxation times and water ADC were analyzed separately for vital and necrotic areas of tumors (study I). Data from all relaxation time measurements were fitted using a single monoexponential decay function. The single cosine function was fitted to B1 data.

4.6 HISTOLOGY AND IMMUNOHISTOCHEMISTRY (STUDIES I-III)

Tissue samples were collected immediately after the sacrifice of animals and tissue samples were immersed in 4 % paraformaldehyde-sucrose for 4 to 6 hours, followed by overnight immersion in 15 % sucrose (Ylä-Herttuala et al., 1990). In study I, tissue samples were collected with disposable sterile pincers in increasing order expected to express the transgenes. Tissue samples were embedded in paraffin and sliced to 5 μm (Microm HM355; Microm). The sections were stained with hematoxylin-eosin (Sigma-Aldrich, Zwijndrecht, NL), CD-34 (clone ME14.7, HyCult Biotech, AA Uden, NL), KI67 (clone MIB-1 Dako M7240, Dako-Cytomation, Glostrup, Denmark), apoptosis (ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit, Merck Millipore, KGaA of Darmstadt, Germany), pericyte α-SMA (clone 1A4, Dako M0851, DakoCytomation, Glostrup, Denmark) and lymphatic vessel hyaluronan receptor-1 (polyclonal rabbit anti-mouse LYVE-1, RealliaTech GmbH, Braunschweig, Germany) stainings. The histological samples in studies I and II were photographed in a Olympus AX70 microscope (Olympus Optical, Japan).

Microvessel density (MVD/ mm²) and total microvascular area (TVA-%) were measured from CD-34-immunostained ovarian tumor sections in a blinded manner from 10 different fields of each tumor at 100 x magnifications. Necrotic areas were avoided when the tumors were being photographed. The total number of KI67 proliferating cells and the numbers of α-SMA pericyte stained vessels were counted from the total area of tumors at 100 x magnifications and tumor apoptotic cells were analyzed at 400 x magnifications. In study III, the histological sections were photographed with Nikon DS-Ri2 microscope and the number of positive cells or stained vessels were counted with NIS-Elements AR 4.30.02 (Nikon, Tokyo, Japan). The sections were analyzed further with AnalySIS (Soft Imaging System, GmbH, Germany) and PhotoShop CS4 (Adobe) softwares.

The anatomy of all stained sections and total number of proliferative tumor cells in KI67 immunohistochemical stainings of tumors were observed in a blinded manner by an expert pathologist in the Department of Pathology in Kuopio University Hospital.

4.7 QUANTITATIVE REVERSE TRANSCRIPTASE-PCR (STUDY I)

Soluble VEGFR2 and sVEGFR3 transgene expressions were detected from muscle, ovary, uterus, kidney, heart, lung, spleen and liver samples in study I. The samples were analyzed from the gene therapy and control groups on days 3, 14 and 28 after the gene transfers. All tissues were snap-frozen in liquid-nitrogen and stored at -70°C for the analysis. Total RNA was extracted with TriReagent (Invitrogen, USA) from 25-100 mg of tissue according to the manufacturer’s instructions. Samples were homogenized in sterile (soft tissue CK14, hard tissue CK28) Precellys Lysing Kit®-tubes (Bertin Technologies, France) with a Precellys 24 (EG-RD-135) -homogenizer. Total RNA samples were treated with DNase (RQI RNase-Free DNase 1 U/μl, Promega, Madison, WI, USA). Reverse transcription in cDNA synthesis
was performed for 0.5 μg of total RNA with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, USA). RT-PCR was performed with TaqMan Gene Expression Assays (Applied Biosystems, USA) (sVEGFR2: Hs00911704_m1, sVEGFR3: Hs01047680_m1). The detection limit for both assays was ten copies.

4.8 CLINICAL CHEMISTRY (STUDY I)

From plasma samples collected on days 0, 3, 7, 14, 21 and 28 the following assays were performed: alanine aminotransferase (ALAT), alkaline phosphatase (AFOS), creatinine (Crea), lactate dehydrogenase (LDH), hemoglobin (Hb), hematocrit (Hct), red blood cells (RBC), white blood cells (WBC), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentrate (MCHC), platelets (PLT) and B-Diff. All assays were performed in the MoVet-laboratory (Kuopio, Finland).

4.9 PROTEIN EXPRESSION, ELISA (STUDIES I, III)

The expression of human sVEGFR2 and human sVEGFR3 (study I) was analyzed with Enzyme-linked immunosorbent assays (ELISA, Quantikine; R&D Systems, Minneapolis, MN) from plasma samples collected at days 0, 7, 14 and 28 after the gene transfers and at the endpoint. Levels were measured after the treatment of AdsVEGFR2 and AdsVEGFR3 as well as when gene therapy was combined with chemotherapy; there was also a control group of AdLacZ.

In study III, the expression of human and mouse VEGFA and Ang2 in ascites fluid in ovarian cancer xenografts was detected from samples collected from animals at the time of death. Ascites was collected with a syringe and frozen in liquid nitrogen, and stored at -70°C prior to the analyses. ELISA (ELISA, Quantikine; R&D Systems, Minneapolis, MN) was performed according to the manufacturer’s instructions.

4.10 STATISTICAL ANALYSES (I-III)

All statistical analyses were performed with IBM SPSS Statistics Software. In studies I and III, the analyses of tumor volumes measured with MRI were performed with mixed model analysis. Otherwise, the significances between the study groups were analyzed with Kruskall-Wallis test and the Mann-Whitney U-test was followed, when appropriate with non-parametric data. The data from survival of the mice (studies II, III) was analyzed with Kaplan-Meier plots of survival and log rank –tests. The correlations were analyzed by the Spearman correlation coefficient test. The results are presented as mean ± standard error of the mean (SEM). Significances are shown as *p< 0.05, **p< 0.01, ***p< 0.001.
5 Results and discussion

5.1 SAFETY OF ADENOVIRAL GENE THERAPY (STUDY I-III)

The safety of adenoviral sVEGFR2 and sVEGFR3 gene therapy was studied in study I as background material for future clinical trials with adenoviral ovarian cancer gene therapy. The body weight of rats in this study was significantly lower only in low-dose AdsVEGFR2 and AdsVEGFR3 and chemotherapy treated group on days 21 (193 ± 17 g) and 28 (198 ± 16 g) after the gene transfers (p< 0.05). In the other groups, the rats’ weights were maintained throughout the follow-up. As the i.v. gene therapy with adenovirus mainly targets liver hepatocytes, toxic effects are possible in addition to signs of an activated immune system (Stone et al., 2007). A summary of the histological alterations is presented in Table 13.

Immediate effects related to adenoviral gene therapy in liver tissue were mild or moderate regeneration with variation in the size and shape of nuclei and some necrosis present on days 14 and 28 after the gene transfers. Liver enzymes ALAT and AFP were transiently elevated in healthy animals when assessed on day 3 and one week after the dose of 1 x 10^{11} vp i.v. gene therapy of AdLacZ. A transient elevation in AFOS was observed on day 7 after the i.v. AdsVEGFR2 and AdsVEGFR3 (2 x 10^{11} vp). In healthy rats, AdsVEGFR2 and AdsVEGFR3 gene therapy at the low dose (2 x 10^{10} vp) was well tolerated, with no sign of liver toxicity or adverse events, also when combined to i.v. chemotherapy of paclitaxel (6 mg/kg) and carboplatin (40 mg/kg). In the groups receiving the low-dose gene therapy, there was no elevation in AFOS activity. The changes observed after the high dose gene transfers in study I reflect the transient stress and strain on liver hepatocytes. In the long-term follow-up of healthy animals, no liver toxicity was observed at 3 months after the AdsVEGFR2 and AdsVEGFR3 gene transfer.

In the subsequent studies performed with mice (studies II and III), ovarian cancer was characterized as a diffuse disease with possible liver metastases, which may have influenced the levels of the assessed liver enzymes. In lung tissues, edema was observed in two animals - one receiving high-dose gene therapy and one from the group low-dose gene therapy with chemotherapy groups. Hemorrhagia was observed in the ovarian tissue of one animal treated with high-dose gene therapy. All of the other observed tissue samples were mainly normal in terms of their histology.

