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SULT1E1 and ER- α in patients with advanced ovarian
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Abstract

Background: Epithelial ovarian cancer (EOC) is the deadliest gynecological cancer, due to the fact that it is mostly diagnosed at advanced stages. The need for new therapies and detection systems is urgent. One new approach may be to target enzymes and receptors that are involved in the estrogen pathway. By regulating the concentration of active estrogens in tumor cells, steroid hormone-inactivating estrogen sulfotransferase (SULT1E1) and estrogen activating steroid sulfatase (STS) may influence the progression of epithelial ovarian cancer (EOC). Therefore, we investigated the expression and prognostic impact of both enzymes together with the estrogen receptors (ER) α/β and the progesterone receptor (PGR) in this tumor entity.

Methods: The protein expression levels of STS, SULT1E1, ER- α/β , and PGR were assessed by immunohistochemistry in a large and well-described advanced stage EOC patient cohort (n=243). By using an automated quantitative microscopic image analysis system (TissueFAXS®, TissueQuest®) the immunoreactive scores (IRS) were calculated. The protein levels were correlated with each other and with clinic-pathological parameters. Finally, the prognostic values of these inactivating or activating enzymes and the hormone receptors were determined.

Results: Multiple Cox regression analysis showed a significant independent impact of ER- α (HR=0.72; 95% CI 0.5-1.0) and SULT1E1 (HR=0.57; 95% CI 0.4-0.9) protein expression on overall survival. ER- α also was associated with a longer progression free survival (HR=0.75; CI 0.56-1.00), while STS did not have an independent impact on overall- or progression free survival.

Summary and Conclusion: In patients with advanced stage EOC, high levels of ER- α and SULT1E1 are favorable prognostic factors for overall survival. Therefore the importance of estrogen signaling in patients with ovarian cancer warrants for further investigations on the role of the estrogen homeostasis as a target for therapeutic intervention and as a biomarker in EOC.

Abstract (Deutsch)

Einleitung: Das epitheliale Ovarialkarzinom (EOC) ist der tödlichste gynäkologische Tumor, da er meistens erst im fortgeschrittenen Stadium diagnostiziert wird. Darum werden dringend neue Therapien und Detektionssysteme gebraucht. Eine Möglichkeit stellen die Enzyme und Rezeptoren des Östrogen-Stoffwechsels dar. Durch die Regulierung der Konzentration von aktiven Östrogenen in Tumorzellen, könnten die Steroidhormon inaktivierende Östrogen Sulfotransferase (SULT1E1) und die Östrogen aktivierende Steroidsulfatase (STS) die Progression von EOC beeinflussen. Deshalb wurden die Expressionslevels von STS, SULT1E1, den Östrogen Rezeptoren (ER) α/β und dem Progesteron Rezeptor (PGR) in einer großen Patientenkohorte ermittelt und durch statistische Analyse der prognostische Einfluss auf EOC eruiert.

Methoden: Die Proteinexpression von STS, SULT1E1, ER- α/β und PGR wurden durch die Anwendung von immunhistochemischer Färbung in einer großen und gut charakterisierten Patientenkohorte mit fortgeschrittenen EOC bestimmt (n=243). Für die Berechnung des immunreaktiven Scores (IRS) wurde ein automatisiertes quantitatives mikroskopisches Image Analyse System (TissueFAXS®, TissueQuest®) verwendet. Die Proteinlevels wurden miteinander und mit anderen klinisch pathologischen Parametern korreliert. Schlussendlich wurde die prognostische Wichtigkeit von diesen inaktivierenden und aktivierenden Enzymen sowie der Hormonrezeptoren bestimmt.

Ergebnisse: Die Multiple Cox Regressionsanalyse zeigte einen signifikanten unabhängigen Einfluss von ER- α (HR=0.72; 95% CI 0.5-1.0) und SULT1E1 (HR=0.57; 95% CI 0.4-0.9) auf das Gesamtüberleben. ER- α wurde auch mit einem längeren progressionsfreien Überleben (HR=0.75; CI 0.56-1.00) assoziiert. STS hatte in dieser Studie keinen unabhängigen Einfluss auf das Gesamtüberleben und das progressionsfreie Überleben.

Zusammenfassung und Conclusio: In Patienten mit fortgeschrittenen EOC sind hohe Levels von ER- α und SULT1E1 vorteilhafte prognostische Faktoren für ein längeres Gesamtüberleben. Deshalb ist es wichtig, weitere Forschung im Feld der Östrogen-Signalübertragung in Patienten mit fortgeschrittenen EOC zu betreiben, um die Rolle der Östrogen Homöostase als Target für therapeutische Nutzung und als Biomarker zu bestimmen.

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1 Introduction

1.1 The Ovaries

The two ovaries are part of the female reproduction system. The ovaries are located intraperitoneal at the lateral pelvic wall. They are basically a homolog to the testes in the males and have comparable functions. They produce sex steroid hormones (estrogen and progesterone). The second function is to carry out follicle maturation (Junqueira, 2004). Before puberty the ovaries are small, but during puberty they begin to grow and reach the normal size (approximately 3-4cm length, 1.5-2cm wide, 1-1.5cm diameter). After the menopause, the size of the ovaries starts to decrease again (Mescher, 2009).

1.1.1 Histology and Function

The surface of the ovary is coated in a cubic epithelial monolayer, the **germinal epithelium**. It is covered with microvilli and is also called “Müllner Epithel”. Under the germinal epithelium is a white capsule of tight conjunctive tissue, which is named **tunica albuginea**. The ovarian **cortex** is the next layer of the ovary. It also consists of tight conjunctive tissue but is filled with various smooth muscle cells. The ovarian follicles are embedded here. The internal part of the ovaries is called **medulla ovarii**. It includes loose conjunctive tissue and is traversed by blood vessels, which enter through the suspensory ligament of the ovary.

The **ovarian follicles** are oocytes at all stages of development, surrounded by one or more layers of epithelial cells. During fetal life, the ovaries produce a great number of primordial follicles; they are stored over decades until activation. The process of follicle development is characterised by an increased endocrine production of estrogen, induced by the follicle stimulating hormone (FSH). The secretion of FSH and also luteinizing hormone (LH) from the pituitary gland is stimulated by the gonadotropin releasing hormone (GnRH), which originates from cells in the hypothalamus.

In the follicle phase of the menstruation cycle, approximately 20 primordial follicles are recruited by unknown stimuli and form a unilaminar primary follicle. It already contains the **Zona pellucida**, a thick layer of glycoproteins. Through ongoing proliferation of the follicular cells, a stratified epithelium consisting of granulosa cells develops. Through proliferation of the **granulosa cells**, the primary follicle transforms to the secondary follicle. The granulosa cells

express FSH receptors. By the stimulating effect of FSH, the production of 17β -estradiol (E2) through the aromatase pathway is induced. **Theca cells** express LH- receptors and support the granulosa cells estrogen synthesis by secreting steroid hormones (androgens), which act as estrogen precursors. The next step is the formation of the **cumulus oophorus** and a cavity containing a mucopolysaccharide rich liquid. The follicle is now called tertiary follicle. During this process the estrogen concentration is increased. It starts to inhibit FSH and LH as a negative “feedback”. This estrogen excess is the reason for the domination of one follicle and it causes a stimulation of the LH production. The estrogen level starts to decrease and under the influence of LH, the ovum leaves the follicle.

After the ovulation, the “luteal phase” begins with the conversion of the empty follicle into the progesterone-producing **corpus luteum**. This is important for the maintenance of the pregnancy and similar to estrogen, it is responsible for the inhibition of GnRH. The low GnRH level causes a degeneration of the corpus luteum. As a consequence, the progesterone and estrogen level decreases and initiates the menstrual bleeding (Böcker et al., 2008; Mescher; 2009; Silbernagel and Despopoulos, 2007; Leidenberger et al., 2009).

1.2 Ovarian Cancer

1.2.1 Epidemiology

In Austria, ovarian cancer has a 4% incidence. Statistically, for a 75 year old woman, the risk of developing ovarian cancer is 0.9%. In 2010, 675 women were diagnosed with ovarian cancer, and 438 died from this disease. It is the most fatal gynaecological tumor (Statistik Austria, 2014). Overall, ovarian cancer was the 9th most frequent cancer in women in 2010 (after breast-, colon-, lung-, uterus-, blood building system-, pancreas-, skin- and thyroid cancer).

Generally, ovarian cancer is characterised by a very poor prognosis and consequently the 5 year overall survival (OS) is only 46%. This is partly due to the fact that these tumors often develop high resistance to anticancer chemotherapy. Furthermore, the bad prognosis is also based on the diagnosis at a very late stage. At advanced stages, the tumor has already reached other organs beyond the pelvic cavity (WHO, 2014).

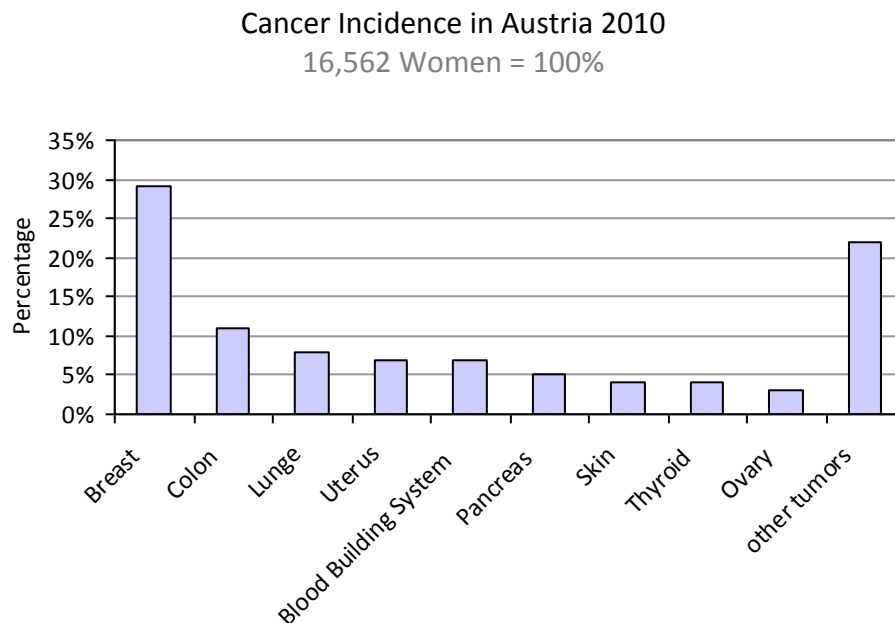


Fig. 1.2.1: Cancer Incidence in Austria

This figure shows the cancer incidence of women (%) in Austria in the year 2010. Remarkable is that ovarian cancer has a low incidence compared to breast or uterus cancer, but still is the most deadly gynaecological malignancy (Statistik Austria, 2014).

As illustrated in Fig.1.2.1, the incidence and mortality of ovarian cancer decreased in the last years. In 1986, 841 new cases of ovarian carcinoma were registered and in 2010, only 675. This reduction occurred in all age groups, but mostly in women at the age of 50 to 64 (Statistik Austria, 2014). This can be explained by the introduction of the oral contraceptive pill (OCP) which is commercially available since the early 60's and is in widespread use. Data proved that OCPs have a protective effect against ovarian cancer (Narod et al., 1998). Additionally, the classification of different ovarian carcinomas and the adaptation of novel therapies also resulted in a decreased mortality (Cancer Research UK, 2014).

It is also notable that in developing countries the incidence of ovarian cancer is nearly twice as low as in industrial countries. The reason seems to be the fact, that the birth rate is higher in developing countries and a high number of pregnancies protects from ovarian cancer (IARC, 2013; Whittermore et al., 1992).

Malignant Neoformation of the ovary
Age standardisation rates of 100,000 people
(WHO-Worldpopulation, 2001)

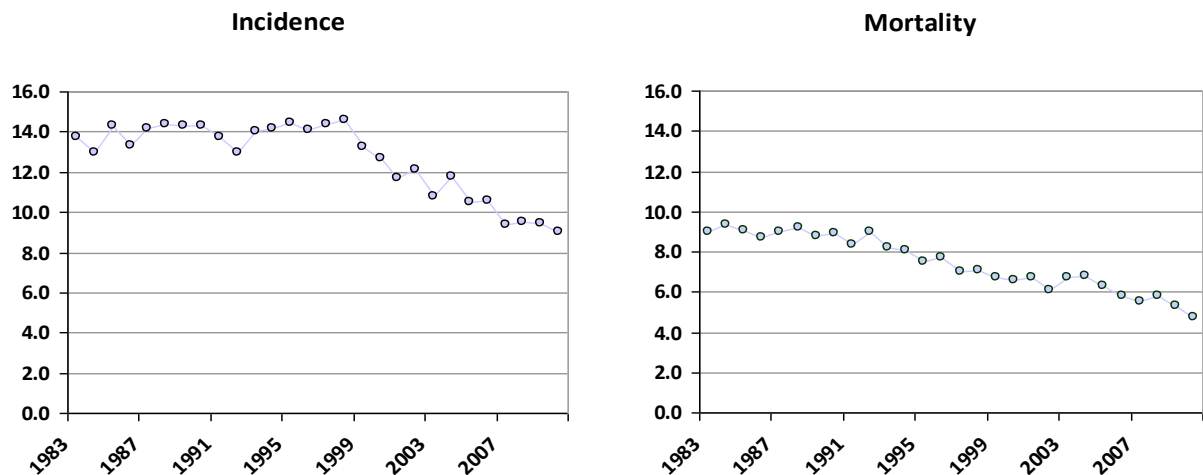


Fig. 1.2.2: Malignant Neoformation of the Ovary

The left graph shows the incidence of ovarian cancer and in comparison the right graph represents the mortality. (Statistik Austria, 2014)

1.2.2 Aetiology

Until now, the aetiology of the formation of ovarian cancer is not fully understood. There are hereditary influences that increase the risk of ovarian cancer, but also lifestyle related factors including tobacco smoking or obesity may play an important role. Moreover the endogenous hormonal environment of the ovaries especially gonadotropins, estrogens and androgens, but also inflammatory diseases can increase the risk of developing ovarian cancer (Hunn et al.; 2012).

Hereditary Factors

It is assumed that approximately 89% of all ovarian carcinomas occur sporadically, only about 11% are caused by genetic predispositions (Sogaard et al.; 2006). However, a family history of ovarian cancer is the most confident and important risk factor and this knowledge is essential in the prevention of ovarian cancer (Schildkraut et al.; 1989). The discovery of BRCA1 and BRCA2 mutations confirmed that a certain percentage of women develop ovarian cancer as a consequence of a genetic predisposition (Berchuck et al.; 1996). Relatives from families suffering from the hereditary Lynch Syndrome also have a high life time risk for developing ovarian cancer (Schorge et al.; 2010).

BRCA1 and BRCA2

Breast Cancer Antigen 1 and 2 (BRCA1 and BRCA2) are important proteins for the repair of DNA double strand breaks and due to this fact they are called tumor suppresser genes. Moreover they are also involved in the control of cell cycle check points and in chromosomal segregation during mitosis and meiosis. Germline mutations in one of this genes increase the genomic instability and this consequently results in a higher risk of developing cancer. By the age of 70 a germ line mutation in BRCA1 leads to a 30% to 40% increased ovarian cancer risk and a mutation in BRCA2 to a 10% to 15% higher risk (O'Donovan et al.; 2010).

HNPCC (hereditary nonpolyposis colorectal cancer, Lynch Syndrome)

It is characterised through a mutation in mismatch repair genes, namely MSH2, MLH1, PMS1, and PMS2. People with the Lynch Syndrome have a high life time risk of developing colon cancer. Additionally, 10% to 15% of hereditary ovarian cancers are caused by Lynch Syndrome and these tumors occur at a younger age (< 48 years). They are non-serous tumors. Similar to BRCA1 and BRCA2 germ line mutation, HNPCC leads to genomic instability and increases the risk of developing ovarian cancer (Ketabi et al.; 2011).

Reproductive

Reproductive factors like pregnancy, breastfeeding and the use of OCP influence the life time risk of developing ovarian cancer.

Studies have shown that pregnancy has a protective influence; moreover this effect increases by 10% to 16% with each pregnancy (Purdie et al., 2003; Risch et al., 1983; Whittermore et al., 1992). Another interesting fact is that pregnancy after the age of 35 is two times more protective than at the age of 25 (Whiteman et al.; 2003).

Ness et al. found an increased risk of developing ovarian cancer for women suffering from infertility (Ness et al., 2002). Application of drugs used for the treatment of infertility, like GnRH antagonists or clomiphene, seems to increase the risk (Choi et al., 2007).

Breastfeeding may also have an influence on the risk of developing ovarian cancer. Data suggest that the risk decreases for women that do breastfeeding. Furthermore, it is mostly depending on the duration of application. A significant decrease occurs by women that do breastfeeding for 18 months or longer (Titus-Ernstoff et al., 2010; Jordan et al., 2012).

Progestins are synthetic analogs to Gestagenes and are important components of the oral contraceptive pill (OCP) (Rodriguez et al., 1998).

OCPs or more specific progestins inhibit ovulation. They have an apoptotic effect on the ovarian surface epithelium cells (OSE) and therefore eliminate cells that have irreparable damages and could lead to malignant transformations (Canman et al., 1994). Several studies proved that women who take OCPs for more than three years have a 30% to 50% decreased life time risk of developing ovarian cancer (Narod et al., 1998).

All these protective factors including breastfeeding, pregnancy and OCPs cohere with a repression of the ovulation. Since every ovulation goes along with an inflammation of the OSE and a damage of the DNA, this can lead to mutations of tumor suppressor genes and/or oncogenes. It may initiate tumor growth (Levanon et al., 2008).

The hormone hypothesis

It is known that the OSE is permanently exposed to hormones including gonadotropins (FSH and LH), estrogen and progesterone. These hormones are involved in the activation or inhibition of proliferation pathways. This permanent exposure can cause the development of ovarian cancer (Levanon et al., 2008).

Gonadotropins

These hormones are produced in the pituitary gland and stimulate the endocrine hormones of the ovary. Gonadotropins are regulated by the GnRH secreted by the hypothalamus (Böcker et al., 2008). Cramer et al. investigated the role of gonadotropins (follicle stimulating hormone and luteinizing hormone) in ovarian cancer development. They suggested a positive effect of circulating gonadotropins during menstruation or menopause on the formation of EOC (Cramer et al., 1983). Later investigations do not fully support the “gonadotropin hypothesis” of Cramer, because gonadotropin showed both, a stimulating and a suppressing effect on OSE (Choi et al., 2007; Levanon et al., 2008).

Estrogens and Progesterone

Estrogen and progesterone are the most important female sex steroid hormones. A high amount of estrogen also seems to promote ovarian tumor progression in contrast to progesterone.

For the reduction of menopause ailment an estrogen replacement therapy (ERT) or ERT in combination with progestin hormone replacement therapy (HRT) is in clinical use. The effect of these therapies on the formation and progression of ovarian carcinoma is still in discussion and clinical data are overall very controversial (Coughlin et al., 2000; Riman et al., 2002; Garg et al., 1998; Hein et al., 2013).

A recent study with 909.946 women from denmark showed an increasing risk for HRT users for endometroid and serous ovarian cancer but not for the muscinous and clear cell subtype. It seems that there is a risk variation among the different subtypes (Beral et al., 2007; Morch et al., 2012).

Lifestyle

Several studies have indicated that obesity is an important risk factor. Obesity does not only increase the risk of ovarian cancer, it is also associated with a poorer prognosis (Webb, 2013). Data suggest that women with a low body mass index (BMI) have an up to 40% reduced risk compared to women with a high BMI (Purdie et al., 2001). Women that suffer from obesity generally have an increased production of androgens, which leads to a higher estrogen concentration in the body (McLemore et al., 2009).

The influence of cigarette smoking and diet is still not fully defined. In the ovaries, nicotine and its metabolites have been detected. This suggests that smoking could have a direct damaging effect on the DNA in ovarian tissue and consequently enhance the risk of developing ovarian cancer (Zenzes et al., 1997).

Studies among diet show a risk reduction by eating green leafy vegetables but overall data are inconsistent (Kushi et al., 1999).

1.2.3 Histological Classification and Pathogenesis

With a frequency of 70% the most common and also the most deadly EOC is the high-grade serious carcinoma (HGSC), followed by the endometrial carcinoma (EC) and the clear cell carcinoma (CCC) with 10%, the muscinous carcinoma (MC) with 3% and the low-grade serous

carcinoma (LGSC) with less than 5% frequency (Gilks and Prat, 2009). In the past years the classification of the very heterogeneous group of ovarian carcinomas is coming more and more into focus of research, in order to find the best subtype-specific therapy.

Many immunohistochemical and genetic analyses have been done to detect differences and identify features of the subtypes. Köbel et al. performed biomarker analyses and they came to the conclusion that some biomarkers e.g. Ki-67 (cell proliferation marker), Wilms tumor 1 (WT1) and also cancer antigen 125 (CA125) show significant differences in expression rates between the subtypes and therefore can be used as subtype specific biomarkers (Köbel et al., 2008).

Overall the subtypes show different behaviours, mutations, origins, markers and prognosis. Due to the fact that the subtypes respond differently to the common therapies, a characterisation of the tumor type is very important for a successful treatment (Erickson et al., 2013).

