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DISSERTATION

Polymorphism and expression of IL-10 in serum and ascites from patients with advanced ovarian cancer

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1 Introduction

1.1 Ovarian cancer

Ovarian cancer is the 5th most frequent malignant disease of the European woman. Due to poor prognosis, it is the leading cause of death related to gynecologic tumors (1). In Germany, about 8,000 women are diagnosed every year as having a malignant ovarian tumor. Because no effective screening method is available until now and due to the poor clinical behavior most of the patients are diagnosed in FIGO stage III or IV (1, 2).

In Europe the highest incidence of ovarian cancer is in the Scandinavian countries, the lowest one being in the south European countries.

As screening methods bimanual pelvic examination, ultrasonography and measurement of the CA-125 serum levels were investigated in large trials. Unfortunately the methods are not specific and sensitive enough to fulfill the requirements for an efficient screening test (3, 4, 5). The new study presented at ASCO in 2009, which included 1442 patients from 10 countries, was designed in order to determine the benefits of early treatment based only on the elevation of CA-125 compared to delayed treatment until clinically indicated. Although the treatment was started 5 months earlier in the immediate arm, the study showed no significant impact on the overall survival between the two arms (hazard ratio 1.01, 95% CI 0.82-1.25, p = 0.91) (6).

Oskay-Oezcelik et al. presented at ASCO the results from a multi-institutional survey about cancer care in ovarian cancer patients. The study included 1060 patients. According the patients' opinion, the main objective for the follow-up is the early detection of relapse and a prolongation of overall survival (95.8%). More than 90% get CA-125 measurements, being the procedure with highest anxiety but also the most important procedure for the patient (7).

CA-125 antigen is a useful serum marker to monitor the patients with ovarian cancer during chemotherapy, but the changes in chemotherapeutical treatment or the diagnosis of the recurrence should not be made only on the basis of CA-125 levels (5, 8). The changes of CA-125 levels in serum during chemotherapy are a good marker to follow the treatment efficiency (4). On the other hand, the value of CA-125, as a

marker, for the initial diagnosis, is limited because of its elevation in a multiple benign disorders like endometriosis, benign ovarian tumors, peritonitis and other benign diseases (3). Other strategies include the transvaginal ultrasonography (TVS) and the color doppler imaging. The TVS shows the morphology of the ovary or of the ovarian mass and other pelvic pathology. The abnormal neovascularization detected by color doppler imaging can suggest the presence of a malignant process (4). These methods may improve the specificity for detecting ovarian cancer, but they cannot establish a certain diagnosis of malignancy. The efficiency of three-dimensional ultrasonography as a screening tool for ovarian cancer should be investigated in further trials.

Studies have tried to characterize the group at risk for ovarian cancer, using the molecular biology; hence they investigated the involvement of different genes in the ovarian carcinogenesis (9-11, 14, 15).

Various working groups have investigated the microarray technique in the development of a discriminatory proteomic pattern in order to differentiate between benign and malignant ovarian tumors, the early stages and late stages of ovarian cancer (9-11).

The ovarian cancer treatment consists in cytoreductive surgery and chemotherapy. Most of the patients respond well to these forms of treatment, but the majority of them develop recurrence and chemotherapy resistance disease (4). Despite of increased 5-year survival rates in the last period - 37% in 1976 comparing with 41% in 1985 and 45% in 2002- new treatment strategies must be developed (12).

Further studies are needed in order to find a tumor marker, new genetic mutation that may raise the susceptibility of a possible incipient ovarian cancer, or to describe better the collective of patients having a higher risk for the development of ovarian cancer. Also the discovery of a possible molecular target has resulted in increasing attention being paid to tumor-specific immunological therapy (10).

There are well-described predisposing genetic factors in ovarian cancer, such as the mutation of BRCA-1/2 gene, p-53 and IL-1, PAI, IL-12 gene polymorphisms. Despite increasing knowledge in immunology in solid cancer, the tumorigenesis and the value of genetic aspects are still underestimated (14-19).

1.1.1 Epidemiology

In Europe 61,000 new cases of ovarian cancer are diagnosed each year, most of them, 75%, being diagnosed at the time of diagnosis, FIGO stage III or IV (1, 20).

The incidence of ovarian cancer in Germany is about 14 cases per 100,000 women, which is the average incidence rate in the European countries. Differences between the incidence rates in certain countries and geographical regions were observed. Women from Scandinavia (Finland, Sweden and Denmark), Western Europe, Ireland, Great Britain and USA have the highest risk of developing ovarian cancer. The south European countries (Italy, Greece and Spain) have the lowest incidence rate of all countries in Europe (22). The incidence rates in Africa and Asia are very low. In Japan, only 3-4% new cases of ovarian cancer are registered every year (21, 22).

Ovarian cancer is most often encountered in women aged between 50 and 59 years (22, 23). The median age of diagnosis, between 1992 and 2002, was 62 years of age (21). The risk for ovarian cancer increases with the age of the woman, 8.4% of all the cases of ovarian cancer diagnosed during this period of time were women between 35 and 44 years of age. Overall, 21.1% of women with ovarian malignancy were between 65 and 74 years of age (21).

Seventy five percent of the women diagnosed as having a malignant ovarian tumor have a FIGO stage III or IV disease, and therefore a 35% 5-year survival rate. In contrast, the 5-year survival rate for patients with FIGO stage I disease is about 90% (3).

Of all gynecologic malignancies, ovarian cancer remains the leading cause of death (26). The nonspecific symptomatology and the absence of an effective screening test are responsible for the highest mortality rate due to ovarian cancer (23).

Ninety to ninety five percent of ovarian malignancies are sporadic, in those cases a hereditary factor could not be identified. Only five to ten percent of the ovarian tumors can be classified as hereditary malignancy, most of them (75%), being part of the hereditary ovarian and breast cancer (27).

1.1.2 Etiology and risk factors

Etiology

Dietary factors

The increasing incidence rate of ovarian cancer among women originating from Asian or African countries, but living in Europe or America, has suggested the involvement of nutrition as a contributing factor to the carcinogenesis (28-29). Like other types of cancer (breast cancer, endometrial cancer), the pathogenesis of ovarian cancer seems to be related with a diet rich in saturated fatty acids (29) and also with excessive weight (28).

Studies showed that the intake of animal fat is associated with an increased risk for ovarian cancer, whereas the intake of vegetables with a lower risk (28, 29). It seems that no association between ovarian cancer and alcohol consumption was proved (30).

Genetic factors

Studies showed that the involvement of the genetic factor is involved in about 5-10% of all cases of ovarian cancer (27, 51). There are three clinical forms of hereditary ovarian carcinoma: “site-specific” ovarian cancer, ovarian and breast hereditary cancer and hereditary nonpolyposis colorectal cancer (HNPCC; Lynch II) syndrome (31). Site-specific ovarian cancer and the breast ovarian hereditary cancer are associated with the mutation of BRCA-1 and BRCA-2 genes. The mutation of BRCA-1 gene increased the risk of ovarian cancer by 20% to 60% and the mutation of BRCA-2 gene increased the risk by 10% to 35% (32-34). These genes act as tumor suppressors maintaining the cellular proliferation and the DNA structure unaltered.

Usually BRCA-associated ovarian cancers have different clinical and pathological features as compared to sporadic ovarian cancer. They seem to have a more favorable history than the sporadic ones (4). The prophylactic bilateral salpingo-oophorectomy is the best preventive treatment for the patients who bear a BRCA mutation.

In the study published by Kauff et al. 199 women with BRCA mutation-negative hereditary site-specific breast cancer were included in order to determine if they still have an increased risk of developing ovarian cancer. The data indicates that these women have an increased risk for breast cancer (19 new cases of breast cancer were

diagnosed, whereas only 6.07 were expected), but their risk for ovarian cancer remains unchanged (one case of ovarian cancer was diagnosed, whereas only 0.66 was expected) (34).

In the hereditary nonpolyposis colorectal cancer syndrome, mutations in the following four genes are more frequently observed: *hMSH2* (chromosome arm 2p), *hMLH1* (chromosome arm 3p), *hPMS1* (chromosome arm 2q), and *hPMS2* (chromosome arm 7p) (7). Mutation of these genes increases the risk of ovarian cancer by 3.5-fold, as does the risk for malignancy in the gastrointestinal tract (35).

Incessant ovulation

Postulated by Fathalla in 1971 (36), the incessant theory reveals that an increased ovulation rate is associated with an incidence of ovarian cancer. Fathalla showed that the absence of ovulation is associated with low risk of epithelial ovarian cancer. This theory does not apply to the germ cell and mesenchymal tumors. The impact of ovulation on the incidence of ovarian cancer was explained as an overexposure of the ovarian epithelium to the estrogens contained in the follicular liquid and the minor trauma suffered by the ovarian surface during ovulation.

Further studies have used this theory to explain the protection provided by pregnancies, breastfeeding and the use of contraceptives against ovarian cancer (37, 38).

The gonadotropin hypothesis

This refers to the increased incidence of ovarian cancer in patients with increased levels of androgens, especially androstendione. In vitro and in vivo studies showed that gonadotropins may initiate and stimulate the growth of epithelial ovarian cancer. Also FSH and LH receptors in the ovarian epithelium and in ovarian cancer tissue were described (37).

The carcinogens and the ovarian cancer

The exposure of the perineum to talc has increased the risks for ovarian cancer. The same holds true for asbestos (39, 41, 42). This is explained by the inflammation caused at the ovary site by these carcinogens. The inflammation seemed to favor the development of ovarian cancer due to the alteration in the epithelial surface, the oxidative stress and the accumulation of the prostaglandins and cytokines (40).

Different authors suggested that exposure to talc is not involved in all types of epithelial ovarian cancer and if it occurs before the first pregnancy it is a stronger risk factor for ovarian cancer (41, 42).

Many risk factors have been described not knowing exactly if they act alone or in conjunctuon with others in the development of cancer.

Endometriosis and the ovarian cancer

Epidemiologic, histopathologic and molecular data suggest that endometriosis does have a malignant potential. Brinton's retrospective cohort study included 2491 patients with invasive ovarian cancer, who were diagnosed between January 1, 1978 and December 31, 1998. All women were born after 1936. Endometriosis seemed to predispose to the development of ovarian cancer, with the association restricted to endometrioid and clear cell malignancies. Five or more years after the diagnosis of endometriosis, the RRs (95%CI) were 2.9 (1.2-7.1) for ovarian cancer, 2.53 (1.19-38) for endometrioid and 3.37 (1.24-9.14) for clear cell malignancies (43). In 2006 the Swedish cohort studies were expanded by Melin et al. in order to evaluate the risk ratios with longer follow-up and calculation of updated standardized incidence ratios. There was no risk for overall cancer, but an increased risk for ovarian cancer (1.43 (95% CI 1.2-1.7)), endocrine tumors, non-Hodgkin lymphoma and brain tumors (44).

A causal relationship between endometriosis and ovarian cancer should be recognized, but the possibility that ectopic and eutopic endometrium undergo malignant transformation should be considered.

Common pathogenetic factors were also described for both diseases, including familial predisposition, genetic alterations, immunobiologic, cell adhesion, angiogenic and hormonal factors (43). High amounts of IL-1 were found in peritoneal fluid of women with endometriosis and ovarian cancer. TNF alpha also seems to be over-expressed in serum in both patient groups (45).

Age

The mean age of the patients diagnosed with ovarian cancer is 62 years old. The incidence of ovarian cancer increases with the age (52). The incidence of ovarian cancer is highest after 75 years, about 62-65 at 100,000 women (21).

Studies showed that the age-adjusted incidence rate varies according to the histological type (46). Mucinous tumors appear most frequently between the third and

the fourth decades of age, serous tumors between the fourth and the fifth decades, endometrioid tumors between the fifth and the sixth decades and clear cell tumors between the fifth and the seventh decades. Germ cell tumors represent more than 60% of the ovarian neoplasms diagnosed in children and adolescents and one third of them are malignant (46, 47).

Menopausal status

The majority of the studies showed no significant correlation between the age of menarche or menopause and the risk for ovarian cancer (48, 49).

In a multiethnic case-control study it was demonstrated that the involvement of the menopausal status in the development of ovarian malignancies depends on the histological type of cancer. For example, the risk for non-mucinous tumors was described as being associated with the menstruation years (odds ratio=1.5 for the highest vs. the lowest quartile) and lifetime ovulatory cycles (odds ratio=2.8 for the highest vs. the lowest quartile) (50).

Parity

The impact of birth on ovarian cancer was discussed in many studies but the role of parity as a risk factor for ovarian cancer is still not clear.

Some studies showed that a higher parity is a protective factor for ovarian cancer (49). The same holds true for women who had breastfed, only that the period of breastfeeding doesn't seem to correlate with the incidence of ovarian cancer. Incomplete pregnancies don't have any protective role (49). The time since last birth seems to influence the risk for ovarian cancer (50).

Oral contraceptives

The use of oral contraceptives seemed to protect against ovarian cancer in dependence on dosage and duration (about 10% risk reduction per year) (53, 54). The ideal time of the suppression of ovulation it is not known yet. Studies showed that the use of oral contraceptives for more than 6 months protect against ovarian malignancies (54).

Infertility

The role of infertility as a risk factor for ovarian cancer has been and still is a very controversial subject. First of all because these women are also the ones that use fertility drugs, and it is very difficult to separate the role of these two risk factors (56).

One study made in USA in 1998, by Rodriguez et al, showed that ovarian cancer death rates were 40 percent higher among nulliparas with self-reported infertility than in nulliparas that never tried to become pregnant. This result showed that infertility is an independent risk factor for ovarian cancer (55).

Hormone therapy

Many studies tried to prove the role of hormone replacement therapy in the natural history of ovarian cancer.

Garg et al made a review in 1998 of the studies and articles published between January of 1996 and August of 1997, regarding this subject. The conclusion was that hormone replacement therapy seems to raise the risk for ovarian cancer. The ever postmenopausal use of hormones raises the risk for ovarian cancer. Use of this therapy for more than 10 years is associated with the biggest risk of ovarian cancer (OR 1.27; 95% CI 1.00, 1.61) (57).

The use of unopposed estrogen replacement therapy seems to be more related with the incidence of the following histological types of ovarian cancer: endometrioid or clear cell epithelial ovarian tumors (OR 2.56; 95% CI 1.32-4.94). Also the risk seems to be greater in women who did not undergo any surgery involving the genital tract than in the ones with a history of hysterectomy or tubal ligation (58, 59).

Sterility Treatment

According to the incessant ovulation theory, the use of fertility drugs is likely to raise the risk of ovarian cancer, because of their capacity of stimulating ovulation (36, 38). Controversy surrounds the role of fertility drugs in the development of ovarian cancer (55, 56, 60).

In 1996, Bristow reviewed four case-control studies and three retrospective cohort studies. The data suggests that fertility drugs are not involved in the history of ovarian cancer (60).

Smoking

In a case control study developed between 1976 and 2001, the association between smoking and the incidence of different types of ovarian cancer was analyzed. 709 cases of ovarian cancer were enrolled in this study. The results showed that smoking is a risk factor for the development of mucinous ovarian cancer, but not for other histological types of cancer (61).

Similar results were obtained in other studies. Also it seems that the risk for ovarian cancer increases with the number of cigarettes. It is also higher in patients that are currently smoking than in patients that stop smoking (62).

Ethnicity

Ovarian cancer seems to be more frequent in the northern part of Europe and in the USA and very rare in the Asia and Africa. Many studies tried to find out if there is a significant connection between the incidence of ovarian cancer, ovarian cancer mortality and race.

It seems that the mortality rate due to ovarian cancer is more elevated in white patients, followed by black women. The Asian/Pacific and Icelandic women appear to have the lowest mortality rate (63).

There might be a difference in the geographical distribution of ovarian cancer, which can be due to genetics factors, environmental factors or due to the diagnostic methods.

1.1.3 Histological classification of epithelial tumors

Ovarian tumors are divided into different subgroups depending on the histogenesis: epithelial tumors (originating in the epithelial celom), germ cells tumors (derived from the oocyte), sex cord stromal ovarian tumors (originating in the gonadal stroma) and metastatic tumors (Table 1). Epithelial tumors represent 70 to 90 percent of ovarian malignancies, and they are generally regarded as ovarian cancer (4). These tumors are classified as benign ovarian tumors, borderline and invasive carcinoma.

Ovarian carcinoma represents about 90% of all malignant tumors of the ovary. Ovarian carcinoma is subclassified into different histological subtypes, such as serous-papillary, mucinous, endometroid, clear cell, carcinosarcoma, mixed or undifferentiated

carcinoma (71). The serous subtype comprises approximately 40-53% of all ovarian carcinoma. The mucinous and the endometroid ones represent 7-15% and 15-22%, respectively, of all ovarian cancers (4). Others histological forms such as clear cell tumors (4-10%), mixed tumors (2%) and undifferentiated carcinoma (5-17%) are very rarely encountered (71).

Hess et al, showed on 27 mucinous and 57 other histological types of ovarian cancer that advanced mucinous ovarian cancer patients have a poorer response to platinum-based first-line chemotherapy, along with a worse survival compared with other histological subtypes (66).

An exploratory analysis conducted of 3 prospective randomized trials (AGO-OVAR 3, 5, and 7) investigating platinum-taxane based chemotherapy regimens in advanced ovarian cancer conducted between 1995 and 2002 showed that residual tumor mass, age, performance status, grade, FIGO stage, and histology, namely the mucinous subtype, were the only independent prognostic factors (68).

In the retrospective study published by Pignata and co-workers, all patients with a recurrent platinum-sensitive ovarian cancer treated between 2000 and 2002 in 37 Italian centers were included. This study analyzed twenty patients with mucinous histotype and 388 patients with other histological types. The mucinous patients were diagnosed with lower tumor grading ($p = 0.0056$) and less advanced FIGO stage ($p = 0.025$). At time of recurrence, there was a statistically significant difference in performance status (worse in mucinous, $p = 0.024$). About 20% of patients underwent secondary cytoreduction in both groups, the number of patients who were optimally debulked in the mucinous group being lower ($p = 0.03$). The response rate to the second line chemotherapy was lower in mucinous than in non-mucinous group (36.4% vs 62.6%, respectively, $p = 0.04$). Median time to progression and overall survival were worse for mucinous ovarian cancer (67).

Border line tumors of the ovary are made up of cells whose histological properties are similar to those of the malignant ones, except the aggressive way to grow. The borderline disease comprises tumors that share an excellent prognosis, despite certain histological features that suggest cancer (69). These tumors tend to occur in younger women. The diagnosis is made microscopically (70). Borderline tumors were described for every epithelial tumor type (65). The serous borderline tumors represent about 55% of all borderline tumors. The prognosis in stage I is excellent, the 15 years survival rate being of 99%. The mucinous type is encountered in

about 40% cases of ovarian borderline disease, the 15-year overall survival rate being 97%. The borderline disease is considered to be a different entity and not a premalignant status, although studies showed that the mucinous subtype could be an intermediary form, between benign and malignant tumors (69). For the serous and endometroid type there are no data to suggest such a hypothesis.

Table 1 *Classification of Malignant Ovarian Tumors (WHO 2003)*

I		**Epithelial Ovarian Tumors**
	1	Serous (Endosalpingeal)
	2	Mucinous (Endocervical)
	3	Endometrioid (Endometrial)
	4	Clear cell ("mesonephroid" Müllerian)
	5	Brenner (Transitional)
	6	Mixed epithelial (Mixed)
	7	Undifferentiated (Anaplastic)
	8	Unclassified Mesothelioma
II		**Ovarian Germ Cell Tumors**
	1	Dysgerminoma
	2	Teratoma
	3	Endodermal sinus tumor
	4	Embryonal carcinoma
	5	Polyembryoma
	6	Choriocarcinoma
	7	Mixed forms
III		**Sex Cord-Stromal Tumors**
	1	Granulosa stromal cell tumors
		1.1 Granulosa cell tumor
		1.2 Tumors in the thecoma-fibroma group
	2	Androblastomas: Sertoli-Leydig cell tumors
	3	Gynandroblastoma
	4	Unclassified
IV		**Uncommon Ovarian Tumors**
	1	Small Cell Carcinomas
	2	Lipoid Cell Tumor
	3	Sarcomas
	4	Unclassified
V		**Metastatic Tumors**
	1	Gynecological Tumors
	2	Non-gynecological Tumors
	3	Krukenberg Tumors
	4	Other gastrointestinal tumors
	5	Carcinoid Tumors
	6	Lymphoma and Leukemia

1.1.4 Grading

Surface epithelial tumors are also sub-classified based on the pattern of cellular differentiation and tumor grade. According to the grade of differentiation, there are three types of ovarian tumors: G1, very well differentiated, G2 well differentiated and G3 poorly differentiated to undifferentiated (70).

1.1.5 The natural history of ovarian cancer

Ovarian cancers spread through the body using, in the very early phase, the peritoneal surface. At the beginning, the ovarian cancer involves only the pelvic serosa, but in advanced stages the mesenterium, the paracolic gutter, the diaphragm, hepatic serosa can also be affected by the tumor (4).

The involvement of the lymphatic sector occurs mainly through the following pathways: using the ligamentum infundibulopelvicum to involve the paraaortal lymph nodes or using the cardial ligament to spread to the pelvic lymph nodes (common iliacal, iliacal external and internal, hypogastric and obturatory) (4).

1.1.6 FIGO and TNM classifications

Table 2 *Carcinoma of the ovary: FIGO nomenclature (FIGO Annual Report 2003)*

Stage I		Growth limited to ovaries
	Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface, capsule intact.
	Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surface, capsule intact.
	Ic	Tumor either stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites with malignant cells, or with positive peritoneal washings.
Stage II		Growth involving one or both ovaries with pelvic extension
	IIa	Extension and/or metastases to the uterus and/or tubes.
	IIb	Extension to other pelvic tissues.
	IIc	Tumor either stage IIa or IIb, but with tumor on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.

Stage III		Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equal stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.
	IIIa	Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologically proven extension to small bowel or mesentery.
	IIIb	Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameters; nodes are negative.
	IIIc	Peritoneal metastasis beyond the pelvis > 2cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV		Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to stage IV. Parenchymal liver metastasesare classified as stage IV.

Table 3 *Stage grouping for ovarian cancer (UICC 2003)*

FIGO Stage	UICC T	N	M
Ia	T1a	N_0	M_0
Ib	T1b	N_0	M_0
Ic	T1c	N_0	M_0
Iia	T2a	N_0	M_0
IIb	T2b	N_0	M_0
IIc	T2c	N_0	M_0
IIIa	T3a	N_0	M_0
IIIb	T3b	N_0	M_0
IIIc	T3c	N_0	M_0
	Any T	N_1	M_0
IV	Any T	Any N	M_1

1.1.7 Prognostic factors

Under the term „prognostic factor“ should be considered the special feature of the malignant tumor which characterizes the expected disease behaviour, independent of received therapy (14). Prognostic factors have to help to determine how aggressive the tumor is and to choose the therapy options.

Despite the fact that surgery has become aggressive and radical and chemotherapy has developed a lot, the results of treatment for ovarian cancer are not satisfactory. The understanding of the prognostic factors and the discovery of new ones might help to achieve a better treatment. Thanks to the development of new biomolecular techniques and due to the progress made in understanding the history of ovarian cancer, new potential molecular prognostic factors are currently being studied (14). Two groups of prognostic factors were described: the conventional ones such as stage, grading, postoperative tumor mass, age, lymph node status, ascites and the new molecular biological factors such as Her-2-status, PAI-I, MMP, VEGF and CD24 which are discussed in different controversial studies (14).

Tumor classification FIGO

The FIGO staging is the most important prognostic factor for ovarian cancer. The 5-year survival rate correlates very well with the FIGO stage. The FIGO stage I disease, with well or moderately differentiated tumors is associated with a 5 year survival rate of about 95%, FIGO stage II with 65%, FIGO stage III with an overall survival rate of 14-30% and stage IV with a five year survival rate of 0-20% (71, 74).

Some studies defined a new entity, the so called early-stage disease with unfavorable characteristics, i.e. means the FIGO stage I tumors but undifferentiated or mucinous or clear cell type tumors, which are treated like the stage FIGO II tumors (4). The 5 year survival rate in patients with unfavorable-prognosis early-stage ovarian cancer is approximately 80% (4).

The rupture of the capsule may lead the FIGO stage Ia to FIGO stage Ic. Controversies surround the role of iatrogenic rupture of the tumor capsule and the spread of the disease. A study made in Sweden showed no difference between the survival rates of patients with rupture of the capsule during the surgery and the ones without (75). In contrast to this, other working groups showed a worse prognosis for the patients who experienced an iatrogenic capsule rupture (75). The general opinion is that an iatrogenic rupture of the capsule during surgery should be avoided (4).

The residual mass after primary surgery

The improvement in the survival rate in patients who underwent radical surgery with no post-operative residual tumor gives this parameter a high clinical relevance. The postoperative residual tumor mass is a major independent prognostic factor. The

complete surgical extirpation of the malignant tissue has several benefits, including: reduction of poorly vascularized tumor, whereupon the curative effects of chemotherapy are limited, smaller tumor masses (82), removal of drug-resistance clonogenic cells and enhancement of host immunocompetence (82).

Meigs and Griffits, in 1935 and 1975, were among the first to postulate the importance of the tumor residual for the overall survival of patients with primary ovarian cancer. Several trials confirmed the role of residual mass as an independent prognostic factor in patients with primary ovarian cancer (83, 84).

In the metaanalysis of Bristow and co-workers, the impact of cytoreduction and other prognostic factors on the overall survival were analyzed. They included 81 cohorts of 6885 patients with FIGO stage III and IV. The results showed that each 10% increase in maximal cytoreduction was associated with a 5.5% increase in median survival time. Maximal cytoreduction was significantly associated with median survival time, even when controlled using multivariate analysis (p=0.001) (85).

The role of cytoreductive surgery in recurrent ovarian cancer is not so clear. Until now there are no clinical trials to confirm this data (82).

The presence of ascites

The presence of ascites has a negative effect on the survival of the patient. It is more frequent in FIGO stages III and IV (86).