The safety of AdsVEGFR2 and AdsVEGFR3 was studied in multiple organs of healthy rats. Gene therapy did not exert any toxic effects on the kidneys i.e. there were normal creatinine levels in all groups, nor were there any major changes in the lactate dehydrogenase levels (study I). In hematological analyses, transient leukocytosis and neutrophilia were observed in the group receiving the high dose of gene therapy within one week after the gene transfers. Gene therapy did not have any significant effect on the numbers of red blood cells, levels of hemoglobin, hematocrit or platelet count (study I). Previous studies have detected some signs of activated leukocyte production in bone-marrow by VEGF2 and VEGFA, which may be reflected in our findings e.g. the transient leukocytosis (Larrivee et al., 2003; Podar and Anderson, 2005).

In study III, the possibility of liver toxicity with adenoviral dose of 1 x 10^{11} vp was suggested by the local necrosis observed after AdsVEGFR2 and AdsTie2 gene therapy in 33 % of the mice at the endpoint (Figure 4, Table 10). Chemotherapy did not expand the degree of liver necrosis. In study III, the safety of AdsTie1 and AdsTie2-gene therapy was also confirmed and no liver toxicity related to therapy was observed in healthy nude mice (n=6, unpublished data). AdsNRP1 and AdsNRP2 gene therapy did not evoke any liver toxicity (study II).
Our results do point to the possibility of liver toxicity after the adenoviral \textit{i.v.} gene transfers (Figure 5). Similar results have been presented by Mahasreshti et al. (2003) in ovarian cancer xenografts, while \textit{i.v.} gene transfer with AdsVEGFR1 resulted in liver toxicity with a viral dose of $1 \times 10^{10}$ pfu. Previously, with our xenografts, some regenerative alterations and local necrosis in liver samples have been detected at the end of the follow-up, when treated with combination of AdsVEGFR1, AdsVEGFR2 and AdsVEGFR3 (Sallinen et al., 2009) and when combined with chemotherapy of carboplatin and paclitaxel (Sopo et al., 2012).

Clinical trials with adenoviral vectors support the high tolerability of viral therapy, and no significant adverse events have been encountered. In a clinical trial in patients with advanced malignancies, Ad5CMV-p53 therapy at a viral dose of $1 \times 10^{12}$ vp, administered \textit{i.v.} three times daily did not result in severe toxicity (Tolcher et al., 2006). The safety of adenoviral gene therapy has been revealed also in clinical studies. In a 8-year follow-up study in patients with coronary artery disease and treatment with coronary angioplasty and stenting, the local intracoronary adenoviral VEGF-A gene transfer did not increase the incidence of major adverse effects or other diseases as compared to controls (Hedman et al., 2009).

In the studies presented in this thesis, the ovarian cancer mouse xenografts were medicated with the maximally tolerated doses of adenoviral vectors to detect the possible side effects caused by gene therapy. This result supports the possibility of exploiting adenoviral AdsVEGFR2 and AdsVEGFR3 gene therapy and chemotherapy in the treatment of ovarian cancer. With adenoviral \textit{i.v.} therapy, a transient stress in liver cells was detected, although the side effects in other tissues were relatively mild and the results from clinical chemistry and hematology were not abnormal. In summary, according to our studies and previous preclinical and clinical studies, adenoviral gene therapy with soluble receptors seems to be potentially safe should it be used in the treatment of ovarian cancer.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Histology of liver tissues in hematoxylin-eosin stainings. A) Normal liver tissue 10 days after the gene transfer of AdsVEGFR2 and AdsTie2 (study III). B) Liver tissue with high necrosis (80 $\%$, n=1) five days after the gene transfer of AdsVEGFR2 and AdsTie2 (study III). Scale bar= 100 $\mu$m.}
\end{figure}
Table 13. Histological alterations in analysed tissues after the gene therapies.

<table>
<thead>
<tr>
<th>Group</th>
<th>Study</th>
<th>Animal</th>
<th>Total viral dose (vp)</th>
<th>Tissue</th>
<th>Alteration</th>
<th>Number of abnormalities n (%) from animals</th>
<th>Time point</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdLacZ, chemo</td>
<td>I</td>
<td>Rat</td>
<td>$1 \times 10^{11}$</td>
<td>liver</td>
<td>mild/moderate regeneration</td>
<td>6 (60)</td>
<td>14, 28</td>
</tr>
<tr>
<td>High dose sR2, sR3</td>
<td>I</td>
<td>Rat</td>
<td>$2 \times 10^{11}$</td>
<td>ovary</td>
<td>hemorrhagia</td>
<td>1 (6)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lung</td>
<td>edema, hemorrhagia</td>
<td>1 (6)</td>
<td>28</td>
</tr>
<tr>
<td>Low dose sR2, sR3, chemo</td>
<td>I</td>
<td>Rat</td>
<td>$2 \times 10^{10}$</td>
<td>lung</td>
<td>mild regeneration</td>
<td>1 (6)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High dose sR2, sR3, chemo</td>
<td>I</td>
<td>Rat</td>
<td>$2 \times 10^{11}$</td>
<td>liver</td>
<td>mild regeneration</td>
<td>8 (53)</td>
<td>14, 28</td>
</tr>
<tr>
<td>sR2, sTie2</td>
<td>III</td>
<td>Mouse</td>
<td>$1x 10^{11}$</td>
<td>Liver</td>
<td>80% liver necrosis in 1 animal 5 days from GT, 60% liver necrosis in 1 animal 7 days from GT</td>
<td>4 (33%)</td>
<td>Endpoint</td>
</tr>
<tr>
<td>2 x sR2, sTie2</td>
<td>III</td>
<td>Mouse</td>
<td>$2x (1x 10^{11})$</td>
<td>Liver</td>
<td>50% liver necrosis in 2 animals at days 27 and 35 from GT</td>
<td>5 (42%)</td>
<td>Endpoint</td>
</tr>
<tr>
<td>CMV, chemo</td>
<td>III</td>
<td>Mouse</td>
<td>$1x 10^{11}$</td>
<td>Liver</td>
<td>necrosis (2-5 %)</td>
<td>3 (25 %)</td>
<td>Endpoint</td>
</tr>
</tbody>
</table>

5.2. ANTIANGIOGENIC AND ANTILYMPHANGIOGENIC GENE THERAPY IN OVARIAN CANCER XENOGRAFTS (STUDIES I-III)

5.2.1 Survival after the adenoviral gene therapy (Studies I-III)

In the mouse model of ovarian cancer, AdsVEGFR2 and AdsVEGFR3 -gene therapy significantly prolonged the survival of mice (mean survival 30 ± 2 days) compared to AdLacZ-controls (24 ± 1 days, P=0.020) or other groups (AdsVEGFR2, AdsVEGFR3 and chemotherapy 23 ± 1 days, P=0.007 or AdsNRP1 and AdsNRP2 24 ± 2 days, P=0.009) (study II, Figure 6, Table 14). The survival advantage was achieved with AdsVEGFR2 and AdsTie2 -gene therapy alone and when combined with chemotherapy in study III with prolonged mean survival of 29 ± 2 days compared to only AdsVEGFR2 and AdsTie2 -gene therapy (20 ± 3 days, P=0.017). Altogether, the survival of the mice with ovarian cancer was prolonged if they had been treated with the adenoviral gene therapy of AdsVEGFR2, AdsVEGFR3 or AdsVEGFR2, AdsTie2 and chemotherapy.

The survival of the control groups of AdLacZ and AdCMV treated mice in studies I-III were consistent (mean 24 ± 1 days and 23 ± 2 days), highlighting the comparability of the results between the studies. In study III, the survival of mice with ovarian cancer was prolonged as a result of chemotherapy, and the survival was even longer in groups when chemotherapy was combined with AdCMV (mean 28 ± 2 days) or AdsVEGFR2 and AdsTie2 -gene therapy (mean 29 ± 2 days) as compared to only AdsVEGFR2 and AdsTie2-gene therapy treated mice (20 ± 2 days). This result emphasizes the positive impact of chemotherapy in promoting the survival of mice with ovarian cancer xenografts. Interestingly in study II, when AdsVEGFR2 and AdsVEGFR3 gene therapy was combined with chemotherapy, the survival was shortened (mean 23 ± 1 days) compared to mice treated with only gene therapy (mean survival 30 ± 2 days). The role of combined chemotherapy and adenoviral gene therapy in survival is somewhat unclear, as chemotherapy exerted conflicting effects on survival in studies II and III. In study III, the proliferation of ovarian tumor cells (KI67) was increased after AdsVEGFR2 and AdsTie2
gene therapy, although no similar response was seen after AdsVEGFR2 and AdsVEGFR3 gene therapy in study II. This may be related to the reduced chemotherapy response in ovarian cancer xenografts and may explain the poor survival results obtained in study III. Further studies will be needed to evaluate the role of VEGFR2, VEGFR3 and Tie2 signaling in ovarian cancer and also in tumor cell proliferation.