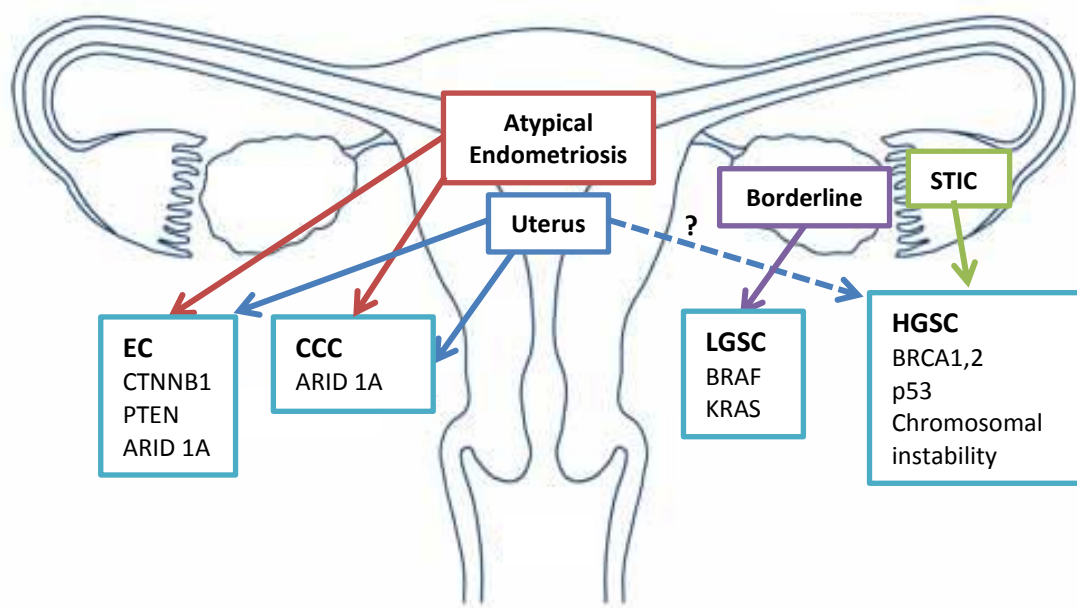


Fig. 1.2.3: The Origins and significant Mutations of the Ovarian Cancer Subtypes

This figure shows the classification of the ovarian cancer subtypes based on their key mutations and origins. Mucinous carcinoma is not shown on this figure due to the fact that it is still unclear from which tissue it develops. Endometrial cancer (EC) as well as clear cell carcinoma (CCC) seems to derive from atypical endometriosis or from the uterus. Low-grade serous carcinoma (LGSC) has its origin in borderline tumors of the ovary in contrast to high-grade serous carcinoma (HGSC), which is assumed to derive from serous tubal intraepithelial carcinoma (STIC) (Gilks and Prat, 2009).

High-grade serous carcinoma (HGSC)

This subtype is the most aggressive and lethal one. It is usually detected in an advanced stage and after responding to standard platin and taxane based chemotherapy the majority of patients will experience a recurrence and develop a resistance to the therapeutic drugs (Erickson et al., 2013; Kessler et al., 2013). A very early transcoelomic spread is common (Prat, 2012). The pathological morphology of HGSC is heterogeneous; mostly it shows a strong stratified epithelial growth with slit-like lumen within the tumor areas. The appearance of mononuclear giant cells with immense sized nuclei is also common (Köbel et al., 2008). In contrast to LGSC, HGSC have a very high mitotic rate and arise as solid growth tumors. Characteristics are genetic changes in the p53 gene, a loss of function mutation (LOF) in BRCA1 and 2, and a high chromosomal instability (Gilks and Prat, 2009; Prat, 2012). Additionally, Köbel et al. detected a high immune reactivity of estrogen receptors (ER) in two thirds of the patients (Köbel et al., 2009).

It is still not clear from where HGSC arrive. The old model predicts that by forming inclusion cysts, the ovary epithelium is the origin of HGSC. However, latest studies have given a hint that the fallopian tube could be the source of malignant cells in a significant number of cases. It is known that serous tubal intraepithelial carcinomas (STICs) have mostly the same p53 mutations, express the same oncogenes and also have similar phenotypic characteristics as HGSC (Kessler et al., 2013). In 67% of HGSC also the existence of STICs was proven (Carlson et al., 2008). STICs are mostly associated with BRCA1 and 2 mutations (analogous to HGSC) (Cass et al., 2005).

Some questions still remain open including how the STIC cells transform and then migrate to the ovary and form the HGSC.

Low-grade serous carcinoma (LGSC)

LGSC is a very rare subtype with less than five percent appearance. In earlier stages the prognosis is good but in an advanced stage it is less favorable. The five year survival is longer than in HGSC but this newly characterised type does not respond to conventional chemotherapy in most cases. As HGSC, this subtype also often spreads trans-coelomicly, but it has no aggressive behavior (Crispens et al., 2002). In contrast to HGSC, it has a micropapillary structure and often appears with psammoma bodies. These are calcium incorporations that are formed by necrotic tumor cells. LGSC cells have uniform nuclei and a much lower mitotic rate

than HGSC. Genetically, there is no chromosomal instability and the presence of BRAF and KRAS mutations is common in most patients. These two oncogenes are involved in cell growth and proliferation. LGSC does seem to be associated to BRCA1 and BRCA2 germline mutations (Singer et al., 2002; Prat et al., 2012). In LGSC ER also was expressed in a lot of patients (Köbel et al., 2009).

The origins of LGSC are benign or borderline tumors that show a micropapillary structure and transform finally to invasive LGSC. LGSC rarely transforms to HGSC (Gilks and Prat, 2009).

Endometrioid carcinoma (EC)

Mostly affected by EC are women in their perimenopausal age. The EC of the ovary appears in 28% of cases bilateral and in 15% to 20% in combination with carcinomas of the endometrium. EC has the most favorable prognosis of all subtypes, due to the fact that it is usually diagnosed at an early stage. It is mostly confined to the pelvis and shows a good response to conventional chemotherapy. The typical structures are round or tubular glands, lined with stratified nonmucin-containing epithelium. In 50% of the patients, even a squamous differentiation of the tumor is observed. Interestingly, also clear cells are found in EC but overall the architecture looks more like HGSC than CCC (Gilks and Prat, 2009; Prat, 2012).

Mutations in the cell-cell adhesion gene β -catenin (CTNNB1) and the tumor suppressor gene phosphatase and tensin homologue gene (PTEN) are very common in EC and are assumed to be early events in tumor progression. PTEN plays a crucial role in the activation of apoptosis and via inactivating it the apoptosis inhibiting signaling pathway PI3K-AKT is initiated (Sato et al., 2000; Prat, 2012). ECs are mostly immunoreactive for ER (Köbel et al., 2009). In a study of 455 ovarian carcinomas, Wiegand et al. identified a mutation in the tumor suppressor gene AT-rich interactive domain-containing protein 1A (ARID1A) in 30% of EC and in none of the HGSC tumors. ARID1A is a part of the SWItch/Sucrose NonFermentable (SWI-SNF-A) complex, a big multiprotein chromatin remodeling complex that is involved in the enhancement and repression of transcription. It is supposed that this mutation is a key step of adjacent endometriosis transforming into ovarian EC (Wiegand et al., 2010; Lemon et al., 2001).

Mucinous carcinoma (MC)

MC is with 10% is the third most common EOC, but only 20% are malignant. Only 15% to 35% response to the standard platin and taxane based chemotherapy (Gilks and Prat, 2009; Prat,

2004). Characteristic for this tumors is the large size >13cm and the unilateral appearance. Normally MC is confined to the ovaries. Histological key features are the goblet cells, but in some tumors they are very rare. Overall MC is a very heterogeneous group of tumors, a coexistence of benign, borderline and invasive and/or noninvasive carcinoma is common. In most of the cases they are confined to the ovary and do not show surface involvement (Gilks and Prat, 2009; Prat, 2012). Lately the MC was classified in two groups, the “expansive type”, which shows no obvious stromal invasion and the “infiltrative type” in which a stromal invasion was observed. Generally, the “expansile type” has a more favorable outcome than the “infiltrative type”.

The presence of mutations in the oncogene KRAS are widely spread and are supposed to be an early event in the development of MC. In 10% to 20% amplifications of the human epidermal growth factor receptor 2 (HER2) were found. In these patients, a therapy with the human monoclonal antibody Trastuzumab is recommended.

This subtype is negative for ER. It still remains open from where these subtypes develop (Gilks and Prat, 2009; Prat, 2012; Köbel et al., 2009).

Clear cell carcinoma (CCC)

This subtype accounts approximately for 10% of all EOCs. It is mostly diagnosed at stage I or II but has a less favorable outcome than the other subtypes discovered at these stages, because it has a low chemosensitivity. Interestingly, these tumors have a much better prognosis if they are associated with endometriosis. Furthermore it is assumed that CCC arises from endometriosis. Usually these tumors are confined to the pelvis (Gilks and Prat, 2009; Komiyama et al., 1999; Prat, 2012). Typically for this subtype is the presence of various complex papillae, the membrane basement, which is rich of hyaline, expands the core of the papillae. In approx. 20% of the patients, hyaline bodies are visible. The mitotic rate is (after LGSC) the lowest of all subtypes (Prat, 2012). No abnormalities in the BRCA genes or a chromosomal instability were detected in CCC until now. But recently Wiegand et al. observed in about 50% of the patients ARID1A mutations (Wiegand et al., 2010). 95% of the patients are negative for ER (Köbel et al., 2009).

1.2.4 Staging and Grading

The staging is essential for the prognosis and choice of therapy for the patient. The higher the stage of the tumor is the shorter is the survival time. Normally, the staging is done after surgery.

The common staging systems are the systems declared from the “Federation Internationale et d’Obstetrique Federation” (FIGO) and the “American Joint Committee on Cancer system” (AJCC=TNM). These two systems are similar and categorize the tumors based on affliction of the ovaries and extension of the tumor to the pelvis, the lymph nodes and to other organs (metastasis).

FIGO		TNM
	Primary tumor cannot be assessed	TX
0	No evidence of primary tumor	T0
I	Tumor confined to the ovaries	T1
IA	Tumor limited to one ovary, capsule intact no tumor on ovarian surface no malignant cells in the ascites or peritoneal washings	T1a
IB	Tumor limited to both ovaries, capsules intact no tumor on ovarian surface no malignant cells in the ascites or peritoneal washings	T1b
IC	Tumor limited to one or both ovaries with any of the following capsule ruptured, tumor on ovarian surface, positive malignant cells in the ascites or positive peritoneal washings	T1c
II	Tumor involves one or both ovaries with pelvic extension	T2
IIA	Extension and/or implants in uterus and/or tubes no malignant cells in the ascites or peritoneal washings	T2a
IIB	Extension to other pelvic organ no malignant cells in the ascites or peritoneal washings	T2b
IIC	IIA/B with positive malignant cells in the ascites or positive peritoneal washings	T2c
III	Tumor involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph nodes metastasis	T3/N1
IIIA	Microscopic peritoneal metastasis beyond the pelvis	T3a
IIIB	Macroscopic peritoneal metastasis beyond the pelvis 2cm or less in greatest dimension	T3b
IIIC	Peritoneal metastasis beyond pelvis more than 2cm in greatest dimension and/or regional lymph nodes	T3c/N1
IV	Distant metastasis beyond the peritoneal cavity	M1

Tab. 1.2.1: FIGO and TNM Staging System for Ovarian Cancer

This table shows the official ovarian cancer staging of FIGO and AJCC.

Through microscopic observation of ovarian cancer samples, the differentiation grade of the tumor cells can be identified. Generally, the more the tumor cells are different from the cells of

origin, usually, the more aggressive and faster growing the tumor is. This implies that the patient has a less favorable prognosis with a higher grading and a higher stage tumor. The grading system contains four stages “G1 to G4” for malignant tumors, one stage for borderline tumors “G0” and GX for not evaluable grades (Sato et al., 2003).

Grading of ovarian cancer	
GX	The grade cannot be evaluated
G0	The tissue is considered borderline cancerous.
G1	The tissue is well differentiated
G2	The tissue is moderately differentiated
G3-G4	The tissue is poorly differentiated or undifferentiated

Tab. 1.2.2: Grading of Ovarian Cancer

The stages G1 to G4 represent how far the tumor cells are differentiated from the origin cells.

1.2.5 Diagnosis

Due to the fact that at the early stages, when the tumor is confined to the ovaries, patients have no or few symptoms, approximately 70% of ovarian cancers are diagnosed at advanced stages. This is a serious problem because the five year survival in women with advanced disease is 20% to 30% and this is much lower than the rate for women where the cancer is still defined to the ovaries. The latter have a five year survival rate of 70% to 90% (Ledermann et al., 2013; Ozols et al., 2005). Therefore, it is essential to find and establish new screening methods for an earlier detection of ovarian cancer.

Symptoms that are seen at all stages are abdominal or pelvic pain, constipation, diarrhea, urinary frequency, vaginal bleeding, abdominal distension and fatigue. Further symptoms that are only observed in advanced stage of disease are ascites, bloating, nausea, anorexia, dyspepsia etc. because of the expanding abdominal masses (Ledermann et al., 2013, Goff et al., 2007).

The first step of the diagnosis is normally an accurate anamnesis of the patient. Very essential is the information about the patients, e.g. if there is a history of ovary dysfunctions. Also the family history is important because of known cases of ovarian cancer in the family.

For a clinical diagnosis the palpitation of the pelvis is required. By the use of this method the tumor or bigger amounts of ascites can be detected manually.

The most common imaging procedure in the diagnosis process of ovarian cancer is the transvaginal or/and transabdominal sonography, a method based on ultrasonic inspection. It is

not established as a conventional screening method, due to the fact that ovarian cancer can develop fast in between two sonography appointments within a year. With this method the tumor size, structure of the tumor, appearance of ascites and metastases can be observed. For further investigations of the spread of the tumor and also of metastases a Computer tomography (CT) is applied.

These imaging methods are applied before operation and are essential for a proper evaluation of the tumor expansion, structure and metastasis (Bühling and Friedmann, 2009).

The most reliable blood serum marker for ovarian cancer is the cancer antigen 125 (CA 125). But this marker only detects 80% of all ovarian cancers. Moreover nearly all HGSC express high detectable amounts of CA 125 but only 60% of the subtype MC expresses it. The problem is that ovarian cancer is a very heterogeneous disease with different clinical characteristics and several molecular changes. Therefore it would be important to find a set of tumor markers to detect all subtypes in early stage disease (Lu et al., 2004).

1.2.6 Prognosis

The most significant prognostic factors for ovarian cancer are histological type, tumor grading, age, general condition and postoperative residual tumor.

Ovarian cancers are a very heterogeneous group of tumors and therefore are subdivided into various types based on their origin and appearance. The subtypes CCC and MC mostly do not response well to chemotherapy and have a less favorable prognosis in contrast to serous and endometrial carcinomas (du Bois et al., 2009).

Also age seems to play a crucial role. That is proved by a metaanalysis demonstrating that patients younger than 65 have a significantly higher five survival rate of 64% than women older than 65 (Thigpen et al., 1993).

Another important prognostic factor is the tumor stage. The five year survival rate for patients with FIGO stage I is 80% to 90%, with stage II 60% to 80%, with stage III 20% to 25% and with stage IV 5% to 10% (Tingulstad et al., 2003).

The general condition can be described through the “Karnofsky Index” (KI). Patients with a KI higher than 70% have a better prognosis in contrast to patients with a KI below 70% (Sehouli et al., 2004).

The most valuable prognostic factor is the presence and size of residual tumor after surgical removal. Bristow et al. carried out a metaanalysis including women with FIGO stage III and IV that received an adjuvant platin and taxane based standard therapy. They came to the conclusion that women with less than 25% residual tumor after operation had a significantly longer survival compared to women with more than 25% residual tumor (Bristow et al., 2002; du Bois et al., 2009).

1.2.7 Therapy

The standard procedure in the therapy of ovarian cancer is primary a cytoreductive surgery and at advanced stages, it is common to start an adjuvant chemotherapy.

Generally for a more exact observation of the ovarian cancer a laparotomy is carried out. This is important to obtain more information for further therapy of the ovarian cancer including tumor spread and staging (Diedrich et al., 2000).

After the laparotomy a radical removal of the tumor takes place. The main ambition is to remove the whole amount of degenerated cells until no residual tumor is left, due to the fact that this is the main prognostic factor of ovarian cancer. In advanced stages if necessary also surrounding tissue can be removed including the tuba uterinea, uterus, omentum and parts of the colon. Furthermore also affected lymph nodes should also be removed (Kreienberg et al., 2009; Pedian and Garcia, 2003).

In women with early FIGO stage and low grading (IA/G1, IA/G2, IB/G1, IB/G2) ovarian carcinoma the five year survival rate after a laparotomy is between 90% and 95%. With adjuvant chemotherapy no significant improvement was observed in this group (Bühling and Friedmann, 2009).

Starting with FIGO stage IA with high grading (G3) adjuvant chemotherapy after the operation is recommended (Trimbos et al., 2003).

The success of chemotherapy is mainly interdependent from the size of the residual tumor. The smaller the tumor, the less is the risk of a full recurrence after the chemotherapy (McGuire et al., 1996).

The first line treatment standard therapy is Carboplatin (5-7,5mg/ml/min) in combination with 175mg/m² Paclitaxel for three hours and dependent on the severity of the disease 4 to 6 cycles

every three weeks. Also in use is the combination of Cisplatin and Paclitaxel but due to its side effects which are worse than the combination of Carboplatin with Paclitaxel it is not used as a first line therapy (Neijt et al., 2000).

Often the tumors develop a platin resistance and this results in a relapse in approximately 70% of all patients during the first three years (Friedlander et al., 2011). Patients with platinum resistance or with an early relapse should receive a treatment that improves quality of life and the control of symptoms. For these patients the prognosis is very poor and overall survival is usually lower than 12 months (Parmar et al., 2003). Paclitaxel, topotecan, pegylated liposomal doxorubicin (PLD) and gemcitabine showed in a phase III trial an extension of the lifetime for 3 to 4 months (Raja et al., 2013).

1.3 The Role of Steroid Hormones

Steroid hormones, especially estrogen seems to play a crucial role in the development of hormone dependent cancers including ovarian-, endometrial- and breast cancer.

1.3.1 Estrogen

The most active form of estrogen is 17 β -estradiol (E2), which is produced in premenopausal women mostly by the ovaries and acts as an endocrine circulating hormone. In postmenopausal women the circulating levels are very low, the E2 synthesis in the ovaries decreases dramatically and most E2 is produced from adrenal steroid precursors in peripheral tissues including breast, brain, and liver as well as adipose tissue (Labrie et al., 2003; Suzuki et al., 2005).

The precursor of all steroid hormones is cholesterol. It consists of 27 C-atoms and is synthesized by the liver or is absorbed in the colon from food. Cholesterol is converted over a few steps to pregnenolone and next to progesterone. This is the starting point for all steroid hormones in the adrenal cortex, male (androgens) and the feminine sex hormones (estrogens). Dehydroepiandrosterone-sulfate (DHEA-S), Dehydroepiandrosterone (DHEA), 5 α -androstenediol-sulfate (5-Diol-S) and estrone-3-sulfate (E1-S) are synthesised in the adrenal cortex and released to the blood system (Silbernagel and Despopoulos, 2007). In postmenopausal women the plasma levels of DHEA-S, DHEA and 5-Diol-S are relatively high compared to premenopausal women. In contrast estrone (E1) and E2 plasma concentrations

are decreased. E1-S has a much higher half life time than E1 and E2. In the blood 10% to 30% of estrogen is bound to sex hormone binding globuline (SHBG) and act as reservoir for peripheral formation of E2. SHBGs are synthesised in the liver by the stimulation of estrogen and thyroid hormones. Progesterone inhibits its production (Purohit and Foster et al., 2012; Leidenberger et al., 2009; Audet-Walsh et al., 2011).

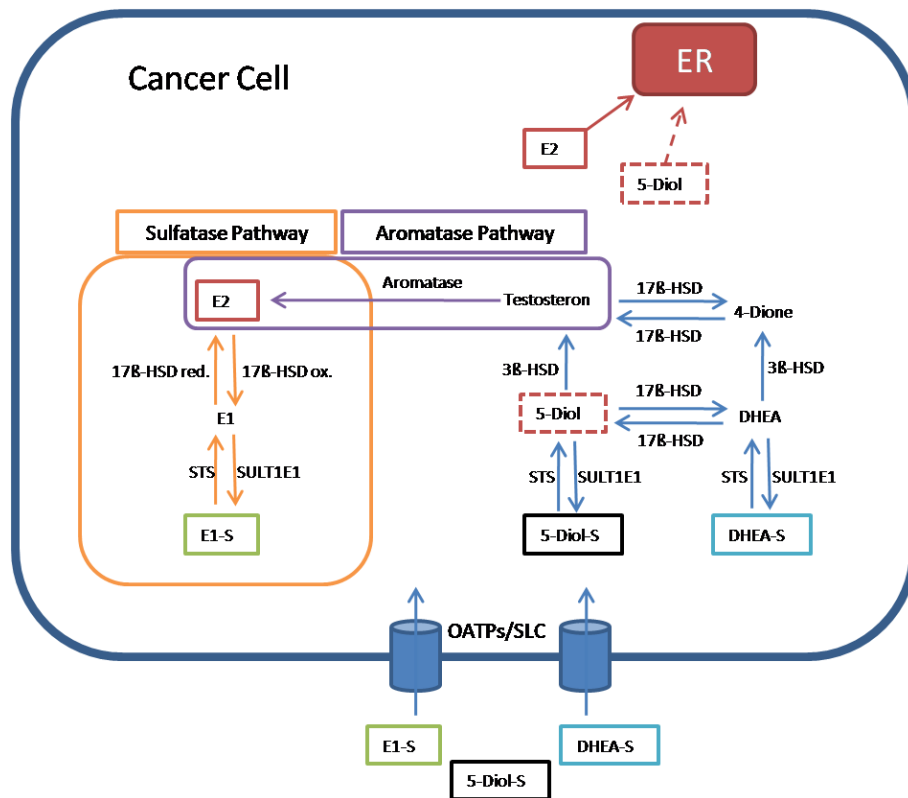


Fig. 1.3.1: The Pathways of Estrogen

Through organic anion transporting polypeptides (OATPs) and other members of the solute carrier family (SLCs) the precursors of 17β-estradiol (E2) namely estrone-3-sulfate (E1-S), androstenediol (5-Diol-S) and dehydroepiandrosterone-sulfate (DHEA-S) are taken up from the blood vessels into the cells. E1-S is converted in the sulfatase pathway through steroid sulfatase (STS) to estrone (E1) and next transformed with the reductive 17β-hydroxysteroid dehydrogenases (17β-HSD red.) to the most active form of estrogens E2, which can bind and activate estrogen receptor (ER). The reverse pathway from E2 to E1-S is operated by the oxidative 17β-hydroxysteroid dehydrogenases (17β-HSD ox.) and the steroid specific sulfotransferase (SULT1E1). For the aromatase pathway testosterone has to be formed by converting 5α-androstenediol-sulfate (5-Diol-S) to 5α-androstenediol (5-Diol) by STS and further to testosterone through 3β-HSD. The second way to form testosterone is by converting DHEA-S to dehydroepiandrosterone (DHEA) by STS and further to androstenedione (4-Dione) by 3β-HSD and in the end to testosterone by 17β-HSD. Testosterone can be transformed by aromatase to E2. Also 5-Diol can bind ER but with a lower affinity (Secky et al., 2013; Labrie, 1991).