Lymph node involvement

Even lymph node involvement give rise to many different opinions. It appears that lymph node metastases are not an independent prognostic factor, even if a systematically lymphadenectomy is required in order to have a correct stadialization (76). Currently, a randomized trial of the AGO (Arbeitsgemeinschaft Gynäkologiesche Onkologie) has initiated a randomized trial comparing systemic lymph node dissection with no lymph node dissection in patients with primary ovarian cancer and no macroscopic postoperative tumor residuals (the LION study).

The involvement of the lymph nodes classifies the disease as a FIGO IIIc stage. Also the presence of tumoral mass having a diameter bigger than 2 cm classifies the disease in the same stage. Better prognosis and three year overall survival for patients with ovarian cancer FIGO IIIc and having positive lymph nodes was described in a

study made by Camino in comparison with those having peritoneal involvement (46% vs. 26%) (76).

The role of lymphadenectomy in the treatment of ovarian cancer is controversial. In a study published by Benedetti Panici and co-workers, 427 patients with FIGO Stage III and IV disease were included. Of these, 216 patients underwent systematic lymphadenectomy and in 211 of them only extirpation of bulky nodes was performed. The results of the study showed a significantly longer progression-free survival for the patients in the first group, 29.4 vs. 22.4 months, p=0.01. There was no significant impact on the overall survival (58.7 vs. 56.3 months) (77). In this study, only 37% of the patients who potentially will benefit from systematic lymphadenectomy had no residual mass after operation. Therefore the limitation of this study could be the very long recruiting period (12 years), during this time the therapy principles have undergone many changes.

In a large international, prospective, randomized trial, the Scottish Randomized Trial in Ovarian Cancer (SCOTROC), the benefits of docetaxel-carboplatin vs. paclitaxel-carboplatin as first line chemotherapy for stage Ic to IV ovarian cancer were analyzed (79). In this study 1077 patients were included, two thirds being treated in the UK and a third of patients were treated in Europe, US and Australasia. The fact that chemotherapy was carefully defined and the success of the performed surgery was not a conditional including factor made the SCOTROC trial an important tool for estimating the variation of the surgical practice internationally and the impact on the progression-free survival (PFS) (79). The results of the study showed that the consequence of a more extensive surgery performed on non-UK patients was a higher rate of optimal surgical outcome (71.3% vs. 58.4%, respectively, p<0.01). As in the previous studies (77, 78) a better PFS was observed among patients that had been optimally debulked (p<0.01). The last observation was that the UK patients having no visible residual disease had a less favorable PFS compared with the non-UK patients who experienced the same surgical results (p=0.01). Statistically significant differencies within the surgical management on UK and non-UK patients were as follows: large bowel dissection (1.9% vs. 16% respectively, p<0.001), pelvic (6.9% vs. 50.8%, respectively, p<0.001) and para-aortic (6.3% vs. 42%, respectively, p<0.001) lymphadenectomy (79).

The differences in the PFS rates between the UK and non-UK optimally debulked patients could be explained by the presence or the lack of the ablation of lymph nodes,

showing once more the important role that systematic lymphadenectomy plays in the treatment of epithelial ovarian malignancies (79).

The clinical morphologic factors at surgery (such as: ascites, adherence or extracystic excrescence) can't be relied on to predict the status of the lymph nodes, 55% of positive lymph nodes being less than 2 mm in size at the time of diagnosis (80, 81). The benefit of systematic pelvic and para-aortic lymphadenectomy consists also in a substantial upstaging of the patients without increased morbidity. Thus systematic lymphadenectomy is recommended for any specific subgroup of patients with intraabdominal disease confined to the ovaries (80).

The histological type

The histological subtype plays an important role in prognosis of ovarian cancer. Studies showed that the presence of clear cell tumors and mucinous tumors correlates with a poor prognosis (4, 88, 90).

The impact of histological type on the prognosis and on the treatment of ovarian cancer remains to be established.

Grading

The degree of differentiation seems to be an independent prognostic factor in patients with FIGO stage I ovarian cancer. In the advanced stages, histological grading loses its significant influence on progression-free and overall survival (70, 88).

Age

One study performed in Norway over a 10-year period on 571 cases of primary ovarian cancers revealed that the only independent prognostic factors for ovarian cancer were: FIGO stage ($p<0.001$), size of the tumor residual mass after the primary surgery ($p<0.001$) and age of diagnosis ($p<0.01$) (88). Another study made in Italy showed that age as a prognostic factor was statistically significant only in the univariate analysis and not in the multivariate one (89).

The performance status

It seems that a poor general status correlates with diminished survival (88). Hemoglobin concentrations influence also the prognosis (90) but until now published data was inconsistent.

The performance status also seems to be a predictive factor for the surgical outcome in recurrent disease. In a study by Eisenkop and co-workers, a better performance status together with other factors such as: the absence of salvage chemotherapy and recurrent disease size of less than 10 cm had a predictive role for the surgical outcome (91, 93).

1.1.8 Screening

To date there is no efficient screening test for ovarian cancer (24). Neither ultrasound examination, nor the determination of CA-125 level in serum, fulfills the requirements for a screening test.

The natural history of the ovarian cancer remains unknown, and so does the time required for the progression from stadium I to stage IV (4). Patients presenting with an early stage of the disease at the time of diagnosis have significantly better survival rates than those with advanced stage disease (25). Patients with correctly diagnosed stage I disease have 5-year survival rates of 90%, in comparison with those with stage III disease, whose survival expectations are extremely poor (15% 5-years survival rate) (3).

It is speculated that greater awareness of specific symptoms and their identification may assist in early detection of ovarian cancer and that reducing diagnostic delays may improve morbidity and mortality. It has therefore been repeatedly attempted to identify specific symptoms and causes of delay that will allow early detection of ovarian cancer (95-98). For instance a large study examined the symptoms of 168 ovarian cancer patients compared with symptoms experienced by healthy women using a case-control design. Specific symptoms distinguished women with ovarian cancer controls. However there were only minor differences in reported symptoms between 37 cases with early and 118 cases with later stage disease (99).

The most frequently diafnostics methods are the bimanual pelvic examination, tumor marker (CA-125) and ultrasound imaging. The bimanual pelvic examination can detect ovarian cancers but more often only in advanced stages, small, early-stage tumors are not detected by pelvic examination. Also this technique can give false positive results when benign adnexal tumors, like functional cysts, are found (4, 95, 98).

In looking for an efficient screening method for ovarian cancer, the potential role of many serum markers such as NB/70K, TAG 72.3, CA 19-9, CA 15-3, CASA and CA-125 (101-103) was tested. Of all of these markers, CA-125 remains the more useful one. CA-125 is elevated in more than 80% of women with advanced ovarian cancer (Stage III-IV) (94) and only in less than 50% of women with stage I (102). CA-125 is elevated in a multiple benign diseases such as: cirrhosis, pancreatitis, pulmonary hamartoma, peritonitis, endometriosis, ovarian benign tumours, pelvic inflammatory disease and uterine fibroids. The sensitivity for epithelial malignancies is about 80%, the specificity is very poor (101, 102). So CA-125 cannot be used alone in determining an incipient ovarian disease. It doesn't have the sensitivity or the specificity required for a screening test (100, 101).

Ultrasound has also been used as a screening test for ovarian cancer. It can detect the ovarian mass, the morphological aspect of cysts (the presence and absence of solid areas, the irregular shape). The criteria for malignancy in ultrasound are: multilocular cysts, internal septations, papillary projections, solid components, bilateral and big tumors and the presence of ascites (4, 98). The color Doppler could detect the abnormal neovascularization, suggesting a malign process (104).

The new tumor marker HE4 seems to add more specificity to older screening methods. The study published by Moore et al enrolled 67 patients with ovarian cancer and 166 with benign diseases. They analyzed the levels of several markers, such as CA-125, SMRP, HE4, CA 72-4, activin, osteopontin, EGFR, HER2. The combination of CA-125 and HE4 proved to have the highest sensitivity 76.4% at a specificity of 95%. This combination added 33.1% to the sensitivity of CA-125 alone, and 3.5% to the sensitivity of HE4 alone. For the patients with stage I disease, HE4 alone had a significantly higher sensitivity than CA-125 alone ($p<0.001$). Of all analyzed tumor markers, HE4 had the highest sensitivity as a single marker (105). In a multicentric study, enrolling 531 patients from 12 different geographic areas in USA, HE4 showed elevated levels in more than 50% of the tumors that do not express CA-125 (106). In this study 352 patients were diagnosed as having a benign disease, 157 with malignancy and 22 with borderline tumors of the ovary. Another study analyzed 129 patients with endometriosis, 14 patients with ovarian cancer, 16 patient with endometrial cancer and 66 healthy women. The HE4 mean serum levels were similar and below the cut-off value of 70pM in both the endometriosis and the control group, but elevated in ovarian cancer and endometrium cancer. CA-125 levels were elevated in oncological

patients, but also in patients diagnosed with endometriosis (107).

1.1.9 Diagnostics

The symptoms that may appear are generall. The most common are abdominal discomfort and bloating, vaginal bleeding, urinary tract symptoms and other gastrointestinal symptoms. The patients ignore these symptoms in most cases (3, 4).
A pelvic examination could find an adnexal mass and ascites. The tumor is usually fixed and hard.

The transvaginal ultrasound can discriminate better than any radiological measurements between benign and malignant ovarian tumors (98). The presence of an ovarian mass detected by ultrasonography, in reproductive age women, without the features of malignancy and with normal CA-125 serum levels, infirms the diagnosis of ovarian cancer (4).

Other investigations such as chest radiographs, CT and RMN are used to evaluate the presence of pleural effusions (in less than 10% of the cases) and the presence of lymph nodes or metastases (exploring the liver, mesentery) (111).

There is no place for routine cystoscopy or colonoscopy, neither method can predict the necessity of a potential bowel resection.

There is no other more effective method than surgical staging available for diagnosis or for predicting the surgical outcome (109, 110).

Ongoing clinical trials are assessing whether new tumor markers, including those generated by proteomic and genomic studies, will prove useful. The latest studies have already showed a difference between serum proteins in the patients with benign and malign ovarian diseases. In order to translate these new markers into clinical routine, further prospective trials are needed (105).

1.1.10 Therapy

There are various trials investigating different possibilities in the management of ovarian cancer. The therapy is adapted according to risk factors and the tumor pattern, thereby the tumor stage being the most relevant factor (72). The modern treatment standard of primary ovarian cancer concept consists of surgery and adjuvant

chemotherapy (73). For recurrent disease, there are until now no therapy standards. Today, there is no effective tool available to replace the surgical staging or to predict the surgical outcome.

Surgery

The standard surgery for ovarian cancer includes total abdominal hysterectomy, bilateral salpingo-oophorectomy, appendectomy, radical omentectomy, systematic pelvic and aortic lymphadenectomy, biopsies from peritoneal surface, ascites aspiration for cytological analysis (119).

When involved, the bowel segments can be resected and then an end-to-end anastomosis may be performed, the tumor mass on the peritoneal surface and on the diaphragm should also be removed, the main objective being a residual tumor of less than 1 cm and if possible less than 0.5 cm in maximum diameter (109-110, 112, 113).

The role of surgery in primary ovarian cancer is widely accepted (83-87). Meigs was the first to emphasise the role of cytoreductive surgery. Further studies have shown that survival depends on the postoperative residual tumor mass (83, 84). It is a well accepted conclusion that the postoperative residual tumor is the most important prognostic factor that influences the progression-free and overall survival (82, 85).

Previous studies showed that patients who underwent optimal cytoreduction with a residual tumor less than 2 cm maximum diameter, the median survival was 25 to 40 months; whereas in patients with a residual mass greater than 2 cm maximum diameter, the median survival was only 10 to 18 months. The optimal cytoreductive surgery is to reduce the residual mass to less than 1cm in maximum diameter (4).

The results of the metaanalysis by Bristow and co-workers have shown that the residual mass was a very significant prognostic factor in ovarian cancer, FIGO stage III and IV. Every increase in the percentage of maximal cytoreductive surgery from 20% to 30 % was associated with an increase in median survival time of 1.5 months. Even after the multivariate analysis was performed, the cytoreductive surgery still remained an independent significant prognosis factor ($p=0.001$) (85).

In contrast to primary ovarian cancer, the role of cytoreductive surgery in patients with relapsed ovarian cancer is still unclear (82).

These are the main reasons why the surgery represents in majority of the cases the first therapeutical option. The few cases of advanced ovarian cancer treated first with neoadjuvant chemotherapy, are the ones with poor performance status or stage IV

with retrocrural or supraclavicular lymph nodes, mediastinal metastases and parenchymal lung metastases (4). The metaanalysis by Bristow et al. included 26 studies with a total of 1336 patients who received neoadjuvant chemotherapy instead of primary cytoreductive surgery. In the same study, three randomized trials and six non-randomized trials of interval cytoreduction following suboptimal initial surgery were analyzed. Maximal primary cytoreductive surgery remains the standard of care for the majority of women with ovarian cancer. Neoadjuvant chemotherapy can be applied in the limited number of patients classified by experienced ovarian cancer surgeons as optimally unresectable. Current data suggest that the survival outcome is better when cytoreductive surgery is performed instead of neoadjuvant chemotherapy (114). Studies suggested that even women with stage FIGO IV with parenchymal liver disease may benefit from an optimal cytoreductive surgery (109, 112, 113).

Harter and co-workers showed that both optimal surgery and state-of-the-art chemotherapy contribute independently to the outcome in primary ovarian cancer (82).

Chemotherapy

Adjuvant chemotherapy is a very important method of treatment for ovarian cancer (82).

Many types of chemotherapy were investigated in recent years (122). Currently the state-of-the-art therapy for the first-line treatment is considered to be platinum-paclitaxel combination. Ongoing studies evaluate 3 different strategies to improve efficacy of first-line treatment: (a) incorporation of further non-cross-resistant drugs into first-line regimens; (b) modification of the sequence of treatment modalities; (c) modifications of dose intensity with either intraperitoneal therapy or high-dose chemotherapy with peripheral stem cell support (126).

Several trials tried to establish the dose and the number of cycles that are more efficient in the therapy of ovarian cancer. Extending the treatment with cisplatin-based therapy from 5 or 6 cycles to 10 or 12 cycles did not improve the progression-free or the overall survival, it only increased the toxicity (120, 121). In 1980, it was shown that taxanes (Paclitaxel, Docetaxel) possess a significant activity against platinum-resistant ovarian cancer (4, 123, 124).

Despite the latest achievements in the field of surgery and chemotherapy, the majority of patients experience recurrence. Women with recurrent ovarian cancer are classified into responder and non-responder patients, depending on the response to the

first platinum therapy. Late toxicity, including neurotoxicity, limits the administration of a new paclitaxel-platinum therapy in sensitive patients who experience recurrence. New trials have studied other different chemotherapy combinations in order to obtain a better PFS and a better quality of life (125). Chemotherapy available in the second line setting is not curative, the aim of the treatment being controlling the disease, prolonging survival and maintaining quality of life.

In a randomized study made by the Arbeitsgemeinschaft Gynäkologische Onkologie Ovarian Cancer Study Group, the National Cancer Institute of Canada Clinical Trials Group, and the European Organisation for Research and Treatment of Cancer Gynaecological Cancer Group, the combination of Gemcitabine and Carboplatin showed a better PFS and better response rates than in the group treated with Carboplatin alone ($p= 0.0031$ and $p= 0.0016$) (125). The hematological toxicities were higher in the gemcitabine group, no significant differences were observed regarding the non-hematological toxicities (eg. Alopecia, hematotoxicity).

For platinum resistant ovarian cancer, Topotecan, Pegylated Doxorubicin and Gemcitabin seem to be the most active agents (126, 127).

1.2 Interleukin-10 involvement in tumor development

1.2.1 Cancer immunology

The human body usually acts against the non-self-cells or against altered self-cells through active defenses that comprise the immune system (164). This hypothesis is the milestone for cancer immunotherapy.

The immune response is composed of: the innate and the adaptive immune responses. The adaptive responses are specific for particular pathogens, closely regulated and develop the memory for the pathogen and they usually don't destroy normal tissue. They are adaptive, therefore they need time to build. On the other hand, the innate immune responses develop very quickly, but are not so closely regulated.

The immune system includes the B-cells and the T-cells. The B-cells are responsible for the umoral immunity, they produce antibodies that are capable of neutralizing and destroying different injuries of the body. They usually recognize the

antigen in its native form. The T lymphocytes are responsible for the cellular immunity and they are able to recognize only the antigens that are captured, processed and presented on the surface of the cell by the MHC molecules .

Immune antitumoral response can be divided into two parts:

- the induction phase, which includes the antigen uptake, the transformation and presenting to the T-cell by the antigen presenting cells
- the effector phase, the direct destruction of the tumor cells by CD8+ T cells, the release of reactive oxygen by macrophages, toxic products by eosinophils or the activation of the antibody-mediated cytotoxic mechanism.

The presence or the absence of the tumor specific antigens triggers the immune system. There are many types of antigens: products derived from the mutated gene, tissue-specific differentiation antigens, which are present in both the normal and the malign tissue, over-expressed antigens and embryonic antigens. The antigens may be specific and may differ from the normal tissue in order to obtain a therapeutic effect from the immune response.

Furthermore, antigens are ingested by APCs, here they are processed and presented on MHC class I or II in order to be recognized by T-lymphocytes.

The two MHC classes differ because of their distribution in the tissues; they bind to different T cell subtypes, determining different types of effects. They also follow different intracellular pathways (3, 138).

The MHC I class molecules interact with the T CD8+ cells and the MHC II class with CD4+ cells. So the MHC molecules start a cytotoxic activity, in the meantime the class II molecules stimulate the T CD4+ cells to help the antitumoral defense.

After the uptake of the antigen, the APCs process the antigens and present them on the surface of the cells together with the MHC class. This is the only way in which T cells may recognize the tumor antigens. Any disturbance in this mechanism potentially diminishes the body’s capacity to react against the tumor. One way for tumors to escape the immune surveillance can be obtained by disturbing the process of generating and expressing the MHC-peptide complexes (130). They might also down-regulate the expression of particular class I loci (130).

Most of the tumor cells express MHC class I molecules, some even MHC class II, but they don’t express the co-stimulatory molecules which are supposed to be critical

and to induce the activation of T cells (4). Immunostimulatory cytokines seem to help initialize the T cell response.

The most effective APCs are the mature and maturing dendritic cells, named so because of their stellate or branch-like appearance (132). They have a hematopoietic origin. The immature DCs are spread in all tissues, but when they became mature they migrate to the T-cell rich zones, where they activate them (4). They have two key roles: first, they can stimulate T cells initializing the primary immune responses and second, they can support tolerance to self-antigens (4, 133).

Immature DCs phagocytate live cells, dying cells or necrozing cells, tumor and infected cells (134). Different stimuli are needed in order to activate the DCs. After maturation they gain the capacity to express the MHC molecules, the co-stimulatory molecules and chemokine receptors, but they lose their ability to take up antigen. Cytokines are also involved in the process of DCs maturation. Some of them, such as IL-10, TGFβ (transforming growth factor) hamper the maturation of DCs (4, 135). The antigens captured by immature DCs are processed, then stocked in endosomes and expressed on the cell surface of mature DCs, together with increased levels of MHC class I and II complexes with antigenic peptides (4).

Mature DCs migrate to the lymphoid organs and stimulate the T cells, determining also, the type of function they will develop. They can transform the activated naive CD4+ T cells into Th1 CD4+ cells, Th2 CD4+ T cells or regulatory T cells (136).

Cytokines are very important in the process of DCs maturation, especially the IL-12. High levels of IL-12 stimulate the DCs maturation, whereas low levels are associated with the absence of maturation. IFN-γ stimulates the production of IL-12 and IL-10 inhibits this process (137).

IL-10, TGFβ and PGE2 promote tumor growth by dampering the DCs' maturation and their migration to lymph nodes, inhibiting in this way the T-cells activation and antitumoral response (4, 141).

The immune system plays a crucial role in the body's defense against malignancies. Tumor cells are recognized and destroyed primarily by the cellular immune responses. Unfortunately, malignant tumors usually hold some modalities of escaping the immune surveillance, such as expressing antigens that are self-proteins shared with normal cells (4) or by producing certain cytokines that inhibit the DCs' maturation and T-cell activation.

1.2.2 The cytokines network

Interleukin-10 was first described 15 years ago as a cytokine synthesis inhibitory factor (140). Cytokines are low molecular weight proteins, which regulate the intensity and duration of an immune response (140). They are active at very low concentrations. A typical feature of cytokines is their pleiotropy and redundancy. Every cytokine has numerous functions, and one function is often mediated by several different cytokines creating a cytokine network (142). A cytokine's function depends on its concentration in the tissue, the target cells and on the activating signal. Cytokines usually act by binding to the specific cytokine receptor ligands and can initialize a signal transduction and a second messenger pathway. The results of these actions can be: mitotic division, migration, growth, differentiation or apoptosis of the target cells (140).

Cytokines can be produced by every cell in the body, such as: monocytes, lymphocytes, keratinocytes and tumor cells. They act in a complex network, by inhibiting their own synthesis or that of other cytokines or cytokines receptors, by changing their function in the presence of other cytokines. Their production can be caused also by the antigen-specific or non-antigen-specific stimuli involving the B and T cells.

The CD8+ T cells, also called cytotoxic cells, act by destroying the target cells and by secreting the IFN-gamma. The subtypes of T helper cells (Th1, Th2, Th3 and Th0) in humans are responsible for: promoting B cell mediated umoral responses and secreting pro-inflammatory cytokines, such as: IL-2, IFN-gamma and TNFß, as also anti-inflammatory cytokines such as: IL-4, IL-5, IL-6 and IL- 10. They are also responsible for the production of GM-CSF (granulocyte-macrophage colony-stimulating factor) and TGFß (tumor growth factor ß) (128, 129, 131, 139).

Many cytokines (IL-1, IL-2, IL-6, IL-8, IL-10, IL-11, IFN−gamma, TNFalpha, TGFbeta, GM-CSF) were described in the normal ovarian tissue and also in the malignant ovary and they are said to play an important role in the pathology of ovarian function and ovulation (142).

There are two possibilities for the cytokines to influence tumor development. They might represent growth factors, stimulate metastasis or tumor angiogenesis or they might block the immune response against tumor cells. The cytokines expressed by

the normal ovary are expressed also in the malignant one, only there might be a shift in the balance of expression of these cytokines (142).

The presence of elevated serum levels of IL-6 and M-CSF seems to correlate with worse prognosis. Also high levels of TGF-beta and IL-10 at the site of the tumor help tumors escape immune recognition (142).

1.2.3 Structure and function

The molecule of IL-10 is a homodimer made up of 160 amino acids and having a molecular weight of 18.5kDa. The tertiary structure of the human IL-10 is that of a V-shaped homodimer (143). Each arm of this dimer is composed of six alpha-helices. The structure of Interleukin 10 is very similar to that of interferon γ and Epstein-Barr virus protein BCRF1 (146, 147).

Initially, Interleukin-10 was reported to be produced only by Th2 lymphocytes. Previous studies showed that other cells are able to produce IL-10 too, such as: B lymphocytes, mast cells, macrophages, eosinophils, keratinocytes and tumor cells (145). In vitro, the IL-10 production is increased by the administration of lipopolysaccharide. Other investigators indicate that inflammatory stimuli such as IL-2, IL-6, TNF-alpha, IFN-γ have the same effect. On the other hand, IL-4, IL-13 and even IL-10 down-regulate the production of IL-10 (143).

The IL-10 receptor is very similar to IFNγ receptor and has a molecular weight of 90 to 110 kDa (143). It is made up of two different receptor chains, IL-10 R1 and IL-10 R2. The interaction between IL-10 and its receptor occurs in 2 steps, the first one is binding with higher affinity to the first receptor chain and the second is binding with low affinity to the second receptor chain (150). The cytosolic domain of the receptor is very similar to the one of IL-6, IL-10 being actually capable of recruiting the signal transduction pathways utilized by IL-6 (143). Janus kinase/STAT 1 and STAT3 mediate the signal transduction of IL-10 by binding to specific sites on nuclear DNA.

IL-10 R1 is expressed by most of the hematopoietic cells, although it was observed on non-hematopoietic cells too. Its gene is located on the chromosome 11q23 (140). The IL-10R2 is the accessory subunit for signaling and it is expressed in most tissues (140).

Studies also showed that IL-10 has anti-inflammatory effects, by suppressing the release of pro-inflammatory cytokines (IL-1, IL-6, IL-8, GM-CSF and TNFα), the

secretion of IFN γ by NK cells and antigen presenting cell maturation (145, 149). The release of the nitric oxide and toxic oxygen radicals from the macrophages is also down-regulated by IL-10 (143). Interleukin-10 down-regulates the expression of MHC class II molecules on the cell surface of APCs, including dendritic cells. It also dampers T cells proliferation, the production of IL2 and it might cause T cells anergy. All of these actions tend to produce immunosuppression.

Some authors suggest that the simple definition of IL-10 as an anti-inflammatory cytokine does not reflect its pleiotropic role. At the beginning of the innate immune response, IL-10 acts like a pro-inflammatory cytokine, having also chemoattractive effects. Later, IL-10 acts to limit the inflammatory process and in this way to reduce the damage of the surrounding tissue (128, 153).

IL-10 possesses also immunostimulatory actions by being a potent stimulator of NK cells, participating in this way to eliminating the pathogen (139, 151). IL-10 also has the ability to recruit the monocytes and the macrophages and in this way it is able to stimulate the antibody-dependent cell-mediated cytotoxicity and phagocytosis of opsonized particles (145). It stimulates the differentiation of B-cells into antibody-secreting cells.

The main effect of IL-10 remains its ability to reduce inflammation. The experiments on mice show that IL-10 knock-out mice develop a chronic inflammatory bowel disease, the IL-10 transgenic mice show mucosal inflammation and the development of autoimmune diabetes and increased T-cell mediated rejection of cancer (140).

The ability of IL-10 to down-regulate the immune response was studied also in bone marrow and organ transplantation (140). Studies showed that elevated levels of IL-10 prior to bone marrow transplantation (BMT) were associated with lower incidence of graft-vs.-host disease (GVHD), but the administration of IL-10 at or after the BMT showed little beneficial effect or even stimulated rejection (140). This showed that the effects of IL-10 are dependent on the time of administration and on the type of treatment performed.