Surprisingly the mean survival with AdsVEGFR2 and AdsTie2 was shorter than that of the AdCMV controls (20 ± 2 days vs. 23 ± 2 days) and these animals also had the lowest median survival (17 days) in study III. Similar to previous studies with SKOV3m ovarian cancer xenografts, significantly shortened survival has been observed with AdsTie1 and AdsTie2 -gene therapy (19 ± 3 days). In that study, the macroscopic alterations in liver as well as elevated ALAT values were also detected, reflecting the possibility of hepatotoxic effects of AdsTie1 and AdsTie2 -gene therapy (Sallinen et al., 2011). These results may reveal the possibility of toxic effects related to AdsTie2 gene therapy and the unfavourable survival in our mouse model of ovarian cancer. In previous studies with the same ovarian cancer xenografts, Sallinen et al. observed the prolonged survival of mice with combined gene therapy of AdsVEGFR1 and AdsVEGFR3 (mean 55 ± 16 days) (Sallinen et al., 2009) as well as in another study with AdsVEGFR1, -3 and AdsTie2 (mean 30 ± 2 days) (Sallinen et al., 2011). The survival benefit of AdsTie2 gene therapy in ovarian cancer needs to be clarified, although when combined with soluble VEGF-receptors, it seems to relate to improved survival. AdsNRP1 and AdsNRP2 did not have any significant effect on the survival of the mice, indicating their indirect impact on survival in ovarian cancer xenografts. As far as we are aware, no preclinical animal studies with ovarian cancer and soluble neuropilins have been published.

In healthy rats with a normal immune response, no immediate deaths were encountered related to i.v. adenoviral gene therapy was. When AdsVEGFR2 and AdsTie2 -gene therapy was administered twice, the immediate death of mice with ovarian cancer appeared after the second i.v. gene transfer (total viral dose, 2 times of 1 x 10^{11} vp/ml). In this group, the survival of mice was 8.6 ± 2.6 days after the second gene transfer and seven from 12 mice had to be sacrificed within one week after the second gene transfer. The curtailed survival of mice may be related to the possible toxic effect of viral gene therapy, but it has also to be noted that these mice had a more advanced stage of the disease at the time of second i.v. gene transfer, which may have reduced their tolerance of repeated gene therapy.

In our studies with ovarian cancer gene therapy, the human SKOV3m ovarian cancer cell line was used; this cell line is known to display high aggressivity with relatively similar characteristics as found in human ovarian cancer. This cell line has been derived from a patient with ovarian cancer, with the tumor cells being collected from peritoneal ascites. Furthermore, the cell line was transplanted subcutaneously into nude mice and the SKOV3m cell line was developed by transplanting these tumors into the peritoneal cavity of another nude mouse. One could speculate that the high aggressivity of our ovarian cancer model in mice and the rapid progression of the disease mean that this model may not be optimal for investigating the effects of repeated i.v. gene therapy.

In conclusion, since there was a prolongation of the survival of the mice receiving the combined chemotherapy of paclitaxel and carboplatin and adenoviral gene therapy of sVEGFR2 and sTie2, this can be interpreted as evidence that this combination has potential in the treatment of human ovarian cancer (study III). A survival advantage was also achieved with AdsVEGFR2 and AdsVEGFR3 gene therapy (study II).
Ovarian cancer xenografts, significantly shortened survival has been observed (Sallinen et al., 2011), although when combined with ovarian cancer and adenoviral gene therapy of AdsVEGFR2 and AdsVEGFR3 (mean 55 ± 16 days), the effects of repeated gene therapy were more advanced compared to the group receiving only AdsVEGFR2 and AdsTie2 -gene therapy. Chemotherapy exerted a notable effect on survival in study III also in AdCMV and chemotherapy -group.

Table 14. Survival of the mouse ovarian cancer xenografts (studies II and III).

<table>
<thead>
<tr>
<th>Group (study)</th>
<th>Mean survival (days after SKOV3m i.p.)</th>
<th>Median</th>
<th>Min-Max survival (days after SKOV3m i.p.)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdLacZ (II)</td>
<td>24</td>
<td>24</td>
<td>20-32</td>
<td>11</td>
</tr>
<tr>
<td>AdsR2, AdsR3 (II)</td>
<td>30</td>
<td>32</td>
<td>24-33</td>
<td>6</td>
</tr>
<tr>
<td>AdsR2, AdsR3, chemo (II)</td>
<td>23</td>
<td>24</td>
<td>19-26</td>
<td>7</td>
</tr>
<tr>
<td>AdsNRP1, AdsNRP2 (II)</td>
<td>23</td>
<td>22</td>
<td>16-30</td>
<td>6</td>
</tr>
<tr>
<td>AdCMV (III)</td>
<td>23</td>
<td>23</td>
<td>12-40</td>
<td>12</td>
</tr>
<tr>
<td>AdCMV, chemo (III)</td>
<td>28</td>
<td>27</td>
<td>20-39</td>
<td>10</td>
</tr>
<tr>
<td>AdsR2, AdsTie2 (III)</td>
<td>20</td>
<td>17</td>
<td>15-34</td>
<td>10</td>
</tr>
<tr>
<td>AdsR2, AdsTie2, chemo (III)</td>
<td>29</td>
<td>27</td>
<td>21-41</td>
<td>10</td>
</tr>
<tr>
<td>AdsR2, AdsTie2 x2 (III)</td>
<td>27</td>
<td>24</td>
<td>19-46</td>
<td>12</td>
</tr>
<tr>
<td>AdsTie1+AdsTie2 (III)</td>
<td>21</td>
<td>22</td>
<td>17-25</td>
<td>3</td>
</tr>
</tbody>
</table>

5.2.2 Transgene expression after the i.v. gene transfer (Studies I-III)
The safety and biodistribution study with adenoviral vectors demonstrated that mRNA expression of sVEGFR2 and sVEGFR3 transgenes in liver was detectable three days after the gene transfers. Chemotherapy prolonged the transgene expression of sVEGFR2 and sVEGFR3 in liver until day 28 after the gene transfers (study I). The expression of mRNA of transgenes in liver was confirmed with qRT-PCR on day 6 after the gene transfers of AdsVEGFR2 and AdsTie2 (study III). In study III, the mRNA expression of the sVEGFR2 and sTie2 transgenes in liver could be detected until the end of the follow-up, although the expression range was relatively low in some samples. Concomitant administration of
chemotherapy and adenoviral gene therapy changed the transgene expression i.e. there was delayed expression in liver samples (Table 12). The variation in mRNA of transgene expression may be related to different transduction efficacy of adenoviral vectors in xenografts between the animals and as well between the adenoviral vectors.

The production of soluble VEGF-, NRP- and Tie-receptors was confirmed with in vitro and western blotting analysis before the animal studies (data not shown). The levels of soluble proteins in mice were detected weekly with ELISA from plasma samples and, as expected, were under the detection limit before the gene transfer. The analyses of soluble proteins with ELISA succeeded for sVEGFR2 in all experiments (Table 15). Unfortunately, because of technical challenges and detection of mouse endogenous soluble receptors, the analyses for sVEGFR3 or sTie2 were unsuccessful.