For the production of E2, two pathways came into focus, on the one hand the sulfatase pathway and on the other hand the aromatase pathway. The sulfatase pathway uses the inactive adrenal precursor E1-S to produce E2 in contrast to the aromatase pathway which transforms testosterone to E2. The testosterone is also synthesised from adrenal precursors,

namely 5-Diol-S and DHEA-S. The active estrogen E2 and with a much lower affinity 5-Diol activate the nuclear estrogen receptor (ER), which binds to a specific DNA sequence, the estrogen response element (ERE) and induces the transcription of target genes in the cell (Secky et al., 2013; Labrie, 1991).

Gonodale function

Estrogen is essential for the development of sexual characteristics and in the process of follicle maturation (see chapter “1.1.1 Histology and Function”). In the uterus, it is important for the proliferation of the endometrium and of the uterus musculature (myoendometrium). In the cervix, it modifies the mucus, which leads to an easier entrance of the sperms and increases their lifetime. Also the proliferation of the mammary gland is controlled by estrogens and progesterone (Silbernagel and Despopoulos, 2007).

Extragonodale function

Estrogens also have a positive effect by exciting the production of osteoblasts, stimulating the synthesis of collagen type I and other bone proteins (osteocalcin, osteopontin), and inducing cell death of osteoclasts by the activation of ER- α , which leads to an increased expression of the proapoptotic Fas-Ligand. Many studies showed that after menopause women have an increasing risk of developing osteoporosis, which can be explained by the reduction of the estrogen level (Nakamura et al., 2007).

Estrogens stimulate the collagen production, and therefore lead to a younger looking skin, but consequently after menopause and the reduction of estrogen this effect starts to decrease.

Another area of influence is the lipid metabolism. Estrogens lower the concentration of the low density lipoproteins (LDL) and very low density lipoproteins (VLDL). Also an increased level of protective high density lipoproteins (HDL) is induced by estrogens. This explains why women after menopause have a much higher risk of developing atherosclerosis than women in their premenopause (Silbernagel and Despopoulos, 2007; Leidenberger et al., 2009).

Estrogen also promotes the blood coagulation by increasing the concentration of Fibrinogen, factors VII, VIII and X and decreases coagulation inhibiting proteins including protein C, protein S and Antithrombin III (Leidenberger et al., 2009).

1.3.2 Steroid Sulfatase (STS)

STS is a member of the arylsulfatases, which are known for the hydrolysis of sulfate ester bonds in a wide range of substrates. The corresponding gene is located on the X chromosome and contains ten exons. Hernandez-Guzman et al. was the first to isolate STS from the human placenta, purified and crystallized it. This enzyme consists of two membrane-spanning hydrophobic alpha helices that are arranged anti-parallel. The amino acid proline on position 212 acts on the cytosolic side of the endoplasmatic reticulum membrane as turning point. STS is present in the endoplasmatic reticulum of many tissues and is especially high in ovarian granulose cells (Ghosh, 2007; Hernandez-Guzman et al., 2001; Clemens et al., 2000).

It plays an essential role in the synthesis of E2, as shown in Fig 1.3.1, by converting E1-S into more potent E1.

1.3.3 Steroid specific Sulfotransferase (SULT1E1)

SULT1E1 belongs to the family of sulfotransferases (SULT) and is a phase II detoxification enzyme. Ten isoforms of SULT are known to be expressed in humans. Phase II enzymes have a wide range of substrates including hydroxysteroid, thyroid hormones, phenols, arylamines and primary alcohols. Due to the fact that SULT1E1 has the highest affinity of all SULTs to E1 and E2 it is also called estrogen sulfotransferase. It catalyzes the transfer of a sulfonyl group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to nucleophilic groups of their substrates. PAPS are synthesized by PAPS synthesizing enzymes (PAPSS).

SULT1E1 is the antagonist to STS by converting E1 to E1-S and therefore inactivating estrogen. The corresponding gene is located on chromosome 4q3.12, has eight exons and a length of 20kB. The mRNA of SULT1E1 is present in various tissues, including liver, testis, mammary epithelium cells and endometrium (Secky et al., 2013; Honma et al., 2002; Adjei et al., 2003; Xu et al., 2012).

1.3.4 Estrogen Receptor (ER- α , ER- β)

Until 1996 only one ER receptor was known. Then Kuiper et al. cloned a new estrogen receptor, named ER- β from a rat testis. As a consequence, ER was renamed to ER- α . The two ERs are encoded by the genes ESR1 and ESR2 (Kuiper et al., 1996). The estrogen receptors are members of the nuclear receptor superfamily. They belong to the steroid receptors and function as ligand

inducible transcription factors (Beato et al., 1989). They mediate most estrogen induced effects on gene regulation and therefore, are essential for various developmental and functional processes of several tissues/cells (Kumar et al., 2011). The regulation of the target genes happens by binding to ERE and/or other specific co-regulatory proteins (Kumar. et al., 1999). A dimerization of ER is necessary for transcriptional activation (Delaunay et al., 2000).

The nuclear receptor superfamily is defined through five different domains, namely A/B, C, D, E and F. Each domain fulfills another obligation, which is essential for the correct function of the receptor. The A/B domain, also named N-terminal domain (NTD) includes a ligand independent activation function (AF-1). This domain also has a rather poor (20%) homology (ER- α vs. ER- β) (Zhoa C. et al., 2008). This gives a hint that this region takes mainly part to the ER subtype specific actions on target genes. Moreover ER- α seems to have a stronger corresponding AF-1 function than ER- β (Delaunay et al., 2000). The C domain, also called DNA-binding domain (DBD) appears to be responsible for specific DNA binding and also receptor dimerization. This domain shows a high homology of 95% for ER- α and ER- β and therefore is highly conserved. The D-domain is a flexible hinge between DBD and ligand-binding domain (LBD) and therefore is called "Hinge domain". ER- α and ER- β share only 30% homology in the sequence of this domain. It is essential for the transport to the nucleus and contains a nuclear localization signal. The following E-domain, also called LBD is responsible for hormone-dependent activation (AF-2), ligand binding and together with DBD for receptor dimerization. ER- α and ER- β show a homology of 55% for LBD (Tora et al., 1989; Picard et al., 1990). The amino acid sequence of the ligand binding cavity of the two receptors varies only in two positions, what leads to a significant smaller cavity in ER- β . This may be an advantage for receptor subtype specific drugs (Brzozowski et al., 1997; Pike et al., 1999). The function of the F domain is still not clear.

ESR1 the corresponding gene for ER- α is located on chromosome 6q25.1. The receptor is the predominant ER in the uterus, mammary glands, testis, pituitary, liver, kidney, heart and skeletal muscle (Brandenberger et al., 1999; Byers et al., 1997; Couse et al., 1999; Halon et al., 2011). The target genes of ER- α are known for being involved in processes including proliferation and cell survival (Bardin et al., 2004).

The ER- β gene is located on chromosome 14q23.2, consists of eight exons and is approximately 61.2 kB long. Through alternative splicing five isoform, namely ER- β 1-5 can be synthesized by deletion of one or more exons. The translated human ER- β 1 has a length of 530 amino acids

(Kuiper and Gustafsson 1997; Ogawa et al., 1998). ER- β is the predominantly distributed in ovary and prostate (Brandenberger et al., 1999; Byers et al., 1997; Couse et al., 1999). It activates pathways that are anitproliferative and pro-apoptotic (Bardin et al., 2004).

1.3.5 Progesterone

Progesterone consists of 21 C-atoms and is synthesized from cholesterol in the corpus luteum, follicles, placenta and the adrenal gland. During transport through the blood plasma, it is bound to cortisol binding globulin (CBG), because of its lipophilic nature and also to avoid degradation (Silbernagel S. and Despopoulos A., 2007).

Gonodale Functions

Progesterone as an antagonist of estrogen has an antiproliferative effect on specific cells. It acts in part by decreasing the production of ER, and through activation of 17 β -HSD and SULT1E1.

Progesterone plays an important role in the follicle maturation (see chapter “1.1.1 Histology and function”) and, moreover, it is responsible for preparing the female genital tract for pregnancy. It also maintains the pregnancy after fertilization. Progesterone promotes the growth of the uterus musculature (myometrium) and changes the endometrium from a proliferative to a secretory tissue. Progesterone also decreases the myometrium activity during pregnancy. It changes the quality and quantity of the cervix mucus, thus preventing the entrance of sperms into the uteri and the tubes (Silbernagel and Despopoulos, 2007; Leidenberger et al., 2009).

Extragonodale functions

High doses of the degradation product of progesterone, namely pregnenolone, in the central nervous system can stimulate epileptic seizures. It also has a thermogenic effect, which leads to an increase of the basal body temperature and seems to be the cause for depression before the menstruation and at the end of the pregnancy.

In the lipid metabolism it acts against estrogen by the stimulation of the lipoprotein lipase and affects, therefore, an increased storage of fat.

In the kidney, it enhance the effect of aldosterone which leads to an increased excretion of NaCl (Silbernagel S. and Despopoulos, 2007; Leidenberger et al., 2009).

1.3.6 Progesterone Receptor (PGR)

This PGR is a member of nuclear receptor superfamily, and is responsive to progesterone. The PGR is present in two isoforms, namely PR-A and PR-B. They are encoded by one gene but the transcription is carried out by two different promoters. Apart from the lack of a 164 amino-sequence at the N-terminal end in PR-A, the two PGRs are identical. PR-B is responsible for the transcriptional activation of responsive genes. It can be inhibited by the other isoform PR-A (Vegeto et al., 1993; Kastner et al., 1990; Li and O'Malley, 2003; Lonard and O'Malley, 2012). PR-A and PR-B can act by homo- or heterodimerization and are synthesized in equal amounts in healthy epithelial cells (Mote et al., 2002).

PGR consists of four domains, namely the ligandbinding domain (LBD), the Hinge-Region, the DNA-binding domain (DBD) and the N-terminal domain (NTD). The Hinge-domain represents the flexible link between the LBD and DBD (Hill et al., 2012).

1.4 The Relevance of STS, SULT1E1, ER and PGR in Ovarian Cancer

There is evidence that estrogens play a role in the progression of ovarian cancer. An overexpression of STS will lead to an increased level of E2 and this may contribute to cancer progression. In postmenopausal women, the local conversion from circulating low potency estrogens and other precursors including DHEA to high potency estrogens could promote ovarian cancer progression. But there are variations in the amount of enzymes in the “estrogen pathways” in healthy ovaries as well as in patients with ovarian cancer.

STS was detected by IHC in 30% of serous-, in 70% of CCC and in 8% of the mucinous ovarian carcinomas (Okuda et al., 2001). Ovarian cancer studies showed that a longer progression free survival is significantly associated with lower STS levels. That can be explained by the fact that through a high expression of STS, more E2 is synthesised (Chura et al., 2009; Secky et al., 2013). Also breast cancer studies showed a similar effect of STS. Even its activity in breast cancer correlated with the E2 serum levels. High SULT1E1 expression is associated with smaller tumors, a better prognosis and a negative lymph node status (Utsumi et al., 1999; Miyoshi et al., 2003; Poission Pare et al., 2009; Kirilowovas et al., 2007). Another evidence that SULT1E1 acts as a tumor suppressor gene is based on a study with Xenograft models carried out by Xu et al. They found out that an overexpression of SULT1E1 and PAPSS inhibited estrogen dependent

cell growth by downregulating c-myc, cyclin D1, bcl-2 and therefore induces apoptosis (Xu et al., 2012).

Studies with aromatase inhibitors in ovarian cancer were not promising so far, which leads to the conclusion that the sulfatase pathway is the main pathway for estrogen activation in ovarian cancer. This is already proved for breast cancer. The activity of STS in breast cancer is at least 50 fold higher than in healthy tissue, which could lead to an approximately ten times higher amount of E1 through the sulfatase pathway (Stanway et al., 2007; Purohit and Foster 2012).

Stanway et al. published a study with the STS inhibitor STX64 and showed a reduction not only of estrogen, but also of androstenedione levels in the serum. He came to the conclusion that 4-dione is not released directly from the adrenal gland, but is synthesised by STS peripheral by the conversion of DHEA (Stanway et al., 2007).

Overall high E2 levels are often observed in ovarian cancer patients (Kirilovas et al., 2007). E2 also increases the mobility and metastasis of ovarian cancer through the inhibition of cell-cell adhesion. This process is exclusively regulated by the ER- α dependent pathway (Park et al., 2008). Furthermore this effect also was observed in breast cancer by Al Sarakbi et al (Al Sarakbi et al., 2006).

ER- α seems to initiate cell proliferation and progression of the ovarian cancer. In contrast ER- β is associated with a proapoptotic and antiproliferative effect. This hypothesis is supported by various studies such as one of the group of Fujimoto et al. who found that a high level of ER- α and a low level ER- β is associated with a worse prognosis. Indeed, a balanced ratio between ER- α and ER- β seems to be important for a proper function of the receptors in healthy tissue (Fujimoto et al., 2000; Li et al., 2003; Lindgren et al., 2004). Halon et al. found that high level of ER- α is associated with a longer overall- and progression free survival (Halon et al., 2011). The reduction of ER- β in tumor tissues compared to healthy tissues were found in estrogen dependent tumors, lead to the assumption that the loss of ER- β seems to promote tumor progression (Skiris et al., 2003; Iwao et al., 2001; Horwath et al., 2001). Further the mRNA levels of ER- β are significantly associated with longer disease-free survival and a longer overall survival. The reduction of ER- β significantly correlated with the hypermethylation of the ER- β promoter, causing an inhibition of gene translation (Suzuki et al., 2008). Another proof for the protective effect of ER- β is that this isoform is the dominant estrogen receptor in healthy ovaries and borderline tumors. But in all serous tumors and also in metastasis, ER- α is dominant

and ER- β weak or absent. This leads to the conclusion that the gradual reduction of ER- β during tumor progression (from normal to borderline to malign tissue to metastasis) is a continuous process and important for malignant transformation and cancer cell proliferation (Chan et al., 2008; Rutherford et al., 2000). Similar findings with respect to ER- α and ER- β were found for breast cancer (Skiris et al., 2003; Rody et al., 2005).

Lee et al. showed that PGR is a biomarker for a better prognosis and higher overall survival in ovarian cancer (Lee et al., 2005). Also, PGR levels are significantly lower in benign, borderline and malignant ovarian tumors than in healthy tissue (De Stefano et al., 2011; Lindgren et al., 2004).

Taken together, progesterone has been proved to decrease the proliferative effect of estrogen, inhibit OCE penetration and inflammation by suppressing ovulation and also initiates apoptosis in tumor cells (Lee et al., 2005).

1.5 Organic Anion Transporting Polypeptides (OATPs)

The cell membrane provides a natural mechanical barrier for cells to prevent the uncontrolled movement of endogenous and exogenous substances between the environment and the cell interior. Very small molecules and ions like O₂ or CO₂ are able to diffuse through the membrane according to lipophilicity, although the contribution of specific channels is discussed (Wang et al., 2007). However, bigger molecules, particularly with a more hydrophilic nature need special transport proteins that are embedded in the membrane. Transporter proteins are classified to their mode of action: passive transporters transport compounds according to the electrochemical gradient (facilitated transport) and active transporters mediate the transport of compounds against the chemical and/or electrical gradient. The active transporters are classified into two groups: the primary active transporters, which directly use ATP or GTP hydrolysis for providing energy and the secondary active transporters that function as cotransporters or exchangers. In the latter, the transport of two compounds is coupled and one of them is shuttled against the chemical and/or electrical gradient (Dopson et al., 2009; Svoboda et al., 2011).

In 1994, Jaquemin et al. discovered for the first time a member of the OATP family in a rat liver. This family consists of sodium independent transporters that are located in the plasma membrane (Jaquemin et al. 1994; Buxhofer-Ausch et al., 2013). Until now 300

OATPs(human)/oatps(non-human) from at least 40 species were identified in the animal kingdom.

In humans, OATPs are found in epithelial and endothelial cells in nearly every organ as well as in blood cells. Some OATPs seem to be organ specific and others are expressed ubiquitously. They transport a wide range of substrates mostly amphipathic organic anions with a weight more than 300Da including prostaglandins, thyroid and steroid hormones, their conjugates, bile acids, drugs, and xenobiotics. Based on this information OATPs could be targets in cancer treatment (Hagenbuch and Gui, 2008; Hagenbuch and Stieger, 2013).

1.5.1 Nomenclature and Classification

The classification of the OATP superfamily (>25% identity) is based on the homology of the DNA sequence. This system was established by the HUGO Gene Nomenclature Committee (HGNC). The solute carrier family acts as passive transporters or secondary active transporters and contains 48 SLC families. One of them is the family SLC21, also called SLCO(human)/slco(non-human) family. The SLCO/slco system describes genes and their corresponding proteins are known as OATP/oatp.

The OATPs/oatps that have at least sequence 40% homology are subdivided in the six families OATP1, OATP2, OATP3, OATP4 OATP5 and OATP6. With more than 60% homology according to their protein structure they are classified again into subfamilies and are marked in the end with a letter e.g. OATP1A, OATP2B and OATP4A. Until now 6 members of human OATPs were found and they are present in all six families (Hagenbuch and Meier, 2004; Hagenbuch and Gui, 2008).

OATP1	OATP2	OATP3	OATP4	OATP5	OATP6
OATP1A2 OATP1B1 OATP1B3 OATP1C1	OATP2A1 OATP2B1	OATP3A1	OATP4A1 OATP4C1	OATP5A1	OATP6A1

Tab. 1.5.1: Overview of the six human OATP Superfamilies (Hagenbuch and Gui, 2008)

1.5.2 Structure and Function

OATPs consist of 643 to 722 amino acids. Based on computer modelling techniques and the application of molecular dynamic simulations, a structure of twelve transmembrane helices with five intra- and six extracellular loops was proposed. The C-terminal as well as the N-terminal end is located at the cytoplasmic side. The twelve transmembrane domains are

arranged to a pore, which has a positive electrostatic potential that transports negatively charged compounds (Hagenbuch and Meier, 2004; Meier-Abt et al., 2005).

There are several sequences that give a hint how the transporters operate. The “OATP superfamily signature” is a highly conserved 13 amino acids long sequence at extracellular domain three and transmembrane domain six. Until now it is not known if this specific OATP signature plays a role in the function (Svoboda et al., 2011).

The intracellular loops three and five have some conserved potential N-glycosylation sites, with OATP2A1 mutations studies the importance of glycosylation for the function and the correct membrane localisation of OATPs was shown (Lee et al., 2003).

On loop five, ten conserved cysteine residues are present, which are similar to the zinc finger domains of DNA-binding proteins. Hänggi et al. came to the conclusion that they are usually disulfide bonded and moreover by OATP2A1 mutation studies they could show that these bonds are important for a proper expression of the transporter, and also for protein-protein interactions (Hänggi et al. 2006).

Some OATPs contain a C-terminal PDZ (Post synaptic density, separate junction-protein Discs, tight junction-protein Zonula occludentes-1) consensus sequence and the group of Wang showed that it is essential for the interaction with the PDZK1 protein and therefore for plasma membrane localisation (Wang et al., 2005).

OATP5A1

In this thesis the main focus is on one of the less characterised members of the OATP family, namely OATP5A1. It has a predicted protein mass of 848 amino acids and a molecular mass of approximately 92kDa. The corresponding gene *SLCO5A1* is located on chromosome 8q13.3. So far no data exist which compounds OATP5A1 transports. But it is known that it has a high mRNA expression in brain, heart, skeletal muscle, ovary, breast cancer and bone cancer (Hagenbuch and Meier, 2004; Wlcek et al., 2008; Svoboda et al., 2011). Also an expression of OATP5A1 in primary liver cancer and in metastases from colon cancer in the liver was observed lately (Wlcek et al., 2011). Furthermore also in satraplatin resistant lung cancer an expression of OATP5A1 was found. It seems as OATP5A1 is mainly involved in this process (Olszewski-Hamilton et al., 2011). Kindla et al. detected plasma membrane bound OATP5A1 in epithelial cells of the lactiferous ducts in healthy breast tissue. In cancerous breast tissue they found the transporter at the plasma membrane and in the cytoplasm (Kindla et al., 2011).

Interestingly, the expression profile on www.proteinatlas.org shows highest amounts of OATP5A1 in cortical cells of the adrenal gland and in follicle cells of the ovary. Regarding the transport of hormones OATP5A1 might be as important as other OATP family members.

But there is also evidence of functional studies and transcriptional analysis that OATP5A1 is a non-classical OATP, which is involved in reorganisations of the cellular shape including differentiation and migration (Sebastian et al., 2013).