The presence of proinflammatory cytokines (TNFα, IL-1, IL-6, GM-CSF, IL-8) in the synovial tissue and liquid of patients with rheumatoid arthritis raised the suspicion that IL-10 might be used as a therapeutic method. In fact the systemic administration of IL-10 in mice, ameliorated collagen-induced arthritis (140).

In systemic lupus erythematosus, IL-10 seems to stimulate the production of autoantibodies by B cells, so it may be involved in the induction and maintenance of the pathologic process. Overexpressed IL-10 may explain the autoantibody formation and worse outcome of patients with myasthenia gravis, Grave's disease, Sjogren syndrome, polymyositis, bullous pemphigoid and systemic sclerosis (143).

Psoriasis is typically characterized by inflammation of the skin and it shows a regression of the pathologic process when IL-10 receptors are induced (143).

Inhibiting the eosinophils, secretion of proinflammatory cytokines and IgE, IL-10 may be used as a therapeutic intervention in asthma (143).

Interleukin 10 shows multiple actions in the pathogenesis of several diseases, some of these actions are opposites. This fact shows once more the pleiotropic function of cytokines. The detailed involvement of IL-10 in pathogenesis is still not well understood so far.

1.2.4 Interleukin-10 and cancer

The observation that IL-10 is overexpressed in many types of cancer raised the suspicion that it may create an immunosuppressive microenvironment in favor of the tumor (128).

Studies performed on experimental cancer models show that IL-10 has multiple antitumoral effects (150). These properties are explained by the increasing NK activity and by the stimulation of the accumulation of macrophages and neutrophils. It seems that the suppression of tumor growth is achieved also by the inhibitory effects on angiogenesis and invasiveness through induction of metalloproteinase inhibitors (155).

IL-10 transfected cell lines from adenocarcinoma of the mamma, ovary and the melanoma of the mouse have an increased immunogenicity accompanied by an important, strong lymphocyte- and antibody-dependent immune memory (156-158). So, the experiments made on mice's tumors show an antitumoral activity for IL-10, being a mediator for tumor regression.

The role of IL-10 in human cancers is not well understood. Studies demonstrate a poor prognosis for patients with tumors that have an overexpressed IL-10 (140).

In the tumoral microenvironment, IL-10 seems to be produced by the tumoral cells, maybe with the purpose of stimulating the tumoral growth and of helping the tumor to escape immune recognition (159).

IL-10, through hampering the classical APC maturation, preserves their antigen-uptake function. Based on this pathway, working groups suggested that this antigen-loading phase may contribute to the innate immune reaction to a pathogen (128). It promotes the angiogenesis and tissue repair. At the same time, IL-10 inhibits the recruitment and activation of immune mediators, which create and maintain a chronic inflammatory state within the tumor microenvironment. The administration of a secondary proinflammatory stimulus like IL- 2 seems to stimulate the APC´s activation, and the accumulation of chemoattractants, co-stimulatory molecules and cytokines that stimulate the immune cells to proliferate. These findings suggest that IL-10 might have a facilitator role in pre-conditioning tumors to immune recognition (128).

Studies also investigated the potential influence of therapeutic procedures on the levels of IL-10. Gianotti and co-workers published a study in which 45 patients with stomach, colorectal and pancreas malignancies and 40 controls were enrolled. They evaluated how the expression of IL-10 varies with the type of exeresis of the neoplasm. Blood was collected before and after the surgical act. The results showed significantly ($p=0.022$) lower IL-10 levels after radical surgery and no change after palliative surgery. The levels of IL-10 did not depend on the localization of the tumor, they correlated only with the histological type. The results suggest that IL-10 might be produced by the tumor cells or by the tumor infiltrating lymphocytes or macrophages in order to escape the immune surveillance (115). These results were confirmed by another study performed by Galizia and co-workers (116). Expression of IL-10 and IL-6 was determined in 50 patients with histologically proven colorectal cancer and 25 patients with no malignancy. Blood was also collected before and after the surgery. The results showed a significant association between IL-10 levels and surgical outcome ($p=0.0005$). Circulatory IL-10 significantly dropped after the operation in patients, regardless of wether they underwent a curative or a non-curative surgery; the difference was that in the case of curative surgery the levels remained low even on day 16, whereas in the other group, the values dropped immediately after surgery but were still higher than in controls. Six patients who underwent curative surgery but had persisting high levels of IL-10 developed later recurrence. The data showed that IL-10 serum levels were a significant predictive factor for the recurrence ($p=0.025$) (116). In the multivariate

analysis the extent of primary tumor, the Dukes' stage, number of metastatic resected nodes, basal IL-10 serum levels, and preoperative CEA serum levels were demonstrated to be the only independent covariates which influence disease-free survival. This result supports the hypothesis that the tumoral microenvironment represents an important IL-10 source (115, 116) and the potential role of IL-10 as a monitoring tool, even in patients with minimal (microscopic) tumor residuals.

1.2.5 Interleukin-10 polymorphism

The human IL-10 promoter gene is located on the first chromosome (1q31-32). At least 49 IL-10 associated polymorphisms were described in the literature (Fig. 1) (163). Of these 46 are SNPs, two are microsatellite polymorphisms and one is a small (3 bp) deletion (163).

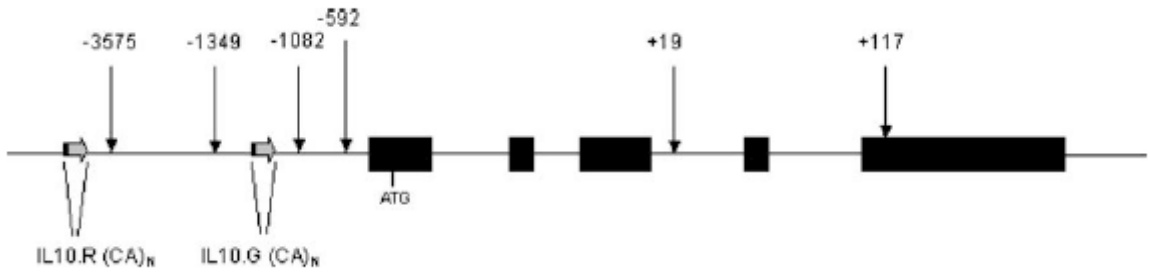


Figure 1 *IL-10 gene showing SNPs and microsatellite polymorphism*

The most investigated polymorphisms of IL-10 promoter gene remain the ones situated at loci: -1081 G to C allele, -819 C to T allele and at -592 C to A allele. The -819C and -592C genotype are inherited together, and the same is valid for -819T and -592A. These SNPs usually combine and form the following haplotypes: GCC, ATA, ACC and GTA, the later being extremely rare (163).

IL-10 G genotypes 136/136 and IL-10 R genotypes 112/114 and 114/116 were found to be involved in the development of multiple myeloma (167). The variations in the microsatellite regions were also shown to determine a difference in the IL-10 expression. The R2, G14 genotype is associated with increased IL-10 production, whereas the R3, G7 with decreased production (162).

Studies also showed an ethnic distribution of the SNPs. The European population showed a more frequent GCC genotype (50%), whereas the Asian people present with this haplotype only in a few cases (less than 5%) (163, 165).

The variability of cytokine secretion was found to be determined by genetic factors in about 50-75% of the cases. Other factors that might influence the expression are: infection, smoking, body mass index and gender (163).

The expression of IL-10 *in vitro* seems to be determined primarily by polymorphism at the -1082 position. Studies showed the association of -1082 alleles G and A with a low (AA), medium (AG), high (GG) IL-10 production (144). The presence of the GCC or ATA haplotypes was associated with increased or decreased IL-10 serum concentrations, respectively. Until now, due to the controversial results obtained it was difficult to establish the exact way in which genotype might influence IL-10 production (140).

The linkage between different haplotypes and the severity of, and/or susceptibility to diseases was analyzed by many scientists. The most important association was described for the systemic lupus, where high levels of IL-10 and the corresponding alleles seem to play a causal or exacerbating role (140). IL-10 polymorphism also was found to be important in the therapeutical response in patients with hepatitis C and in the survival of patients with meningococcal disease (140).

Until now, more than 20 studies regarding 13 malignancies were published (Tab. 4), but only one regarding the impact of the polymorphism on ovarian cancer (163).

Wu et al. analyzed the IL-10 polymorphism in 220 patients with gastric cancer and 230 healthy controls. The results showed a significant association between the IL-10 GCC haplotype and the presence of malignant disease. Also when the stratification analysis was performed the high IL-10 production genotype was more often present in advanced stages and in cardia localization. Smoking, H. pylori infection and IL-10 genotypes (OR 2.54, 95% CI 1.24-5.61) were the only independent risks factors for gastric carcinoma in this study (160).

Among the gynecological malignancies, IL-10 polymorphism was examined in patients with cervical cancer, breast cancer and even ovarian cancer (161, 164, 171, 172, 174).

Table 4 *IL-10 polymorphisms and malignant diseases cancers (modif. after Howell et al)(163)*

Malignancy	Analyzed Polymorphisms	Cases	Controls	Association	Allele or haplotype	Authors
Ovarian Cancer	-1082, -819	182	223	No	-	Bushley et al (164)
Ovarian Cancer	-1082, -819, -592	147	129	Survival (increased) Optimal tumor debulking	-819 CC -592 CC	Braicu et al(190)
Cervical cancer	-1082	77	69	Susceptibility	-1082 AG	Stanczuk et al (144)
Cervical cancer	-1082, -819, -592	144	179	No	-	Roh et al (175)
Breast cancer	-1082	144	263	No	-	Smith et al (171)
Breast cancer	-1082	125	100	Susceptibility	-1082 AA	Giordani et al (172)
Breast cancer	-592	500	500	Protection	-592 AA	Langseblehner et al (173)
Breast cancer	-3575, -1349, -1082, -592, +19, +117	2000	2000	No	-	Bulpitt et al (174)
Cutaneous malignant melanoma (CMM)	-1082, -819, -592	153	158	Susceptibility, advanced stage, greater tumor thiskness Greater tumor thickness Non-invasive growth phase	-1082AA ACC/ACC, ACC/ATA, ATA/ATA -1082 -GCC/GCC	Howel et al (168)
CMM	-1082, -819, -592	42	48	Survival (shorter)	ACC/ATA	Martinez-Escribano et al (169)
Prostate cancer	-1082	247	263	Susceptibility	-1082 AA	McCarron et al (169)
Gastric carcinoma	-1082, -819, -592	220	230	Susceptibility, advanced stage	GCC	Wu et al (160)
Gastric carcinoma	-1082	150	220	Association with EBV-negative gastric carcinoma	-1082 G allele	Wu et al (160)
Gastric carcinoma	-1082, -819, -592	188	212	Susceptibility	ATA-haplotype	El-Omar et al (176)
Squamous cell carcinoma of skin (post renal transplant)	-1082, -819, -592	70	70	Susceptibility Protection	GCC, ATA	Alamartine et al (198)
Hepatocellular carcinoma (post HBV infection)	-1082, -819, -592, +117	230	792	Susceptibility, accelerated age of onset	ACCT haplotype	Shin et al (177)
Renal cell carcinoma	-1082	166	161	Susceptibility	-1082 AA	Havranek et al (177)
Multiple myeloma	IL-10 G, IL-10 R	73	109	Susceptibility Protection	IL-10 G136/136, IL-10 R 112/114, IL-10 R 114/116	Zheng et al (167)
Myelodisplasia acute myeloid leukemia	-1082, -819, -592	150	Up to 1000	No	-	Gowans et al(179)
Non-Hodgkin's lymphoma	-1082, -819, -592	126	302	Susceptibility to aggressive disease	-1082 AA, ATA, ACC	Cunningham et al (180)

Non-Hodgkin's lymphoma (in AIDS patients)	-592	139	1011	Susceptibility	-592	Breen et al (181)
Diffuse large B-cell lymphoma	-1082, -819, -592	199	112	Susceptibility. Higher complete remission, DFS and OAS	CC 1082G	Lech-Maranda et al (181)
Hodgkin's disease	-1082, -592	149	111	No	-	Munro et al (183)
Acute lymphoblastic leukaemia	-1082	135	-	Protection from poor response to Prednisone treatment	-1082 GG	Lauten et al (184)

1.2.6 Interleukin-10 and ovarian cancer

It is well described that inflammatory cells are found in the malignant tissue. The analysis of these cells revealed the presence of cytotoxic and helper T cells, natural killer cells, macrophages and neutrophils. The exact pathway in which they modify the history of the tumor remains to be clarified. It is also known that patients have very different immunological mechanisms involved in the pathogenesis of the malignant disease.

The discovery of the tumor infiltrating lymphocytes showed that they are functionally deficient, but they regain most of their lytic function "in vitro", when IL-2 is added; this suggest that the lack of response against tumor cells due to inadequate immunological activation. An explanation for this lack of activation might be obtained by studying the cytokines produced at the site of the tumor (166).

Pisa et al examined the expression of IL-1α, IL-2, IL-3, IL-4, IL-6, TNF-α, IFN-γ, GM-CSF and G-CSF in malignant ovarian tissue of 11 patients with ovarian cancer and normal ovarian tissue in 8 controls. The results showed a difference in cytokine expression between the malignant ovary and the normal one, more specifically the absence of interleukin 10 and GM-CSF in the normal ovary. The IL-10 mRNA was expressed in 10 of 11 patients with ovarian cancer, whereas none of the eight control patients presented with IL-10 mRNA expressed in ovarian tissue. Furthermore, there are some differences in the degree of T-cells infiltration in the normal and malignant ovary. Infiltrating T cells were observed in all malignant tissue and in most, but not all of the benign ovaries. This feature might explain the differences between the cytokine profiles of the two tissues (166).

Berger et al investigated the expression of IL-10 in four cell lines: OVCAR-3, SKOV-3, CAOV-3 and OAW-42. They observed that OVCAR-3 presented IL-10 specific m-RNA (185).

The observation that ovarian cancer remains confined to the peritoneal cavity even in advanced stages generate the hypothesis that this behavior might be explained by a local phenomena of immunosuppression. Santin et al reported significantly higher levels of IL-10 ($p<0.001$) in ascitic fluid than in plasma of 28 patients with ovarian cancer. The controls (10 patients) showed no detectable IL-10 levels (186). These data suggest once more that the major source of IL-10 is located in the abdominal cavity, and might be represented by the ovarian tumoral cells themselves or by the tumor infiltrating lymphocytes (TIL) or tumor associated lymphocytes (TAL) (186).

In another study performed by Zeimet et al. involving 76 patients with ovarian cancer, ascitic levels of IL-12, together with FIGO staging and residual mass were the only independent prognostic factors. In this study the levels of IL-10, IL-12, were more elevated in the ascites of the patients with ovarian cancer in comparison to circulatory levels. In the univariate analysis, high ascitic levels of neopterin, TNF-α and IL-12 were associated with poor overall and disease-free survival. Multivariate Cox regression analysis showed residual disease, FIGO stage, age at first diagnosis and ascitic-fluid IL-12 levels to be the only independent prognostic factors ($P < .03$ for disease-free and $P < .01$ for overall survival (16).

The results published by Bushley et al. in 2004 regarding the role of IL-10 polymorphisms (-1082 and -819 loci) in the pathogenesis of ovarian cancer revealed no significant influence on disease-free and overall survival (164).

1.3 Study objectives

Due to its very poor prognosis, ovarian cancer is the leading cause of death caused by gynecological malignancies. Despite new achievements in surgical and systemic treatment, the therapeutically results remain unsatisfactory. In order to better define the group at risk and the predictive factors for the surgical and therapeutical outcome, different working groups are trying to define new prognosis factors.

Primary aim: The main aim of this study was to determine the role of IL-10 as a prognostic and predictive factor by determining its expression in serum and ascites and its promoter polymorphism in ovarian cancer patients. We found particularly that

studying the expression of IL-10 in ascites is of very big importance, because ascites is the direct environment and it might reflect better the tumor microbiology.

The secondary aims are:

1. Differences in expression of IL-10 in serum and ascites in patients with and without ovarian cancer.
2. What is the pattern of IL-10 polymorphism in cancer and non-cancer patients?
3. Correlation of IL-10 polymorphism and expression in serum and ascites with established prognostic factors.
4. Influence of IL-10 polymorphism on the susceptibility for ovarian cancer.
5. Correlation between the IL-10 expression in serum and ascites and the polymorphisms analyzed.
6. Does the expression or the polymorphism of IL-10 have an impact on the overall or progression-free survival rates?

2 Methods

This prospective, mono-institutional study was reviewed and approved by the Clinical Review Board and Ethics Committee of the Charité Medical University Berlin. All samples were obtained from the prospective Tumor Bank Ovarian Cancer (TOC) (IMO) (118). Patients with histologically confirmed ovarian cancer were recruted for this trial. Written informed consent was provided by each patient. Borderline ovarian tumors are different tumor entities from epithelial ovarian cancer and were therefore excluded from this study (69).

2.1 The collective of patients

Most of the patients that were diagnosed with a primary ovarian carcinoma underwent laparotomy, together with bilateral adnexectomy, hysterectomy, omentectomy, appendectomy and pelvic and paraaortic lymph adenectomy with the aim of maximal cytoreduction. In the case of patients with a relapse of ovarian tumor, a tumor reduction and a maximal tumor debulking were performed.

In this study, the “IMO” (Intraoperatives Mapping des Ovarialkarzinoms), a systematic surgical and histopathological tumor documentation system was used. This provided the information regarding the exact tumor FIGO stage, the tumor subgroup and made the documentation of the therapeutically results more effective (118) (Fig. 2). Tumor spread, ascites volume and residual tumor mass were documented in detail systematically.

After discharge, the patients were re-examined in the ambulatory section of Campus Virchow (Ovarsprechstunde), every three months during the first three years and every six months after the end of the third year of follow-up. During the examinations a complete status was obtained and documented.

The clinical and follow-up data were collected into a database (Software SPSS Inc., Chicago). The period between the first surgery and the date of death or the last follow-up was considered the overall survival. The period between the current surgery and the next recurrence was regarded as the progression free survival.

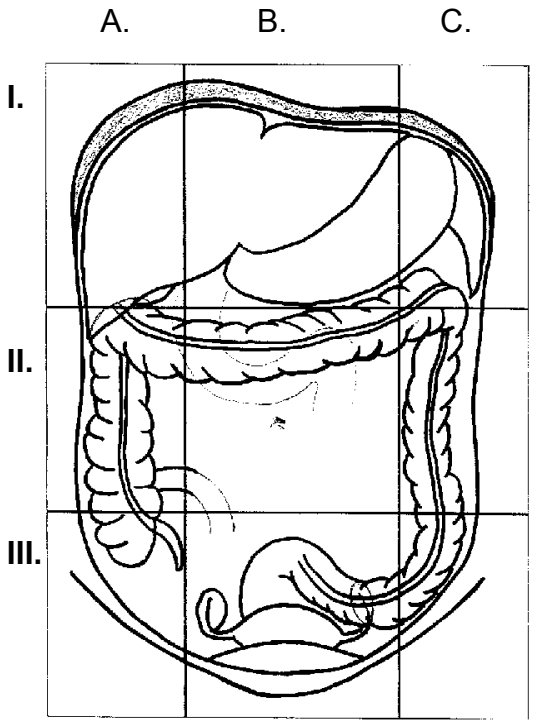


Figure 2 *The intraoperative mapping of ovarian cancer (IMO) (118)*

2.2 Collection of blood and ascites samples

Drawing blood and ascites collection was performed just before the surgery. Blood was obtained before any other blood transfusion, when needed, in order not to contaminate the DNA probes. Ten milliliters of blood was drawn from antecubital vein from each patient. Serum and ascites probes were centrifuged 3000 rpm and 1500 rpm for 10 min and 15 min, respectively, then stored at -80°C until analyzing.

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay is a biochemical technique used generally in immunology for analyzing different biological samples in order to detect the presence of an antibody or an antigen (148). It utilizes two antibodies, one of which is specific to the antigen and the other is coupled to an enzyme (148). The use of the second antibody gives the assay its "enzyme-linked" name and causes a chromogenic

or fluorogenic substrate to produce a signal. Because the ELISA can evaluate the presence of antigen or the presence of antibody in a sample, it is useful both for determining serum antibody concentrations and for detecting the presence of antigen (148).

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative reading for a sample. The cut-off between positive and negative will be determined. Generally, two or three times the standard deviation is often used to distinguish positive and negative samples. In quantitative ELISA, the optical density (fluorescent units) of the sample is interpolated into a standard curve which is typically a serial dilution of the target (152).

The technique, called "sandwich" ELISA, was performed by using the following steps: preparing a surface to which a known quantity of antibody is bound, applying the antigen-containing sample to the plate, washing the plate so that unbound antigen is removed, applying the enzyme-linked antibodies which are also specific to the antigen, washing the plate so that unbound enzyme-linked antibodies are removed, applying a chemical which is converted by the enzyme into a chromogenic signal, viewing the result: if it is colored, then the sample contains antigen (152).

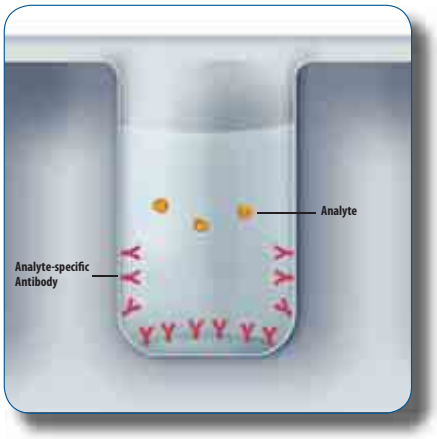
Serum and ascites IL-10 concentrations were measured using enzyme-linked immunosorbent assay kit (ELISA) according to the manufacture's protocol (Quantikine, R&D Systems Inc., Minneapolis, MN). All samples were measured in duplicate (154).

A monoclonal antibody specific for IL-10 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-10 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-10 is added to the wells. Following the wash phase to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound in the initial step. The color development is stopped and the intensity of the color is measured (Fig. 3) (152).

Assay Principle

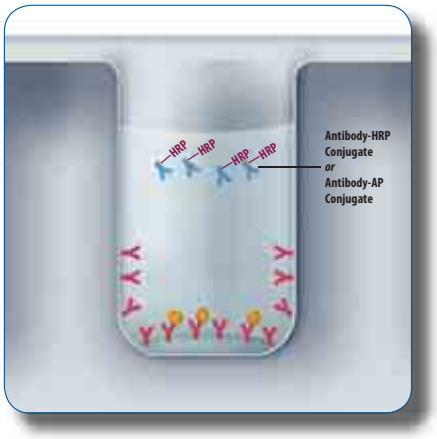
Quantikine, Quantikine HS, Quantikine IVD, QuantiGlo, and Surveyor colorimetric or chemiluminescent sandwich ELISAs

Step 1



Analyte-specific antibody (capture antibody) is pre-coated onto a microplate. The sample is added and any analyte present is bound by the immobilized antibody.

Step 2

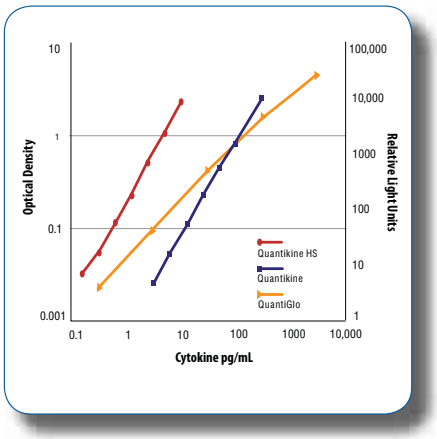


An enzyme-linked analyte-specific detection antibody binds to a second epitope on the analyte forming the analyte-antibody complex.

Step 3

Substrate is added and converted by the enzyme, thereby producing a colored product or light emission in direct proportion to the amount of analyte bound in the intial reaction (signal increases as concentration increases).

Step 4



Quantikine ELISAs typically offer a dynamic range starting in the low pg/mL and cover three logarithms.

Quantikine HS offers the best sensitivity via color amplification, but the smallest dynamic range.

QuantiGlo offers the best combination of sensitivity and dynamic range. A microplate luminometer is require to run these assays.

Figure 3 *Principles of Enzyme-Linked Imunosorbent Assay (154)*

The reagents used in this procedure were (154):

- IL-10 Microplate(Part 890227) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against IL-10.
- IL-10 Conjugate(Part 890232) - 21 mL of polyclonal antibody against IL-10 conjugated to horseradish peroxidase.
- IL-10 Standard(Part 890233) - 2.5 ng of recombinant human IL-10 in a buffered protein base with preservatives, lyophilized.
- Assay Diluent RD1W(Part 895117) - 11 mL of a buffered protein base with preservatives.
- Calibrator Diluent RD6P(Part 895118) - 21 mL of animal serum with preservatives.
- Wash Buffer Concentrate(Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.
- Color Reagent A(Part 895000) - 12.5 mL of stabilized hydrogen peroxide.
- Color Reagent B(Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).
- Stop Solution(Part 895032) - 6 mL of 2 N sulfuric acid.

After bringing them to the room temperature the reagents were prepared as following (154):

- *Wash Buffer*- If crystals were formed in the concentrate, it was warmed to room temperature and mixed gently until the crystals were completely dissolved. 20 ml of Wash Buffer Concentrate was diluted in distilled water to prepare 500 ml of Wash Buffer.
- *Calibrator Diluent RD5C (1X)*– 20 ml of Calibrator Diluent RD5C (5X) Concentrate was diluted into distilled water to yield 100 ml of Calibrator Diluent RD5C (1X).
- *Substrate Solution*- Color Reagents A and B were mixed together in equal volumes within 15 minutes of use. They were protected from light. 200 µL of the resultant mixture was required per well.
- *IL-10 Standard*– The IL-10 Standard was reconstituted with 5 ml of Calibrator Diluent RD6P. This reconstitution produced a stock solution of 500 pg/ml.

The standard sat for a minimum of 15 minutes with gentle agitation prior to making dilutions.