The protein levels were below the detection limits on day 28 in all groups reflecting the transient production of soluble proteins in liver (study I). In study I, the VEGFR2 level in plasma was highest on day 7 with the high dose AdsVEGFR2 and AdsVEGFR3 gene therapy (1.27 ± 0.36 ng/ml). With low dose gene therapy, the sVEGFR2 levels in plasma were elevated on day 7 (0.72 ± 0.38 ng/ml). In study III, the sVEGFR2 protein levels were elevated already by day 7 (2.55 ± 0.39 ng/ml, N=22) after AdsVEGFR2 and AdsTie2 gene therapy, although the highest protein levels were measured on day 21 in gene therapy group (3.80 ± 2.26 ng/ml, N=3) and on day 35 in gene- and chemotherapy group (6.42 ± 0.55 ng/ml, N=2). The prolonged expression of soluble proteins in plasma was observed with chemotherapy (study I), as the sVEGFR2 protein expression lasted one week longer with combined therapy of AdsVEGFR2 and AdsVEGFR3 and chemotherapy in study I and furthermore the highest protein levels after AdsVEGFR2 and Tie2 gene therapy were observed at day 35. The prolonged expression of sVEGFR2 may be related to prolonged survival in chemotherapy and AdsVEGFR2 and AdsVEGFR3 gene therapy group (study II). Despite the presence of the protein, there was no survival benefit with prolonged sVEGFR2 expression in plasma observed in study II with AdsVEGFR2 and AdsTie2 gene therapy. Nonetheless, increased expression levels of VEGFR2 and Ang2 in the circulation are very significant in the progression of ovarian cancer as well as with its poor prognosis in a similar manner to the increased levels of VEGF (Sallinen et al., 2014). Thus it would be important to develop targeted therapies against these growth factors and their receptors (Brunckhorst et al., 2014; Spannuth et al., 2009)

In our studies, the level of soluble VEGFR2 was transiently higher than 1 ng/ml when the high gene therapy dose of 1 x 10^11 vp was used; this is believed to be the effective level of soluble VEGF-proteins in plasma if one wishes to exert antiangiogenic or antilymphangiogenic effects in the tumor vasculature (Takei et al., 2007). This putative effective level of sVEGFR2 in plasma was not attained with low dose gene therapy (1 x 10^10 vp), indicating that the high viral dose is more likely to achieve a therapeutic response in epithelial ovarian tumors. Interestingly, the levels of sVEGFR2 in plasma were notably higher when mice were treated with AdsVEGFR2 and AdsTie2 gene therapy as compared to AdsVEGFR2 and AdsVEGFR3 therapy (Table 12). This indicates that angiopoietin and VEGF-signaling have some potentiating signaling pathways and in ovarian cancer, their signaling is connected to each other.

These results indicate that we achieved only transient expression with adenoviral-associated production of soluble receptors, which may also be related to the possibility of activated cellular immunity as a response to the viral therapy (Alzuguren et al., 2015; Langford et al., 2009; Suda et al., 2003; Yang et al., 1994). In addition, the other challenges encountered with adenoviral gene therapy are vector targeting to cells other than the targets, and the possibility of activated immune response leading to decreased levels of effective viral vectors (Bessis et al., 2004). Both in our experiments as well as in other preclinical cancer studies, the mice with ovarian cancer were immunodeficient. Immunodeficient mice are unable to mount an active immune response, and this is useful when developing gene therapies. Furthermore, in study I, chemotherapy prolonged the
transgene expression in plasma for one week, the results may reflect the higher transduction efficacy of viral vectors in liver cells after chemotherapy. In clinical settings, prolonged transgene expression may be beneficial in the treatment of ovarian cancer patients and this will favour the combined AdsVEGFR2 and AdsVEGFR3 gene- and chemotherapy. Recent studies have described new strategies to improve the vector targeting, and to inhibit the local or systemic adverse effects (Chira et al., 2015b). The expression of the adenoviral targets, i.e. the CAR-receptors in tissues such as in liver, has been claimed to be relevant in adenoviral mediated gene therapy and its efficacy in ovarian cancer (Kim et al., 2002).

Table 15. Expression of sVEGFR2 in plasma samples after combined gene therapies and chemotherapy. The animals used were rats in study I and mice in study III.

<table>
<thead>
<tr>
<th>Therapy (study)</th>
<th>Detected protein</th>
<th>Highest gene expression in plasma (day)</th>
<th>The level of soluble receptor in plasma (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sR2, sR3 (high; 1 x 10¹¹ vp) (I)</td>
<td>sVEGFR2</td>
<td>7</td>
<td>1.27 ± 0.36</td>
</tr>
<tr>
<td>sR2, sR3 (low; 1 x 10¹⁰ vp) (I)</td>
<td>sVEGFR2</td>
<td>7</td>
<td>0.72 ± 0.38</td>
</tr>
<tr>
<td>sR2, sR3 (high), chemo (I)</td>
<td>sVEGFR2</td>
<td>7</td>
<td>0.75 ± 0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0.59 ± 0.26</td>
</tr>
<tr>
<td>sR2, sR3 (low), chemo (I)</td>
<td>sVEGFR2</td>
<td>7</td>
<td>0.67 ± 0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0.88 ± 0.040</td>
</tr>
<tr>
<td>sR2, sTie2 (1 x 10¹² vp) (III)</td>
<td>sVEGFR2</td>
<td>21</td>
<td>3.80 ± 2.26</td>
</tr>
<tr>
<td>sR2, sTie2 (1 x 10¹¹ vp), chemo (III)</td>
<td>sVEGFR2</td>
<td>35</td>
<td>6.42 ± 0.55</td>
</tr>
</tbody>
</table>

5.2.3 Inhibited growth of ovarian tumors with gene therapy (Studies II, III)
Mice developed the intraperitoneal tumors within 10 days after the SKOV3m cell injections. The size of tumors when examined in the first MRI on day 10 was 217 ± 33 mm³ in study II and 173 ± 14 mm³ in study III. At the end of the follow-up, the most effective treatment for reducing tumor growth i.e. 50 % lower tumor weights, was obtained after AdsVEGFR2 and AdsTie2 -gene therapy (1.83 ± 0.40 g) as compared to AdCMV and chemotherapy-controls (3.68 ± 0.31 g, P<0.01) (study III) (Figure 7).

According to results assessing the tumor volumes measured by MRI at weekly intervals, significantly reduced tumor growth was observed already at one week after the AdsVEGFR2 and AdsTie2 -gene therapy (660 ± 91 mm³) as compared to AdCMV-group (983 ± 93 mm³, P=0.048 study III). Parallel results were also observed two weeks after the gene transfer (1061 ± 159 mm³ in AdsVEGFR2, AdsTie2 -gene therapy group and 1680 ± 194 mm³ in AdCMV-group, P=0.021). In study II, AdsVEGFR2 and AdsVEGFR3 -gene therapy significantly reduced the tumor growth four days after the gene transfer in comparison to AdLacZ-controls (407.3 ± 113.7 mm³ vs. 960.4 ± 86 mm³, P=0.004). The effect of adenoviral antiangiogenic gene therapy in tumor growth reflected the production of soluble receptor proteins in plasma, and we postulate that high protein production was the reason for the inhibition of tumor growth during the two weeks after the gene transfers.

The impact of VEGFR coreceptors AdsNRIP1 and -2 (3.07 ± 0.37 g) on tumor growth was not significant as compared to AdLacZ controls (3.40 ± 0.44 g). The combined AdsTie1 and - 2 gene therapy (2.79 ± 0.58 g) or dosing gene therapy twice with AdsVEGFR2 and AdsTie2 did not significantly reduce the tumor growth as compared to AdCMV or AdCMV with chemotherapy (unpublished data). The poor effect on tumor growth inhibition may be related to the relatively short survival of the mice in these groups after the gene transfers,
especially after the second gene therapy dosing (26.6 ± 2.55 days, 8.6 ± 2.6 days after the second gene transfer).

In the studies of this thesis, none of the mice with ovarian cancer were totally cured with antiangiogenic gene therapy and chemotherapy. The potential of AdsTie2 to reduce growth of tumors has been presented in previous studies with AdsVEGFR1 and AdsVEGFR3 in ovarian cancer xenografts (mean at the end of the follow-up 2.3 ± 0.25 g) (Sallinen et al., 2011). The potential of AdsTie2 to reduce growth of ovarian tumors was evident when it was combined with AdsVEGFR2 (1.83 ± 0.40 g). Our results are in line with the data from another study, where AdsVEGFR2 restricted the growth of ovarian tumors in mice (Wu et al. 2006). The efficacy of combined AdsVEGFR2 and AdsTie2 gene therapy in restricting tumor growth implies there is a synergistic effect, when targeting both VEGF and angiopoietin-related signaling pathways.