Isidor et al. found that the deletion of *SLCO5A1* and *SULF1* (encoding the gene for heparin sulphate 6-O-endosulfatase 1) at chromosome 8q13 leads to the very rare mesomelia-synostoses syndrome (MSS). This autosomal-dominant disease results in mesomelic limb shortening, acral synostoses and multiple congenital malformations. *SULF1* is involved in the regulation of various growth factors during the development of the embryonic skeletal (Isidor et al., 2010).

1.6 Relevance of OATPs in Ovarian Cancer

OATPs are involved in the transmembrane transport of a great variety of compounds including cytostatic compounds and other drugs such as statins, but also of endogen substrates such as cyclic nucleotides, steroid hormones and their conjugates as well as prostaglandins. By transporting these substrates they regulate the cellular levels of these compounds and this may influence tumor development, progression and treatment.

OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1 and OATP4A1 transport estrogen precursors, which seem to be involved in tumor progression. In chapter “1.4 The Relevance of STS, *SULT1E1*, ER and PGR in Ovarian Cancer” the influence of estrogen on ovarian cancer progression is discussed.

Also prostaglandins (PGs) are substrates of OATP1A2, OATP1B1, OATP2B1, OATP3A1 and OATP4A1. PGs are involved in several biological functions in the human body including regulation of blood pressure, contraction and relaxation of smooth muscle cells in the trachea, renal functions, reproductive functions and others. Their activities also provide cell growth and differentiation. Proinflammatory PGs, especially PGE₂, were also found to contribute carcinogenesis and progression of cancer in the gastro-intestinal system, pancreatic, gallbladder, colon, lung, prostate, breast, cervix and ovary (Hagenbuch and Gui, 2008; Wang and Dubois, 2006; Allaj et al., 2013).

OATPs can also influence cancer chemotherapy. For example, paclitaxel, used in first line treatment of ovarian cancer is a substrate for OATP1B3. Expression and activity of the transporter can influence the level of Paclitaxel in the cell (Smith et al., 2005).

Under physiological conditions, expression of some OATPs is confined to certain tissues or organs. However, under pathological conditions such as inflammation and tumor growth, the normal expression profile is modified. For instance, OATP1B3 only expressed in normal liver, is detected in colon-, mamma-, prostate-, lung-, and ovarian carcinoma cells (Marin et al., 2009). OATP6A1 is normally expressed in testes, it was also found in lung- and brain tumors (Lee et al., 2004; Buxhofer-Ausch et al., 2013). Al Sarakbi et al. observed that high levels of OATP2A1 are associated with a higher tumor grade in breast cancer (Al Sarakbi et al., 2006).

Based on these findings, OATPs might be valuable as tumor markers for the progression and the outcome of a chemotherapeutic intervention in ovarian cancer (Hagenbuch and Gui, 2008).

2 Aim of the Study

Epithelial ovarian cancer is the deadliest of all gynaecological cancers. This is partly due to the fact that it is generally detected at an advanced stage. Patients do not show any symptoms in early stages and no reliable screening techniques are available. It is a very heterogeneous disease and the need for novel subtype specific therapies, tumor targets and markers is urgent. Also some questions about the aetiology, the origin and the formation of ovarian carcinoma still remain open and more research is highly indicated.

As long as the tumor is confined to the ovaries (FIGO stage I) and a minor pelvic extension (FIGO stage II), the five-year overall survival is relatively high (80% to 90% and 60% to 80%, respectively). But this changes dramatically when the tumor spreads and forms metastasis beyond the pelvis (FIGO stage III and IV). The five-year overall survival decreases in FIGO stage III to 20% to 25% and in stage IV to 5% to 10%.

Ovarian cancer responds well to chemotherapy initially, but it acquires drug resistance early to the first line chemotherapeutic treatment with platin-derivatives and taxanes. This is a major problem, but the mechanisms leading to resistance are not fully understood.

Since reduced accumulation of drugs in cancer cells prevents cytostatic effects, transport proteins mediating drug uptake play an important role in the resistance of cancer cells. Transport proteins from the organic anion transporting polypeptides (OATP) family were identified in transporting cytostatic drugs into cells. But they also carry endogenous substrates such as cyclic nucleotides, steroid hormones and their conjugates as well as prostaglandins, which could all be important for cancer growth. Therefore, by transporting these substrates and regulating their intracellular levels, OATPs may influence tumor progression and sensitivity to treatment.

Most OATPs are located in healthy tissue in certain organs but appear at different levels and other areas under pathological conditions including cancer. This suggests OATPs play a role in the uptake of compounds important to cell growth and tumor progression. Therefore, OATPs could be valuable markers for cancer progression and also work as targets for anticancer therapy.

In addition to transport proteins, enzymes, which are effective in increasing the amount of active estrogens, like steroid sulfatase (STS), in combination with the altered expression of

estrogen receptors α and β (ER- α and ER- β) could be important for the progression of ovarian cancer.

Therefore, the aim of this thesis is to study OATPs, enzyme and ER expression in ovarian cancer. First, the location and distribution of OATP5A1, which is one of the less characterised members of the OATP-family, should be investigated in malign ovary tissue from patients with ovarian cancer at advanced stages. Paraffin-embedded sections will be stained by immunohistochemistry and immunofluorescence. The distribution of OATP5A1 in these specimens will be analysed with an automatic microscope TissueFAXS® and the corresponding HistoQuest® software. The calculated IRS values will be correlated to important prognostic clinical data of patients, including age, presence of residual tumor after removal, quantity of ascites, response to chemotherapy and survival. In the next step, the enzymes of the sulfatase pathway, STS and sulfotransferase 1E1 (SULT1E1) as well as the nuclear receptors ER- α and PGR will be examined using the paraffin-embedded tissue sections. Specimens from a large cohort of ovarian cancer patients will be stained with appropriate antibodies and the distribution of the proteins will be evaluated with methods as described for OATP5A1.

This study aims to show the presumed importance of OATP5A1, STS, SULT1E1, ERs, and progesterone receptor (PGR) in ovarian cancer. The data should produce insight on the importance of drug uptake transporters, steroid hormones metabolizing enzymes and steroid hormone receptors in ovarian cancer. In the future, better understanding of mechanisms important for therapeutic applications could lead to an improved outcome of different chemotherapy regimens for ovarian cancer.

3 Materials and Methods

3.1 Patients

Paraffin sections of ovarian cancer were collected for diagnostic purposes. Remaining sections were provided for the EU project “Ovarian cancer - Diagnosing a silent killer” (OVCAD, 2008-2010). In this project Berlin (Charité/Campus Virchow-Klinikum, University of Berlin, Germany), Hamburg (Institute of Tumorbiology, University Clinic Hamburg-Eppendorf, Germany), Leuven (Division of Gynecological Oncology, University Hospital Leuven, Belgium), Innsbruck (Department of Obstetrics and Gynecology, Medical University Innsbruck, Austria) and Vienna (Department of Obstetrics and Gynecology, Medical University Vienna, Austria) are involved. The ethical committee gave the OVCAD project the permission to establish a tumor database (EK 260/2003 and EK 366/2003) and also to “Characterise new molecular prognostic factors for patients with advanced epithelial ovarian cancer” (EK 793/2011).

In this thesis, 243 ovarian cancer patient samples (tissue microarrays) were used to stain STS, SULT1E1 and PGR. ER- α was stained by Dr. Stefanie Aust (Department of Obstetrics and Gynecology, Medical University Vienna, Austria). She provided the ER- α stained microarrays for the quantitative image analysis.

3.2 Indirect Immunohistochemistry

Materials

- Xylol (Merck, Darmstadt, DE)
- 100% Ethanol (Merck, Darmstadt, DE)
- 20x Epitope Retrieval Solution DEPP-9 pH 9 (Eubio, Wien, AT)
- 1x PBS pH 7.4
- Tween® 20 (Sigma-Aldrich Chemie GmbH, Steinheim, DE)
- Dako Pen (DakoCytomation, Glostrup, DK)
- UltraVision LP Detection System (Thermo Fisher Scientific, Fremont, US)
 - UltraVision Protein Block
 - HRP Polymer

- DAB Plus Chromogen
- DAB Plus Substrate
- ChemMate™ Hematoxylin (DakoCytomation, Glostrup, DK)
- BSA (PAN™ BIOTECH GmbH, Aidenbach, DE)
- H₂O_{bidest}
- Cover Glass 24x 50mm, Thickness no.1 (VWR International, Radnor, PA)
- Fluoromount-G (Southern Biotech, Alabama, US)

Antibodies

Antibody	Dilution	Company
OATP5A1 [HPA05062]	1:20	Atlas Antibodies, Stockholm, SE
SULT1E1 [12522-1-AP]	1:200	Proteintech Group, Chicago, US
STS [N1C3]	1:400	GeneTex, Michigan, US
PGR [SP2]	1:50	Thermo Scientific, Fremont, US
ER-α [1D5]	-	DakoCytomation, Glostrup, DK

Tab. 3.2.1: List of Antibodies used in Immunohistochemistry

Devices

- Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
- Microscope Zeiss AXIO Imager Z1, Axio Cam HRc2 Color CCD Digitalcamera, Autovision 4.6 Software (Zeiss, Jena, DE)
- TissueFAXS® and HistoQuest® Software (TissueGnostics GmbH, Vienna, AT)

This method is applied to analyse the localization and distribution of the target protein in the cell or/and tissue. Immunohistochemistry (IHC) is based on an antigen-antibody reaction. For labelling the proteins, a direct or indirect method can be applied.

The direct method is based on a primary labelled antibody, which can be detected directly. In the indirect IHC, the primary antibody binds on the target protein and the secondary antibody bind to the primary antibody. The secondary antibody is marked with dextran polymolecules on which horseradish peroxidase (HRP) and antibodies are bound. By the addition of an electron donor, the chromogen, the peroxidases form an enzyme-substrate complex, and this leads to an oxidation of the chromogen. The oxidation of the chromogen (e.g. 3,3'-diaminobenzidine, DAB) causes a brown staining for the antigen. The nuclei are stained blue with Haematoxylin.

The disadvantage of this method and also of the indirect immunofluorescence is that the cells/tissue need to be fixed e.g. with formaldehyde and this might prevent epitopes from binding to the antibody. Therefore, antigen retrieval has to be done. Additionally, cells/tissue have to be permeabilized to allow the antibody to bind to intracellular proteins.

Before the staining, the tissue samples have to be fixed in Paraffin, cut and mounted on a glass slide. Two different methods of deparaffinisation were used depending on the antibody.

Before staining against the targets STS, SULT1E1, OATP5A1, PGR the slides were submerged for two minutes in Xylol and 100% Ethanol. For the deparaffinisation, the slides were incubated for ten minutes in DEPP-9 Buffer in the microwave and after that cooled down for twenty-five minutes at room temperature.

Before staining with the antibody against ER-alpha, slides were submerged three times for ten minutes in Xylol, two times for five minutes in 100% Ethanol, one time five minutes in 96% Ethanol, one time in 70% Ethanol, one time for five minutes in 50% Ethanol and in the end one time for five minutes in 30% Ethanol. After washing the slides three times for five minutes in 1xPBS + 0.05% Tween they were cooked for twenty minutes in citrate buffer.

Thereafter for all antibodies, slides were washed three times for three minutes in 1x PBS + 0.05% Tween, the tissue on the slides were surrounded with Dako Pen and labelled with the date of the staining, the used antibody and the dilution of the antibody. Afterwards the slides were washed again three times for three minutes in 1xPBS + 0.05% Tween. Then the tissues were covered with "UltraVision Protein Block" for seven minutes at room temperature in a humid box. During the blocking the antibody was diluted and centrifuged (14.000xg; one minute; 4°C). After the blocking the antibody was added on the tissue and incubated overnight at 4°C (OATP 5A1, SULT1E1, PGR) or for one hour at room temperature (STS).

Again the slides were washed three times three minutes in 1xPBS + 0.05% Tween. Afterwards the tissues were covered in HRP Polymer and incubated for thirty minutes at room temperature in a dark and humid box. A last time the slides were washed three times three minutes in 1xPBS + 0.05% Tween. During the last washing steps "DAB Plus Chromogen" and "DAB Plus Substrate" were mixed in the ratio 1:40. After the washing steps the prepared DAB solution was added for three to ten minutes on the tissue for the visualization of the target protein. The slides were washed four times one minute in H₂O_{bidest}. The nuclei were stained with heamatoxylin for five

minutes. After that the slides were washed in tap water, embedded in one drop Fluoromount and covered with a coverglass.

3.3 Indirect Immunofluorescence

Materials

- Xylol (Merck, Darmstadt, DE)
- 100% Ethanol (Merck, Darmstadt, DE)
- 20x Epitope Retrieval Solution DEPP-9 pH 9 (Eubio, Vienna, AT)
- 1x PBS pH 7.4
- Tween® 20 (Sigma-Aldrich Chemie GmbH, Steinheim, DE)
- Dako Pen (DakoCytomation, Glostrup, DE)
- BSA (PAN™ BIOTECH GmbH, Aidenbach, DE)
- 4',6-Diamidin-2'-phenylindoldihydrochlorid, DAPI (Roche Diagnostics GmbH, Bael, CH)
- H₂O_{bidest}
- Cover Glass 24x 50mm, Thickness no.1 (VWR International, Radnor, PA)
- Fluoromount-G (Southern Biotech, Alabama, US)

Antibodies

Antibody	Dilution	Company
OATP5A1 [HPA05062]	1:50	Atlas Antibodies, Stockholm, SE
CK19 [AP06201PU-N]	1:500	Acris Antibodies, DE
CD20 [RB-9013-P]	1:100	Thermo Scientific, Fremont, US
Anti-mouse Alexa 568	1:1000	Invitrogen Life Technologies, Carlsbad, California, US
Anit-rabbit Alexa 488	1:1000	Invitrogen Life Technologies, Carlsbad, California, US

Tab. 3.3.1: List of Antibodies used for Indirect Immunofluorescence

Devices

- Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
- Microscope Zeiss AXIO Imager Z1, Axio Cam HRc2 Color CCD Digitalcamera, Autovision 4.6 Software (Zeiss, Jena, DE)
- TissueFAXS® and TissueQuest® Software (TissueGnostics GmbH, Vienna, AT)

The principle of the indirect immunofluorescence (IF) is similar to the indirect immunohistochemistry, but instead with HRP the secondary antibody is marked with an immunofluorescent dye (fluorochrom). After the staining the immunofluorescence can be detected with fluorescent microscopy by exciting the fluorochroms with defined wavelengths. The nuclei were stained with DAPI, a blue fluorescent dye, which binds directly on the DNA and can be detected at an emission maximum of 461 nm. The advantage of this technique is that a staining of more than one target protein is possible by marking the secondary antibodies with different fluorochroms. That is a very essential for localisation of different proteins simultaneously.

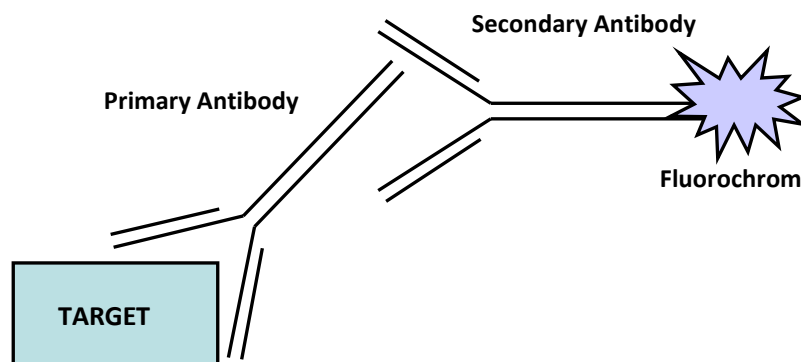


Fig. 3.3.1: Principle of Indirect Immunofluorescence

This figure shows the simplified principle of an indirect immunofluorescence staining. The primary antibody binds specific on the target protein. The fluorochrome marked secondary antibody binds the primary antibody. The secondary antibody can be detected by fluorescence microscopy.

The paraffin slides were already cut and transferred to a glass slide. First, the slides were submerged shortly in Xylol and 100% Ethanol. Then they were incubated for ten minutes in DEPP-9 Buffer in the microwave and, thereafter, allowed to reach room temperature (for approx. twenty-five minutes). After washing the slides three times for three minutes, the tissue was carefully surrounded with DAKO-Pen. The tissue was permeabilized by incubating with PBS + 0.02% Tween for ten minutes. In the next step, blocking was done with 5% FCS in PBS for thirty minutes. During the blocking, the primary antibody dilutions were prepared. When the blocking was finished, the antibody dilutions were added and the slides were incubated overnight in a humidifier chamber at 4°C.

On the next day, slides were washed three times for ten minutes in 1xPBS + 0.05% Tween. Afterwards they were incubated with the secondary antibody for one hour at room temperature. After that, the procedure has to be done without direct light because of bleaching of the fluorochromes. Then the slides were washed three times for ten minutes and they were incubated in 1:1000 DAPI solution for ten minutes. Finally, the slides were washed once with H₂O_{bidest} and mounted with Fluoromount.

3.4 Cell Culture

AD293

The cell line AD293 is derived from the human embryonic kidney cells (HEK)293 cell line, which was genetically modified for better adhesion and plaque formation properties (Zhang J. et al., 2006).

- **AD293 5A1:** AD293 cells were transfected with a vector that contains the SLC05A1 gene and a Geneticin resistance gene
- **AD293 EV:** AD293 cells were transfected with an “empty Vector”. This vector contains a Geneticin resistance gene only.

Materials

- RPMI-1640 medium with L-glutamine, sodium bicarbonate R8758 (SIGMA-ALDRICH, Buchs, CH)
- G418 desulfate salt solution (SIGMA-ALDRICH, Buchs, CH)
- Fetal Bovine Serum FBS superior (Biochrom AG Biotechnologie, Berlin, DE)
- 10x Trypsin - EDTA solution Nr. 59418 (SIGMA-ALDRICH, Buchs, CH)
- 1x PBS pH 7.4
- Safe-seal tips (Biozym Scientific GmbH, Oldendorf, DE)
- Pasteur pipettes (SIGMA-ALDRICH, Buchs, CH)
- 25cm² cell culture flasks (TPP, Trasadingen, CH)
- Pipettes 10ml, 25ml (Biozym Scientific GmbH, Oldendorf, DE)

Devices

- Lamina flow (Thermo Fisher Scientific, Asheville, US)
- Heraeus cytoperm 2 (Thermo Fisher Scientific, Asheville, US)
- Olympus CK2 inverted microscope with phase contrast (Spach Optics, Rochester, NY)
- Hettich Rotante centrifuge (Hettich Bäch, CH)

To ensure an optimal growing of the cells, they were cultivated in 25cm² flasks in about 4ml medium (RPMI 1640 + 10% FBS + 440µg/ml G418) at a temperature of 37°C, 95% humidity and 5% CO₂. Before working with the cells, all the solutions were brought to room temperature. Every second day, the cells were inspected under the microscope for confluency. When the confluency of the cells was reaching 70%, the medium was replaced by aspirating the old and add the fresh medium. When the cells reached a confluency, they were splitted. First, the old medium was aspirated and the cells were washed with 4ml of PBS. The PBS was aspirated and for the detachment the cells were incubated in trypsin-containing medium for approximately three minutes in the incubator. Afterwards, 1ml of fresh medium was added to the cell-trypsin suspension. Next, the solution was filled into a vial and centrifuged for three minutes at 450g. The supernatant was aspirated and the pellet was resuspended in 1ml fresh medium. 3-5 drops of the cell suspension were pipetted into a new 25cm² flask and cultured with 4ml fresh medium.

3.5 Indirect Immunofluorescence Staining of Cells

Materials

- Cover Slips 15mm, Thickness no.1 (VWR International, Radnor, PA)
- 1x PBS pH 7.4
- PFA (Sigma-Aldrich Chemie GmbH, Steinheim, DE)
- NH₄Cl 50mM (Merck, Darmstadt, DE)
- BSA (PANTM BIOTECH GmbH, Aidenbach, DE)
- TritonTMX-100 (Sigma-Aldrich Chemie GmbH, Steinheim, DE)
- 4',6-Diamidin-2'-phenylindoldihydrochlorid, DAPI (Roche Diagnostics GmbH, Basel, CH)
- Fluoromount-G (Southern Biotech, Alabama, US)
- Cover Glass 50mm, Thickness no.1 (VWR International, Radnor, PA)

Antibodies

Antibody	Dilution	Company
OATP5A1 [HPA05062]	1:50	Atlas Antibodies, Stockholm, SE
Anit-rabbit Alexa 488	1:1000	Invitrogen Life Technologies, Carlsbad, California, US

Tab. 3.5.1: List of Antibodies used for Immunofluorescence Cell Staining

Devices

- Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
- Microscope Zeiss AXIO Imager Z1, Axio Cam HRc2 Color CCD Digitalcamera, Autovision 4.6 Software (Zeiss, Jena, DE)

First the cells have to be cultivated in a 24-well plate on cover slips until a confluence of 90% is reached. The medium was removed and the cells were washed two times for five minutes with PBS. To fix the cells, they first were incubated for fifteen minutes in 4% (w/v) PFA in PBS and afterwards for five minutes in 50mM NH₄Cl. Next the cells were washed one time with PBS. After blocking with Blocking Buffer (1% BSA; Triton™X-100; in 1x PBS) for one hour the cells were incubated with the primary antibody for one hour. The cells were washed four times for ten minutes. The secondary antibody was added for one hour, from this point on every work step has to be full filled in the dark. Afterward the cells were washed two times for ten minutes with PBS. For the cell nucleus staining the cells were incubated for fifteen minutes in a 1:1000 DAPI solution. The cells were washed two times for five minutes with H₂O_{bidest} mounted with one drop Fluoromount and covered with a cover glass.