- Using the polypropylene tubes 500 µl of the appropriate Calibrator Diluent was pipetted into each tube.

The stock solution was used to produce a dilution series and each tube content was mixed thoroughly before the next transfer. The undiluted standard served as the high standard (500 pg/ml), the appropriate Calibrator Diluent served as the zero standard (0 pg/ml). The process is presented below (Fig. 4) (154).

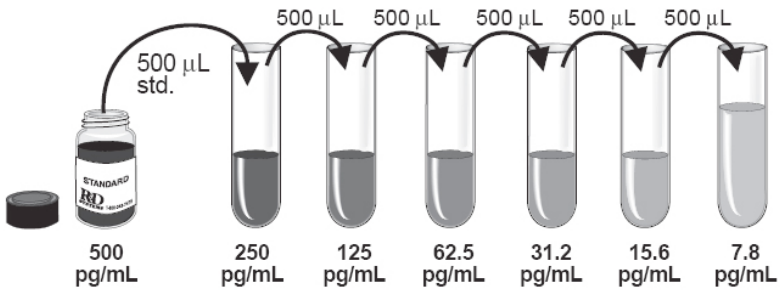


Figure 4 *The dilution of the IL-10 standard (154)*

The following steps were accomplished in order to obtain the ELISA results (154):

1. After all reagents, working standards and samples were prepared, the excess microplate strips were removed from the plate frame, and they were returned to the foil pouch.

2. Then 50 µl of Assay Diluent RD1W were added to each well and after that 200 µl of standard, control, or sample was added per well. Using an adhesive strip, the plate was covered and incubated for 2 hours at room temperature.

3. Each well was aspirated and washed. The process was repeated three times. Each well was washed by filling it with Wash Buffer (400 ∫l) using a multi-channel pipette. After the last wash, any remaining Wash Buffer was removed by aspirating.

4. 200 µl of IL-10 Conjugate was added to each well. The plate was covered with a new adhesive strip and then incubated for 2 hours at room temperature.

5. The aspiration was repeated and a wash procedure similar to the one described in step 3 was used.

6. 200 µl of Substrate Solution was added to each well and incubated for 30 minutes at room temperature.

7. 50 µl of Stop Solution was added to each well and the optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

2.4 DNA extraction

DNA was extracted from the whole blood samples using a QIAamp DNA Blood Midi Kit protocol. The reagents reagents and their preparation are listed below:

- QIAGEN Protease stock solution was stored at -20°C when not used.
- QIAamp DNA Blood Midi Kits: 4.4 ml distilled water was pipeted into the vial of lyophilized QIAGEN Protease provided in the QIAamp DNA Blood Midi Kit
- Buffer AL was stored at room temperature, 25°C Mix Buffer AL was shaken thoroughly before use.
- Buffer AW1 was also stored at room temperature. I added 25 ml of ethanol (96%) to concentrated Buffer AW1 before using the kit for the first time. The final volume of Buffer AW1 was 44 ml.
- Buffer AW2 was stored at room temperature, too. 40 ml ethanol (96%) was added to Buffer AW2 concentrate before using the kit for the first time. The final volume of Buffer AW2 was 57 ml.

The next steps were performed in order to obtain the DNA:

1) First 200 µl QIAGEN Protease was pipetted into the bottom of a 15 ml centrifuge tube and then 1/2ml blood was added and mixed briefly.
2) 2.4 ml Buffer AL was added, it was mixed thoroughly by inverting the tube 15 times, followed by additional vigorous shaking for at least 1 min. and then incubated at 70°C for 10 min.
3) 2 ml ethanol 96% was added to the sample and mixed by inverting the tube 10 times, followed by additional vigorous shaking.
4) At the previous step half of the solution obtained was transferred onto the QIAamp Midi column placed in a 15 ml centrifuge tube and then centrifuged for 3 min at 1850 x *g* (3000 rpm).

5) The QIAamp Midi column was removed, the filtrate was discarded, and the QIAamp Midi column was placed back into the 15 ml centrifuge tube. The remainder of the solution from step 3 was loaded onto the QIAamp Midi column and centrifuged again at 1850 x *g* (3000 rpm) for 3 min.
6) The QIAamp Midi column was then removed, the filtrate discarded, and the QIAamp Midi column placed back into the 15 ml centrifuge tube.
7) 2 ml Buffer AW1 was added to the QIAamp Midi column and than centrifuged at 4500 x *g* (5000 rpm) for 1 min.
8) 2 ml Buffer AW2 were added to the QIAamp Midi column and centrifuged at 4500 x *g* (5000 rpm) for 15 min.
9) The QIAamp Midi column was placed in a clean 15 ml centrifuge tube, and the collection tube containing the filtrate discarded.
10) 300 µl distilled water - equilibrated to room temperature (15–25°C) - was pipetted directly onto the membrane of the QIAamp Midi column. The product was incubated at room temperature for 5 min, and centrifuged at 4500 x *g* (5000 rpm) for 2 min.
11) In order to obtain a maximum concentration, the eluate containing the DNA was reloaded onto the membrane of the QIAamp Midi column. Then I incubated at room temperature for 5 min and centrifuged at 4500 x *g* (5000 rpm) for 2 min.

The extracted DNA was introduced with Eppendorf Pipettes in 1.2 ml Eppendorf-tube and at -20 °C stored until further analysis was performed.

2.5 Determining the DNA concentration using UV-spectrophotometry

The DNA concentration was tested on an UV spectrophotometer for absorbance at wavelengths of 260 nm and 280 nm. The spectrophotometer uses a crystal cuvette.

The results indicate a purified DNA free from contamination, when the A260: A280 ratio for the sample, using the spectrophotometer, is between 1.80 and 2.

2.6 Polymerase Chain Reaction (PCR)

PCR is used to amplify a short, well-defined part of a DNA strand. This can be a single gene, or just a part of a gene. In December 1983, Kary Mullis invented the polymerase chain reaction, for which he was awarded the Nobel Prize in Chemistry in 1993 (187).

PCR requires several basic components such as: DNA template, or cDNA which contains the region of the DNA fragment to be amplified, two primers, sense and antisense, which determine the beginning and end of the region to be amplified, Taq polymerase, which copies the region to be amplified, deoxynucleotides-triphosphate, from which the DNA-Polymerase builds the new DNA buffer, which provides a suitable chemical environment for the DNA-polymerase. The name Taq came from the first thermostable DNA polymerases that was obtained from *Thermus aquaticus* and was called "Taq."

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands — often not more than fifty and usually only 18 to 25 base pairs long nucleotides that are complementary to the beginning and end of the DNA fragment to be amplified. They anneal by adhering to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand.

The first part of PCR consists of a denaturing step. The double-stranded DNA has to be heated to 94-96°C in order to separate the strands. During this step the hydrogen bonds that connect the two DNA strands are broken apart. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and are now single-stranded only. After separating the DNA strands, the temperature is lowered so that the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C). A wrong temperature during the annealing step can result in primers not binding to the template DNA at all, or binding at random. Finally, the DNA-Polymerase has to copy the DNA strands. It starts at the annealed primer and works its way along the DNA strand. This step is called extension. The extension temperature depends on the DNA-Polymerase. The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified. A final

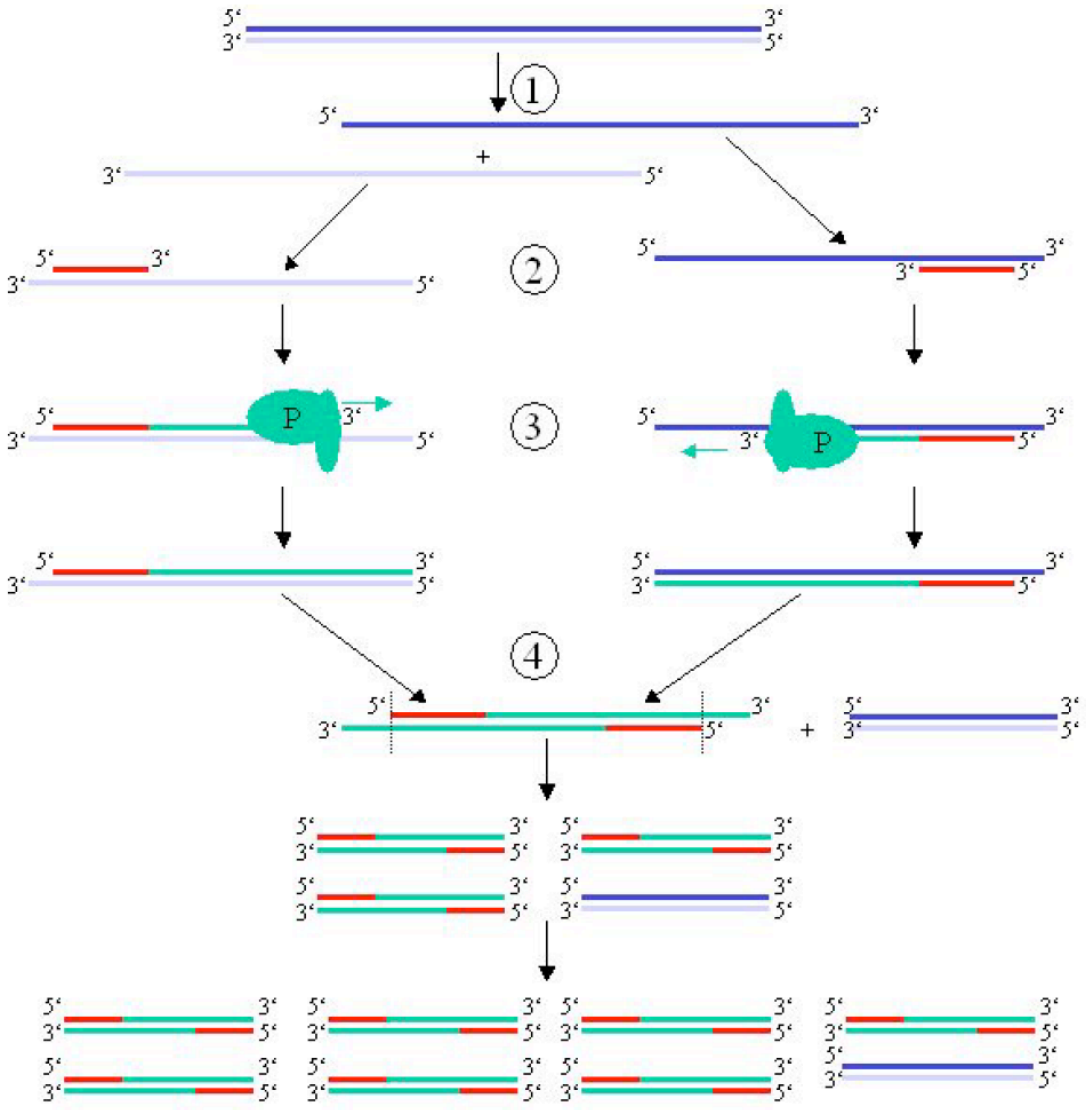
extension step is frequently used after the last cycle to ensure that any remaining single stranded DNA is completely copied (Fig. 5).

There are many subtypes of PCR: Nested PCR, Inverse PCR, Reverse Transcription PCR (RT-PCR), Asymmetric PCR, Quantitative PCR, Quantitative real-time PCR (QRT-PCR), Touchdown PCR, Colony PCR.

In the current study, the polymerase chain reaction (PCR) was performed in order to determine the transformation from guanine to adenine at the position -1082. The oligonucleotide primers: forward, 5'-AACCCAACTGGCTCTCCTTA-3' and reverse, 5'BIOTIN-GCTGGATAGGAGGTCCCTTA-3' (Gene Bank accession no. U75291) were used to generate a 154-bp-long PCR product. The following conditions were respected: an initial denaturing step for 5 minutes at 95°C; 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes.

A C to T transition at the -819 position of the IL-10 gene was searched using the following PCR conditions: an initial denaturing step for 5 minutes at 95°C; 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds; and a final extension at 72°C for 5 minutes. The oligonucleotides primers used to amplify a 171-bp-long genomic DNA fragment were: forward, 5'BIOTIN-TTCTCAGTTGGCACTGGTGT-3'; reverse, 5'-AACTGTGCTTGGGGGAAGT-3' (Gene Bank accession no. X78437).

The PCR amplification was performed using oligonucleotide primers flanking the 130-bp-long genomic DNA fragment in the -592 position of IL-10 gene. The PCR conditions were as follows: an initial denaturing step for 5 minutes at 95°C; 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 15 seconds; and a final extension at 72°C for 5 minutes. The following primers were used: forward, 5'BIOTIN-TGGAAACATGTGCCTGAGAA-3'; reverse, 5'-CAAGCAGCCCTTCCATTTTA-3' (Gene Bank accession no. X78437).



1. Denaturation at 96°C **2. Annealing at 60°C** **3. Final extension 72°C**

Figure 5 *Principle of PCR (modified according to M. Manske)*

The PCR product can be identified using agarose gel electrophoresis. Agarose gel electrophoresis is a procedure that consists of injecting DNA into agarose gel and then applying an electric current to the gel. As a result, the smaller DNA strands move faster than the larger strands through the gel toward the positive current. The size of the PCR product can be determined by comparing it with a DNA ladder, which contains DNA fragments of known size, also within the gel.

The 2.5% agarose gel was prepared and the electrophoresis was performed at 5-6 V/cm. The ethidium bromide was used to visualize the products. Gels are run until the

bromophenol blue dye front is 4 to 5 cm from the wells. Gells were photographed (Fig. 6).

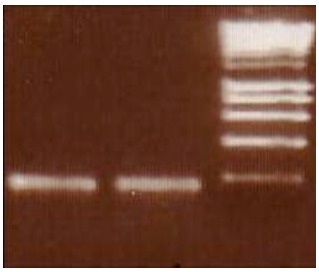


Figure 6 *Products quality visualized with the help of electrophoresis*

Table 5 *Components of the PCR-mixture*

Components	Volumes (µl)	Final concentration
DNA	4	2ng/ µl
Taq-Polymerase	0.12	5 U/ µl
dNTP's	0.18	100 µM
Forward-Primer	0.18	0.4 µM
Reverse-Primer	0.18	0.4 µM
10*BCR Buffer I	1.5	
H_2O	5.84	
Final volume	12	

2.7 Pyrosequencing

Polymorphisms of IL-10 (G/A at -1082, C/T at -819, and C/A at -592) were detected using the Pyrosequencer PSQ 96 and PSQ 96 SNP Reagent Kit (Uppsala Sweden).

The principle of pyrosequencing is based on the following facts. The DNA strand is made up of 4 nucleotides. The double helix structure of DNA is formed by binding the complementary bases (188).

The analysis was performed using a PSQ HS 96A device, which is an automated, highly sensitive and quantitative genetic analysis system based on pyrosequencing technology. Pyrosequencing stands out among rapid genetic analysis

methods in that it always generates true sequence information. This gives per-sample quality control in every run, from which the software makes an automatic quality assessment. Results are therefore unambiguous and eliminate the need to repeat assays or run separate controls. Each result, called a pyrogram, is a quantitative representation of the samples' nucleotide base sequence. Every pyrogram shows the determined polymorphic position and is ideal for quantitative analysis.

The High Sensitivity (HS) light detection system enables small amounts of sample DNA to be analyzed (typically 5-10 µl of PCR product). Consequently, only small volumes of reagents and nucleotides are required, thereby reducing running costs.

Pyrosequencing technology offers greater than 99% reproducibility and greater than 99.997% accuracy in SNP scoring, measured by an independent test.

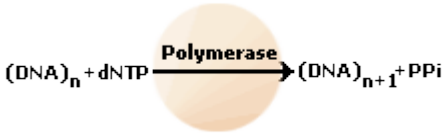
Pyrosequencing uses a four enzyme-cascade system, consisting of four enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and substrates, to produce light whenever a nucleotide forms a base pair with the complementary base in a DNA template strand. Before the analysis, PCR products are converted to single stranded templates.

To build the double helix structure DNA polymerase incorporates the nucleotides into the DNA strand, but only when bases are complementary to each other. The incorporation event releases pyrophosphate, which is converted to ATP. This last product is transformed into visible light in the reaction with firefly luciferase. For each incorporated nucleotide, a peak appears on the pyrogram. If no bound is formed, there is no light and nothing appears on the pyrogram. The amount of light and the height of the peak are proportional to the number of incorporated nucleotides.

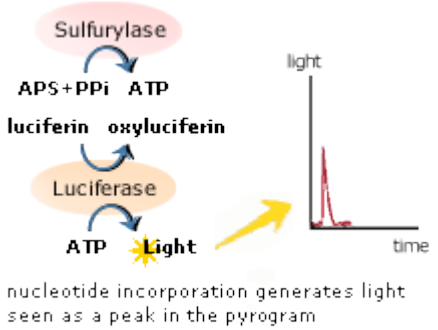
The fourth enzyme, the apyrase degrades each time the excess of base and of ATP, preparing the reaction for the next nucleotide. This way pyrosequencing can detect the sequence of DNA and can perform SNP and mutation analysis (Fig. 7).

Step 1 A sequencing primer is hybridized to a single-stranded, PCR-amplified, DNA template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5´ phosphosulfate (APS) and luciferin.

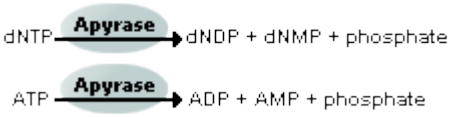
Step 2 The first of four deoxynucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxynucleotide triphosphate into the DNA strand if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.



Step 3 ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram™. Each light signal is proportional to the number of nucleotides incorporated.



Step 4 Apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added.



Step 5
Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase.

As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peak in the program.

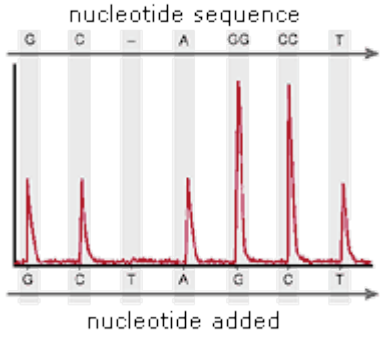


Figure 7 *The scheme of Pyrosequencing (www.pyrosequencing.com)*

Prior to analysis, PCR products were converted to single-stranded templates onto which a sequencing primer is annealed.

After the PCR products were obtained, they are purified by binding to streptavidin-sepharose. Hence sepharose beads are added to the PCR plate, after that, PCR products are immobilized to streptavidin coated sepharose beats (1440 rpm).

Using the Vacuum Prep Workstation (Fig. 8) the samples are prepared for pyrosequencing. PCR plate containing the biotinylated PCR product and the mix is agitated for ten minutes at room temperature. Vacuum is applied and then the beads with immobilized PCR product are picked up by the Vacuum Prep Tool from the PCR-plate and moved to a separate trough, where 70% ethanol is aspirated through the

probes. The Vacuum Prep Tool is then placed in a trough of sodium hydroxide, still with the vacuum pressure switched on, to denature the DNA and to wash out the released single-stranded DNA, while the 5'-biotinylated strand remains immobilized on the beads. Next, the tool is placed in a trough of wash buffer where the strands are rinsed by aspiration. The single-stranded templates are then transferred to a previously prepared PSQ plate containing annealing buffer and sequencing primer. The vacuum pressure is switched off, a gentle shake of the Vacuum Prep Tool releases the beads with attached templates into the 96- well PSQ plate. After the sequencing primer is annealed, this plate is ready for analysis using a Pyrosequencing System.

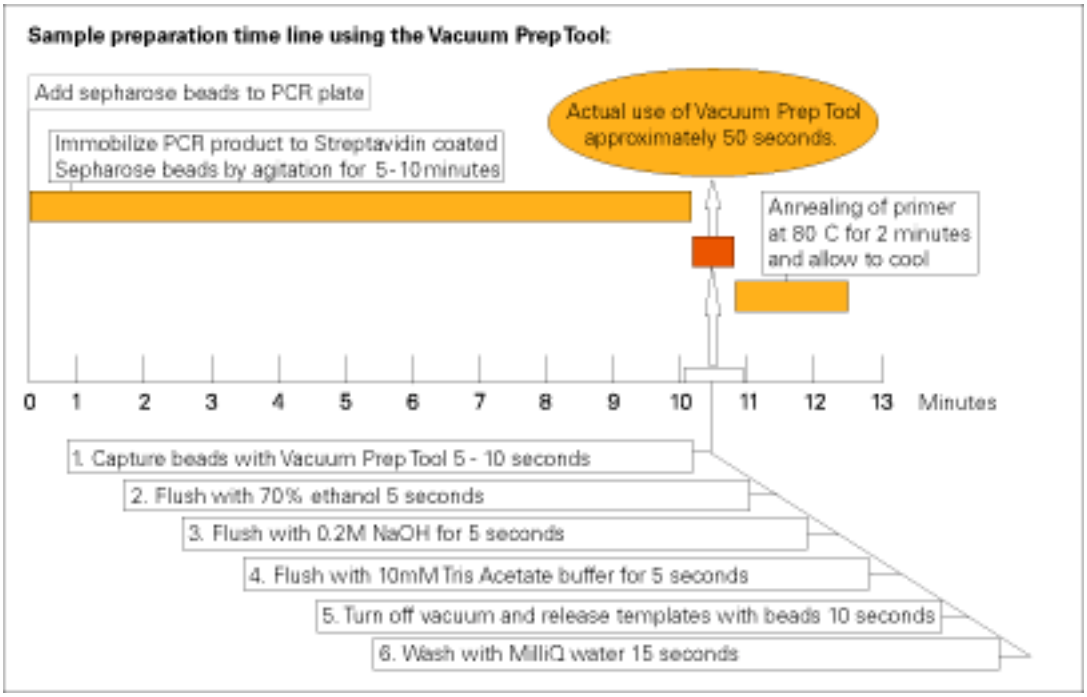


Figure 8 *Sample preparation using Vacuum Prep Workstation (www.pyrosequencing.com)*

The sample preparation takes around 13 minutes, then one plate containing 96 samples is ready to be analyzed in about half an hour.

After single-stranded DNA purification, the template bound to streptavidin-Sepharose is mixed with annealing buffer containing extension primer. After incubation for 2 minutes at 80 ^{o}C, the extension primer: template mixes are subjected to Pyrosequencing in the PSQ HS 96 A instrument (188).

The SNP analysis was performed by using the SNP software. The first step is creating SNP sequences that are entered into the SNP software. The software automatically recommends the most effective order for the dispensation of nucleotides.

We used the following SNP sequences and IL-10 primers:

- for the -1082 position:

  Sequence:

  GACAACACTACTAAGGCTTCTTTGGGA [G/A] GGGGAAGTAG

  Sequencing primer:

  5'-GACAACACTACTAAGGCTTC-3'

- for the -819 position:

  Sequence:

  CAAACTGAGGCACAGAGAT [A/G] TTACATCACC

  Sequencing primer:

  5'-CAAACTGAGGCACAGAG-3'

- for the -592 position:

  Sequence:

  CCAGAGACTGGCTTCCTACAG [G/T] ACAGGCGGGG

  Sequencing primer:

  5'-CCAGAGACTGGCTTCC-3'

The pyrosequencing substance is composed of: 8 units of exonuclease-DNA Polymerase, 40 m-units Apyrase, 100 ng Luciferase, 20 m-units recombinated ATP-sSulphurylase, 2.5 µg DNA-binding protein, 0.1 M Tris-Acetate (pH 7.75), 0.3 mM EDTA, 5 mM Magnesium-acetate, O.1% BSA, 1mM Dithiothreitol, 5 µM Adenosine-5'-Phosphosulfate, 0,4 mg/ml Polyvinylpyrrolidon and 100 /ml D-Luciferin. The procedure comprises several steps and the results are registered by a photomultiplier.

2.8 Statistics

Statistical analyses were performed using the SPSS statistical software package. Continuous variables were reported as medians and 95% confidence intervals, and categoric variables were reported as numbers and percentages. Continuous variables were analyzed using the Mann–Whitney test (two-tailed) and categoric variables were compared using the Chi-Square Test by Pearson and Fisher's exact test. A two-tailed *p* value <0.05 was considered statistically significant. The distribution of allele frequencies was determined using the cross tables.

Overall survival probability was estimated using the Kaplan-Meier product limited method. Correlations between the individual polymorphisms and clinical prognostic factors (e.g. FIGO stage, residual tumor mass, histological type and grading) were analyzed using univariate and multivariate Cox regression models. Odds ratios with 95% confidence interval (CI) were calculated.

To enhance the relevance of the statistical analysis, the following subgroups were summarized: stage I+II and III+IV; histological grading: I+II and III+IV.

3 Results

3.1 IL-10 expression in serum and ascites

3.1.1 Patients characteristics

Collective of patients

From August 2002 to November 2003, 114 women with histologically confirmed ovarian cancer and 30 women without any malignancy were enrolled into this study.

Median age at diagnosis among the patients with malignant disease was 57 years (range 19-88). The median follow-up was 25 months. More than half of the patients (55.3%) were diagnosed with primary ovarian carcinoma, and 44.7% recurrent ovarian cancer. The stage FIGO III was found in 54.1% of the patients and FIGO IV in 29.7%. Only 10 patients (9%) was the disease confined to the ovary, FIGO stage I. The most common histological type was serous-papillary (80.7%) (Tab. 6).

The patients with benign diseases had diagnosis such as: benign ovarian cyst and tumor, endometriosis and myoma. The control group consisted of 30 women with no history of malignancy. Seventeen (56.7%) of them were diagnosed as having a benign ovarian tumor, three (10%) of them endometriosis and four (13.3%) as having benign ovarian cysts and another 2 (6.7%) were diagnosed with myoma. Only 4 (13.3%) of them were healthy donors, from whom blood was obtained (Tab. 7).