One hurdle to the development of antiangiogenic gene therapy in cancer is the potential resistance to therapy. Several hypotheses have been proposed to account for the unfavourable antiangiogenic therapy response i.e. angiogenic tumors adapt to angiogenic inhibitors, mutations in genes encoding the target of the drugs or activation of alternative signaling pathways to reinstate tumor growth (van Beijnum et al., 2015). Additional challenges are reinitiation of tumor angiogenesis by bone-marrow derived pro-angiogenic cells, increased pericyte coverage of tumor microvessels, stabilization of vessels and reduced efficacy of anti-VEGF therapies, and activation of the normal vasculature to invasion by the tumors (Bergers, Hanahan 2008). Recently a connection has been observed between bone-marrow derived myeloid cells and tumor cells after the antiangiogenic therapy. Accordingly, the damage in tumor vasculature induces hypoxia in the tumor tissue, leading to upregulation of myeloid cells and the increased presence of myeloid cells in treated tumors. Myeloid cells are known to increase angiogenesis by inducing the production of angiogenic and tissue-remodelling factors (Ferrara, 2010; Shojaei and Ferrara, 2008).

![Figure 7. Summary of the total dissected tumor weights of mice at the endpoint. AdsVEGFR2 (sR2) and AdsTie2 (sTie2) significantly reduced the tumor growth as compared to AdCMV and chemotherapy in study III (P<0.01).](image-url)
5.2.4 Effects of gene therapy on ascites formation (Studies II, III)

Gene therapy with AdsVEGFR2 and AdsTie2 had a significant effect on reducing the formation of peritoneal ascites (0.80 ± 0.36 ml) as compared to AdCMV controls (2.4 ml ± 0.56 ml, P=0.029) (Figure 8). Ascites formation was significantly reduced, when AdsVEGFR2 and AdsTie2 -gene therapy was combined with chemotherapy with paclitaxel and carboplatin (0.19 ml ± 0.077 ml), reducing the amount of ascites by 90 % as compared to controls of AdCMV (2.4 ml ± 0.56 ml, P=0.01). Ascites formation was also significantly reduced in the gene- and chemotherapy combination group, when compared to AdCMV and chemotherapy (1.1 ml ± 0.30 ml, P=0.003). These results are presented in study III of this thesis and in Figure 8.

In study II, the AdsVEGFR2 and AdsVEGFR3 -gene therapy with chemotherapy did not significantly reduce the accumulation of ascites as compared to AdLacZ-controls (1.75 ml ± 0.72 ml vs. 3.58 ml ± 1.36 ml). Although not statistically significant, the amount of ascites at the endpoint was 49 % lower in AdsVEGFR2 and AdsVEGFR3 -gene therapy group than in AdLacZ-control group. Chemotherapy with AdsVEGFR2 and AdsTie2 reduced the amount of ascites by 79 % as compared to only AdsVEGFR2 and AdsTie2 gene therapy and by 32 % when added to gene therapy of AdsVEGFR2 and AdsVEGFR3, indicating the greater effect to inhibit ascites when chemotherapy is combined to gene therapy. AdsTie1 and -2 gene therapy slightly reduced the ascites formation as compared to CMV-controls. Repeated gene therapy with AdsVEGFR2 and AdsTie2 had a more effective role in reducing the accumulation of ascites (1.39 ± 0.39 ml), but the effect was not greater than seen with a single dose of AdsVEGFR2 and AdsTie2 gene therapy (1.33 ml ± 0.49 ml) or gene therapy with chemotherapy (0.28 ml ± 0.10 ml, P=0.006, unpublished data). In study III, AdsVEGFR2 and AdsTie2 gene therapy reduced the amount of bloody ascites in addition to the accumulation of ascites; in the gene therapy group, the number of bloody ascites at the endpoint was lower (30 % of mice) as compared to controls of AdCMV and AdCMV with chemotherapy (80 % of mice), although the difference between the groups was not statistically significant.

Chemotherapy inhibited the ascites formation reflecting the reduced growth of tumor cells. In clinical trials, chemotherapy plays a valuable role in the treatment of malignant ascites as palliative therapy (Sangisetty, 2012). The high production of VEGFs from tumor cells has been linked to the formation of malignant ascites by increased blood vessel permeability lining the peritoneal cavity (Nagy et al. 1995). For example, VEGF-inhibitors e.g. i.v. aflibercept or i.p. catumaxomab, have been effective in reducing the formation of ascites in clinical trials (Berek et al., 2014; Gotlieb et al., 2012). The reduced ascites formation has been attributed in many studies to inhibition of VEGFR-signaling, for example with the VEGF-Trap and VEGF-antibody, bevacizumab (Kipps et al., 2013; Smolle et al., 2014). Nonetheless, the relevance of alternative signaling pathways in addition to VEGFR has been presented in several antiangiogenic studies in solid tumors and ovarian cancer (Hata et al., 2004; Madsen et al., 2012). In a preclinical study in mice with ovarian cancer, Souza et al. described the potential of soluble Tie2 and sVEGFR1 to inhibit ascites accumulation and slow the growth of diffuse intraperitoneal tumors as well as prolonging the survival of the animals (D’Souza et al., 2010). In another experiment, the levels of VEGF in ascites were significantly lower than in untreated mice with ovarian cancer after i.m. recombinant-AAV therapy (Isayeva et al., 2005). Furthermore, a lower amount of hemorrhagic ascites was observed after i.p. rAAV gene therapy, indicating that antiangiogenic therapy can influence the permeability of peritoneal blood vessels (Isayeva et al., 2006). In previous studies with single therapies of AdsVEGFR2 or sTie2, the formation of ascites was also inhibited in ovarian cancer xenografts (Sallinen et al., 2011, 2009). According to our results, the role of VEGFR2, VEGFR3 and Tie2 have a role in ascites formation in ovarian cancer. Furthermore, we hypothesize that the blockade of these two alternate antiangiogenic signaling pathways may be beneficial in antiangiogenic ovarian cancer therapies.
5.2.5 Cellular effects of gene- and chemotherapy in ovarian tumors (Studies II, III)

In our preclinical model of ovarian cancer mouse xenografts, the intraperitoneal tumors were highly aggressive, atypical high grade 3 serous adenocarcinomas with variable sizes of nuclei and limited stroma. After the AdsVEGFR2, AdsVEGFR3 or AdsNRP1, AdsNRP2 gene therapies, the tumor tissue had been partly replaced with fibrosis. The histology of ovarian tumors is presented in Figure 9.

The proliferation of tumor cells measured by KI67 was significantly lower when treated with AdsVEGFR2 and AdsVEGFR3 gene therapy (30-45 %, mean 38 %) and when combined with chemotherapy (25-55 %, mean 37 %) as compared to AdLacZ-controls (45-90 %, mean 67 %, P=0.002 and P=0.002). According to the values of KI67 observed by the pathologist, the proliferation of tumor cells in AdCMV group was 20-60 % (mean 42 %), interestingly the AdsVEGFR2 and AdsTie2 -gene therapy increased the tumor cell proliferation (30-80 %, mean 49 %) and this was slightly reduced when combined with chemotherapy (25-70 %, mean 43 %); these results were not statistically significant. According to other studies, the activated tumor cell growth and increased aggressivity of tumor cells may occur and the tumor cells may activate compensatory mechanisms to combat the inhibited angiogenic signaling e.g. these may be due to activation of alternative signaling pathways in cancer cells or in the tumor microvessels (Adham et al., 2010). The potential of increased NRP1 expression has been postulated as one of these alternative signaling pathways. Thus, in the future, the combined therapy with AdsVEGFR2, AdsTie2 and AdsNRP1 would be beneficial when utilizing ovarian cancer xenografts.

The necrotic areas in tumors were evaluated with histological stainings and DW-MRI. The amount of necrosis in the tumors was highest when we applied repeated gene therapy of AdsVEGFR2, AdsTie2 (38.9 ± 8.9 %, n.s.). Chemotherapy increased the necrosis when combined to AdCMV (11.3 ± 5.6 % without chemotherapy vs. 21.5 ± 6.9 % with chemotherapy) or AdsVEGFR2, AdsTie2 gene therapy (7.5 ± 4.2 % vs. 12.2 ± 8.5 %). No statistically significant differences were observed in the extents of tumor necrosis. Gene- or chemotherapy did not exert any significant effect on number of apoptotic cells in the tumors.