3.6 TissueFAXS®/HistoQuest®

The TissueFAXS® is an automated fluorescence and bright light microscopic system consisting of a microscope, and powerful computer, high resolution screens. The TissueFAXS® scanning software from the company TissueGnostics is used. This system allows automatically scanning of whole stained tissue slide. The TissueFAXS® used for the experiments described is equipped with 4 objectives (1x, 2.5x, 20x, 40x) and offers 5 channels with different wave-length for immunofluorescence evaluation of the following dyes DAPI, FITCS, Texas Red, Cy5, Cy7. After scanning virtual images, a quantitative analysis can be made with the corresponding

HistoQuest® software (immunohistochemistry staining) or TissueQuest® software (immunofluorescence staining).



Fig. 3.6.1: TissueFAXS® System and TissueQuest® Software (TissueGnostics GmbH, Vienna, Austria)

In this master thesis the HistoQuest® Version 3.0.3.173 and Version 4.0.4.0133 software was used for quantitative analysis of immunohistochemistry staining of paraffin-embedded sections. The program recognises cells by their blue-stained nuclei (haematoxylin staining). First, the program creates virtual cells based on the nuclei with a cell identification logarithm. In the second step, it identifies the HRP-antibody marked target protein by the brownish DAB staining.

At the beginning of the analysis with the HistoQuest® software, a definition of the groups is made: Stroma, tumor and immune cells are defined and “Regions of Interest” (ROI’s) have to be drawn manually according to the classified groups. After analysis of all ROI’s, positive cells have to be identified and a “cut-off” for the negative cells has to be set manually. This is based on comparing positive stained to negative stained cells. The program calculates the amount of positive cells in percent/groups. The “Mean of Mean Intensity” (MoMI) of the target protein staining in a cell can also be calculated by the software. For an evaluation of the amount of immunoreactive protein per sample, the percentage of positive cells has to be multiplied with MoMI. This value is called “Immune Reactive Score” (IRS).

3.7 Isolation of RNA

The isolation of good-quality RNA is a critical step before the RT-PCR. In this thesis, a phenol-chloroform based extraction was used. It is essential to do all working steps at 4°C and in an RNase free environment to avoid the degradation of the RNA.

Materials

- 1x PBS pH 7.4
- PeqGOLD TriFast (PEQLAB Biotechnologie GmbH, Erlangen, DE)
- Chloroform (Merck, Darmstadt, DE)
- Isopropanol (Merck, Darmstadt, DE)
- 70% Ethanol (AnalaR NORMAPUR®, West Sussex, UK)
- GIBCO distilled, RNase/DNase free H₂O (Life Technologies, Carlsbad, CA)

Devices

- Nanodrop Spectrophotometer ND-100 (Thermo Fisher Scientific, Asheville, US)
- Centrifuge 5415R (Eppendorf AG, Hamburg, DE)
- Thermomixer comfort (Eppendorf AG, Hamburg, DE)

The cells lines (AD293 EV and AD293 5A1) were cultured in a six well plate until they reached a confluence of 90%. After removing the medium, they were washed twice with ice cold PBS. Next, cells were resuspended in 1ml peqGOLD TriFast and incubated for five minutes at room temperature. For phase separation, to every ml of peqGOLD TriFast, 0.2ml chloroform was added and the solution was mixed. After five minutes incubation at room temperature, the samples were centrifuged for fifteen minutes at 12000g to allow phase separation. At the bottom is the red phenol-chloroform phase, on the top, the colourless aqueous phase and in between, the interphase. The RNA can be collected from the aqueous phase, while the proteins, DNA and lipids remain in the phenol-chloroform phase and in the interphase.

For RNA precipitation, the aqueous phase was transferred to a new vial and 750µl isopropanol was added per ml peqGOLD TriFast. The mixture was incubated for ten minutes on ice. The samples were centrifuged again for fifteen minutes at 12000g. After the isopropanol was removed, the RNA pellet was washed with 70% ethanol and centrifuged for five minutes at 12000g. For a better solubility of the RNA, it was heated to 55°C for three minutes. Next the

RNA was dissolved in 25-80µl RNase/DNase free H₂O. It was again heated to 55°C for ten minutes.

Finally the RNA content (ng/µl) and the purity (260/280) were measured with the Nanodrop.

3.8 Reverse Transcription

Materials

- GIBCO distilled, RNase/DNase free H₂O (Life Technologies, Carlsbad, CA)
- High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA)
 - RT Buffer
 - dNTP Mix (100mM)
 - RT Random Primers
 - MultiScribe™ Reverse Transcriptase

Devices

- Biometra personal cycler (Gatt-Koller, Innsbruck, AT)

This reaction was performed using the “High Capacity cDNA Reverse Transcription Kit”. By reverse transcription, RNA is converted into complementary DNA (cDNA). Random primers and the enzyme Reverse Transcriptase are needed to synthesize the cDNA. These primers are approx. 20-25 base pairs long and complementary to the nucleotides.

1µg RNA in 10µl H₂O was prepared for the reverse transcription.

Material	Amount
RT Random Primers	2µl
RNase/DNase free H ₂ O	4.2µl
MultiScribe™ Reverse Transcriptase	1µl
RT Buffer	2µl
dNTP Mix	0.8µl
In total	20µl

Tab. 3.8.1: Reverse Transcriptase Mixture for one Sample

10µl Master Mix was added to each RNA sample. This mixture was incubated and the following program was started in the Biometra personal cycler:

- Activation of the Reverse Transcriptase at 25°C for ten minutes
- Reverse transcription at 37°C for 2 hours
- Deactivation of the Reverse transcriptase at 85°C for five minutes
- Storing the cDNA at 4°C unlimited

To obtain a concentration of 10ng/µl 80µl of RNase/DNase free H₂O were added. The diluted cDNA was stored at -20°C.

3.9 Quantitative Real Time PCR-qPCR

Reagent and Material

- 2x TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA)
- GIBCO distilled, RNase/DNase free H₂O (Life Technologies, Carlsbad, CA)
- TaqMan Primer/Probes (Life Technologies, Carlsbad, CA)
 - OATP5A1
- MicroAmp Clear Adhesive Film (Life Technologies, Carlsbad, CA)
- MicroAmp Fast Optical 96-well Reaction Plate (Life Technologies, Carlsbad, CA)

Devices

- A7900HT-Fast Real Time PCR System with SDS 2.4 Software Film (Life Technologies, Carlsbad, CA)
- Heraeus Megafuge40 (Thermo Scientific, Fremont, US)
- SPSS version 19.0 (IBM STS Statistics, New York, US).

The real-time PCR (qPCR) is a fluorescence based method for the rapid quantitative measurement of DNA amplification with high sensitivity and specificity. The reverse transcription to synthesize cDNA is the necessary step previous to the qPCR.

qPCR is performed in a thermo cycler with the possibility for an excitation of the samples and detection of the emitted fluorescent light. During the amplification steps (denaturation,

annealing and elongation) simultaneous measurement of the fluorescence takes place. For the quantification of DNA, two systems are used: SYBR Green or TaqMan. The latter is based on FRET (Förster resonance energy transfer). The SYBR Green binds the DNA, thereby the intensity of the dye increases by 1000x. The more DNA is amplified during qPCR, the more fluorescence can be detected. SYBR Green is a DNA intercalating dye. A melting curve analysis has to be done after to assure that the PCR product is the specific one.

TaqMan probes belong to the group of FRET probes. The FRET probes have specific nucleic acid sequences that are complementary to the target sequence. They are equipped with a quencher on one side and a reporter on the other. During the annealing step, the Taq-polymerase, which also has a 5'-3' exonuclease activity, hydrolyses the target DNA bound FRET probe. This causes a separation of the quencher and reporter and results in increased reporter fluorescence that can be detected in the detection system (A7900HT-Fast Real Time PCR System).

qPCR with AD293 EV and AD293 5A1 to determine the amount of OATP5A1 was also done with TaqMan probes.

The plate was closed with an adhesive optical film, centrifuged for 1min at 200rpm and afterwards analysed with the A7900HT-Fast Real Time PCR System with the following programme:

- two minutes at 50°C
- ten minutes at 95°C
- fifteen seconds at 95°C (40 times)
- one minute at 60°C (40 times)

The Real-Time PCR Software from ABI was used to calculate the Ct-values. With the Excel 2003 software, the $2^{-\Delta\Delta CT}$ values were calculated.

3.9.1 The Human Drug Transporter PCR Array

Material

- Human Drug Transporter PCR Array (Quiagen GmbH, Hilden, DE)
- SYBR Green MM (Quiagen GmbH, Hilden, DE)
- GIBCO distilled, RNase/DNase free (Life Technologies, Carlsbad, CA)
- MicroAmp Clear Adhesive Film (Life Technologies, Carlsbad, CA)

Devices

- A7900HT-Fast Real Time PCR System with SDS 2.4 Software Film (Life Technologies, Carlsbad, CA)
- Centrifuge
- PCR Array Data Analysis Software (Quiagen GmbH, Hilden, DE)

The “human drug transporter PCR Array” contains 84 transporter gene primers on a prepared 96 well plate. In this thesis we investigated the transporter expression profile in the cell lines AD293 EV and AD293 5A1.

Material	Amount
cDNA	1µl
SYBR Green	12.5µl
RNase/DNase free H ₂ O	11.5µl
In total	25µl

Tab. 3.9.1: Human Drug transporter PCR Array Mixture for one Sample

After adding the samples, the plate was closed with an adhesive optical film, centrifuged for one minutes at 200rpm and analysed with the ABI PRISMTM Sequence Detector 7900.

The data were analysed with the “PCR Array Data Analysis Software”.

4 Results

4.1 STS, SULT1E1, ER- α and PGR in Ovarian Cancer

Microarray specimens from 243 OVCAD patients were applied to study enzymes and receptors for the estrogen metabolism. STS, SULT1E1, ER- α , and PGR were stained by immunohistochemistry. Because of bad quality of some tissues and not available clinical data of some patients for STS 107 patients, for SULT1E1 114 patients and for ER- α , finally 110 patients from the initially 243 patients were appropriate for a statistically analysis. For getting a homogeneous study collective, the study cohort was reduced to patients with serous subtypes. For the statistical analysis, the cohort was divided at the median of the IRS of each marker. Strong staining and a high number of stained cells give a high IRS values (IRS>median). These samples are described as “high”, e.g. STS-high. Weak or no staining’s (IRS<median) are marked with “low”, e.g. STS-low and in-between there is the intermediate IRS (median).

In the chapter “4.1.1, from STS, SULT1E1, and the receptors ER- α and PGR, representative IHC staining of each target with “high” and a “low” IRSs are shown (Fig. 4.1.1-4.1.4).

4.1.1 Histological Observation of STS, SULT1E1, ER- α and PGR

STS in serous carcinoma

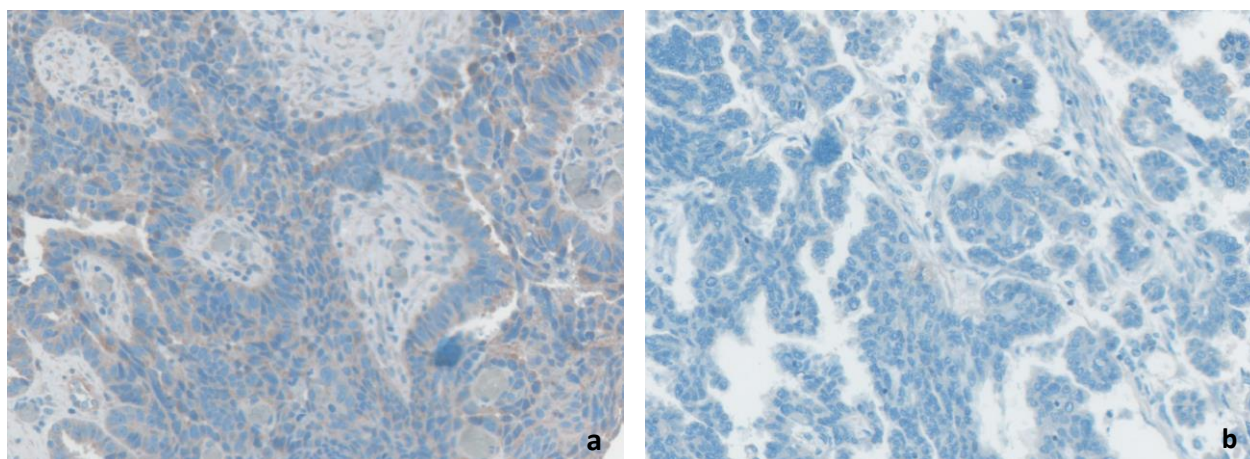


Fig. 4.1.1: Representative IHC Staining in Samples from two Patients, STS-high (a) and STS-low (b)

(a) Sample B051 shows a strong cytoplasmic staining in the tumor cells. A weak staining in the tumor stroma is also notable. (b) In the patient B025 no staining for STS was observed. The tumor as well as the tumor surrounding stroma is STS-negative.

SULT1E1 in serous carcinoma

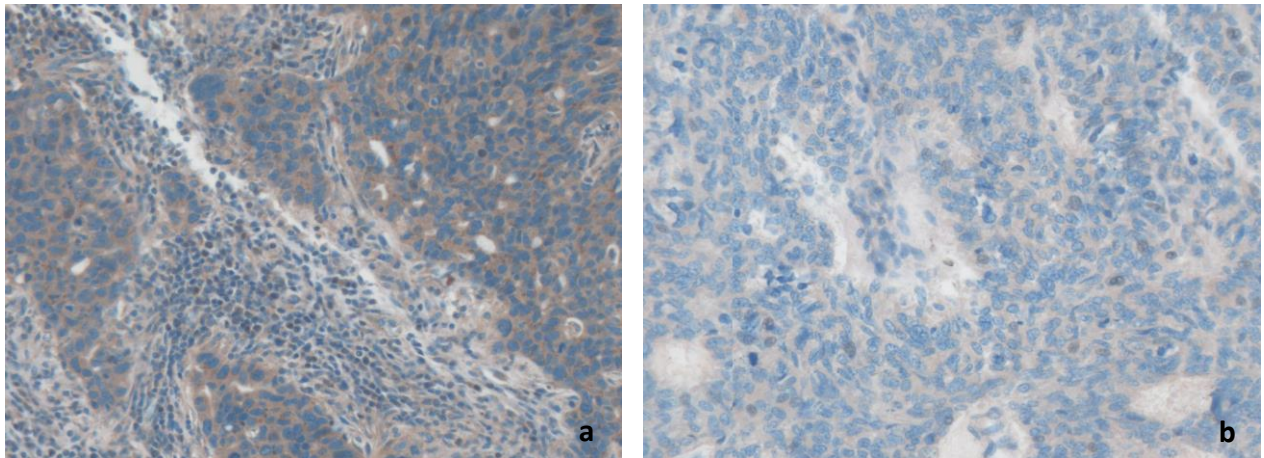


Fig. 4.1.2: Representative IHC staining in Samples from two Patients, SULT1E1-high (a) and SULT1E1-low (b)

(a) In the sample from patient H007, a very strong cytoplasmic tumor staining is observable. In the tumor stroma, there are clusters of immune cells and a part of them are also stained positive for SULT1E1. Interestingly, some nuclei of tumor- and also immune cells are stained too. (b) The sample from patient B044 shows a weak tumor staining. Also a few nuclei are stained.

ER- α in serous carcinoma

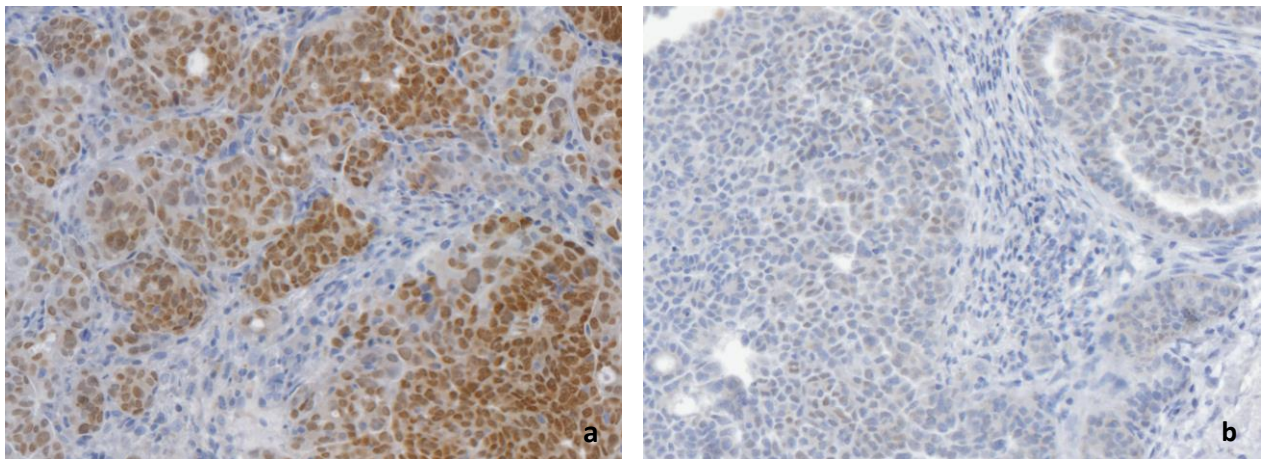


Fig. 4.1.3: Representative IHC Staining in Samples from two Patients, ER- α -high (a) and ER- α -low (b)

The active ER- α is characterised through a strong tumor cell nucleus staining like illustrated in Patient L083 (a). A weak cytoplasmic staining is also observable. The tumor stroma shows no ER- α staining at all. In comparison to the ER- α -high patient L083, patient V036 (b) has a very weak nucleus staining. Similar to patient L038, hardly any staining is visible for tumor stroma.

PGR in serous carcinoma

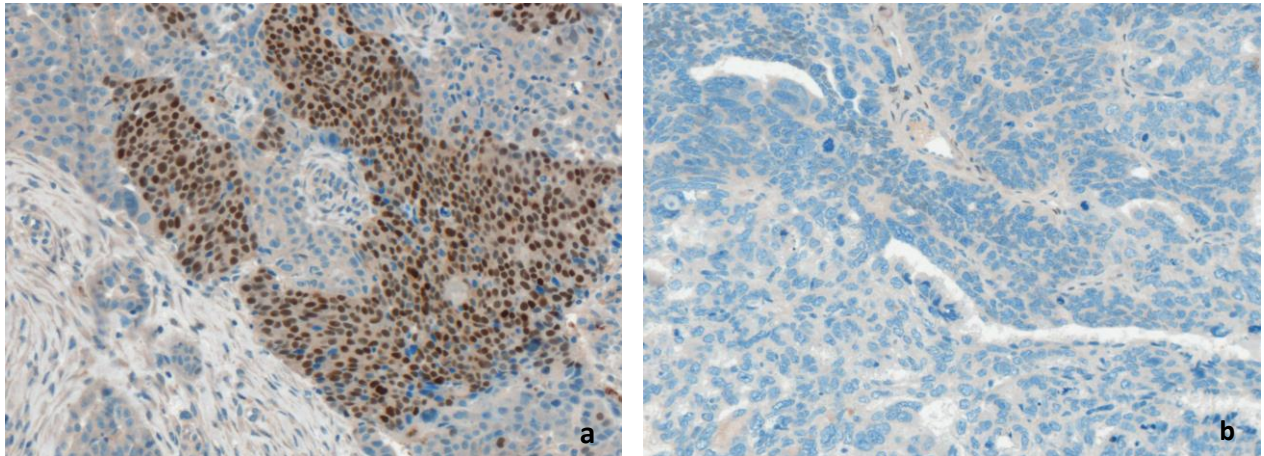


Fig. 4.1.4: Representative IHC Staining in Samples from two Patients, PGR-high (a) and PGR-low (b)

Equal to ER- α the active PGR is also present in the nuclei, like illustrated in patient L173 (a). The tumor stroma is mostly very weak or negative stained. In patient B044 (b) only a few weak stained nucleus were observed.

Summary

The data from the manual evaluation of STS staining in Fig. 4.1.1 show that the enzyme is mostly present in the cytoplasm of tumor cells. Only weak staining is seen in cells in the tumor stroma. In serous as well as in mixed epithelial and endometrioid tumors, STS was present in many samples. Also in some immune cells, a cytoplasmic staining for STS was seen.

SULT1E1 was mostly observed in the cytoplasm of tumor cells but interestingly, it is occasionally visible in the nuclei as well. As shown in (Fig. 4.1.2) also immune cells are positive for SULT1E1 and the enzyme is located in the nuclei. In patient B044, also a surprisingly strong nuclei staining was found in tumor, which otherwise did not show SULT1E1 at a higher level.

As shown in Fig. 4.1.3 ER- α is mostly present in the nuclei of tumor cells. Overall a weak staining of the cytoplasm of tumor cells is observable in some images. The strong staining of nuclei in images from patient L083 suggests that ER- α is highly active. In some patients, only a cytoplasmic staining was found what indicates that the receptor is present in the inactive form. A weak cytoplasmic staining was observed in serous, mixed epithelial and clear cell carcinomas, as well as strong and weak nuclei staining. In MC tumors, ER- α stained nuclei were not detectable.

Similar to ER- α , PGR is present in its active form in the nucleus of the tumor cells. The staining in the nucleus was detectable in a part of serous carcinomas (Fig. 4.1.4) as well as in mixed epithelial carcinomas. The tumor stroma was in these subtypes mostly weakly stained. For the other subtypes, no data were available.

4.2 Statistical Analysis of STS, SULT1E1 and ER- α Levels in Ovarian Cancer Samples

The impact of STS, SULT1E1, ER- α on progression free survival (PFS) and overall survival (OS) was determined by the univariate and multiple Cox proportional-Hazards regression model analyses. The Cox-model is used for multivariate analyses when the simultaneous effect of multiple influencing variables on a time-dependent target variable (PFS and OS) has to be observed. Thereby the hazard ratio (HR) shows if the effect of the variable is favorable (<1) for the PFS and OS, respectively, or not (>1).