Table 6 *Ovarian cancer patients characteristics*

Parameters	**Value**
Age at treatment in years, median (range)	57 (19-88)
Tumor status, n (%)	
• primary	63 (55.3)
• recurrence	51 (44.7)
Histology, n (%)	
• serous-papillary	92 (80.7)
• endometroid	10 (8.8)
• mucinous	8 (7)
• mixed/others	4 (3.5)
Grading, n (%)	
• I-II	54 (47.4)
• III-IV	60 (52.6)
FIGO Stage, n (%)	
• I-II	18 (15.8)
• III-IV	96 (84.2)
Ascites volume, n (%)	
• none	3 (2.6)
• ≤500 ml	54 (47.4)
• >500 ml	57 (50)
Postoperative residual tumor mass, n (%)	
• macroscopically tumor free	48 (44)
• ≤1 cm	30 (27.5)
• >1 cm	31 (28.5)
• no data	5
Lymph node status, n (%)	
• positive (N1)	28 (24.6)
• negative (N0)	28 (24.6)
• not determined (Nx)	58 (50.8)
Recurrence status	
No relapse after surgery	51 (44.7)
Relapse after surgery	62 (55.3)
Follow-up period months (median/range)	25 (0-225)

Table 7 *Control group patients' characteristics*

Parameters	**Value**
Age at treatment in years, median (range)	48.5 (24-76)
Diagnosis, n (%)	
• Benign ovarian tumor	17 (56.7)
• Benign ovarian cyste	4 (13.3)
• Endometriosis	3 (10.0)
• Myoma	2 (6.7)
• Healthy women	4 (13.3)
Ascites/peritoneal fluid volume, n (%)	
• None	25 (83.4)
• ≤ 500 ml	4 (13.3)
• > 500 ml	1 (3.3)

Age distribution

The median age at diagnosis for patients with ovarian cancer was 57 years (range 19-88) (Tab. 8).

Table 8 *Age distribution among the ovarian cancer group*

	Mean (years)	Median (years)	Range (years)	Number of patients
Primary OC	58.43	58	33-88	63
Recurrent OC	53.02	54	19-79	51
OC	56.01	57	19-88	114

Residual tumor mass

Almost half (44%) of the patients were macroscopically tumor free after surgery, 27.5% presented a residual mass smaller than 1 cm and another 31 (28.4%), a residual mass that exceed 1 cm in the largest diameter.

Of all 63 patients diagnosed with primary ovarian cancer 31 (49.2%) benefit from optimal cytoreductive surgery (no residual tumor mass). Sixteen patients (26.7%) presented a residual mass smaller than or equal to 1 cm (Fig. 9).

Among patients with relapsed ovarian cancer, 34.7% (17) were disease-free and 35.3% (18) have had a residual mass bigger than 1 cm after the surgical treatment.

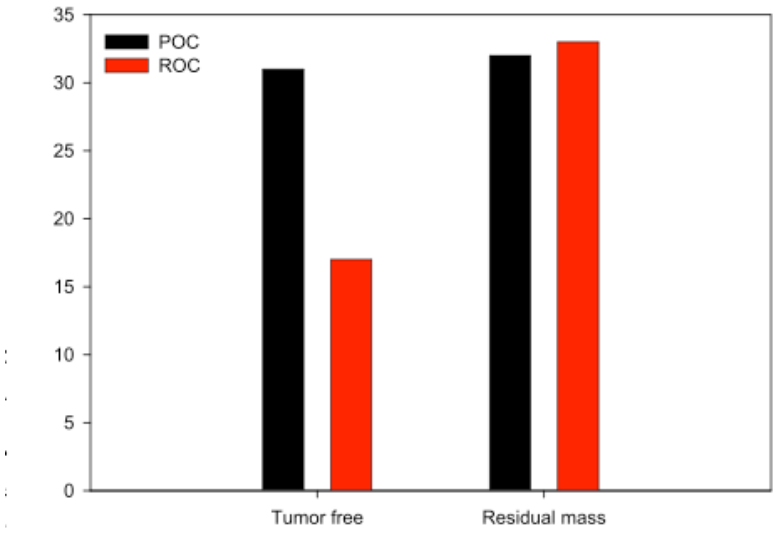


Figure 9 *Rates of optimal cytoreduction among patients with primary (POC) and recurrent (ROC) ovarian cancer*

Histological type

Of 114 patients with ovarian cancer, 92 (80.7%) patients presented serous-papillary histology. Due to the low incidence of other histological types, the mucinous, the endometroid and mixed tumors were grouped together and compared with the serous-papillary histology (Tab. 9).

Table 9 *Histological classification of ovarian carcinoma*

Histology	Diagnosis	
	Primary OC (63 patients)	Recurrent OC (51 patients)
Serous-papillary	49 (77.8%)	43 (84%)
endometroid	10 (15.9%)	
Mucinous	4 (6.3%)	4 (8%)
Clear cells		1 (2%)
Mixed/others		2 (6%)

Staging

Only 6 (9.5%) patients from the primary ovarian cancer group had a disease confined to the ovary, FIGO stage I (Tab. 10).

Table 10 *FIGO-stages in the ovarian cancer group*

FIGO-Stage	Diagnosis	
	Primary OC (63 patients)	Recurrent OC (51 patients)
I	6 (9.5%)	4 (8.3%)
II	5 (7.9%)	3 (6.3%)
III	33 (52.4%)	29 (56.3%)
IV	19 (30.2%)	15 (29.2%)

Grading

Approximately half (50.8%) of the patients with primary ovarian cancer and 52% of patients with relapse had a poorly differentiated tumor. Only 1 (1.6%) patient with primary disease and 5 patients (8.7%) with relapse showed a highly differentiated carcinoma of the ovary (Tab. 11).

Table 11 *Grading of patients with primary and recurrent carcinoma of the ovary*

Grading	Diagnosis	
	Primary OC (63 patients)	Recurrent OC (51 patients)
I	1 (1.6%)	5 (8.7%)
II	30 (47.6%)	20 (39.1%)
III	32 (50.8%)	26 (52.2%)

Lymph nodes involvement

Of patients with primary ovarian cancer, 33.3% (21) had negative lymph nodes. On the other hand, in the recurrent ovarian cancer group, only 13.7 % had no lymph nodes involvement. The results are summarized in the table below (Tab.12).

Table 12 *Lymph node involvement depending on recurrence status*

Lymph node status	Primary OC (%)	Recurrent OC (%)
N_0	21 (33.3%)	7 (13.7%)
N_1	17 (27%)	11 (21.6%)
N_x (not determined)	25 (39.7%)	33 (64.7%)

CA-125

For 32 patients there was no preoperative determination of CA-125 levels in serum. Of these, 20 (31.7%) were diagnosed with primary ovarian cancer and 12 (23.5%) with relapsed malignancy (Tab. 13).

Table 13 *CA-125 levels in serum from patients with ovarian cancer*

CA-125	Diagnosis	
	Primary OC (63 patients)	Recurrent OC (51 patients)
>35 UI/ml	38 (60.3%)	32 (62.7%)
≤35 UI/ml	5 (7.9%)	7 (13.7%)
Not determined	20 (31.7%)	12 (23.5%)

Ascites

Of patients with primary carcinoma of the ovary, 9 (14.3%) patients had no ascites, 24 (38.2%) less than 500 ml and 30 (47.5%) more than 500ml. The patients with relapse had ascites in 72.6% of the cases, 41.2% (21) had ≤ 500 ml and 31.4% (16) more than 500 ml. Fourteen patients (27.4%) had no ascites (Fig. 10-11).

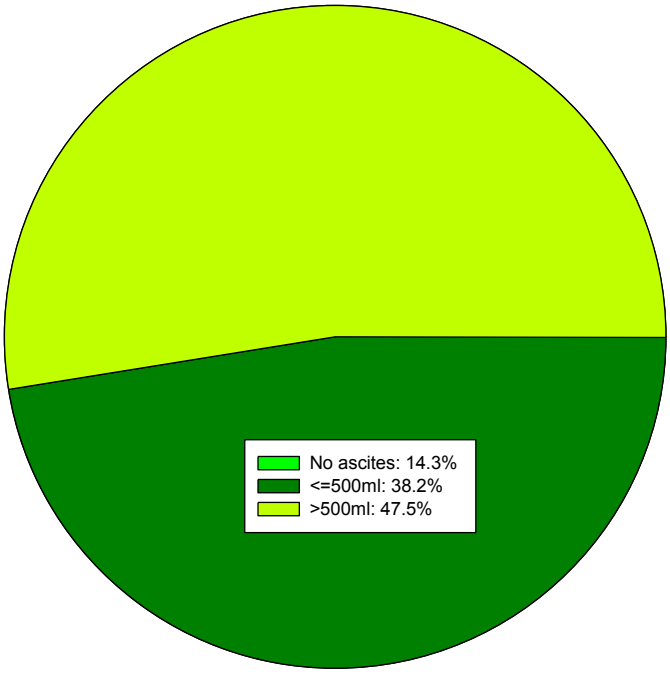


Figure 10 *Incidence of ascites among primary ovarian cancer patients*

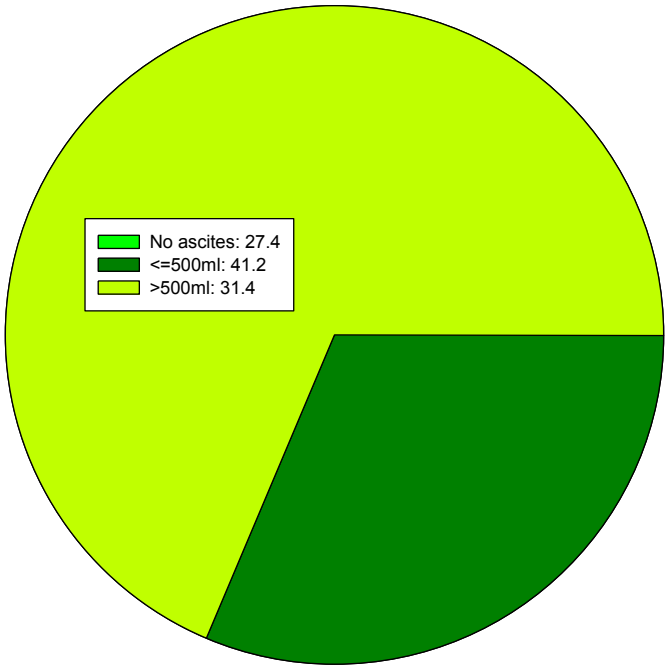


Figure 11 *Incidence of ascites among recurrent ovarian cancer patients*

3.1.2 Levels of IL-10 in serum and ascites in the ovarian cancer and control groups

The following levels of IL-10 were detected in the serum (median= 10.01 pg/ml; range 7.8 to 500.000 pg/ml) and in the ascites (median = 45.46 pg/ml; range 7.8 to 389.4 pg/ml) of ovarian cancer patients, and in serum samples (median= 9.39 pg/ml; range 7.8 to 62.8 pg/ml) and in peritoneal fluid (median= 32.90 pg/ml; range 7.8 to 88.72 pg/ml) of the patients without any malignancies (Tab. 14).

Significant correlation between the concentration of IL-10 in ascites (p=0.029) and serum level of IL-10 (p=0.002) was found in the ovarian cancer group and control group (Fig. 12 and 13).

It should be mentioned that only 5 women from the control group presented ascites, most of them having a small quantity of peritoneal fluid. In contrast, about 91 patients with malignancies had ascites at the time of the diagnosis.

Table 14 *IL-10 expression in ovarian cancer and control groups*

Analyzed group/IL-10 concentration	Mean (pg/ml)	Median (pg/ml)	Minimum (pg/ml)	Maximum (pg/ml)	Mean standard error
			Serum		
OC	26.07	9.95	7.8	500.0	6.49
CG	10.77	7.80	7.8	62.8	2.101
			Ascites		
OC	70.47	45.46	7.8	389.48	7.23
CG	30.96	18.34	7.8	88.72	8.41

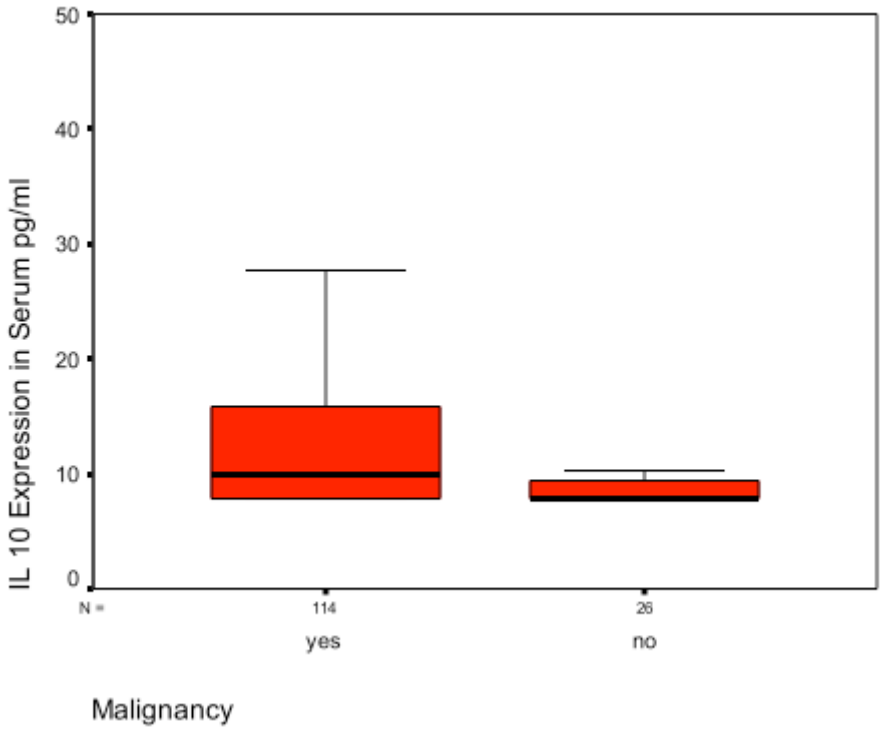


Figure 12 *The difference between IL-10 expression in serum from patients with and without malignancy*

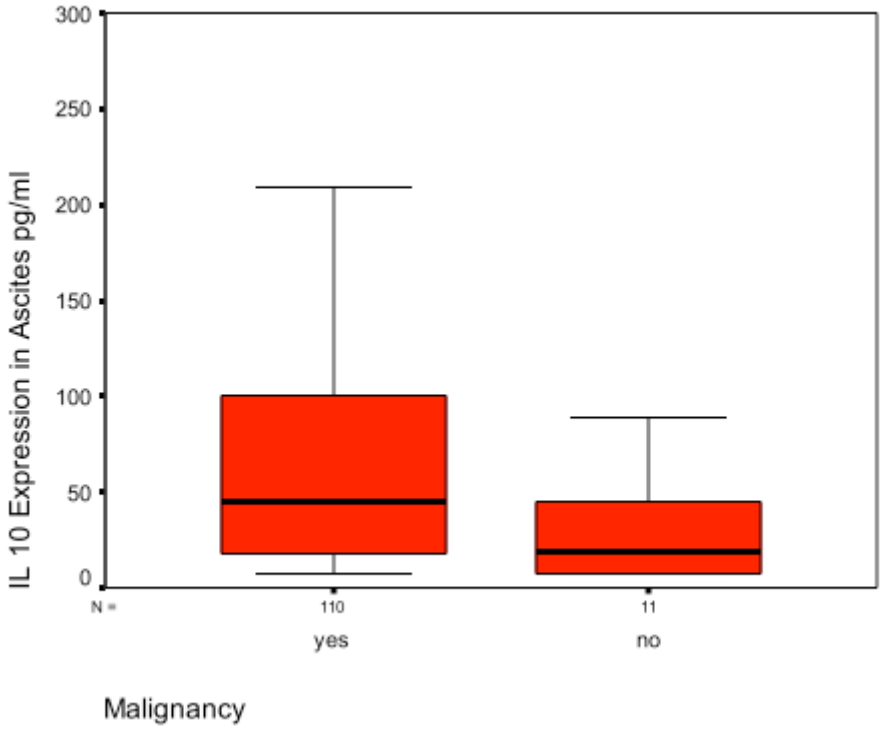


Figure 13 *The difference between IL-10 expression in ascites from patients with and without malignancy*

3.1.3 Expression of IL-10 in serum and ascites in patients with ovarian cancer

In this study 63 and 51 patients with primary and recurrent ovarian cancer, respectively, were included.

An analysis of the correlation between the expression of IL-10 in serum and ascites in primary and relapsed ovarian carcinoma showed no statistically significance (p=0.48 and p=0.35, respectively).

The median concentration of this cytokine in the serum of patients with primary ovarian cancer was 9.77pg/ml (range 7.8-500) and 10.10pg/ml (range 7.8-500) in patients with recurrent ovarian cancer, showing that IL-10 was more expressed in primary disease cases, but did not reach statistical significance (p=0.84). The levels in ascites were also higher in the primary ovarian cancer group, median 53.5pg/ml (range 7.8-389.48), than in the recurrent disease, median 35.20pg/ml (range 7.8-18.34) (Tab. 15), but again without statistical significance (p=0.097).

Table 15 *The expression of IL-10 in serum and ascites according to the time of diagnosis*

	Tumor Typ					
	primary		reccurency		benign	
	IL 10 Ascites pg/ml	IL 10 Serum pg/ml	IL 10 Ascites pg/ml	IL 10 Serum pg/ml	IL 10 Ascites pg/ml	IL 10 Serum pg/ml
Mean	80,714	28,728	56,760	22,803	30,967	10,776
Median	53,500	9,779	35,200	10,103	18,342	7,804
Minimum	7,800	7,800	7,800	7,800	7,800	7,800
Maximum	389,481	500,000	344,328	500,000	88,729	62,800
Number of patients	63	63	51	51	30	30
Standard Deviation	82,941	70,318	63,605	68,795	27,925	10,716
Standard Error of Mean	10,450	8,859	9,278	9,633	8,420	2,102

In patients with primary ovarian cancer, the mean values of IL-10 in ascites and serum were: 80.71pg/ml and 28.72pg/ml, respectively. By performing the correlation using the Spearman test, significant differences were found between the expression of this cytokine in ascites and serum, p=0.021. Significant results were obtained when comparing the expressions in the recurrent ovarian cancer collective, p=0.024. The IL-10 was more expressed in ascites than in the sera of the patients (Fig. 14).

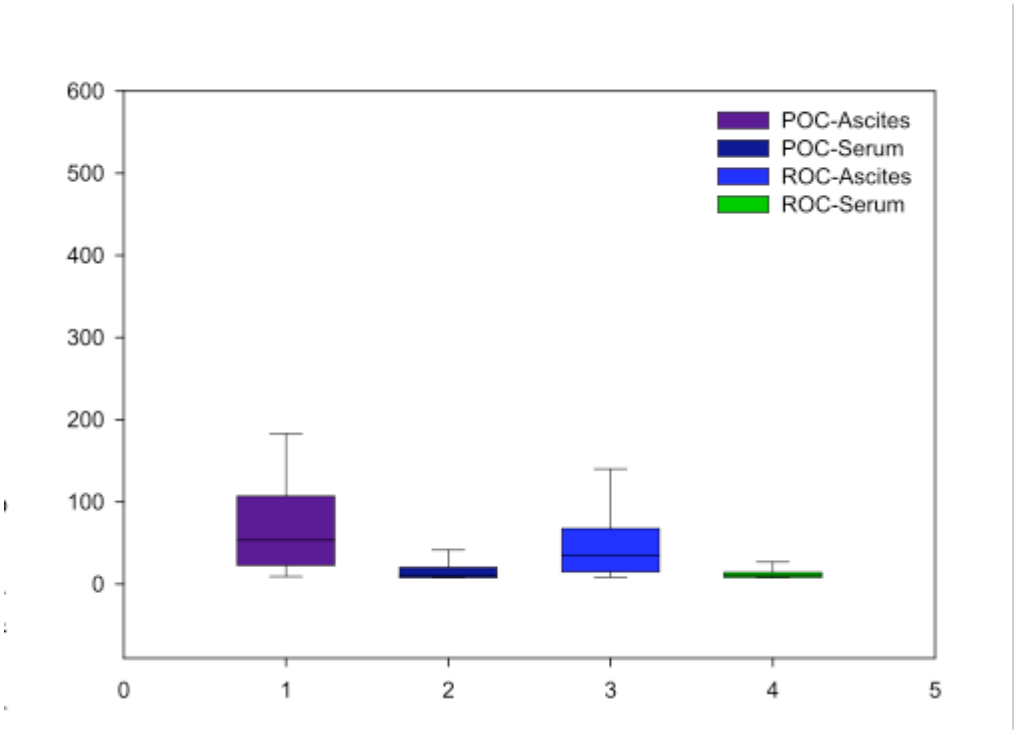


Figure 14 *Concentrations of IL-10 in serum and ascites depending on the recurrence status*

3.1.4 Correlation between established clinical prognostic factors and the expression of IL-10

Also the classical clinical prognosis factors, such as age at diagnosis, FIGO stage, histological type, grading, recurrence status, quantity of ascites, residual tumor mass and lymph node status were compared with the expression of IL-10 in serum and ascites (Tab.16)

.

Table 16 *Correlation between "conventional" prognostic factors and IL-10 ascites and serum level*

Parameters (number of patients)	Serum, p-value	Ascites, p-value
Age (114)	0.52	0.7
FIGO Stage (114)		
I and II vs. III and IV	0.8	0.71
Histological type (114)		
Serous-papillary vs. others	0.1	0.72
Grading (114)		
I and II vs. III and IV	0.85	0.12
Ascites volume (110)		
≤ vs. >500 ml	**0.03**	**<0.001**
CA-125	0.56	**0.03**
Residual tumor mass (111)		
• macroscopically tumor free	0.58	**0.009**
• ≤1cm		
• >1cm		
Lymph node status (56)		
N0 vs N1	0.23	0.47
Recurrency		
• Present	0.89	0.26
• Absent		

The IL-10 levels in ascites correlated with the ascites volume (p<0.001), the preoperative CA-125 level (p=0.03) and with the postoperative residual tumor mass (p=0.009) (Fig. 15-17).

No statistically significant differences were found in the correlation between IL-10 level in serum and all prognosis factors and IL-10 expression in ascites and age at diagnosis, FIGO stage, recurrence status, histological grading, histological type, grading and lymph nodes status, except with the volume of ascites (p=0.03).

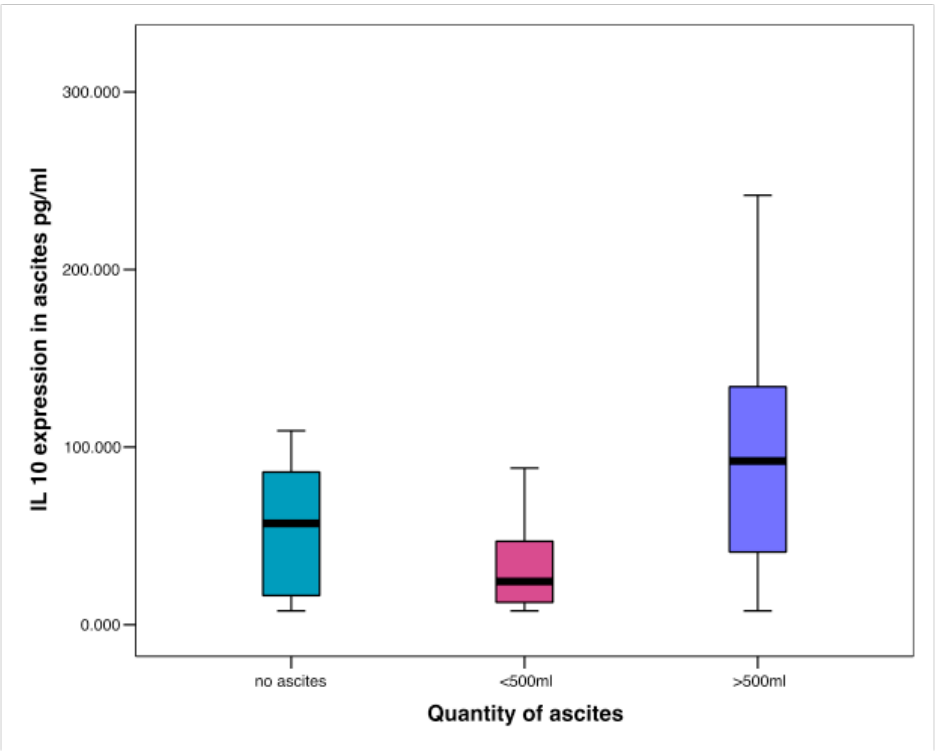


Figure 15 *Correlation between expression in ascites and ascites volume, p<0.001*

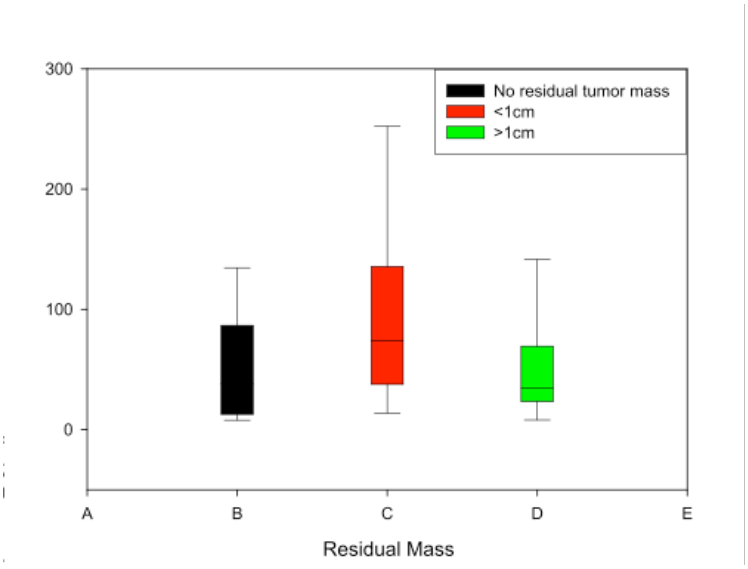


Figure 16 *Correlation between expression in ascites and residual tumor mass, p=0.009*

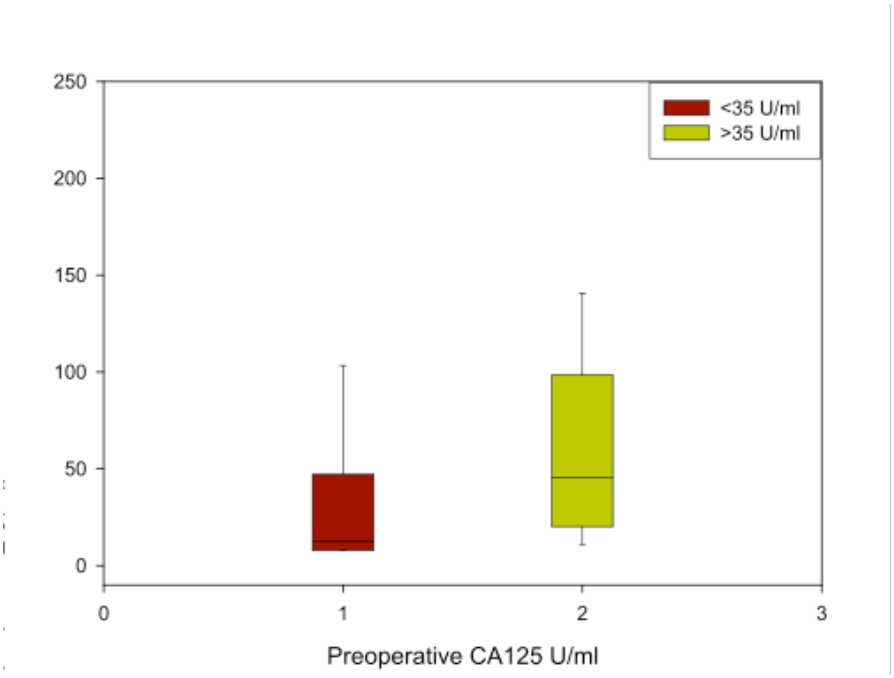


Figure 17 *Correlation between expression in ascites and CA-125, p=0.03*

When the statistical analysis was performed separately for the primary and recurrent ovarian cancer, the following results were obtained (Tab. 17):

- For primary ovarian cancer, the expression of IL-10 in ascites significantly correlated with recurrence after surgery (p=0.022), grading (p=0.049), preoperative CA-125 (p=0.014) and ascites volume (p<0.001). No association was found between the circulatory levels and clinical prognosis factors.
- For recurrent ovarian cancer, the expression of IL-10 in ascites correlated with residual tumor mass (p=0.019) and ascites volume (p=0.008). The expression in serum was significantly associated with recurrence (p=0.002), histological type (p=0.034) and lymph node involvement (p=0.032).