Figure 8. Summary of the volume of ascites fluid in the different groups at the endpoint in mice. AdsVEGFR2 (sR2) and AdsTie2 (sTie2) gene therapy with chemotherapy was clearly effective at reducing the formation of ascites in study II. AdsVEGFR2 and AdsVEGFR3 with chemotherapy also showed a trend towards being effective in reducing the formation of ascites (n.s.).
tumors at the endpoint, although the number of apoptotic cells in tumors was higher after the AdsVEGFR2 and AdsTie2–gene therapy (34.0 ± 11.1 /mm²) or AdsVEGFR2 and AdsTie2–genetherapy and chemotherapy (34.5 ± 15.1 /mm²) and AdCMV (37.0 ± 8.6 /mm²) in comparison to AdCMV and chemotherapy (15.8 ± 4.5 /mm²). The result indicates that tumor cells tend to undergo to necrosis instead of apoptosis as a result of chemotherapy. As a conclusion, chemotherapy increased the destruction of the tumor cells and furthermore, the effect was potentiated when combined with adenoviral gene therapy.

Figure 9. Histology of ovarian tumors A) HE staining of high grade serous ovarian cancer with high mitotic activity and variable sizes of nuclei. B) HE staining of ovarian tumor with highly necrotic area (star). C) KI67 staining of proliferative tumor cells. D) Apoptotic cells in ovarian tumor after the AdCMV and chemotherapy (arrow). Bar=100 µm.

5.2.6 Antiangiogenic and antilymphangiogenic effects of gene therapy (Studies II, III)
The microvessel density (MVD) and the total area of microvessels (TVA) in tumors were analyzed. AdsVEGFR2 and AdsVEGFR3 gene therapy exerted the most effective antiangiogenic effect on tumor microvessels with significantly reduced amounts of microvessels being observed after the therapy (MVD 48.7 ± 4.50 /mm² and TVA 1.24 ± 0.11 %, vs. control of AdLacZ 84.5 ± 10.0 /mm² and 3.23 ± 0.70 %, P=0.001 and P=0.005) (Figure 10). In addition, the combined AdsVEGFR2, AdsVEGFR3 and chemotherapy had a significant antiangiogenic effect on tumor microvessels (MVD 59.7 ± 4.20 /mm², P=0.002 and TVA 1.2 ± 0.10 %, P=0.003), like AdsNRP1 and -2 (MVD; 62.0 ± 4.60 /mm², P=0.026 and TVA; 1.00 ± 0.31 %, P=0.013) (study II). In study III, the antiangiogenic effect was evident with AdsVEGFR2 and AdsTie2–gene therapy (MVD 44.0 ± 10.2 /mm² and TVA 1.01 ± 0.30 %) and when combined to chemotherapy (MVD 42.0 ± 7.02 /mm² and TVA 1.23 ± 0.22 %), as compared to AdCMV or AdCMV and chemotherapy groups. In study III, no statistically significant antiangiogenic effect in ovarian tumors was reached, which may be related to the increased aggressivity of the ovarian cancer cell line used in this study and the lower therapy effect of AdsVEGFR2 and AdsTie2 as compared to AdsVEGFR2 and AdsVEGFR3–gene therapy in study II. In addition, the increased tumor cell proliferation present in study III after AdsVEGFR2 and AdsTie2 therapy may have impacted on the poor antiangiogenic therapy response.
When comparing the control groups of studies II and III, MVD and TVA were notably higher in AdLacZ treated mice (84.5 ± 10.0 /mm² and 3.23 ± 0.70 %) compared to AdCMV-controls (MVD; 58.2 ± 8.52 /mm² and TVA; 1.39 ± 0.19 %), which may indicate that tumor growth had possibly been activated after i.v. AdLacZ, although the mechanism is unknown. The total vascular area of tumor vessels was significantly higher when gene therapy of AdsVEGFR2 and AdsTie2 (2.33 ± 0.30 %) was administered two times in comparison to AdCMV (1.39 ± 0.19 %, P=0.028) or AdCMV and chemotherapy (1.41 ± 0.33 %, P=0.025), AdsVEGFR2 and Tie2 (1.01 ± 0.29 %, P=0.007) and AdsVEGFR2, AdsTie2 and chemotherapy (1.22 ± 0.22 %, P=0.016) (unpublished data, study III). The formation of neutralizing antibodies against adenoviruses may have lead to a reduced efficacy with this kind of repeated viral gene therapy (Kimball et al., 2010). High aggressivity of ovarian cancer and the possibility that the general condition of the mice had declined after the second gene therapy may also be related to poor antiangiogenic effect of repeated gene therapy.

In our previous studies with the ovarian cancer mouse model, the efficacy on antitumor growth by blocking these two signaling pathways has been revealed with the combination of AdsVEGFR1, -3 and AdsTie2 (Sallinen et al. 2011). Unfortunately in this aggressive model of ovarian cancer, no significant differences in microvessel density or total vascular area of tumors were evident in study III with the AdsVEGFR2, AdsTie2 gene therapy. In study II, the antiangiogenic effect as reflected in the lower number of microvessels in the tumors, was observed with AdsVEGFR2 and AdsVEGFR3 -gene therapy. The result reflects the higher antiangiogenic effect with VEGF-signaling related therapy instead of combined VEGF- and angiopoietin therapy. Further studies will be needed to confirm the relevance of Tie-angiopoietin signaling pathway in ovarian cancer angiogenesis. One of the problems encountered in gene therapy trials has been the low transduction efficacy of viral vectors, therefore the development of vectors with higher transduction efficacy would be beneficial (Kanerva et al., 2002b).

The coverage of tumor microvessels with pericytes in tumors did not change significantly as a result of the gene therapies. However, chemotherapy reduced the pericyte coverage with AdCMV and chemotherapy (26.05 ± 4.87 /mm²) or AdsVEGFR2 and AdsTie2 and chemotherapy (43.03 ± 14.4 /mm²) compared to only AdCMV (71.48 ± 27.72 /mm²) or AdsVEGFR2 and AdsTie2 -gene therapy (64.28 ± 25.03 /mm²) in study III. There was an extensive range of pericyte coverage in ovarian tumor microvessels i.e. from 7.3 up to 345.2 /mm². Ang-Tie2 is considered to have a role in regulating the pericyte coverage in tumor microvessels. Stabilization of pericyte coverage in tumor microvessels is believed to increase the delivery of chemotherapeutic agents into tumors, and to further improve the therapeutic effects (van Beijnum et al., 2015). Interestingly in study III, the coverage of pericytes was reduced after the chemotherapy and in these groups, the formation of ascites was also reduced. In this study, the gene therapy with AdsVEGFR2 and AdsTie2 did not enhance the pericyte coverage in tumor microvessels, in contrast to the expected outcome.

In study III, the microvessel density of LYVE-1 positive lymphatic vessels in tumors was lower in the following groups; AdCMV and chemotherapy (2.7 ± 0.61 /mm²), AdsVEGFR2 and AdsTie2 -gene therapy (3.0 ± 0.45 /mm²) or gene and chemotherapy (2.2 ± 0.39 /mm²) treated mice in comparison to AdCMV controls (3.9 ± 1.0 /mm²). Furthermore, in this experiment, the lymphatic vessels were mainly located in the periphery of the tumors and these kinds of vessels were detected in all analyzed tumors. TVA of lymphatic vessels was higher in chemotherapy treated groups of AdCMV and chemotherapy (0.31 ± 0.10 %) and AdsVEGFR2, AdsTie2 and chemotherapy (0.39 ± 0.17 %) as compared to the non-chemotherapy groups of AdCMV (0.12 ± 0.03 %) or AdsVEGFR2, AdsTie2 (0.20 ± 0.07 %) (unpublished data). VEGFR2 and Tie2 have not been thought to play any major roles in lymphangiogenesis where VEGFR3 is more relevant. The results from these studies indicate that VEGFR2 and Tie2 may also have some role in lymphangiogenesis, although this will need to be clarified.
The efficacy of chemotherapy in tumor cells is related to the delivery of chemotherapeutic agents and this demands an efficient circulation. Contradictory results from antiangiogenic studies when combined with chemotherapy have been encountered in many studies, while a response for treatment of tumors in PFS and OS was obtained only when chemotherapy was added to the antiangiogenic gene therapy (Jain et al., 2006). It has been hypothesized that antiangiogenic therapy may increase the normal vasculature in tumors and further increase blood flow. This may be linked to the activation of compensatory mechanisms in the endothelial cells of tumor microvessels in response to the antiangiogenic therapy. It should be noted that this kind of therapy is mainly targeted to only one endothelial growth factor receptor. As the development of new microvessels in tumors is regulated by numerous growth factors and their receptors, this could explain why there is possibly activation of additional signaling pathways after antiangiogenic therapy. This may lead to a poor therapeutic effect or even to activated angiogenesis. The increased number of pericytes in microvessels are thought to decrease the vascular permeability and further to improve the delivery of chemotherapeutic agents into tumors (Carmeliet and Jain, 2011; Dickson et al., 2007; Jain, 2005).