For the determination of the patient characteristics the patient cohort was divided into two groups at the median of the IRS, namely low ($<\text{median}$) and high ($>\text{median}$) expression. In the analysis, only serous ovarian carcinoma patients were included ($n=94$), due to the fact that the different histological subtypes derive from different origins and are very heterogeneous. In all three groups (STS, SULT1E1 and ER- α) the median age at time of cytoreductive surgery was 57 years (range 27-83). The median observation time was 41 months (range 1-69). During this period 52 patients died (45.6%) and 82 received a tumor recurrence (71.9%).

4.2.1 Clinical Data from Patients, investigated for STS Expression in Tumor Samples

n=107	STS-low: n (%)	STS-high: n (%)	p-value
Patient Cohort	54 (50.5)	53 (49.5)	
Age			
≤ 55 (n=46)	22 (47.8)	24 (52.2)	
>55 (n=61)	32 (52.5)	29 (47.5)	0.635
Histology			
Serous (n=90)	47 (52.2)	43 (47.8)	
Non-serous (n=17)	7 (41.2)	10 (58.8)	0.403
FIGO			
II (n=4)	0 (0)	4 (100)	
III (n=87)	47 (54)	40 (46)	
IV (n=16)	7 (43.8)	9 (56.3)	0.099 ¹
Grade			
Grade 1&2 (n=22)	9 (40.9)	13 (59.1)	
Grade 3 (n=85)	45 (52.9)	40 (47.1)	0.314
Residual tumor ²			
no (n=77)	40 (51.9)	37 (48.1)	
>0cm (n=29)	14 (48.3)	15 (51.7)	0.736

Tab. 4.2.1: Patients Cohort, divided by STS -low and STS-high levels

Clinical data including age, histology, FIGO stage, grade and the presence of residual tumor were compared in the group with low and high STS levels. High and low STS levels were determined from the median of the IRS of all samples. No significant differences were found for any clinical data.

¹Fisher's Exact Test

²one case is missing

4.2.2 Influence of STS on the PFS in Patients with Serous Ovarian Cancer

Multivariate STS		
Characteristics	HR (CI95%)	p-value
Age (per decade)	1.22 (1.0-1.5)	0.100
FIGO (IV vs. III vs. II)	2.10 (1.1-4.1)	0.026
Grade (3 vs. 1,2)	1.9 (0.9-4.0)	0.082
Residual tumor (yes vs. no)	1.18 (0.7-2.1)	0.579
STS (log*)	1.12 (0.73-1.74)	0.598

Tab. 4.2.2: Multivariate Analysis of STS and PFS with SPSS 19.0

A multivariate analysis of the clinical data including age, FIGO staging, grade, residual tumor, STS on the PFS in the serous ovarian cancer cohort (n=90). Hazard Ratio (HR) with the Confidence Interval (CI) and the p-value are listed.

*The data were logarithmized to reach a normal distribution

FIGO stage: Significant correlation to PFS (bold)

The multivariate analysis under incorporation of clinical parameters (age, FIGO-stage, grade, residual tumor) was carried out to determine the influence of STS on the PFS in patients with serous carcinoma.

The results (Tab. 4.2.2) indicate that STS has no independent significant influence on the PFS. Only the FIGO stage is a significant parameter in the multivariate analysis.

4.2.3 Influence of STS on the OS in Patients with Serous Ovarian Cancer

Characteristics	Univariate		Multivariate STS	
	HR (CI95%)	p-value	HR (CI95%)	p-value
Age (per decade)	1.36 (1.0-1.8)	0.038	1.27 (0.9-1.7)	0.153
FIGO (IV vs. III vs. II)	2.58 (1.3-5.3)	0.009	1.72 (0.8-3.8)	0.173
Grade (3 vs. 1,2)	2.59 (1.0-6.6)	0.048	1.96 (0.7-5.7)	0.217
Residual tumor (yes vs. no)	3.20 (1.7-6.1)	<0.001	2.45 (1.2-5.0)	0.013
STS (log*)	1.05 (0.6-1.8)	0.848	1.07 (0.61-1.89)	0.807

Tab. 4.2.3: Univariate and Multivariate Analysis of STS and OS with SPSS 19.0

This table shows the correlation between age, FIGO stage, grade, residual tumor and STS on the overall survival (OS).

Only the residual tumor load showed a significant influence on the OS (bold) in the multivariate analysis.

*The data were logarithmized to reach a normal distribution

The univariate analysis (Tab. 4.2.3) suggests that the factors age, FIGO-stage, grade and residual tumor have a significant influence on the OS of patients with serous ovarian cancer. These data support the fact that the complete remove of the tumor is the most valuable prognostic factor for OS ($p < 0.0001$).

Referring to the univariate and multivariate analysis in Tab.4.2.3 STS expression has no significant effect on the OS.

4.2.4 Clinical Data from Patients, investigated for SULT1E1 Expression in Tumor Samples

n=112	SULT1E1-low: n (%)	SULT1E1-high: n (%)	p-value
Patient Cohort	53 (47)	59 (53)	
Age			
≤55 (n=49)	24 (49)	25 (51)	
>55 (n=63)	29 (46)	34 (54)	0.757
Histology			
Serous (n=92)	42 (45.7)	50 (54.3)	
Non-serous (n=20)	11 (55)	9 (45)	0.448
FIGO			
II (n=5)	1 (20)	4 (80)	
III (n=92)	47 (51.1)	45 (48.9)	
IV (n=15)	5 (33.3)	10 (66.7)	0.206 ¹
Grade			
Grade 1&2 (n=27)	12 (44.4)	15 (55.6)	
Grade 3 (n=85)	41 (48.2)	44 (51.8)	0.731
Residual tumor ²			
no (n=82)	38 (46.3)	44 (53.7)	
>0cm (n=29)	14 (48.3)	15 (51.7)	0.858

Tab. 4.2.4: Patients Cohort, divided by SULT1E1 -low and SULT1E1-high levels

Clinical data including age, histology, FIGO stage, grade and the presence of residual tumor were compared in the group with low and high SULT1E1 levels. No significant differences were found for any clinical data.

¹Fisher's Exact Test

²one case is missing

4.2.5 Influence of SULT1E1 on the PFS in Patients with Serous Ovarian Cancer

Multivariate SULT1E1		
Characteristics	HR (CI95%)	p-value
Age (per decade)	1.24 (1.0-1.6)	0.058
FIGO (IV vs. III vs. II)	2.17 (1.1-4.2)	0.023
Grade (3 vs. 1,2)	1.75 (0.9-3.4)	0.103
Residual tumor (yes vs. no)	1.29 (0.7-2.3)	0.401
SULT1E1 (log*)	0.74 (0.47-1.16)	0.185

Tab. 4.2.5: Multivariate Analysis of STS and PFS with SPSS 19.0

A multivariate analysis of the clinical data including age, FIGO staging, grade, residual tumor and SULT1E1 on the PFS in the serous ovarian cancer cohort (n=92). Hazard Ratio (HR) with the Confidence Interval (CI) and the p-value are listed. FIGO stage: Significant correlation to PFS (bold)

*The data was logarithmized to reach a normal distribution

The multivariate analysis of SULT1E1 under the influence of several clinical factors referring to PFS shows no significant effect of SULT1E1 on PFS (Tab. 4.2.5).

4.2.6 Influence of SULT1E1 on the OS in Patients with Serous Ovarian Cancer

	Univariate		Multivariate SULT1E1	
Characteristics	HR (CI95%)	p-value	HR (CI95%)	p-value
Age (per decade)	1.36 (1.0-1.8)	0.038	1.25 (0.9-1.7)	0.162
FIGO (IV vs. III vs. II)	2.58 (1.3-5.3)	0.009	1.68 (0.8-3.8)	0.212
Grade (3 vs. 1,2)	2.59 (1.0-6.6)	0.048	1.93 (0.7-5.0)	0.183
Residual tumor (yes vs. no)	3.20 (1.7-6.1)	<0.001	2.72 (1.3-5.5)	0.006
SULT1E1 (log*)	0.57 (0.4-0.9)	0.023	0.53 (0.32-0.87)	0.013

Tab. 4.2.6: Univariate and Multivariate Analysis of OS (Clinical Data and STS)

This table shows the correlation between age, FIGO stage, grade, residual tumor and SULT1E1 on the overall survival (OS).

SULT1E1 and the Residual Tumor load showed a significant influence on the OS (bold).

*The data were logarithmized to reach a normal distribution

In the univariate ($p=0.023$) as well as in the multivariate analysis ($p=0.013$) SULT1E1 seems to have a highly significant independent impact on OS of patients with serous carcinoma. As illustrated in (Tab. 4.2.6) the presence of residual tumor is an important prognostic factor for the OS.

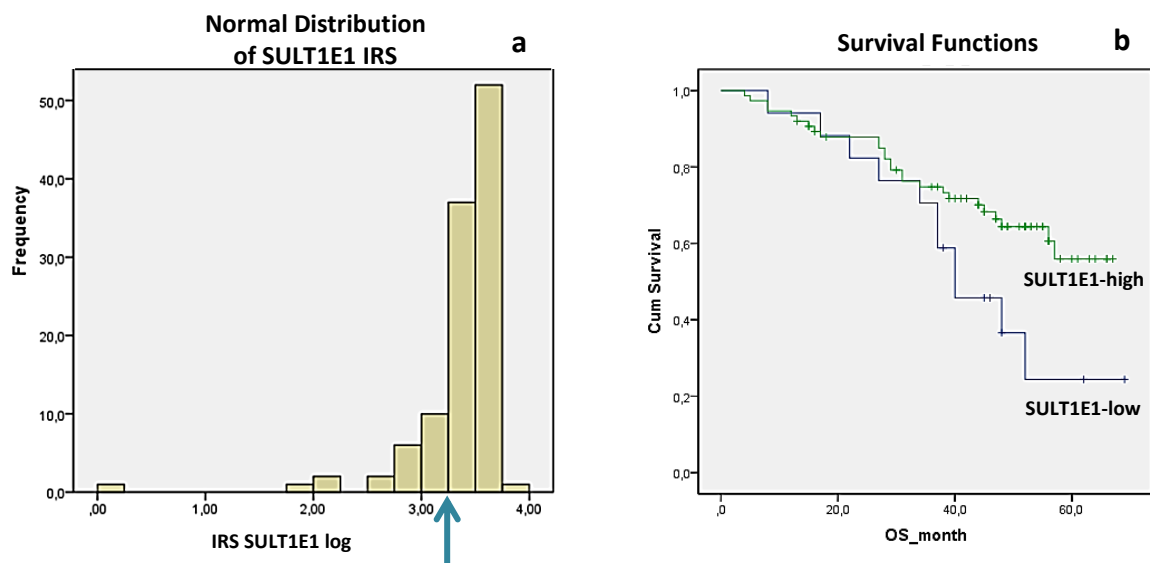


Fig. 4.2.1: Kaplan-Meier estimates the impact of SULT1E1 on OS in Serous Carcinoma

(a) The figure of the logarithmized IRS of SULT1E1 (normal distribution) was used to determine a relevant cut-off to divide SULT1E1 in a low and a high expression group. The cut-off was set at the intersection point (3.25, arrow). (b) This plot illustrates that a high SULT1E1 expression in the tumor is associated with a longer OS in patients with serous ovarian carcinoma ($p=0.046$).

4.2.7 Clinical Data from Patients, investigated for ER- α Expression in Tumor Samples

n=110	ER- α -low: n (%)	ER- α -high: n (%)	p-value
Patient Cohort	55 (50)	55 (50)	
Age			
≤55 (n=47)	21 (44.7)	26 (55.3)	
>55 (n=63)	34 (54)	29 (46)	0.335
Histology			
Serous (n=92)	47 (51.1)	45 (48.9)	
Non-serous (n=18)	8 (44.4)	10 (55.6)	0.606
FIGO			
II (n=4)	1 (25)	3 (75)	
III (n=90)	44 (48.9)	46 (51.1)	
IV (n=16)	10 (62.5)	6 (37.5)	0.393 ¹
Grade			
Grade 1&2 (n=26)	11 (42.3)	15 (57.7)	
Grade 3 (n=84)	44 (52.4)	40 (47.6)	0.369
Residual tumor ²			
no (n=79)	38 (48.1)	41 (51.9)	
>0cm (n=30)	16 (53.3)	14 (46.7)	0.626

Tab. 4.2.7: Patients Cohort, divided by ER- α -low and ER- α -high levels

In this table the patient characteristics including age, histology, FIGO stage, grade and the presence of residual tumor are correlated with ER- α -low and ER- α -high. No significant differences were found for any clinical data.

¹Fisher's Exact Test

²one case is missing

4.2.8 Influence of ER- α on the PFS in Patients with Serous Ovarian Cancer

Multivariate ER- α		
Characteristics	HR (CI95%)	p-value
Age (per decade)	1.24 (0.9-1.6)	0.057
FIGO (IV vs. III vs. II)	2.20 (1.1-4.3)	0.021
Grade (3 vs. 1,2)	1.88 (0.9-3.8)	0.077
Residual tumor (yes vs. no)	1.25 (0.7-2.2)	0.454
ER- α (log*)	0.75 (0.56-1.00)	0.046

Tab. 4.2.8: Multivariate Analysis of ER- α and PFS with SPSS 19.0

A multivariate analysis of the clinical data including age, FIGO staging, grade, residual tumor, ER- α and PFS in the serous ovarian cancer cohort (n=92). Hazard Ratio (HR) with the Confidence Interval (CI) and the p-value are listed.

*The data was logarithmized to reach a normal distribution

A high expression of ER- α seems to have a favorable impact on the PFS (HR=0.75; 95%CI [0.6-1.0]) with a high significance (p=0.046).

4.2.9 Influence of ER- α on the OS in Patients with Serous Ovarian Cancer

Characteristics	Univariate		Multivariate ER- α	
	HR (CI95%)	p-value	HR (CI95%)	p-value
Age (per decade)	1.36 (1.0-1.8)	0.038	1.24 (0.9-1.7)	0.172
FIGO (IV vs. III vs. II)	2.58 (1.3-5.3)	0.009	1.83 (0.8-4.1)	0.138
Grade (3 vs. 1,2)	2.59 (1.0-6.6)	0.048	1.85 (0.7-4.9)	0.212
Residual tumor (yes vs. no)	3.20 (1.7-6.1)	<0.001	2.6 (1.3-5.3)	0.006
ER- α (log*)	0.72 (0.5-1.0)	0.053	0.67 (0.47-0.97)	0.033

Tab. 4.2.9: Univariate and Multivariate Analysis of ER- α and OS with SPSS 19.0

This table consist the univariate and multivariate analysis between age, FIGO stage, grade, residual tumor, ER- α and OS.

*The data were logarithmized to reach a normal distribution

Under the influence of age, FIGO-stage, grade and the presence of residual tumor ER- α seems to be an independent significant prognostic factor for OS (p=0.033). Also the univariate analysis shows the tendency that a high expression of ER- α is associated with a longer OS. This result is also illustrated in the Kaplan-Meier Curve in Fig. 4.2.2 (p=0.037).

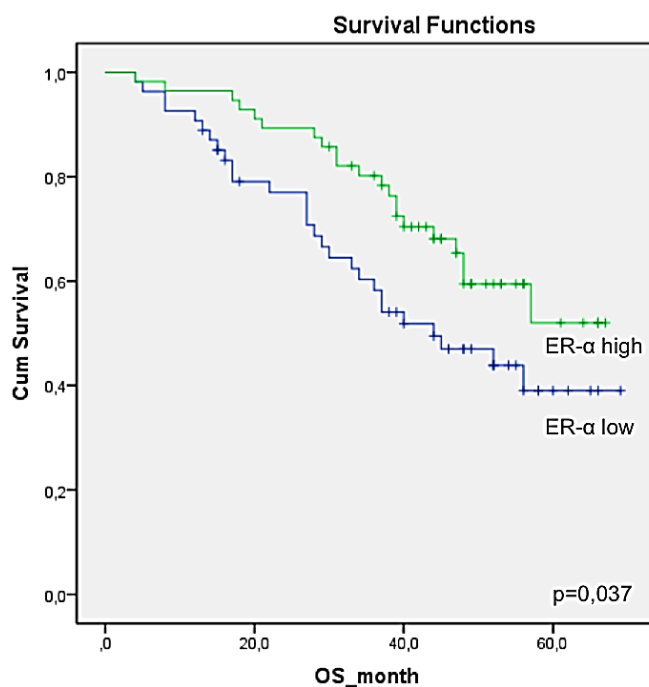


Fig. 4.2.2: Kaplan-Meier estimates the impact of ER- α on OS in Serous Carcinoma

This plot illustrates that a high ER-expression in the tumor is associated with a longer OS in patients with serous ovarian carcinoma (p=0.037).

4.2.10 Cross tabulations of STS, SULT1E1 and ER- α

		STS (log)*	
		High Expression	Low Expression
ER- α (log)*	High Expression	39	25
	Low Expression	14	31

Tab. 4.2.10: Cross Tabulation of STS and ER- α

The cross tabulation of STS and ER- α shows that a high expression of STS is associated with high levels of ER- α (bold – 39 patients). The same tendency is seen in the inverted way (bold – 31 patients).

*The data were logarithmized to reach a normal distribution

As illustrated in Tab. 4.2.10, high levels of STS are associated with high levels of ER- α . This results also show a high significance of $p=0.002$ in the Chi-Square Tests.

		SULT1E1 (log)*	
		High Expression	Low Expression
ER- α (log)*	High Expression	34	29
	Low Expression	16	32

Tab. 4.2.11: Cross Tabulation of SULT1E1 and ER- α

The cross tabulation of SULT1E1 and ER- α shows that a high expression of SULT1E1 is associated with high levels of ER- α (bold – 34 patients). The same tendency is seen in the inverted way (bold – 32 patients).

*The data were logarithmized to reach a normal distribution

The Chi-Square Test shows a significance of $p=0.030$. This results reflect the cross tabulation of STS and ER- α (Tab. 4.2.11).

		STS (log)*	
		High Expression	Low Expression
SULT1E1 (log)*	High Expression	30	23
	Low Expression	19	36

Tab. 4.2.12: Cross Tabulation of STS and SULT1E1

The cross tabulation of STS and SULT1E1 shows that a low expression of STS is associated with low levels of SULT1E1 (bold – 36 patients). The same tendency is seen in the inverted way (bold – 30 patients).

*The data were logarithmized to reach a normal distribution

Also this cross tabulation of SULT1E1 and STS showed a significance $p=0.021$. Low expression of SULT1E1 in the tumor is mostly seen with a low expression of STS. Same tendency is observed with high-high (Tab. 4.2.12).

The progesterone receptor staining did not go well because of the bad quality of the microarrays. Only 56 out of 243 patients were decent for statistical analysis. It was not possible to analyze that small amount of samples and compare it to the rest of the data. Unfortunately time and material was not available to repeat the experiment.

4.3 AD293 5A1 and AD293 EV

The cell line AD293 5A1 is used for the study of the less known transporter of the OATP-family, namely OATP5A1. These studies also include chemosensitivity assays and the investigation of possible substrates of the transporter (done in collaboration with Dr. C. Özvegy, Hung. Acad. Sci. Budapest, HU).

First, it is very important to characterise the SLCO5A1 transfected cell line morphologically, and take a deeper look into the expression profile of OATPs and other transport proteins.

qPCR and immunofluorescence staining analysis of OATP5A1 in the cell lines AD293 5A1 and parental AD293 Empty Vector (EV) cells should give a clear indication of the different expression levels of OATP5A1 at the mRNA and protein level. The cell line transfected with an empty vector has a very weak staining (Fig. 4.3.2), in contrast to the AD293 5A1 cell line, which shows a clear positive staining for OATP5A1 in approximately 40% of the cells (Fig. 4.3.1). Although, all AD293 5A1 cells should have this intense green staining for SLCO5A1, it was interesting to see, that only AD293 5A1 cells expressing the transporter changed their shape to a dendritic cell like shape (cells with long membrane extensions).

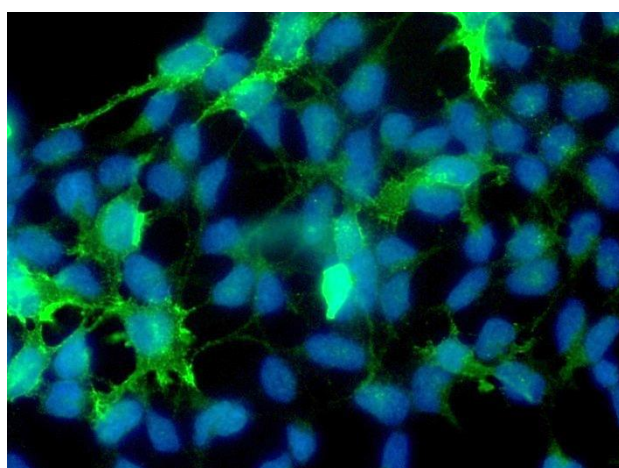


Fig. 4.3.1: AD293 5A1 IF stained with OATP5A1

This picture shows a staining of the cell line AD293 5A1. Blue are the nuclei and green (FITC channel) is OATP5A1.

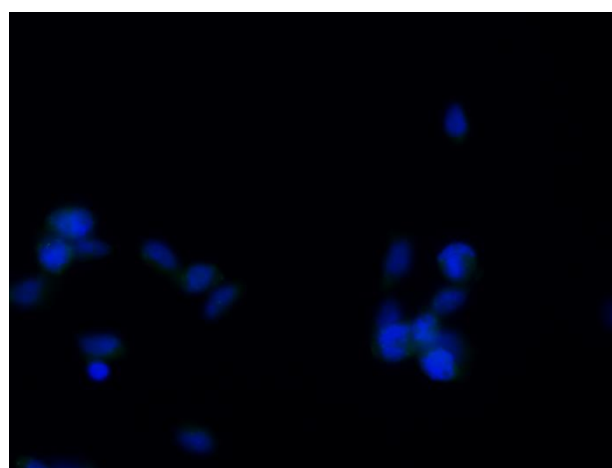


Fig. 4.3.2: AD293 EV IF stained with OATP5A1

In comparison to the AD293 5A1 cells the AD293 EV cell line shows no OATP5A1 staining. Blue are the DAPI stained nuclei and green (FITC channel) is OATP5A1.