Table 17 *Correlation between established prognostic factors and IL-10 ascites and serum level in the two ovarian cancer groups*

Clinical parameters	Primary ovarian cancer		Recurrent ovarian cancer	
	Serum, p-value	Ascites, p-value	Serum, p-value	Ascites, p-value
Age	0.54	0.73	0.86	0.55
FIGO Stage I and II vs. III and IV	0.19	0.39	0.18	0.1
Histological type Serous-papillary vs. others	0.61	0.69	**0.034**	0.23
Grading I and II vs. III and IV	0.39	**0.049**	0.35	0.75
Ascites volume ≤ vs. > 500 ml	0.07	**<0.001**	0.059	**0.008**
CA-125	0.70	**0.014**	0.25	0.88
Residual tumor mass • macroscopically tumor free • ≤1cm • >1cm	0.17	0.21	0.45	**0.019**
Lymph node status (56) N0 vs N1	0.98	0.27	**0.032**	0.85
Recurrence • Present • Absent	0.25	**0.022**	**0.002**	0.25

3.1.5 The predictive value of IL-10 expression in serum and ascites for patients with ovarian cancer

The cut-off values of IL-10 expression in serum and ascites (8,0 pg/ml in serum and 18 pg/ml in ascites) were calculated in order to research the predictive role of IL-10 expression for ovarian cancer.

The cut-off value of 8.0 pg/ml was reported to be useful for the diagnosis of cancer (196) and was thus adopted in this study. In our analyses, the IL-10 serum level of 8.0pg/ml reached a positive predictive value of 84% (95% CI 76-91) and negative

predictive value 29% (95% CI 16-41) with a specificity and sensitivity of 47% (95% CI 29-65) and 70% (95% CI 62-78) respectively (Tab. 18).

Table 18 *Cut-off Level IL-10 Serum 8.000*

Parameters	Value (%)	95% Confidential Interval
Sensibility	70	62-78
Specificity	47	29-65
Positive predictive value	84	76-91
Negative predictive value	29	16-41

3.1.6 The impact of IL-10 expression in serum and ascites on overall and tumor-free survival rates in patients with ovarian cancer

Of the whole collective of patients, more than half (59.6%) arrived at the end of the follow-up period.

Because more than 80% of all patients had a serous-papillary ovarian cancer, we decided not to include the histology in the factors analyzed in the Cox regression.

In the multivariate analysis, IL-10 expression in serum influenced the overall survival for patients with ovarian cancer, irrespective of wether the disease was primary or recurrent. The statistical significance was reached only for the primary ovarian disease patients. The residual mass also turned out to be an independent prognosis factor for the whole collective of patients and for the patients with primary ovarian cancer (Tab. 19).

No significant correlation was found between the IL-10 concentrations in ascites and the overall survival, p=0.09 or disease-free survival, p=0.22, in the univariate or the multivariate analysis (Tab. 19-20).

Table 19 *Cox analysis-multivariate analyses*

	All patients		Primary Ovarian Cancer		Recurrent Ovarian Cancer	
	HR (95%CI)	P	HR (95%CI)	p	HR (95%CI)	p
IL-10 Serum	2,03 (0.93-4.42)	**0.05**	3.42 (1.03-11.3)	**0.03**	3.41 (0.79-14.55)	**0.05**
IL-10 Ascites	-	0.35	-	0.83	-	0.17
FIGO	-	0.58	-	0.55	-	0.58
Residual tumor mass	3.22 (1.52-6.84)	**0.002**	6.11 (1.03-11.3)	**0.001**	-	0.14
Lymph nodes involvement	-	0.49	-	0.45	-	0.77
Ascites volume	-	0.35	-	0.72	-	0.72
Age	-	0.32	-	0.75	-	0.42

In the present study, in the univariate analysis, only residual tumor mass and histology type significantly influenced the overall and disease-free survival rate (Tab. 20).

When the analysis was performed for the two different groups, only the residual mass and IL-10 circulatory level influenced the overall survival in patients with primary ovarian cancer. None of the factors researched affected disease-free survival in patients with recurrent ovarian cancer (Tab. 20).

Table 20 *Kaplan-Meier Analysis*

	Tumor free survival			Overall survival		
	All Patients	POC	ROC	All Patients	POC	ROC
IL-10 Serum	0.46	0.24	0.12	0.18	0.52	0.07
IL-10 Ascites	0.22	0.76	0.9	0.09	0.38	0.14
FIGO	0.77	0.94	0.58	0.33	0.78	0.39
Histology	**<0.001**	0.86	**<0.001**	0.95	0.36	0.76
Grading	0.84	0.31	0.25	0.23	0.29	0.54
Residual tumor mass	0.41	0.15	0.33	**0.004**	**0.004**	0.14
Lymph nodes involvement	0.11	0.38	0.35	0.44	0.10	0.29
Ascites volume	0.56	0.31	0.56	0.77	0.89	0.79
Age	0.11	0.56	0.88	0.71	0.4	0.15

3.2 IL-10 polymorphisms

3.2.1 Patients' characteristics

One hundred forty seven women diagnosed with ovarian cancer and 129 women with no history of malignancy were genotyped for detecting the individual polymorphisms of IL-10.

The characteristics of the women with ovarian cancer are summarized in Tab. 21. The median age at first diagnosis was 56 years. Seventy seven patients (55%) were diagnosed with recurrence of the disease. Most of the patients (60.1%) did not present with recurrence after the operation during the follow-up period.

The control group consisted of 129 women with no history of malignancy. Twenty patients (15.5%) were diagnosed with benign ovarian tumors, 4 (3.1%) patients with leiomyoma, 4 with endometriosis and 101 (78.3%) were healthy donors. Only 5 (3.9%)

patients from the control group presented ascites, only one of them had more than 500 ml peritoneal fluid. The characteristics of the patients of the control group are presented in Tab. 22.

Table 21 *Pyrosequencing-OC Patients Characteristics*

Parameters	Value n (%)
Age median (range)	56 (19-81)
Tumor status	
• primary	70 (47.6)
• recurrence	77 (52.4)
Histology	
• serous-papillary	125 (84.5)
• endometroid	9 (6.1)
• Mucinous	7 (4.8)
• mixed/others	7 (3.9)
Grading	
• I-II	73 (49.6)
• III-IV	74 (50.3)
FIGO Stage	
• I-II	34 (23.1)
• II-III	113 (76.9)
Ascites volume	
• none	55 (37.4)
• ≤500 ml	54 (36.7)
• >500 ml	38 (25.9)
Residual tumor mass	
• tumor free	80 (54.4)
• residual mass	67 (45.6)
Lymph nodes	
• positive (N1)	39 (26.5)
• negative (N0)	40 (27.2)
• not known	68 (46.3)
Recurrence status	
• No recurrence	46 (31.3)
• Recurrence	101 (68.7)
Follow-up median (range)	29 (0-225)

Table 22 *Control group patients characteristics*

Parameters	Number of patients (%)
Age at treatment in years, median (range)	45 (20-89)
Diagnosis	
• Benign ovarian tumor	13 (10.1)
• Benign ovarian cyste	7 (5.4)
• Endometriosis	4 (3.1)
• Myoma	4 (3.1)
• Healthy donors	101 (78.3)
Ascites volume	
• None	124 (95.1)
• ≤ 500 ml	4 (3.1)
• > 500 ml	1 (0.8)

Age distribution

For patients with primary OC, the median age at first diagnosis was 57.5 years (range 34-81), for patients with relapsed OC the median age was 56 years (range 19-79). The age at first diagnosis for control group was 45 years (Fig. 18).

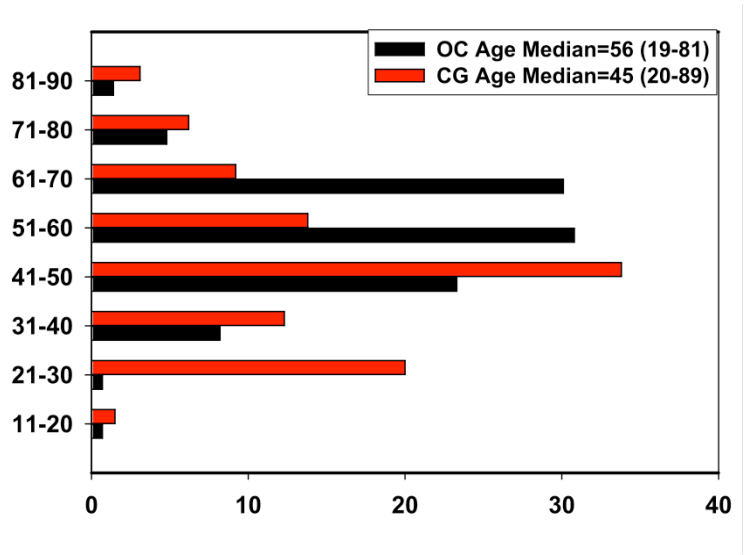


Figure 18 *Age distribution in OC and CG*

Postoperative residual mass

More than half (54.4%) of the patients had no residual tumor mass after surgery, 23.8% presented a residual mass less than or equal to 1 cm and other 32 patients (21.8%) a residual mass larger than 1 cm. Thirty-one (51.8%) of patients with primary ovarian cancer benefit from an optimal cytoreduction. Sixteen patients (26.7%) presented a residual tumor mass less or equal than 1 cm.

Of patients with relapsed ovarian cancer, 34.7% (17) presented no residual tumor mass and 35.3% (18) have had a residual mass larger than 1 cm after the surgical treatment.

Histology

Among all patients diagnosed with ovarian cancer the most frequently encountered histology was serous-papillary (84.5%), followed by the endometrioid type (6.1%) and the mucinous type (4.7%). Other types, such as clear cell and undifferentiated carcinoma were seen in only 1.4% and 2.5%, respectively (Fig. 20).

Among the primary OC the most frequent histological type was serous-papillary in 57 (81.5%) women. The same was true for patients with relapsed ovarian carcinoma. The serous-papillary histology was observed in 67 (87%) recurrent ovarian cancer patients (Fig. 19). To enhance the relevance of the statistical analysis we created two histological groups: serous-papillary and others.

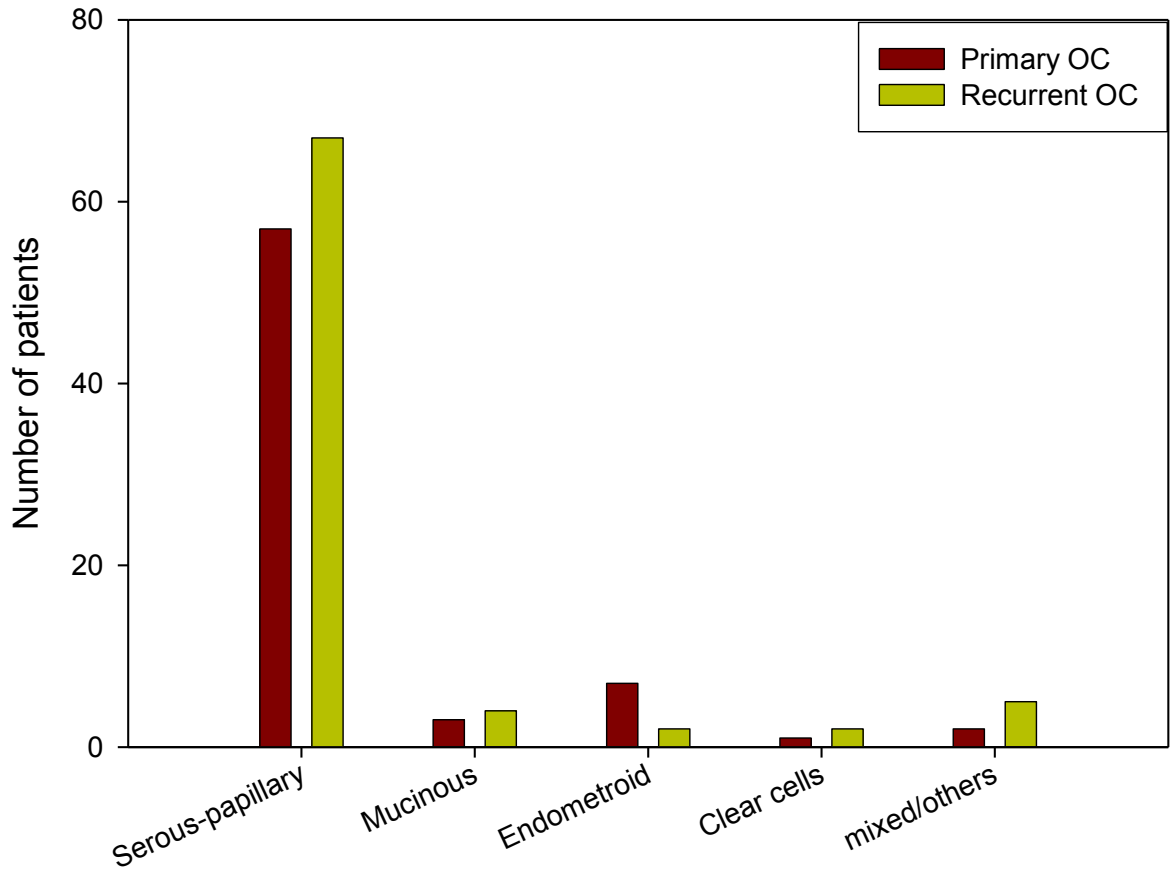


Figure 19 *Histological type breakdown of patients with primary and recurrent ovarian cancer*

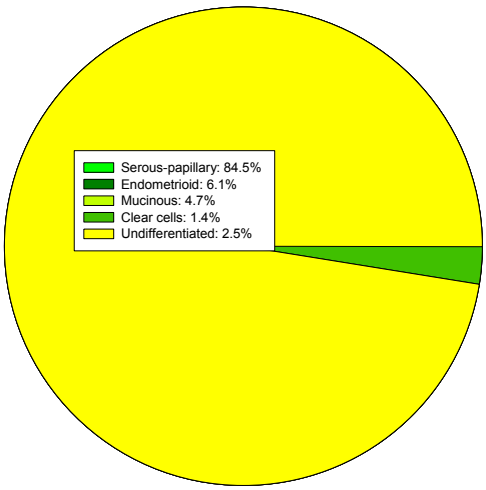


Figure 20 *Histological types in ovarian cancer patients*

FIGO Stage

Half of the patients (35 women) with primary ovarian cancer had a FIGO stage III disease at the time of diagnosis. Only 22.85% (16) had a stage I disease. FIGO stage IV disease was encountered in 18.57% (13) of all patients with primary ovarian cancer (Fig. 21).

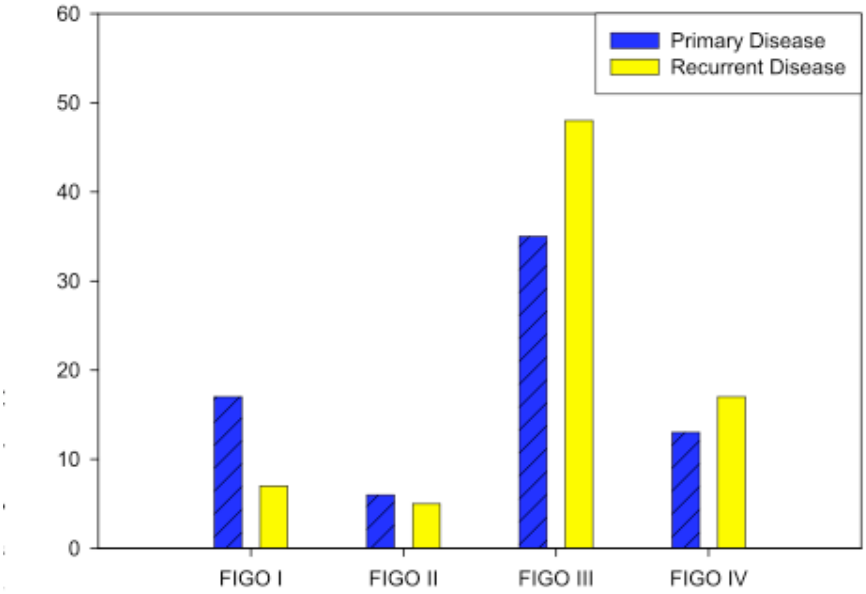


Figure 21 *Incidence of FIGO-Stage depending on recurrent status*

Grading

Only 7.1% of patients that had been diagnosed with primary ovarian cancer had a well-differentiated tumor and 48.6% had a poorly differentiated ovarian carcinoma. Of the group with relapsed ovarian cancer, 10.4%, 37.7% and 51.9% of the patients had a well, good and undifferentiated tumor, respectively (Fig. 22).

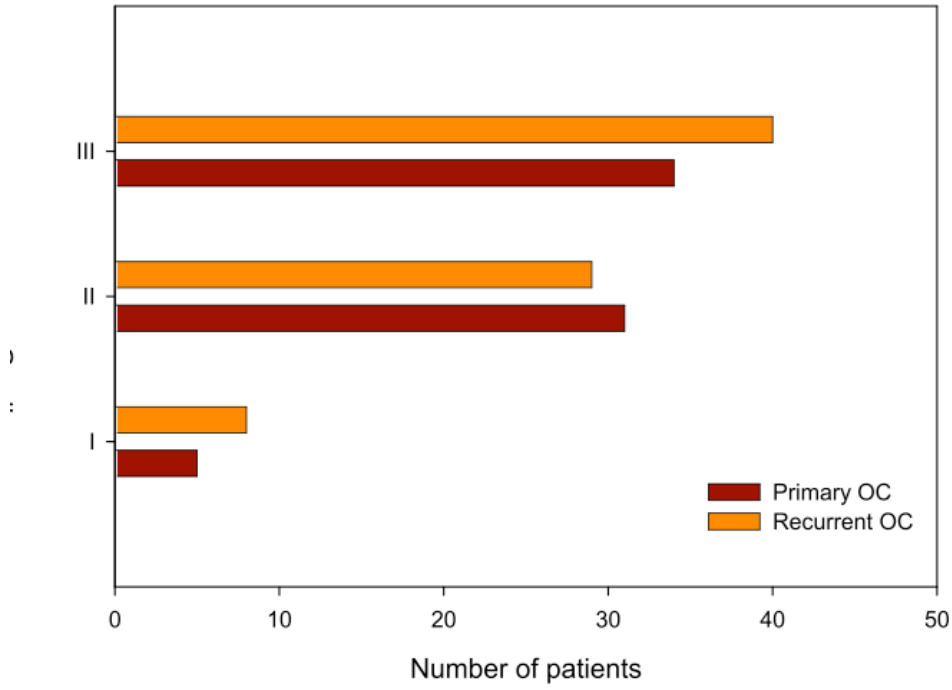


Figure 22 *Grading distribution of patients with primary and recurrent ovarian cancer*

Lymph node involvement

Of patients with primary and recurrent ovarian cancer 31.4% (22) and 59.7% (46), respectively, had no determined lymph nodes involvement. Negative lymph nodes were present in 44.3% (31) and only 10.4% (8) patients with primary and recurrent disease, respectively (Fig. 23-24).

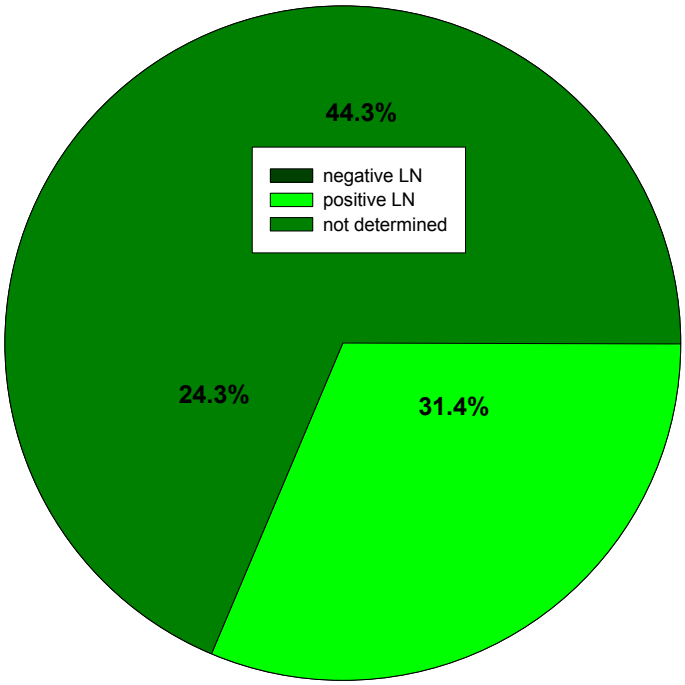


Figure 23 *Lymph nodes involvement in patients with primary disease*

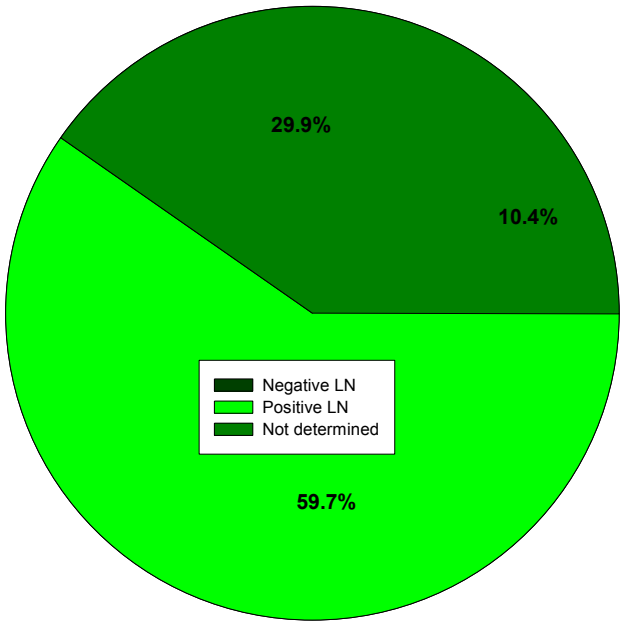


Figure 24 *Lymph nodes involvement in patient with relapsed ovarian carcinoma*

CA-125

Most of the patients had increased levels of CA-125 levels. The results are illustrated below (Fig. 25).

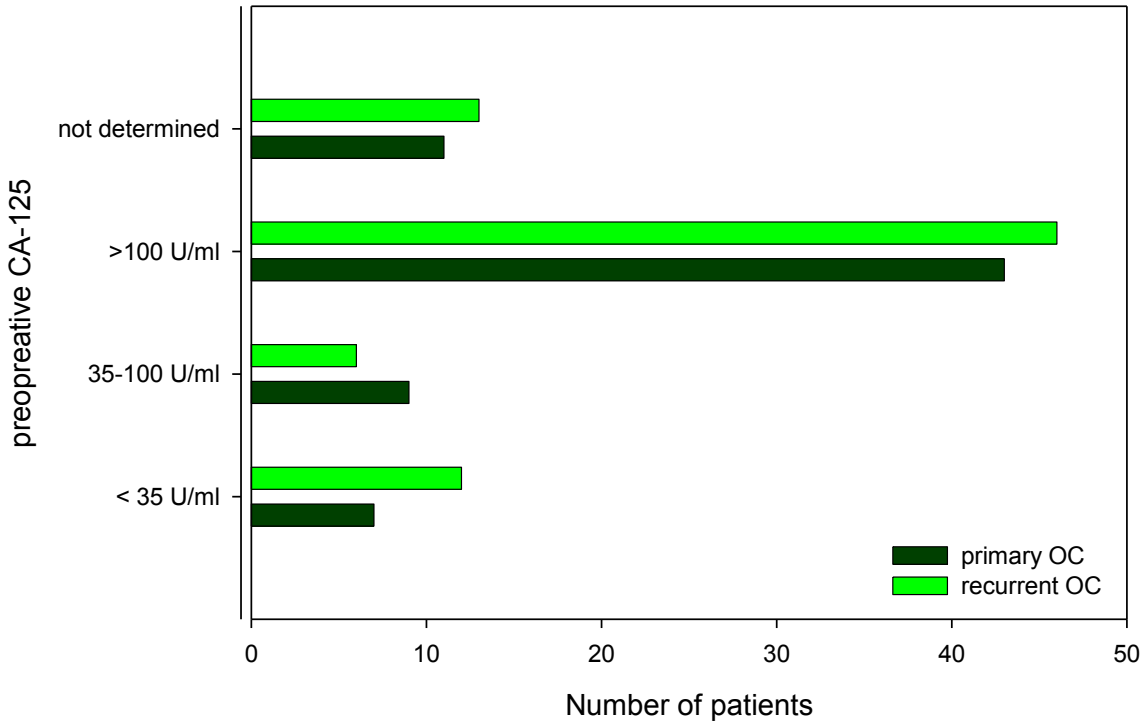


Figure 25 *Preoperative CA-125 serum levels in primary and recurrent ovarian cancer*

Ascites

Overall, 55 (37.4%) patients had no ascites at the time of diagnosis. The presence of ascites was classified into less than or equal to 500 ml, which was the case in 54 (36.7%) patients and more than 500 ml in 38 (25.9%) patients

Among the patients with primary ovarian cancer, 18 (25.7%) patients had no ascites and 24 (34.3%) had more than 500 ml peritoneal fluid. 37 (48.1%) patients with relapsed ovarian cancer had no ascites. Only 14 (18.2%) patients had more than 500ml ascites (Fig. 26, 27).