In our studies, the combined chemotherapy and AdsVEGFR2 and AdsVEGFR3 -gene therapy showed improved antitumoral efficacy of gene therapy in terms of tumor weights (2.8 ± 0.27 g with AdsR2, AdsR3 and chemotherapy; 3.5 ± 0.25 g with AdsR2 and AdsR3) and ascites formation (1.75 ± 0.72 ml with AdsR2, AdsR3 and chemotherapy; 2.59 ± 0.57 ml with AdsR2 and AdsR3) (Figures 6 and 7). In contrast, chemotherapy did not have any effect on the numbers of tumoral microvessels when combined to antiangiogenic therapy of AdsVEGFR2 and AdsVEGFR3 or AdsVEGFR2 and AdsTie2 (Figure 9). In study III, the improved efficacy was not reflected in reduced tumor growth when the animals received AdsVEGFR2 and AdsTie2 -gene therapy and combined chemotherapy (2.5 ± 0.36 g with AdsR2 and AdsTie2 and chemotherapy; 1.83 ± 0.42 g with AdsR2 and AdsTie2), although the amount of ascites fluid was lower when combined to chemotherapy (0.28 ± 0.08 ml with AdsR2 and AdsTie2 and chemotherapy; 1.33 ± 0.40 ml with AdsR2 and AdsTie2). These results indicate that there may be benefits associated with the combination of AdsVEGFR2 and AdsVEGFR3 -gene therapy with chemotherapy in the therapy of ovarian cancer in the model utilizing mouse xenografts.

![Graphs](image)

Figure 10. Microvessel density (/mm²) and total vascular area (%) of tumor microvessels in studies II and III. (sR2, AdsVEGFR2; sR3, AdsVEGFR3; CMV, cytomegalovirus; sNR, soluble neuropilin).
5.2.7 MRI and DW-MRI in the detection of the growth of ovarian tumors and evaluation of the therapeutic response (Studies II, III)

It is difficult to evaluate the effects of gene therapy and evaluate the results as there is a lack of surrogate markers (Rätty et al., 2007). Here, DW-MRI was performed weekly to monitor the effects of gene- and chemotherapy before there were any notable changes in tumor size (Moffat et al., 2005).

The growth of intraperitoneal ovarian tumors in mice xenografts could be detected by MRI on day 10 in studies II and III. The effect of gene- and chemotherapy in intraperitoneal tumors was followed by weekly MRI by estimating $T_1$, $T_2$, $T_{RAFF4}$ and $T_{RAFF4}$ -relaxation times as well as with DW-MRI with apparent water diffusion coefficient (ADC) (Figure 11). After the AdsVEGFR2, AdsVEGFR3 and chemotherapy, the $T_2$-relaxation time was significantly longer on day 8 after the gene transfer ($101 \pm 12.0$ ms) when compared to AdLacZ controls ($82.0 \pm 7.40$ ms, $P=0.005$, study II); this reflected the early molecular changes in tumor tissue as a result of effective therapy. In study II, a significant prolongation in $T_2$-weighted relaxation time was measured in necrotic areas of tumors, which was related to molecular changes taking place in the necrotic areas (Figure 11). This result is in line with other studies where altered $T_2$-relaxation time has been observed in necrotic tissues (Canese et al., 2012). In study III, parallel results were observed on day 8 and 15 i.e. $T_2$-relaxation time was slightly longer in the AdsVEGFR2 and AdsTie2 –gene therapy group compared to the other groups, although the result was not statistically significant. In all relaxation time measurements, slight elevations of analysed parameters were measured within one week after the gene transfers. In study II, on day 4 after the gene transfer $T_{RAFF4}$ -relaxation times and $T_1$-relaxation times on day 8 correlated between necrotic and vital areas of tumors, which is evidence of the difference in water movement between necrotic and vital tumor tissue.

In study III, the effect of gene therapy was monitored with anatomical MRI as a reduced growth of tumors in gene therapy treated groups III and IV two weeks (15 days) after the treatment. Unfortunately, no significant differences from MRI relaxation times or ADC-maps were detected in study III. The problems encountered in this study could be traced to high variation introduced by an individual mouse. Furthermore, in this study, the time of MRI was shortened by applying a spiral readout method which resulted in lower resolution in the final images and extensive variation in the relaxation times. The low resolution in cancer imaging was also a problem when imaging the peritoneal areas, since the movement of the bowel and respiration introduced movement artefacts. It is also possible that the high aggressivity of our intraperitoneal ovarian cancer xenografts was inappropriate for MRI and DW-MRI analyses. It is clear that there should be an optimization of these parameters before embarking on future trials involving MRI.

The ADC value was significantly lower in AdsVEGFR2, AdsTie2 and chemotherapy – group on day 15 after the gene transfer as compared to AdCMV and chemotherapy ($P=0.020$). Instead, after AdsNRP1 and -2 gene therapy, ADC values increased transiently on day 4 after the gene transfer ($1.32 \pm 0.50 \times 10^{-3}$ mm$^2$/s). A significant decrease in ADC values was detected at the 15 day time point in the AdsVEGFR2, AdsTie2, chemotherapy - group as compared to AdCMV, chemotherapy group; this was related to changes in tumor cellularity and the efficacy of gene therapy. In parallel with our results, in an ovarian cancer model involving SKOV3 i.p. xenografts, a reduction in ADC values was observed already by 24-48 hours after the i.v. cisplatin injection (Pisanu et al., 2014b). The difficulties encountered with DW-MRI and ADC values can be attributed to movement artefacts when visualizing the peritoneal disease. Further studies will be needed to optimize DW-MRI as a way of evaluating the gene therapy effect also in ovarian cancer xenografts.
Figure 11. Example from anatomical MRI images, T2-weighted images and ADC-maps in AdsVEGFR2, AdsTie2 and chemotherapy treated mouse with ovarian cancer before the i.v. gene therapy (=day -1), and on days 4, 15 and 21 after the gene therapy. Tumors are shown with arrows. Necrotic areas in tumors are shown with black arrows in the T2-weighted images.
5.2.8 Antiangiogenic factors in ascites (Study III)

The levels of VEGF and Ang2 in ascites were measured in study III by two separate ELISA-assays. Since these proteins are related to poor prognosis and curtailed survival of patients with ovarian cancer and they are used as potential biomarkers for evaluating the stage of cancer, we measured the protein levels in ascites fluid at the end of the follow-up (Liang et al., 2013; Sallinen et al., 2014; Trachana et al., 2016).

AdsVEGFR2 and AdsTie2 -gene therapy increased the mouse VEGF (mVEGF) levels in ascites and this was maintained the gene therapy was combined with chemotherapy (Figure 12). The mVEGF production in ascites was notably activated by antiangiogenic gene therapy of AdsVEGFR2 and AdsTie2. The result indicates that the production of VEGF from human-origin tumor cells is activated by antiangiogenic therapy, possibly by increasing tumor cell hypoxia. This result highlights the possibility that mVEGF may be produced by activated mouse stromal or immune cells after the gene therapy (Zhang et al., 2003). As the human SKOV-3m ovarian cancer cell line was used in our studies, and as expected the VEGF production was induced in the tumor cells, the levels of human VEGF (hVEGF) in ascites of mice were measured. It was anticipated that there would be elevated levels of hVEGF in AdCMV and AdCMV and chemotherapy groups in non-treated circumstances. Human VEGF levels in ascites were elevated also in the groups not receiving antiangiogenic gene therapy (AdCMV and AdCMV with chemotherapy), furthermore the hVEGF production in ascites was increased more efficiently after the AdsVEGFR2 and AdsTie2 gene therapy. Interestingly when AdsVEGFR2 and AdsTie2 gene therapy was combined to chemotherapy, only a slight elevation was observed in hVEGF levels in ascites.