Quantification of OATP5A1 mRNA levels by qPCR revealed a significant increase (Approx. 1200-fold, Fig. 4.3.3) OATP5A1 in the cell line AD293 5A1 in comparison to AD293 EV.

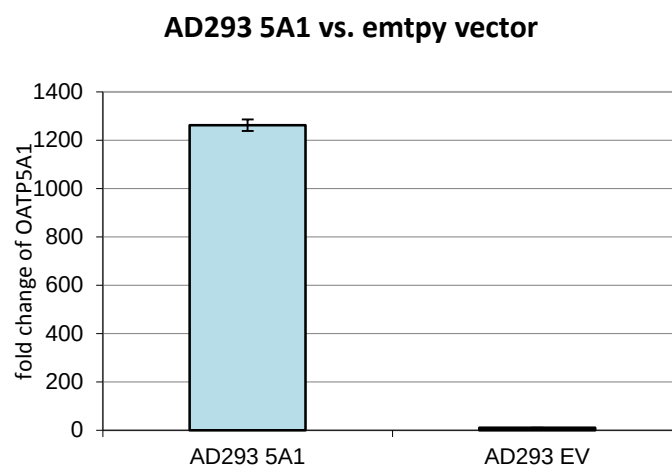


Fig. 4.3.3: qPCR of OATP5A1 of AD293 5A1 and EV

This figure shows the qPCR results of OATP5A1 mRNA levels in AD293 5A1 and AD293 EV. The amount of OATP5A1 mRNA is in the cell line AD293 5A1 1262 folds higher than in AD293 EV.

4.3.1 The Human Drug Transporter PCR Array

The Human Drug Transporter PCR Array analysis was done in the cell line AD293 5A1 and in AD293 EV cells. No significant expression changes of the 84 tested transporters important for drug delivery (29 ABC transporters, 46 SLC transporters, and nine other transporters) was seen.

4.4 OATP5A1 in Ovarian Cancer

4.4.1 Quantitative Analysis of OATP5A1 and correlation studies

After the IHC staining of OATP5A1 in paraffin-embedded tumor samples from eight patients with ovarian cancer, the slides were scanned in with an automated microscopic imaging system (TissueFAXS®). To learn more about the distribution and the expression of OATP5A1 in tumor and tumor stroma the samples were analyzed quantitative with the corresponding HistoQuest® software (Versions: 3.0.3.0173 and 4.0.4.0133).

After calculation of the immunoreactive scores (IRS) for OATP5A1 in tumor and stroma, a statistical analysis using the program GraphPadPrism5 has been carried out. In this pilot study, possible correlation between IRS in tumor and stroma, age of patients, involvement of the lymph nodes, occurrence of metastasis, amount of ascites, and presence of p53 mutation were analyzed (Tab. 4.4.1).

Patient Code	IRS Tumor	IRS Stroma	Age	Lymph-node involvement	Metastasis	Recurrence (in days)	Ascites >500ml
B015	729,74	60,01	51	N1	M1	820	yes
B025	358,03	36,04	41	N0	M0	324	no
B005	40,88	4,55	56	N1	M1	426	no
B040	2204,69	48,63	48	N1	M0	219	yes
B018	564,81	2,31	43	N1	M1	146	no
B024	84,58	396,82	71	N0	M0	1217	no
B001	1534,96	26,33	63	N0	M0	258	yes
B003	409,18	135,55	71	N0	M0	271	no

Tab. 4.4.1: Clinical Data for the OATP5A1 Correlation Studies

Eight ovarian cancer specimens were stained with an antibody against the transporter OATP5A1. The IRS of tumor and stroma, patient age, involvement of the lymph nodes (N0=no involvement, N1=involvement), metastasis (M0=no metastasis, M1=metastasis), recurrence of the tumor, amount of ascites, and the existing of p53 mutations are listed.

The correlation studies showed a significance between IRS of OATP5A1 in the tumor vs. stroma area ($p=0.03$). Also, there was a difference of the IRS in the tumor relation to the amount of ascites in the abdomen ($>$ or $<$ 500ml) ($p=0.007$). For the other clinical parameters, no differences between IRS values in the tumor were found.

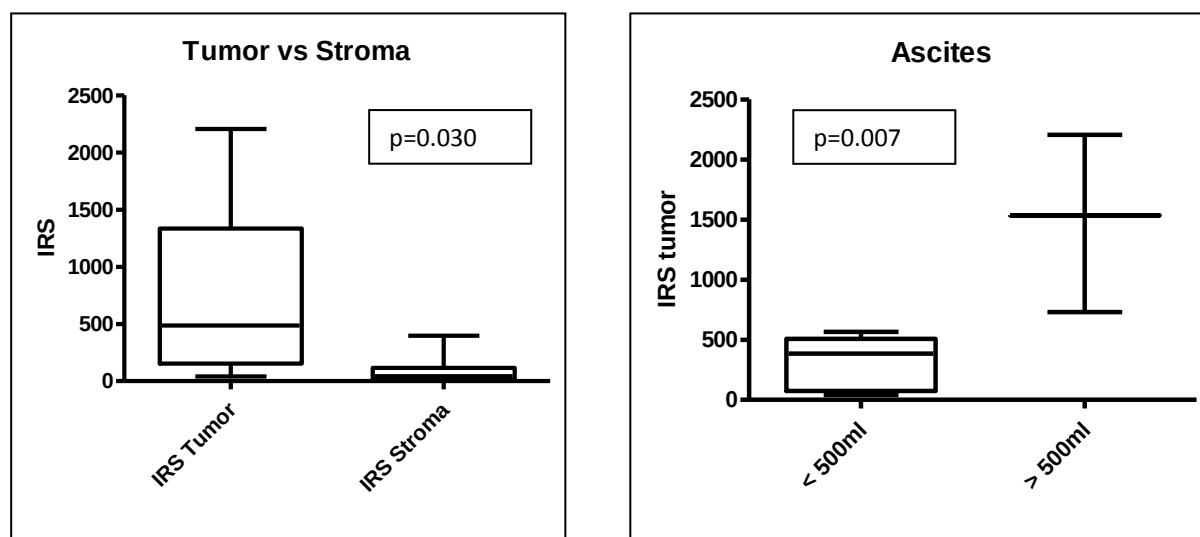


Fig.4.4.1: IRS Tumor vs IRS Stroma (left) and IRS Tumor and Ascites Volume (right)

The diagram shows that OATP5A1 is significantly higher expressed in tumor area than in stroma. Interestingly more OATP5A1 expression was observed in patients with more than 500ml Ascites in their abdomen.

4.4.2 Histological Observation of OATP5A1

Immunohistochemistry of OATP5A1 showed that the transporter is clearly visible in the tumor cells in the cytoplasm and cell membrane in all specimens (Fig. 4.4.2-4.4.9). In a four specimens the staining was very weak, e.g. Patient B024 (Fig. 4.4.7).

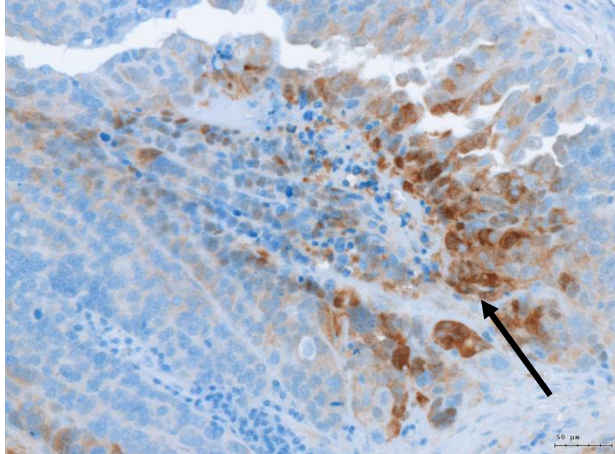


Fig. 4.4.2: IHC of OATP5A1 (Patient B025)

Strong brown staining is seen in tumor cells at areas which are located close to immune cells (cells with small dark blue nuclei). OATP5A1 is located mostly in the plasma membrane and in the cytosol. Only few cell nuclei are stained.

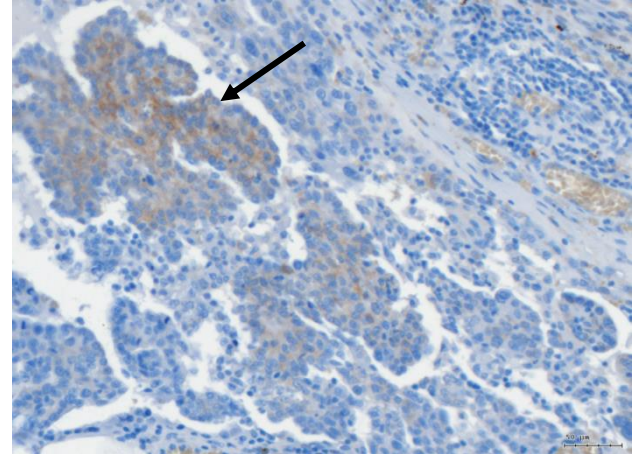


Fig. 4.4.3: IHC of OATP5A1 (Patient B001)

This sample shows staining of the tumor cells, but there was hardly any staining in stroma cells. Only staining of cell membranes and cytoplasm is visible.

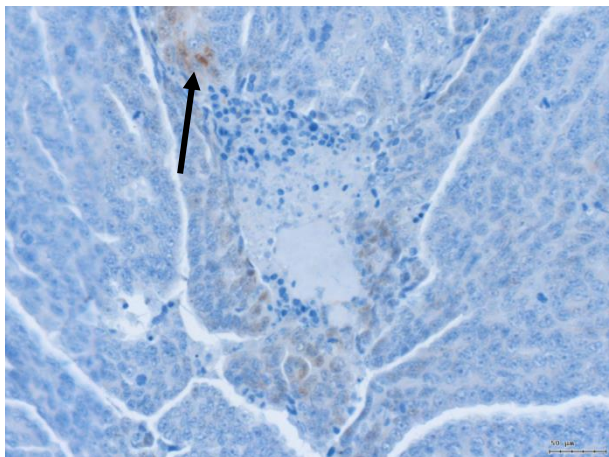


Fig. 4.4.4: IHC of OATP5A1 (Patient B005)

In the tumor cells, membrane and also a nuclei staining is observable in only few cells. The stroma cells are mostly negative.

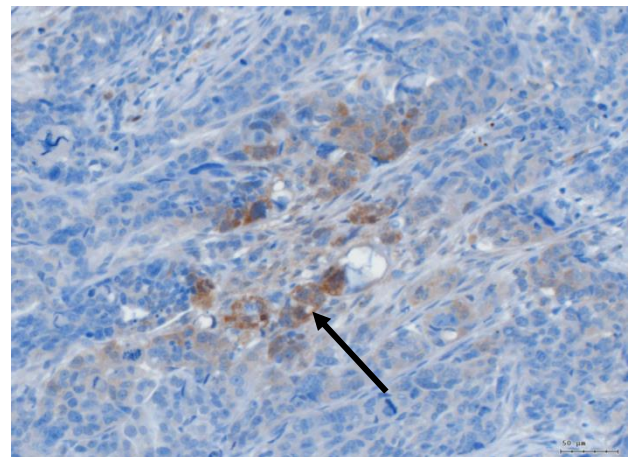


Fig. 4.4.5: IHC of OATP5A1 (Patient B040)

A brown-stained tumor hotspot, where the stroma staining is also strong. The rest of the stroma cells are only weakly stained or not stained at all.

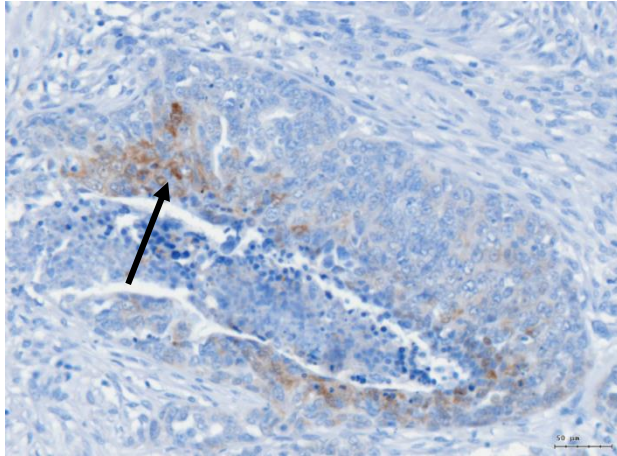


Fig. 4.4.6: IHC of OATP5A1 (Patient B018)

The staining intensity for the transporter is strongest in tumor cells located around the lumen where immune cells are situated. In some cells, nuclear staining is seen. Stroma cells are mostly negative for OATP5A1.

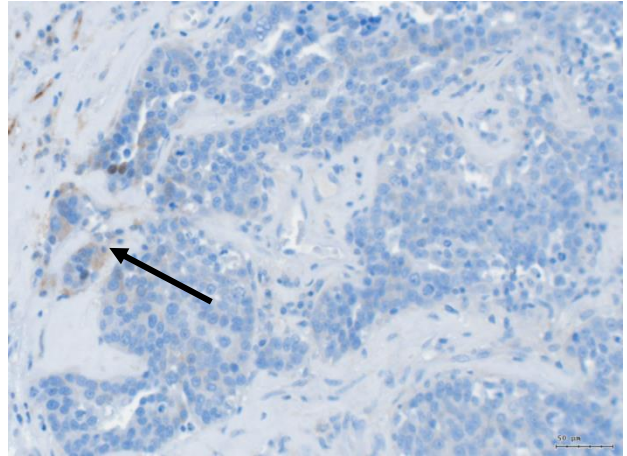


Fig. 4.4.7: IHC of OATP5A1 (Patient B024)

The staining intensity for the transporter is very weak in all areas. In this patient, the stroma staining is stronger than the tumor staining. Nuclear staining is occasionally seen.

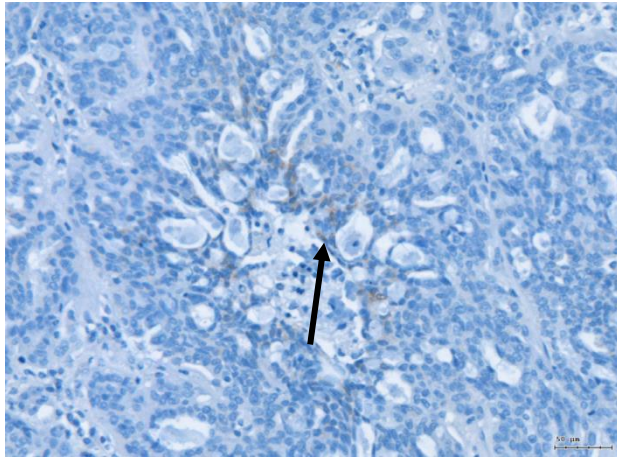


Fig. 4.4.8: IHC of OATP5A1 (Patient B015)

In the tumor cells the membrane staining is dominant and some nuclei are OATP5A1 positive as well.

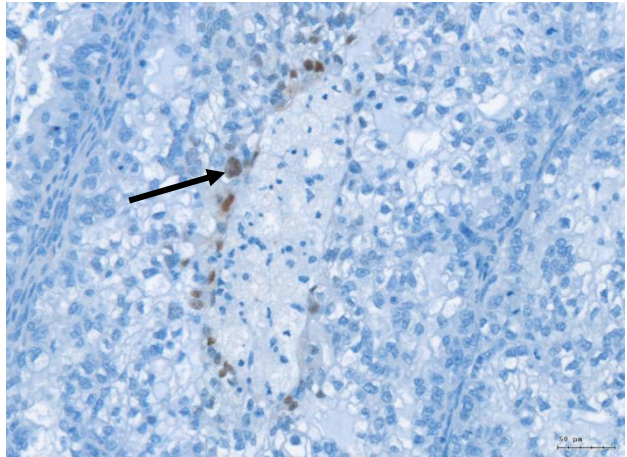


Fig. 4.4.9: IHC of OATP5A1 (Patient B003)

Nucleus staining is seen in tumor cells.

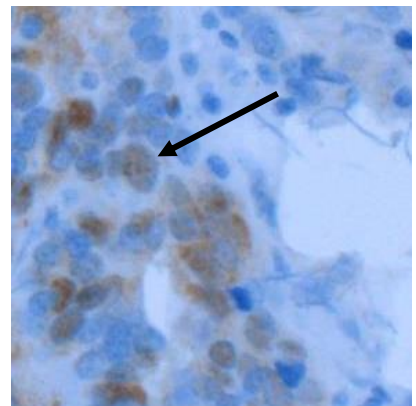
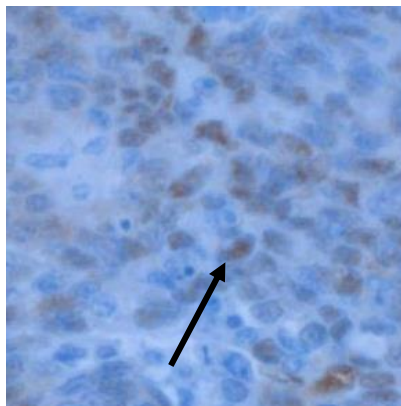
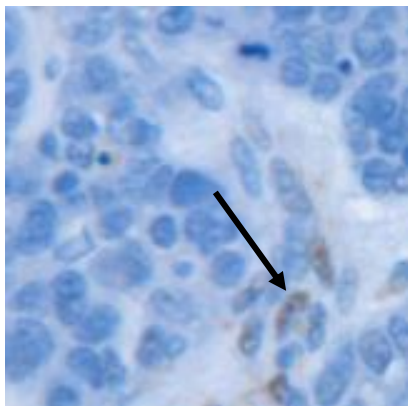


Fig. 4.4.10: OATP5A1 Nuclei staining in Ovarian Cancer

IHC of OATP5A1 in samples from patient **B003**, **B005** and **B025** (from left to right). In the three images, nuclei of the cells are stained (arrow). Nuclear staining for OATP5A1 was observed in samples from all eight patients.

Generally, positive staining for OATP5A1 was seen in tumor cells and, only rarely, in stromal cells. An exception was the tumor of patient B024, with a more intensive staining in the stromal cells. In some patients, OATP5A1 staining was most intensive in cells adjacent to the lumen where also immune cells are concentrated in groups (B025 and B018). It is also interesting to note that the transporter, which should be located in the plasma membrane of the cells, is occasionally seen in nuclei (see tumor sample from patient B003). Immunoreactive OATP5A1 was also seen in the tumor samples from patients B018, B005, B025, B040, and B024, respectively. Nuclear staining was not observed in samples from patient B001 and B015. In the manual inspection of samples, the strongest OATP5A1 immunoreactivity was most prominent in samples from patients B001, B040 and B015, respectively. The results from the quantitative analysis with the software HistoQuest® confirmed this observation.

4.4.3 Immunofluorescence of OATP5A1 and CK19

Double-immunofluorescence staining for OATP5A1 and cytokeratin 19 (CK19), an epithelial marker, was done on paraffin-embedded samples from seven patients. To detect CK19 the secondary antibody labeled with AlexaFluor 568 was used. Therefore, cells with epithelial features appear in red color from CK19 staining after excitation at the appropriate wavelength. OATP5A1 is detected with a rabbit antibody against the transporter and a secondary antibody against the rabbit antibody labeled with AlexaFluor 488. After excitation, the green OATP5A1 staining is visible. In the overlay picture, the red epithelial CK19 and the green OATP5A1 staining in the tumor cells gives a yellow to orange color.

Patient Code	Tumor classification
I002Q	serous
I006Q	serous
I008Q	endometrioid
I011Q	endometrioid
I013Q	serous
2778K	serous
I010Q	no data

Tab. 4.4.2: Samples for OATP5A1 and CK19 Staining

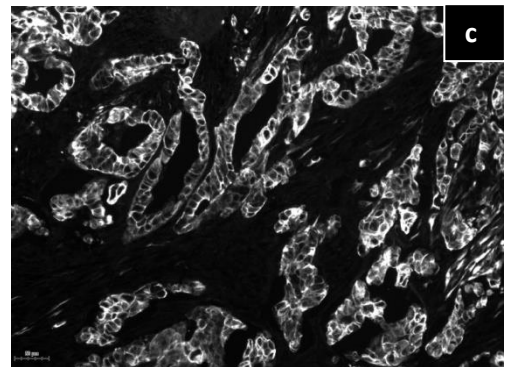
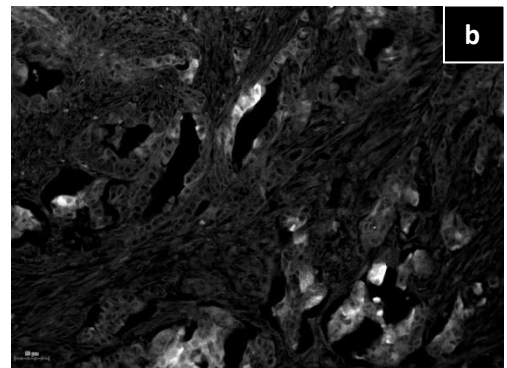
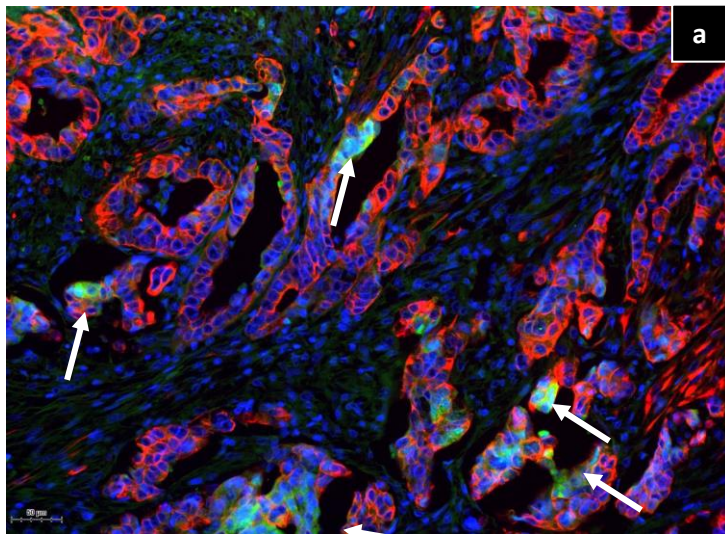


Fig. 4.4.11: IF of OATP5A1 and CK19 (Patient I006Q)

In the green FITC channel OATP5A1 (b) and in the red TexasRed channel the epithelial cell marker CK19 (c) is visible. Picture “a” shows the merged channels including the DAPI stained nuclei in blue. In the merged picture it is clearly visible that OATP5A1 overlaps mostly with CK19 (arrows).