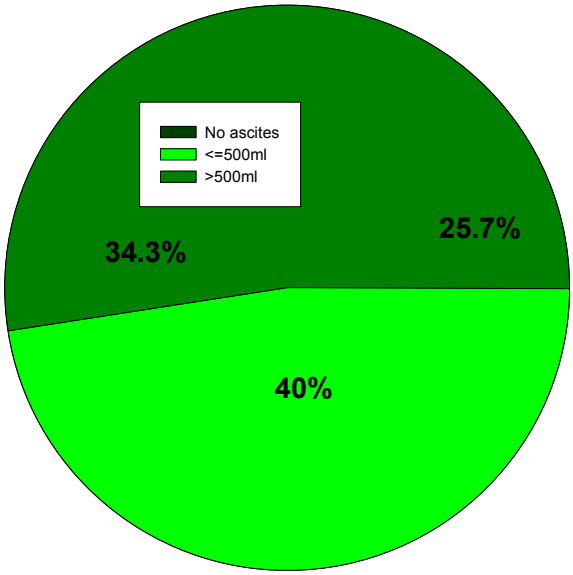


Figure 26 *Ascites volume in patients with primary OC*

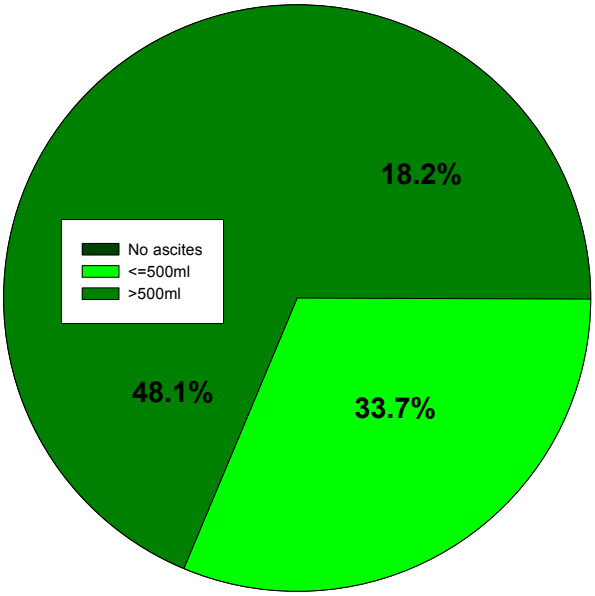


Figure 27 *Ascites volume in patients with recurrent OC*

3.2.2 The IL-10 promoter polymorphism in ovarian cancer group

IL-10 gene was analyzed by using the pyrosequencing method (Fig. 28-35).

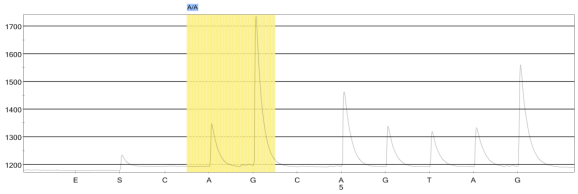


Figure 28 *-1081A/A genotype*

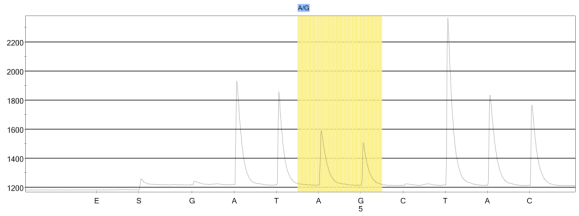


Figure 29 *-1081G/A genotype*

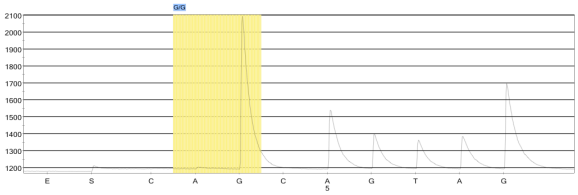


Figure 30 *-1081G/G genotype*

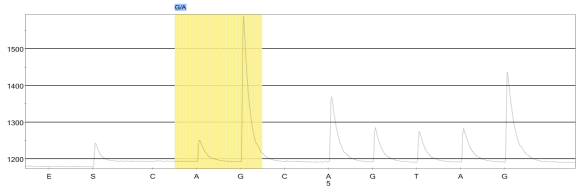


Figure 31 *819C/C genotype*

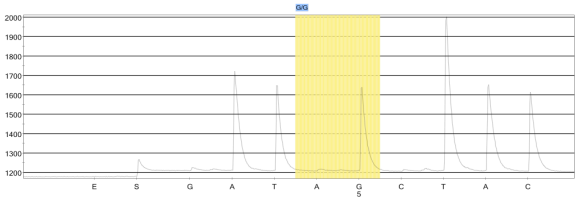


Figure 32 *-819C/T genotype*

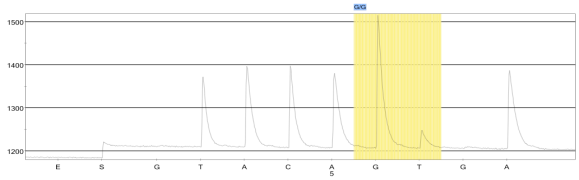


Figure 33 *-592C/C genotype*

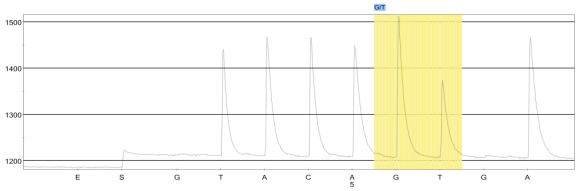


Figure 34 *-592C/A genotype*

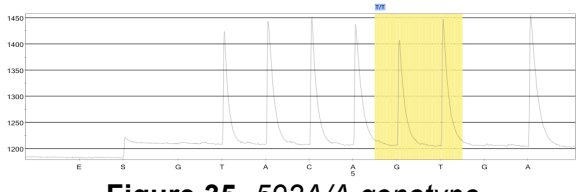


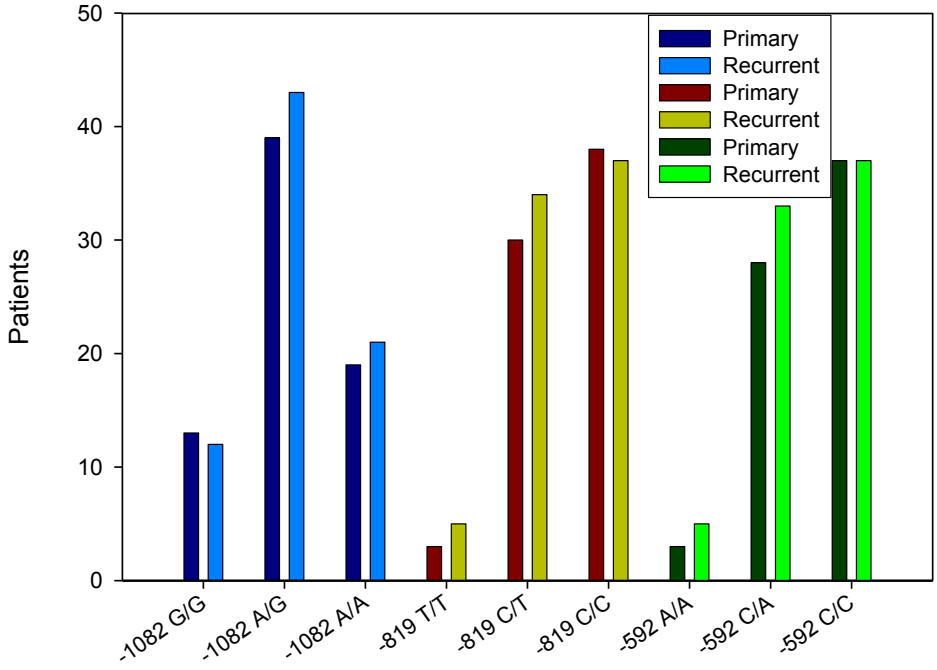
Figure 35 *-592A/A genotype*

The allele distribution in the three determined loci showed no statistically significance between the primary and recurrent ovarian cancer: p=0.896, p=0.717 and p=0.753 for -1082, -819 and -592 loci, respectively (Fig. 36).

No significant association was found in analyzing the distribution of most frequently encountered alleles in Caucasians vs. other polymorphisms: -1082 GG vs -1082 GA*AA, -819 CC vs. -819 CT*TT and -592 CC vs. -592 CA*AA, respectively (Tab. 23).

Table 23 *Allelic distribution in primary and recurrent OC: most common allele vs. others*

Allelic distribution	Primary OC (%)	Recurrent OC (%)	p-value	OR (95% CI)
-1082 GG	13	12	0.65	1.21 (0.51-2.88)
-1082 GA*AA	57	64		
-819 CC	38	37	0.49	1.25 (0.65-2.39)
-819 CT*TT	32	39		
-592 CC	37	37	1.22	1.22 (0.63-2.36)
-592 CA*AA	31	38		



The distribution of allele in primary and recurrent ovarian cancer

Figure 36 *The distribution of allele in primary and recurrent ovarian cancer*

3.2.3 IL-10 promoter polymorphism in ovarian cancer patients and in the control group

No significant correlation was found between the genotype frequencies in the individual loci studied in OC and CG patients. The distribution of the polymorphisms of IL-10 -1082, -819 and -592 is summarized in the table 24. There were no differences in either patient collectives.

The most frequently encountered genotype in the ovarian cancer group was the heterozygote one for the -1082 and -819 positions and the -592C/C genotype.

Table 24 *Allele frequencies in IL-10 gene in women with ovarian cancer and in the control group*

Analyzed polymorphism		Ovarian Cancer	Control Group	p value
IL-10 -1082	G/G	17%	20%	0.950
	G/A	55.8%	56.3%	
	A/A	27.2%	28.1%	
IL-10 -819	C/C	51%	60.2%	0.312
	C/T	43.5%	37.2%	
	T/T	5.4%	4.7%	
IL-10 -592	C/C	51.7%	59.6%	0.437
	C/A	42.7%	35.1%	
	A/A	5.6%	5.3%	

The GCC, ATA, ACC genotypes were observed only in 42 patients in the cancer group and 34 in the control group. No significant difference was found inbetween the distribution of these genotypes. The GCC haplotype was the most frquently encountered one, 25 malignant patients and 20 women from the control group carried this genetic factor, the so called the high-producer genotype. The ATA haplotype was found in 8 ovarian cancer patients and in 6 women from the control group. As for the ACC genotype, 9 patients from the cancer group and 8 patients from the control group carried it. The GTA haplotype, also known as being extremely rare, was not observed in our study, nor in the oncological patients nor in the control group (Fig. 37).

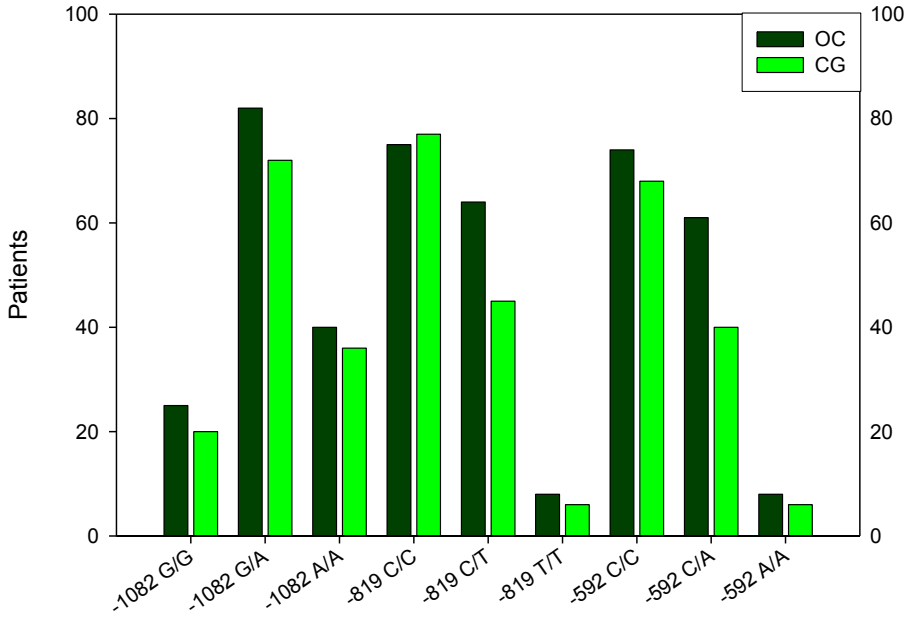


Figure 37 *The distribution of alleles in IL-10 gene in both groups*

Allele distribution in loci -819 and -592 was in linkage disequilibrium; -819 C and -592C were associated, and the same was true for -819T and -592A.

3.2.4 Correlation between IL-10 promoter polymorphism and the established prognostic factors

IL-10 polymorphism in -819 and -592 was significantly associated with the amount of postoperative tumor mass (p=0.036 and p=0.035). C/C allele was more frequently observed in both loci in the patients with optimal cytoreductive surgery, whereas the heterozygote genotype (C/T and C/A respectively) was more frequently encountered in the group with postoperative tumor residuals. When counting the association between polymorphisms and residual mass larger or smaller than 1cm, the same genotype is more frequently observed in the group with residual tumor mass less than or equal to 1 cm in size, for both loci (p=0.010 and p=0.009)(Fig. 38). The presence of A allele in the -1082 region of the IL-10's gene seems to limit the ability to achieve an optimal surgical outcome (p=0.058 G/G vs. A/G*A/A). This observation reached no statistical significance (Tab.25-26).

The patients, who did not benefit from radical surgery, presented more often the heterozygote genotype for the -819 and -592 positions. The presence of T and A alleles in -819 and -592 loci was associated with unsatisfactory surgical results, p=0.009, p=0.010.

Table 25 *IL-10 polymorphism correlates with residual mass*

IL-10 gene loci	Allele	Tumor free patients (%)	Residual mass (%)	P	95% CI
	G/G	18 (22.2%)	7 (10.6%)		
IL-10 -1082	G/A	45 (55.6%)	37 (56%)	0.084	0.074-0.085
	A/A	18 (22.2%	22 (33.4%)		
	C/C	49 (60.5%)	26 (39.4%)		
IL-10 -819	C/T	29 (35.8%)	35 (53%)	0.036	0.007-0.011
	T/T	3 (3.7%)	5 (7.6%)		
	C/C	48 (61.5%)	26 (40%)		
IL-10 -592	C/A	27 (34.6%)	34 (52.3%)	0.035	0.007-0.010
	A/A	3 (3.8%)	5 (7.7%)		

Table 26 *Presence of A, T and A allele in the -1082, -819 and -592 positions reduce the possibility of achieving optimal surgical result*

IL-10 gene loci	Allele	Tumor free patients (%)	Residual mass (%)	P	OR (95% CI)
IL-10 -1082	G/G	18 (22.2)	7 (10.6)	0.058	1.4 (1.04-1.89)
	A/G*A/A	62 (77.8)	59 (89.4)		0.57 (0.29-1.10)
IL-10 -819	C/C	49 (60.5)	26 (39.4)	0.009	1.49 (1.09-2.04)
	T/C*T/T	32 (39.5)	40 (60.6)		0.61 (0.42-0.89)
IL-10 -592	C/C	48 (61.5)	26 (40)	0.010	1.49 (1.08-2.04)
	A/C*A/A	30 (38.5)	39 (60)		0.62 (0.42-0.90)

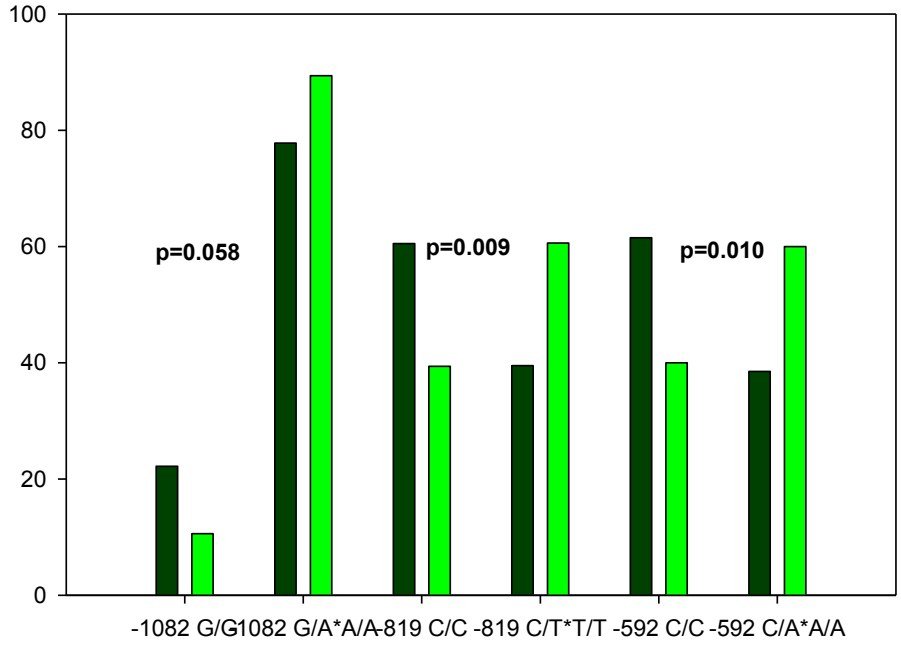


Figure 38 *The IL-10 polymorphism and the possibility of performing cytoreductive surgery*

No association was found between the polymorphisms of IL-10 and the classical clinical prognosis factors such as FIGO stage, histological type, grading, age at diagnosis, recurrence status and lymph node involvement. The results are summarized in table nr. 27.

Table 27 *Association between clinical prognosis factors and the cytokine's gene polymorphisms*

Clinical prognosis factor	**IL-10 -1082**	**IL-10 -819**	**IL-10 -592**
FIGO stage I+II vs. III+IV	0.974	0.441	0.221
Grading I+II vs. III	0.730	0.556	0.571
Histological type (serous-papillary vs. others)	0.173	0.184	0.200
Age at diagnosis	0.950	0.691	0.642
Lymph nodes involvement	0.256	0.201	0.287
Presence of ascites	0.848	0.889	0.879
Reccurrence Status	0.241	0.073	0.088
Residual tumor mass: None vs. Other	0.103	0.036	0.035

When the analysis was performed separately for the primary and recurrent ovarian cancer groups , the presence of C allele in loci -819 and -592 correlated with the surgical outcome only in patients with recurrent disease, p=0.012 and p=0.015, respectively. No other significant association was found.

3.2.5 Correlation between IL-10 promoter polymorphism and the residual tumor mass and overall survival

In the univariate analysis, the genotype C/C of IL-10 gene in -819 and -592 loci was associated with a significantly better overall survival (*p*=0.039 and *p*=0.044, respectively). The presence of the same genotype was associated with a significantly better disease free survival in patients with ovarian cancer (p=0.034) for the -592 locus. The presence of the T or A allele was associated with poor prognosis factor for the patients in the OC group. The IL-10 polymorphism in -1082 locus had no significant impact on the overall and disease-free survival (p=0.067 and p=0.068, respectively)(Fig. 39-44).

Statistical significance wasn't reached when the cohorts of patients with primary and relapsed ovarian cancer were summarized separately (Tab. 28).

Table 28 *The univariate overall and disease free survival results in the two cohorte of oncological patients*

Analyzed loci	Tumor free survival (*p*)	Overall survival (*p*)
	Primary ovarian cancer	
IL-10 -1082	0.341	0.081
IL-10 -819	0.115	0.169
IL-10 -592	0.125	0.165
	Recurrent ovarian cancer	
IL-10 -1082	0.469	0.271
IL-10 -819	0.178	0.115
IL-10 -592	0.208	0.131