Gene therapy with AdsVEGFR2 and AdsTie2 and also AdCMV and AdCMV with chemotherapy increased the mouse Ang2-levels (mAng2) in the ascites fluid, indicating that the activated production of mAng2 was independent of VEGF2 or Tie2 signaling. Instead human Ang2 (hAng2) production in ascites increased as a result of chemotherapy, which may be related to activation of inflammatory cells or increased hypoxia in tumor-related cells after chemotherapy. Furthermore, VEGF2 and Tie2 signaling seemed to exert an influence on hAng2 production, i.e. this was induced after the AdsVEGFR2 and AdsTie2 – gene therapy. The extensive growth of ovarian tumors, increased angiogenesis and loss of pericytes as well as vascular abnormalities have all been related to high Ang2 expression in preclinical studies investigating ovarian cancer (Oliner et al., 2004). This is partly in line with results emerging from the present study, i.e. there was extensive hAng2 expression in ascites at the end of the follow-up in the chemotherapy treated groups. In these groups, the loss of pericytes was analyzed, although the growth of ovarian tumors was restricted.

High VEGF-levels in ascites and serum have been related to an advanced stage of ovarian cancer and poor survival in preclinical and clinical studies (Bandiera et al., 2012; Li et al., 2004). Interestingly the mouse VEGF levels in ascites were remarkably high when the mice were treated with sVEGFR2 and sTie2 gene therapy although the amount of ascites was low at the time of follow-up and the animals’ survival did not correlate with the VEGF or Ang2 levels.

In summary, AdsVEGFR2 and AdsTie2 gene therapy elevated the production of human and mouse VEGF and Ang2 in ascites fluid. Ascites VEGF and Ang2 levels did not correlate with the survival of mice in this study. Finally, these results represent new knowledge about the relevance of VEGF and Tie-signaling in the formation of ascites in ovarian cancer.
In summary, high VEGF levels in ascites and serum were observed. The results emerging from the present study, i.e., there was extensive angiogenic gene therapy exerting angiogenic gene therapy increased the mouse VEGF levels in ascites. Interestingly when AdsVEGFR2 and AdsTie2 gene therapy was combined to chemotherapy increased the mouse Ang2 levels in ascites. This highlights the importance of considering these proteins as biomarkers for ovarian cancer, we expected the VEGF production to be expected when VEGFR and Tie2 signaling was induced after the AdsVEGFR2 and AdsTie2 gene therapy. Anti-VEGF and anti-Tie2 antibodies are used as antiangiogenic factors in ascites fluid. This is partly in line with ovariangliogenic factors in ascites fluid. This is partly in line with our previous studies investigating the levels of VEGF and Ang2 in ascites with ovarian cancer and poor survival in preclinical and clinical studies. The extensive vascular abnormalities in tumor cells, the high Ang2 expression in ascites and serum, indicating that the levels of human VEGF and mouse Ang2 was increased in hVEGF production in ascites was increased after the gene therapy increased the mouse VEGF production in ascites. As the human SKOV-3 ovarian cancer cell line was used in our studies, and they are used as potential biomarkers for ovarian cancer, we measured the protein levels of VEGF and Ang2 in ascites. The mVEGF production in ascites was notably activated by antiangiogenic gene therapy of AdsVEGFR2 and AdsTie2 (Figure 12).

**Figure 12. VEGF and Ang2 levels in ascites.** a) Gene therapy increased the levels of mouse VEGF alone and when combined with chemotherapy (P=0.002**). b,c) With respect to the levels of human VEGF and mouse Ang2, no significant differences were observed. d) Human Ang2 levels were elevated after AdCMV and chemotherapy and AdsVEGFR2 and AdsTie2 -gene therapy combined with chemotherapy as compared to AdCMV (P=0.007** and P=0.023*).
Conclusions and future

I

Gene therapy with AdsVEGFR2 and AdsVEGFR3, and when combined with chemotherapy with paclitaxel and carboplatin, is safe and does not exert any serious adverse events in healthy rats. Intravenously administered gene therapy with $1 \times 10^{10}$ vp, induced a transient stress in liver, but only mild regenerative liver alterations were observed, when a viral dose of $1 \times 10^{11}$ vp was combined with chemotherapy. Combined gene- and chemotherapy is feasible and should be evaluated in future clinical trials in ovarian cancer.

II

AdsVEGFR2 and AdsVEGFR3 gene therapy has a significant antiangiogenic effect on microvessels of ovarian tumors, furthermore the formation of ascites was reduced and the survival of mice was prolonged in our model exploiting ovarian cancer xenografts. MRI with T2-relaxation time measurements and DW-MRI with ADC values represent potential methods with which to evaluate the early molecular alterations occurring in ovarian tumors of mice in response to adenoviral gene therapy and chemotherapy.

III

AdsVEGFR2 and AdsTie2 gene therapy combined with chemotherapy with paclitaxel and carboplatin confers benefits in the treatment of malignant ascites related to ovarian cancer. AdsVEGFR2 and AdsTie2 gene therapy reduces the growth of intraperitoneal tumors in mice with ovarian cancer. In mice xenografts, the levels of mouse and human VEGF and Ang2 in ascites are elevated as a result of antiangiogenic gene therapy. Further studies will be required to clarify the role of VEGFR2 and Tie2 in ovarian cancer. In future preclinical experiments utilizing DW-MRI techniques for visualizing ovarian cancer xenografts, it will be necessary to optimize the protocols.

Results emerging from this thesis demonstrate that gene therapy with AdsVEGFR2, AdsVEGFR3 or AdsTie2 may potentially represent a new approach for treating ovarian cancer with highly aggressive characteristics. It seemed beneficial to combine gene therapy with chemotherapy at least in models with ovarian cancer xenografts; further studies with combined therapies are warranted. In the future, the utilization of long-term expressing viral vectors such as AAVs should be considered as ways to improve the treatment response in ovarian cancer. When considering the future clinical trials with AdsVEGFR2 and AdsVEGFR3 gene therapy, these results indicate that i.v. viral gene therapy seems to be safe as we observed only limited adverse events. On the basis of the results emerging from the first study of this thesis and previous efficacy studies, a phase I clinical trial with AdsVEGFR2 and AdsVEGFR3 is currently under development. Ovarian cancer imaging and development of DW-MRI in preclinical trials did prove to be challenging in our animal model. However, in the future, MRI with relaxation time measurements may offer a reliable means to evaluate the early effects of gene therapy on tumor microvessels and to identify changes occurring at the molecular level.
6 Conclusions and future

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Results emerging from this thesis demonstrate that gene therapy with AdsVEGFR2, AdsVEGFR3 or AdsTie2 may potentially represent a new approach for treating ovarian cancers with highly aggressive characteristics. It seemed beneficial to combine gene therapy with chemotherapy at least in models with ovarian cancer xenografts; further studies with combined therapies are warranted. In the future, the utilization of long-term expressing viral vectors such as AAVs should be considered as ways to improve the treatment response in ovarian cancer. When considering the future clinical trials with AdsVEGFR2 and AdsVEGFR3 gene therapy, these results indicate that i.v. viral gene therapy seems to be safe as we observed only limited adverse events. On the basis of the results emerging from the first study of this thesis and previous efficacy studies, a phase I clinical trial with AdsVEGFR2 and AdsVEGFR3 is currently under development. Ovarian cancer imaging and development of DW-MRI in preclinical trials did prove to be challenging in our animal model. However, in the future, MRI with relaxation time measurements may offer a reliable means to evaluate the early effects of gene therapy on tumor microvessels and to identify changes occurring at the molecular level.


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Ovarian cancer is one of the most insidious malignancies in women. It is often diagnosed with widely disseminated disease and intraperitoneal ascites fluid. Angiogenesis and lymphangiogenesis, i.e. the development of new microvessels and lymphatic vessels in the tumor, are crucial in ovarian cancer development. In this thesis, promising antitumoral effects and safety of adenoviral antiangiogenic and antilymphangiogenic gene therapy in a human ovarian cancer xenograft model and in healthy rats are described. Furthermore, diffusion-weighted MRI (DW-MRI) was used to evaluate the early gene therapy responses in tumors.