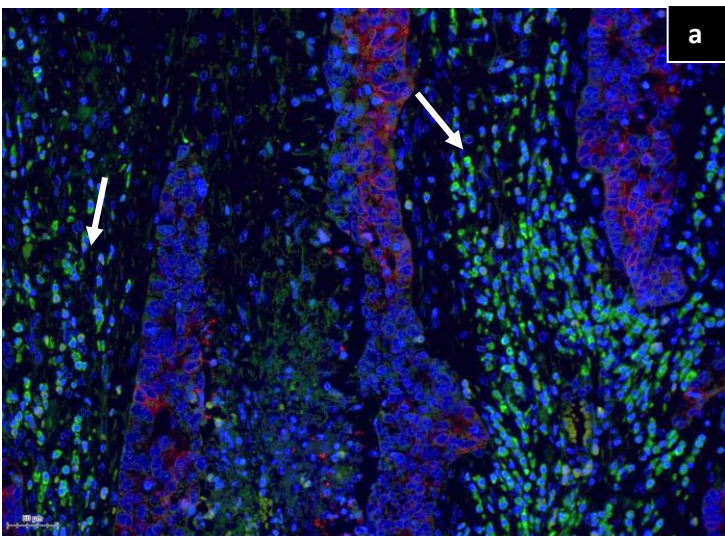


Fig. 4.4.12: IF of OATP5A1 and CK19 (Patient I011Q)

In the green FITC channel OATP5A1 (b) and in the red TexasRed channel the epithelial cell marker CK19 (c) is visible. Picture “a” shows the image of merged channels including the DAPI stained nuclei. This is the only patient that does not have OATP5A1 positive tumor cells. Only immune cells in the stroma are positive for OATP5A1.

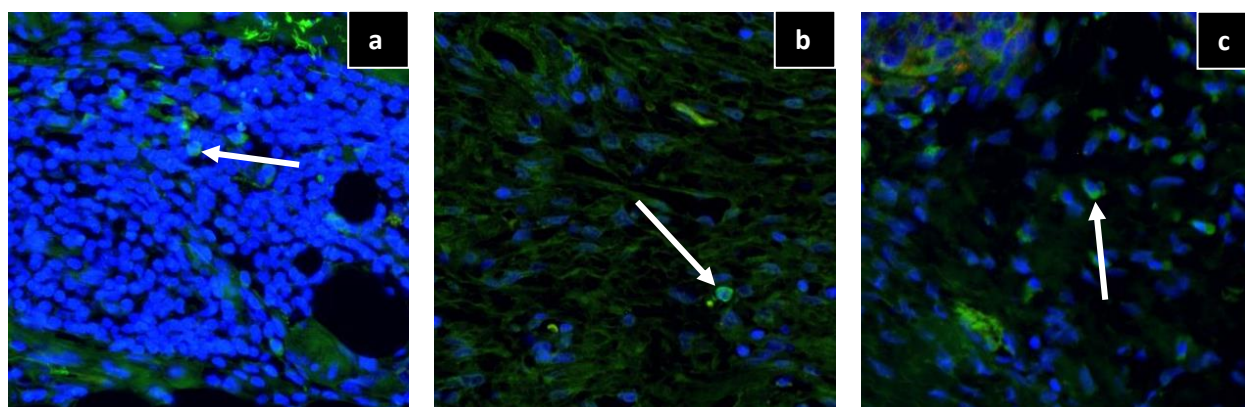


Fig. 4.4.13: OATP5A1 positive Immune Cells in Ovarian Cancer

IF stained tissue samples of the patients I006Q (a), 2778K (b) and I010Q (c). This three pictures show the OATP5A1 (green) stained immune cells (arrows).

In six of the seven patients OATP5A1 was mostly present in the tumor cells (Fig. 4.4.11). The percentage of the OATP5A1 positive cells was consistent with the findings of the IHC. Also some OATP5A1 positive immune cells were detected (Fig. 4.4.13). Only in one patient, no staining of tumor cells was visible. Interestingly, the patient had many OATP5A1 positive immune cells scattered over the whole tissue (Fig. 4.4.12).

4.4.4 Immunofluorescence of OATP5A1 and CD20

Based on the finding of OATP5A1 immunoreactive immune cells, double-immunofluorescence staining of OATP5A1 and of the B-cell marker CD20 was done to investigate whether these immune cells may express the transporter. Samples from four patients with serous tumors were stained with antibodies against OATP5A1 (green) and CD20 (red). (See samples B001, B015, B024 and B040).

Patient Code	Tumor classification
B001	serous
B015	serous
B024	serous
B040	serous

Tab. 4.4.3: Samples for OATP5A1 and CD20 Staining

In samples from all four patients, only few OATP5A1-CD20 double positive cells were found (Fig. 4.4.14).

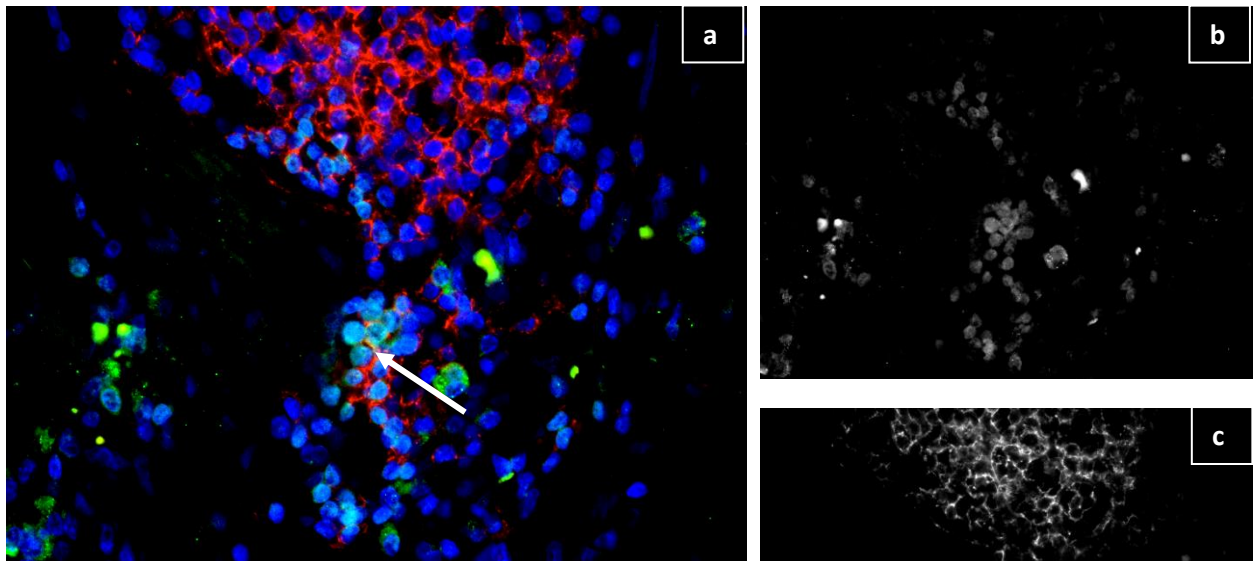


Fig. 4.4.14: IF of OATP5A1 and CD20 (Patient B024)

In green OATP5A1 ("b" only FITC), in red the B-cell marker CD20 ("c" only TexasRed) Picture "a" shows the merged channels including the DAPI stained nuclei. Double positive (OATP5A1⁺/CD20⁺) cells were found in all four patients (arrow).

5 Discussion

5.1 Enzymes and Estrogen Receptors in Ovarian Cancer

The most important aim of this thesis was to investigate the cellular levels of estrogen receptors ER- α , ER- β , STS, and SULT1E1 in paraffin-embedded sections from ovarian cancer patients. This knowledge should allow determination of the influence and relevance of the steroid hormones estrogen and progesterone in the pathogenesis of ovarian cancer. Therefore, microarrays containing 243 advanced ovarian cancer samples were stained by immunohistochemistry and immunoreactive proteins analyzed by the automatic image analysis program HistoQuest® on virtual images from the TissueFAXS®. The IRS of the enzymes STS and SULT1E1 and nuclear receptors ER- α and ER- β was calculated and compared with important prognostic clinical data of patients including age, presence of residual tumor after removal, tumor grading, FIGO staging, overall survival and progression free survival by using the software SPSS 19.0.

Steroid hormones bind the nuclear receptors such as ER- α and ER- β and, therefore, regulate several processes including cell proliferation and differentiation. Remarkable is that the two isoforms of ER have different impacts on the progression of the tumor. We found, that ER- α is the predominant isoform, while ER- β was only marginally present in larger tissue sections and widely undetectable on microarray samples. An independent significant influence of ER- α on the overall survival ($p=0.037$) and progression free survival ($p=0.046$) was achieved in our collective of patients with serous ovarian cancer.

ER- α seems to promote tumor progression in contrast to ER- β , which has an antiproliferative effect (Fujimoto et al., 2000; Li et al., 2003; Lindgren et al., 2004). The expression pattern of the estrogen receptor changes during tumor progression. In healthy ovary tissue ER- β is the predominant estrogen receptor, but in advanced ovarian cancer and metastasis ER- α was found to be the dominant isoform. ER- β was weak or absent in the studies of Rutherford et al., 2000 and Chan et al., 2008; as well as in our study.

Somewhat surprisingly, our study and data from other groups (Burges et al. 2010; Halon et al., 2011 and Aust et al. 2012) showed that high levels of ER- α have a favorable effect on the overall survival and progression free survival of ovarian cancer patients. In younger woman, the

combination of a high expression of ER- α and PGR (and not ER- α alone) has a positive impact on ovarian cancer prognosis (Yang et al., 2009). In our study, there were not enough samples from younger women positive for PGR available to draw a significant conclusion.

It has also to be mentioned that in earlier studies, a correlation between ER- α and PFS and OS were not detected. But this may be caused by the lack of isoform-specific antibodies, which did not discriminate between the isoforms ER- α and - β in the stainings (Lee et al., 2005; Tangjitgamol et al., 2009).

In breast cancers the impact of ER- α is already investigated. In some tumors ER- α is highly expressed and these tumors also have a better prognosis than ER- α negative tumors. An additionally therapy with tamoxifen (antiestrogenic effects) in ER- α positive breast tumors leads to a repression of the tumor growth and a more favorable outcome (Purohit and Foster, 2012). In ovarian cancer several studies showed little or no beneficial effect of tamoxifen on survival, but none of these trials were well designed and patients were not selected by their ER- α status (Williams et al., 2010; Burges et al., 2010, Simpkins et al., 2013). Based on recent data from our and other labs, it would be worth to investigate, whether or not tamoxifen could have a positive effect on survival in a subgroup of ovarian cancer patients that highly express ER- α .

The cellular levels of the enzymes STS and SULT1E1 in the sulfatase pathway were also observed in this study. STS transforms inactive estrogen to more active estrogen, which can bind and activate the nuclear receptors ER- α and - β . We show the importance of SULT1E1, but not of STS for the survival in ovarian cancer.

There are not a lot of data on the role of these enzymes in ovarian cancer. In a study of Chura et al. (2009) a high expression of STS was found to be associated with lower progression free survival in ovarian cancer consisting of different tumor subtypes (LGSC, HGSC, MC, EC, CCC, mixed and undifferentiated tumors). In our studies, for the homogenous group of serous ovarian carcinoma, no independent significant influence of STS on the overall survival or progression free survival was found.

SULT1E1 is the opponent to STS, which means that it is involved in the transformation of active to inactive estrogens. In breast cancer, it is known that a high expression of SULT1E1 is associated with a better prognosis and a negative lymph node status (Utsumi et al., 1999; Miyoshi et al., 2003; Poission Pare et al., 2009). In endometrial cancer, Utsunomiya et al.,

demonstrated that the expression of STS is increased, but SULT1E1 is decreased suggesting that this might increase the levels of active estrogens (Utsunomiya et al., 2004). Xu et al., found in a study in xenograft mice that an overexpression of SULT1E1 in estrogen dependent cancers leads to a reduction of tumor size. Down-regulation of c-myc, cyclin D1 and bcl-2, which leads to an induced apoptosis, was correlated to high levels of SULT1E1 (Xu et al., 2012). In this study it was shown for the first time that SULT1E1 have a highly significant impact on overall survival in patients with serous ovarian carcinoma as evaluated by univariate and the multivariate cox regression ($p=0.023$ and $p=0.013$, respectively).

The cross tabulations of STS, SULT1E1 and ER- α showed that they are associated with each other through high-high expression and also low-low expression levels. This is not unexpected as these proteins share estrogen-dependency and the same pathway. Especially the association of ER- α and STS due to the fact that STS transforms inactive to active estrogen, and high amounts of E2 stimulates ER- α expression.

Conclusion:

The data revealed that SULT1E1 as well as ER- α has a significant effect on overall survival in patients with serous ovarian cancer. Also progression free survival is influenced in a positive way through ER- α . Both proteins might be further studied to evaluate their prognostic power in a larger set of ovarian cancer patients. The data further suggest that patients with high levels of ER- α could profit from antiestrogen therapy such as tamoxifen treatment. Nevertheless, more research needs to be done to emphasize the importance of these enzymes and steroid hormone receptors of the estrogen pathway in ovarian cancer.

5.2 Characterisation of OATP5A1

Since OATPs are active in the cellular uptake of steroid hormones, the characterisation of OATP5A1 in ovarian cancer was the second goal of this thesis. qPCR and the immunofluorescence staining revealed that the OATP5A1 transfected cell line AD293 has as a high expression of OATP5A1 in comparison to AD293 EV (empty vector). Interestingly, only about 20% to 30% of AD293 5A1 cells express the transporter highly and these cells changed their shape into a dendritic cell like shape. Whether the expression of this OATP is cell cycle dependent as suggested by Sebastian et al., (2013) has to be further evaluated. No significant changes were found for the expression of other human drug transporter in the transfected cells using a PCR array, although nearly all transporters showed a tendency to be down-regulated.

This could be explained by the high expression of OATP5A1, which may repress the expression of other solute transporters.

There was also a pilot study of OATP5A1 done to assess the location and distribution of the transporter in ovarian cancer specimen from malign ovary tissue from patients mostly at advanced tumor stage. The immunohistochemical and immunofluorescence staining results showed that the expression of OATP5A1 is relatively low in ovarian cancer. It was mostly observed in the cytoplasm of tumor cells located near blood vessels and necrotic tissue, indicating that it mediate the uptake of an endogenous substrate important for tumor cell growth. This might also explain why a significant association between amount of ascites in the abdomen and IRS of OATP5A1 in the tumor was found.

During the studies OATP5A1 stained immune cells were observed, therefore the B-cell marker CD20 was stained with double immunofluorescence. CD20 is a glycosylated phosphoprotein located on the surface of B-cells. It is involved in the development and differentiation of B-cells. It is expressed by immature and mature B-cells, and is located on the surface (Liang and Tedder, 2001). The knowledge of which transporters are located and functional in immune cells is necessary to understand mechanisms of drug resistance, drug-drug interactions and drug toxicity events (Minuesa et al., 2011). The expression of the efflux transporters of drugs (ABC transporters) in immune cells is well studied but data of OATPs are very rare (Kock et al., 2007). OATP4A1 was detected in macrophages, dendritic cells and monocytes (Skazik et al., 2008). The transporter OATP3A1 was found in CD4⁺ T-cells (Tamai et al., 2000; Janneh et al., 2008). Recent data published by Sebastian et al., suggested that OATP5A1 is expressed in peripheral blood mononuclear cells, monocytes, macrophages and dendritic cells. They could also show that the expression levels of OATP5A1 decrease when monocytes differentiate to macrophages. During differentiation from monocytes to mature dendritic cells the opposite effect was observed. Therefore they suggest that OATP5A1 may is involved in differentiation processes (Sebastian et al., 2013).

In the entire CD20/OATP5A1 stained patient specimens a few double positive cells were found in the tumor stroma. This very rare distribution leads to the conclusion that the transporter is not essentially for CD20 positive B-cells. It would be interesting to see if OATP5A1 is higher expressed in other cells of the immune system.

Conclusion:

So far, only few data exist about the organic anion transporting polypeptide (OATP5A1). This study showed that the transporter is expressed mainly in tumor cells of ovarian cancer but at modest levels. From the specific staining pattern it is evidence that the transporter might be involved in inflammatory processes in ovarian cancer. Based on the data obtained, further and larger investigations on the expression of this particular OATP are planned regarding the identification and biological function.

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8 Abbreviations

4-Dione	androstenedione
5-Diol-S	5 α -androstenediol
17 β -HSD	17 β -hydroxysteroid dehydrogenase

A

ARID1A	AT-rich interactive domain 1A gene
AJCC	American Joint Committee on Cancer system

B

BMI	body mass index
BRCA1, 2	breast cancer antigen 1, 2
BSA	bovine serum albumin

C

CA125	cancer antigen 125
CBG	cortisol binding globulin
CCC	clear cell carcinoma
CD20	cluster of differentiation
cDNA	complementary DNA
CK19	cytokeratin 19
CT	computer tomography
CTNBI	cell-cell adhesion gene β -catenin

D

DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulfate
DNA	Desoxyribonucleic acid
dNTP	nucleoside triphosphate

E

E1	estrone
E1-S	estrone-sulfate
E2	17 β -estradiol
EC	endometrial cancer
EOC	epithelial ovarian cancer
ER- α/β	estrogen receptor α/β
ERE	estrogen response element
ERT	estrogen replacement therapy

F

FIGO	Federation Internationale et d'Obstetrique Federation
FSH	follicle stimulating hormone

FRET	Försters resonance energy transfer
G	
GnRH	gonadotropin releasing hormone
H	
HER2	human epidermal growth factor receptor 2
HDL	high density lipoprotein
HGNC	HUGO Gene Nomenclature Comittee
HGSC	high grade serous tumor
HNPCC	hereditary nonpoyposis colorectal cancer
HRP	horseradish peroxidase
HRT	hormone replacement therapy
I	
IF	immunofluorescence
IHC	immunohistochemistry
IRS	immunoreactive score
K	
kB	kilo bases
KI	Karnofsky index
L	
LBD	ligand binding domain
LDL	low density lipoproteins
LGSC	low grade serous tumor
LH	luteinizing hormone
LOF	loss of function
M	
MC	muscinous carcinoma
MM	master mix
MSS	mesomelia-synostoses syndrome
N	
NTD	N-terminal domain
O	
OATP	organic anion transporting polypeptide
OCP	oral contraceptive pill
OS	overall survival
OSE	ovarian surface epithelium
P	
p53	protein 53
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PAPSS	PAPS synthesizing enzymes

PBS	Phosphate Buffered Saline
PDZ	post synaptic density, separate junction-protein discs, tight junction-protein zonula occludentes-1
PFA	paraformaldehyd
PG	prostaglandin
PGR	progesterone receptor
PLD	pegylated liposomal doxorubicin
PMS1/2	postmeiotic segregation increased 1/2
PTEN	phosphatase and tensin homologue gene
Q	
qPCR	quantitative real time PCR
R	
ROI	region of interest
RPMI	Roswell Park Memorial Institute medium
rt-PCR	reverse transcriptase PCR
S	
SHBG	sex hormone binding globulin
SLCO	solute carrier for organic anions
STIC	serous tubal intraepithelial carcinoma
STS	steroid sulfatase
SULF1	Sulfatase 1
SULT1E1	sulfotransferase 1E1
SWI-SNF-A	swltch/sucrose nonfermentable
T	
TNM	tumor, lymphnode, metastasis
V	
VLDL	very low density lipoproteins
W	
WT1	Willms tumor protein

9 Literature

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10 Curriculum Vitae

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Education

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Specialty Molecular Medicine
Certificate: Master of Science (MSc.)
- 09.2008 - 07.2011** **FH Campus Vienna, Molecular Biotechnology**
Certificate: Bachelor of Science (BSc.)
- 09.2007 – 07.2008** **University of Vienna, Bachelor of Biology**
- 09.1999 – 06.2007** **Bundesrealgymnasium Imst**
Focus on natural sciences (Biology, Chemistry, Mathematics and Physics)
Special Thesis: “The alpine flora and fauna of the Muttekopf area”

Work Experience

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Medical University of Vienna
Master Thesis: “Relevance of estrogen activating STS, inactivating SULT1E1 and ER- α in patients with advanced ovarian cancer”
Supervisor: Dr. Theresia Thalhammer
- 4.11.13 - 9.11.13** **Department of Toxicology**
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Research Stay under the program WTZ Poland 2012/2013
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Internship “Therapy induced senescence in neuroblastoma mouse model”
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- 11.2012 - 12.2012** **Max F. Perutz-Laboratories (MFPL), Vienna**
Internship: “Detection of desmin in the nucleus”
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05.2014

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Poster presentation: "Relevance of estrogen activating STS and inactivating SULT1E1 enzymes and estrogen receptors (ER) α/β in patients with advanced ovarian cancer"