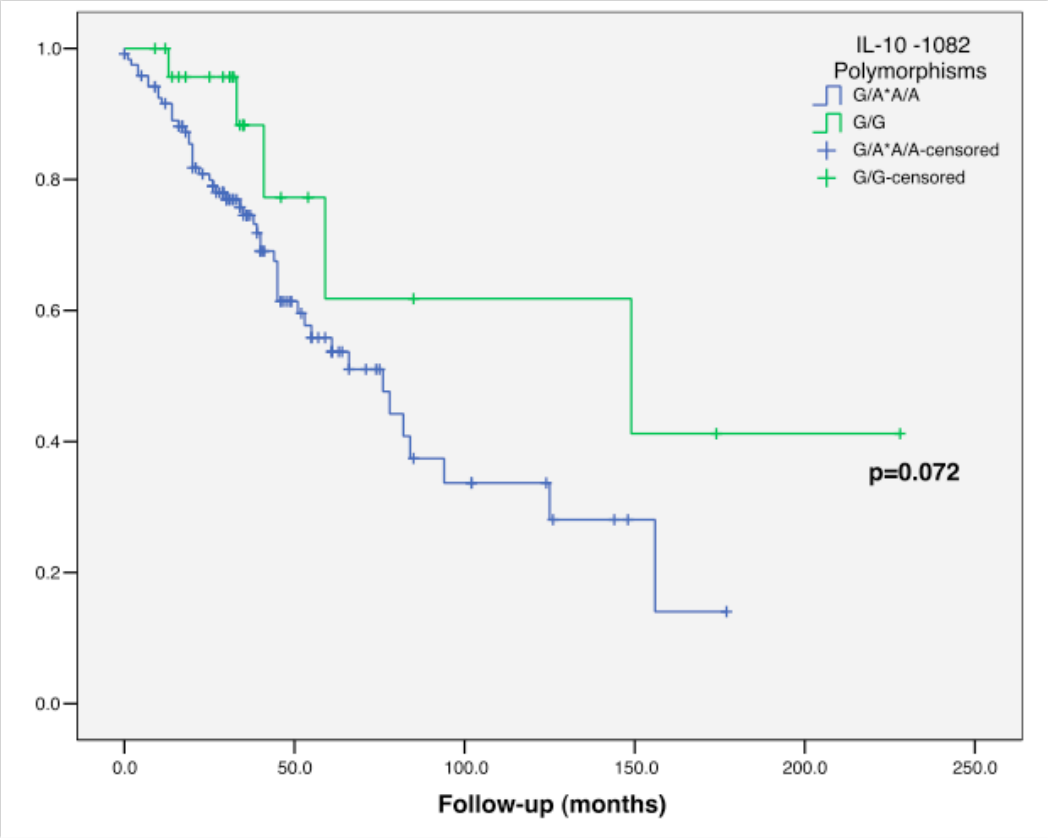


Figure 39 *Overall survival and -1082 polymorphism*

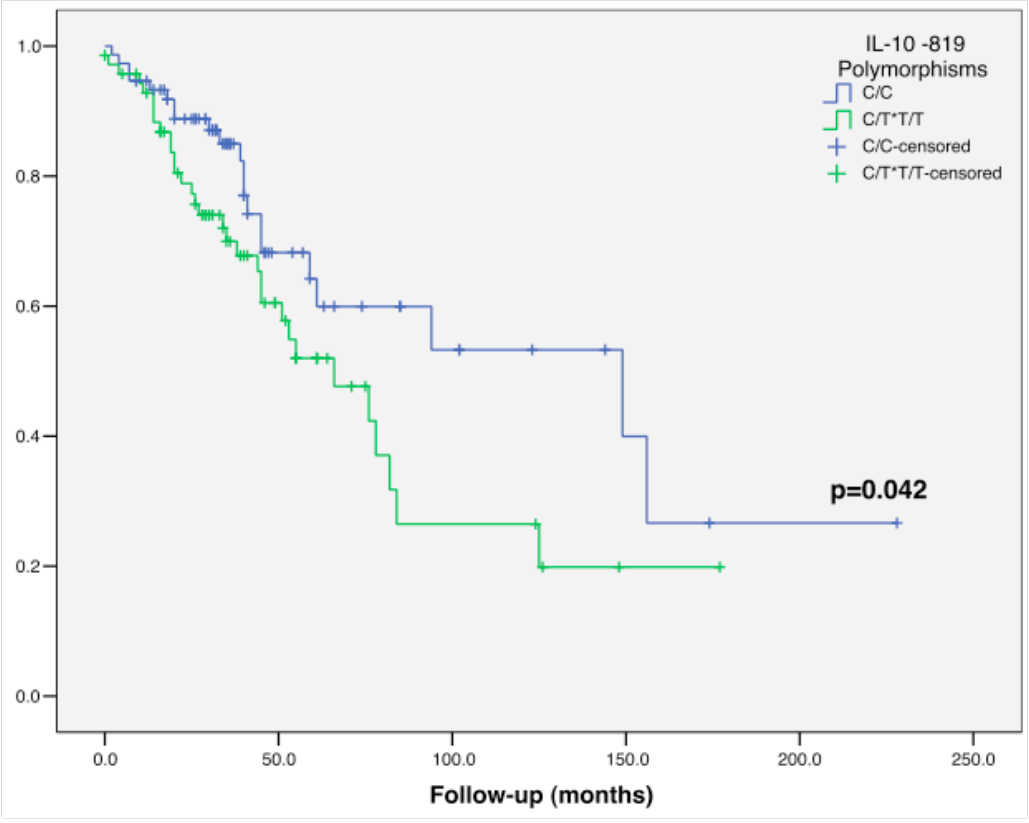


Figure 40 *Overall survival and -819 polymorphism*

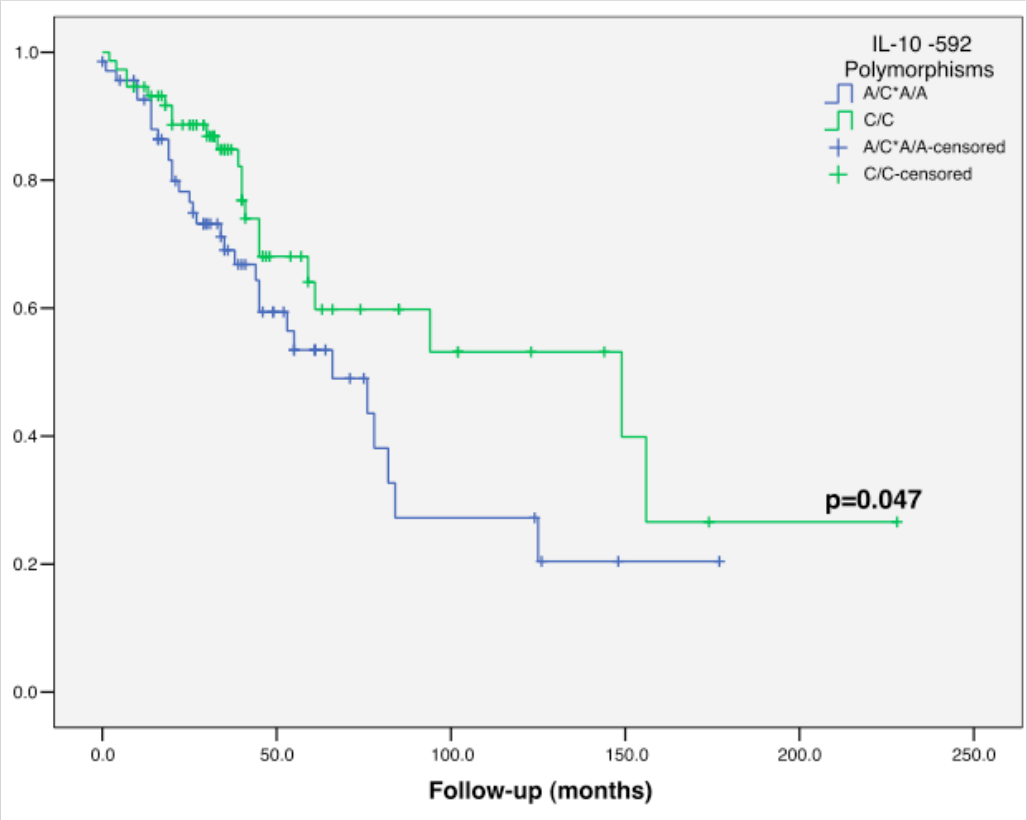


Figure 41 *Overall survival and -592 polymorphism*

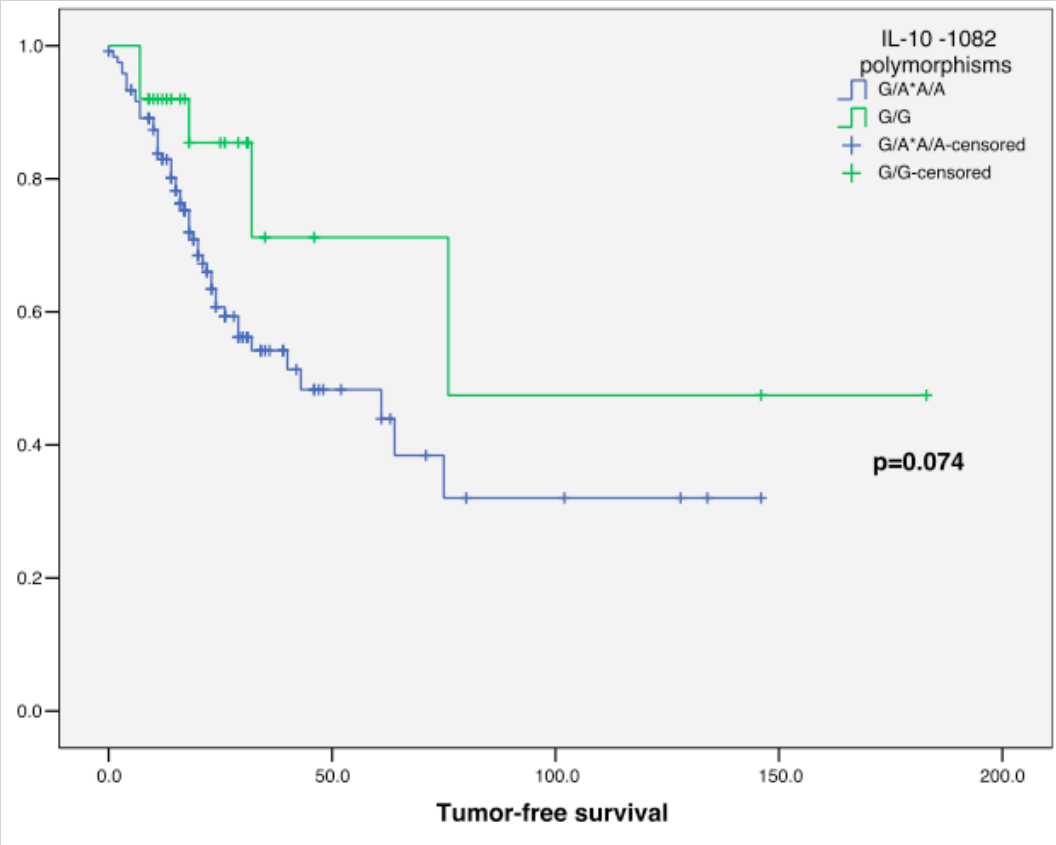


Figure 42 *Disease free survival and -1082 polymorphism*

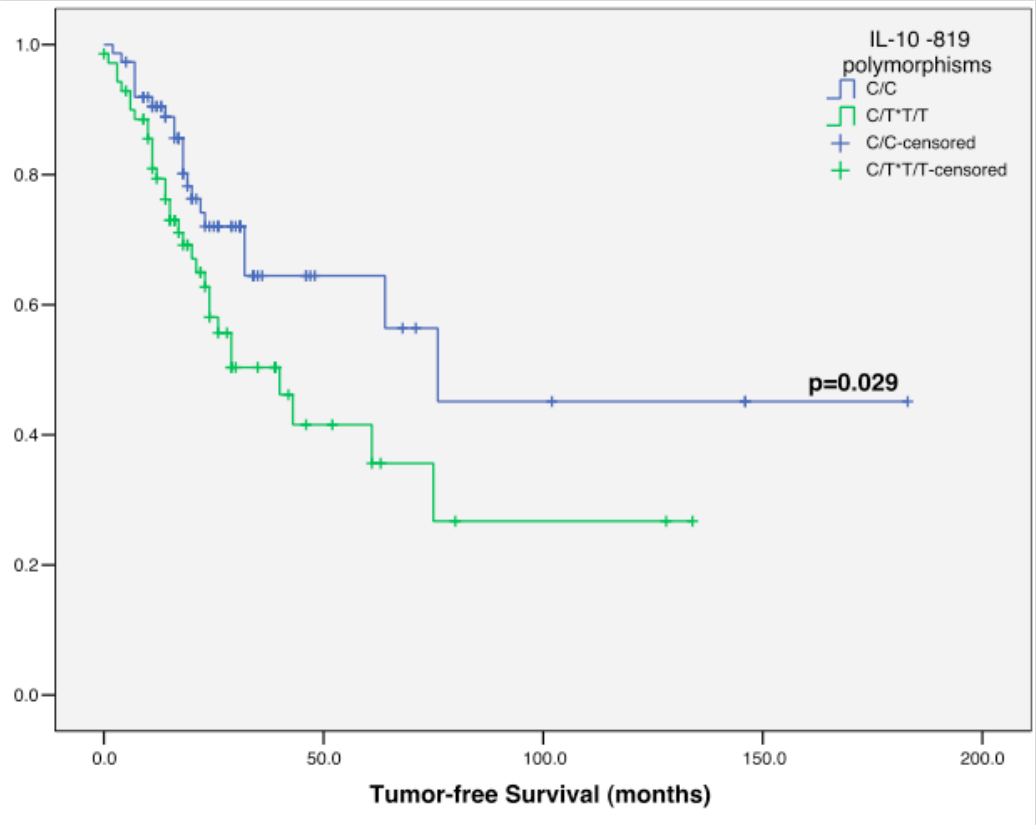


Figure 43 *Disease-free survival depending on -819 polymorphism*

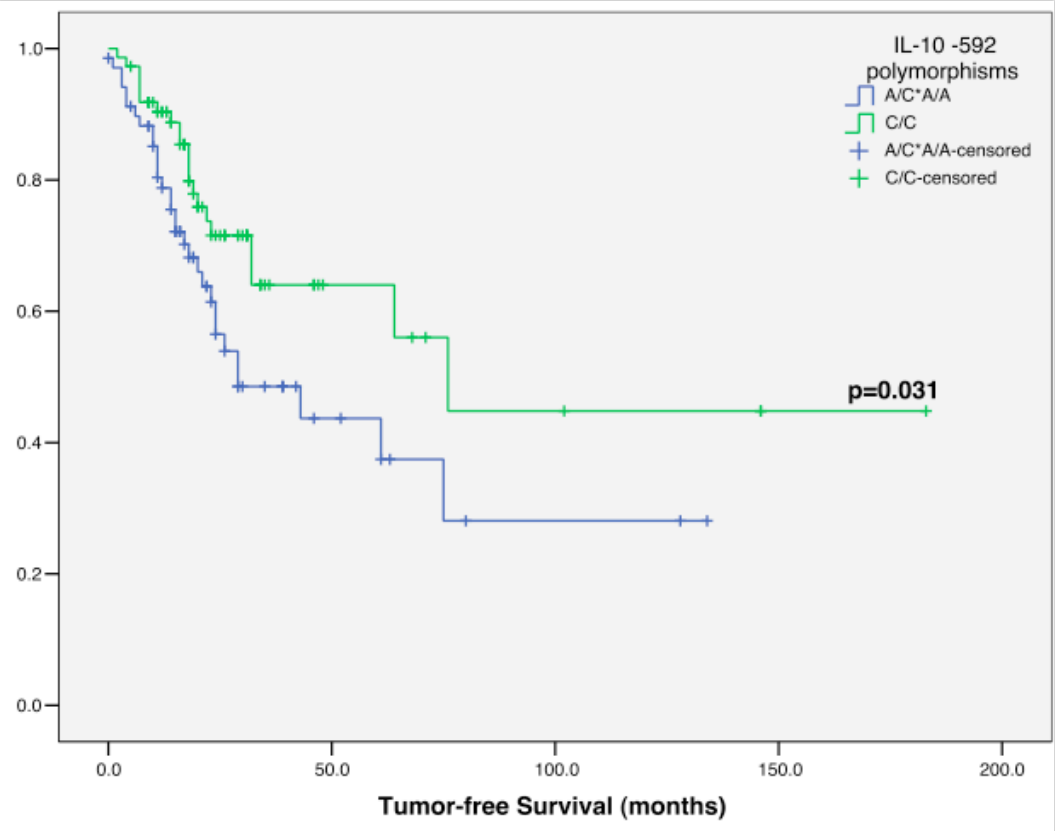


Figure 44 *Disease-free survivals depending on -592 polymorphism*

In the multivariate analysis, ascites volume and residual mass were the only independent prognostic factors for poor overall survival (Tab. 29).

Table 29 *The Kaplan-Meyer analysis and the multivariate Cox Regression for the prognostics factors in ovarian cancer*

	Tumor free survival			Overall survival		
	Univariate	Multivariate		Univariate	Multivariate	
	p*	HR (95%CI)	p†	P*	HR (95%CI)	P†
IL-10 -1082	0.076	-	-	0.087	-	-
IL-10 -819	0.052		0.16	0.037	0.74 (0.40-1.37)	0.34
IL-10 -592	0.059			0.037		
FIGO	0.03	-	-	0.002	-	-
Histology	0.94	-	-	0.74	-	-
Grading	0.3	-	-	0.44	-	-
Residual Mass	0.0001	0.14 (0.04-0.47)	0.001	0.0001	1.93 (0.06-0.84)	<0.001
Lymph nodes	0.02	-	-	0.16	-	-
Ascites	0.05	0.65 (0.32-1.32)	0.24	0.0005	1.98 (0.15-0.69)	<0.001
Age	0.25	-	-	0.05	-	-
CA125	0.11	-	-	0.11	-	-

*Log Rank Test

†MultivariateCox Regression

3.3 Correlation between the expression and the polymorphism of IL-10 in patients with ovarian cancer

3.3.1 Patients' characteristics

Expression and polymorphism of IL-10 were determined in 62 women with ovarian cancer and 18 patients from the control group. The control group comprised women with no malignancy. The median age at diagnosis in the control group was 48 years (range 38-74). Nine (59%) of the patients were diagnosed with benign ovarian tumor, three patients (16.7%) with endometriosis, three with benign ovarian cyst and the remaining thee were healthy donors.

The median age at diagnosis in the ovarian cancer group was 56 years (range 19-81). Half of the patients (31) presented a primary ovarian cancer, the other half of them were diagnosed as having recurrence. Most of the tumors were serous-papillary carcinoma, 4 patients each (6.6%) had other histological types, such as mucinous, endometrioid and undifferentiated tumors. Most of the women in the study group had an advanced tumor stage, 55% FIGO III and 26.7% FIGO IV. Four patients presented well differentiated tumors, histological grading II was presented in 45.8% of the cases and grading III in 47.5%.

Eleven patients did not have ascites at the time of diagnosis, 25 patients had less than 500 ml peritoneal fluid and 25 women more than 500 ml.

Most of them benefited from curative surgery, 50% were macroscopically tumor free after surgery, and 33.3% had a residual mass less than 1 cm. By contrast, 16.7% had a residual mass larger than 1 cm. In most of the cases (69.4%), recurrence after the operation was observed (Tab. 30).

Table 30 *Characteristics of the cancer group*

PARAMETERS	N	%
Follow-up (month)	Median 27.0 / Mean 38.96	Range 0-225
Age at first diagnosis Mean OC (range) CG (range)	51.17 (24-76) 51.17 (24-76)	
Tumor type • primary ovarian cancer • recurrent ovarian cancer	 31 31	 50 50
Histology • serous-papillary • mucinous • endometroid • undifferentiated	 49 4 4 4	 80.2 6.6 6.6 6.6
FIGO • St. I • St. II • St. III • St. IV	 6 5 33 16	 10 8.3 55 26.7
Grading • I • II • III	 4 27 28	 6.8 45.8 47.5
Ascites • no ascites • ≤500 ml • >500 ml	 11 25 25	 18 41 41
Postoperative residual tumor mass • macroscopically tumor free • residual tumor mass	 31 30	 50.6 49.4
LN Status • positive • negative • not known	 14 13 35	 22.6 21 56.5
Recurrence status • no recurrence • recurrence after operation	 19 43	 30.6 69.4

3.3.2 Impact of IL-10 polymorphisms on the expression in serum and ascites

Only one patient from the control group presented the GG genotype, most of the women, 11 (61.11%) in this group, presented the heterozygote genotype and six of them (33.33%) showed only the A allele. In patients having an ovarian malignancy, most of the women, 34 (54.83%) presented the heterozygote genotype, twelve (19.35%) of them only the G allele and 16 (25.80%) women the AA genotype.

The following tables show the incidence of the polymorphisms in the collective of patients and the associated concentrations (Tab. 3-32).

Table 31 *Incidence of polymorphism and associated expression in ovarian cancer group*

IL-10 Genotypes		IL-10 pg/ml	Mean	Median	Minimum	Maximum	Mean standard error
-1082	GG	Ascites	44.94	33.03	7.8	134.02	9.97
	n=12	Serum	12.71	8.59	7.8	40.29	2.79
	GA	Ascites	73.39	38.48	7.8	389.48	16.93
	n=34	Serum	13.37	10.55	7.8	46.27	10.55
	AA	Ascites	109.74	88.19	7.8	344.32	25.37
	n=14	Serum	35.09	12.54	7.8	256.133	12.54
-819	CC	Ascites	70.83	37.11	7.8	389.48	16.22
	n=32	Serum	14.77	10.67	7.8	46.27	1.71
	CT	Ascites	80.79	56.18	11.61	386.71	17.5
	n=28	Serum	21.16	9.98	7.8	256.13	8.79
	TT	Ascites	47.87	47.87	7.8	87.95	40.07
	n=2	Serum	27.10	27.10	12.18	42.02	14.91
-592	CC	Ascites	70.83	37.11	7.8	389.48	16.22
	n=32	Serum	14.77	10.67	7.8	46.27	1.71
	CA	Ascites	83.45	56.63	12.73	386.71	17.98
	n=27	Serum	20.49	9.87	7.8	256.13	8.79
	AA	Ascites	47.87	47.87	7.8	87.95	40.07

	n=2	Serum	27.10	27.10	12.18	42.02	14.91

Table 32 *Incidence of polymorphism and associated expression in control group*

IL-10 Genotypes		**IL-10 pg/ml**	**Mean**	**Median**	**Minimum**	**Maximum**	**Mean standard error**
-1082	GG	Ascites					
	n=1	Serum (n=1)			7.8	7.8	
	GA	Ascites (n=4)	47.91	47.57	7.8	88.72	18.26
	n=9	Serum (n=9)	15.55	9.38	7.8	62.80	5.94
	AA	Ascites (n=2)	44.99	44.99	22.9	57.08	12.08
	n=5	Serum (n=5)	8.05	7.85	7.8	9.06	0.25
-819	CC	Ascites (n=4)	47.91	47.57	7.8	88.72	18.26
	n=8	Serum (n=8)	16.4	9.48	7.8	62.8	6.67
	CT	Ascites (n=1)			57.08		
	n=5	Serum (n=5)	7.99	7.8	7.8	8.75	0.19
	TT	Ascites (n=1)			32.09	32.09	
	n=2	Serum (n=2)	8.43	8.43	7.8	9.06	0.63
-592	CC	Ascites (n=4)	47.91	47.57	7.8	88.72	18.26
	n=8	Serum (n=8)	16.4	9.48	7.8	62.8	6.67
	CA	Ascites (n=1)			57.08		
	n=5	Serum (n=5)	7.99	7.8	7.8	8.75	0.19
	AA	Ascites (n=1)			32.9		
	n=2	Serum (n=2)	8.43	8.43	7.8	9.06	0.63

The polymorphisms of IL-10 correlated significantly with the serum concentration within the ovarian cancer group and the control group (p=0.001) (Fig. 45-47).

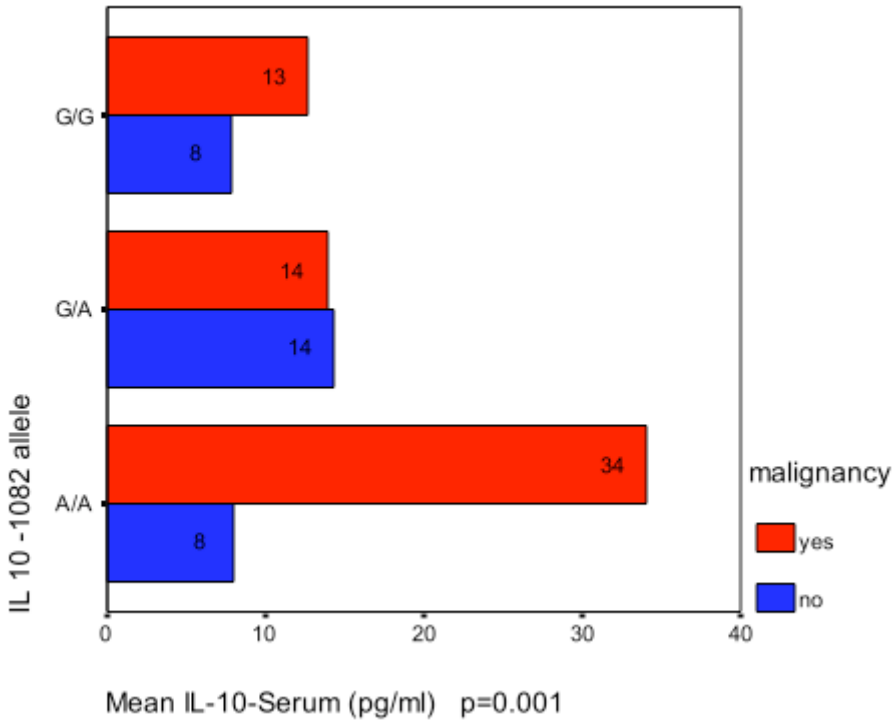


Figure 45 *The A and G distribution in -1082 loci and the associated IL-10 concentrations in serum of OC and CG*

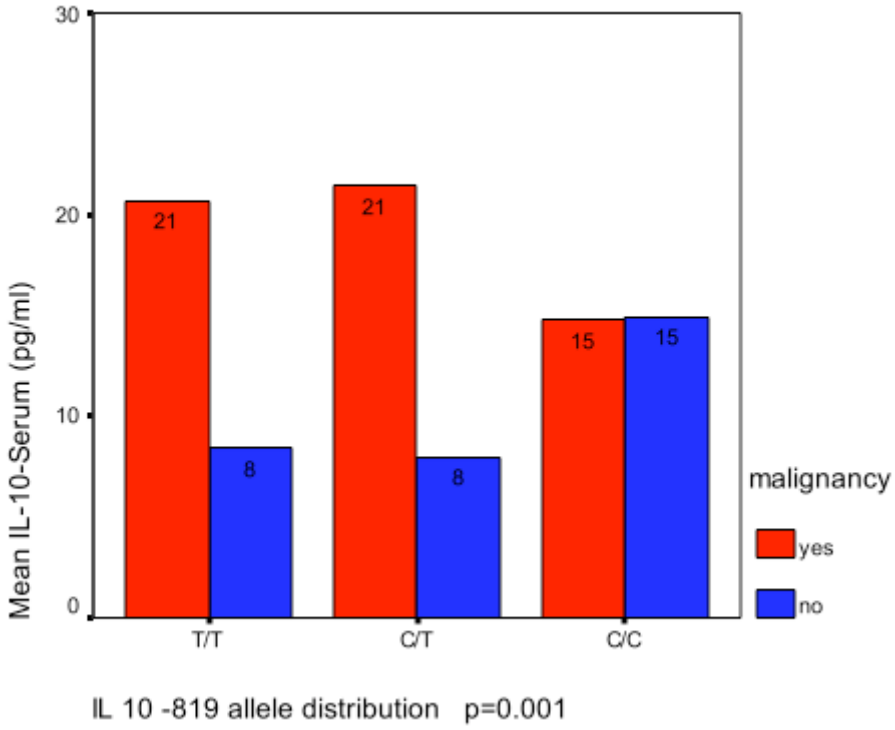


Figure 46 *The C and T distribution in -819 loci and the associated IL-10 concentrations in serum of OC and CG*

The presence of the A/A allele at -1082 position was associated with higher IL-10 expression in patients with ovarian cancer and with the lowest expression in the control group (Fig. 46).

In the -819 position of IL-10 gene, the presence of C/C allele is associated with high circulatory IL-10 levels in the control group and with lower expression in patients with ovarian cancer. The presence of the heterozygote genotype determines the highest IL-10 level in malignant women (Fig. 47).

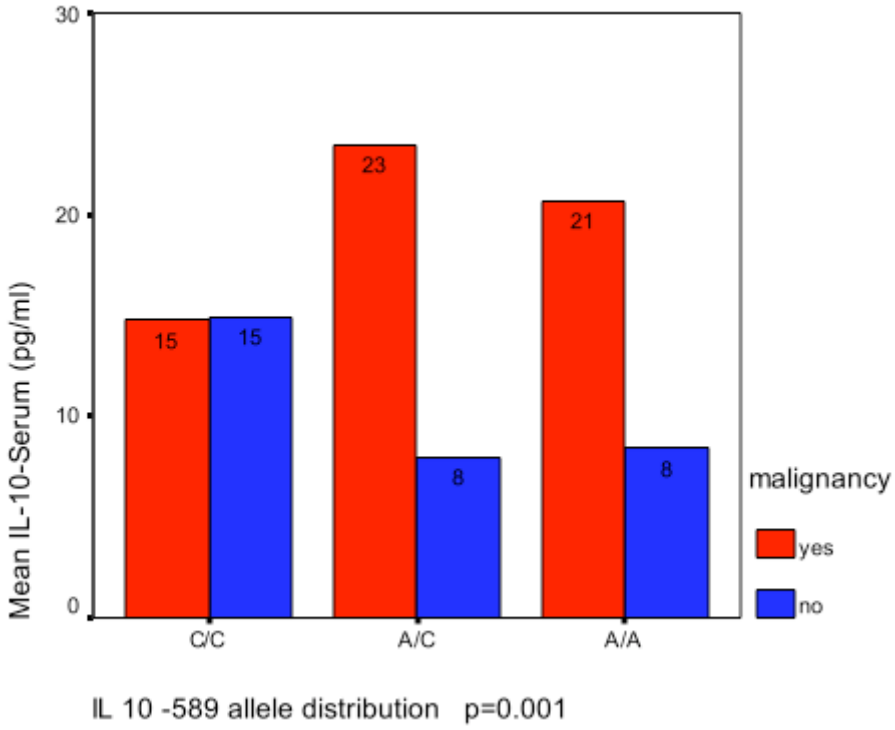


Figure 47 *The C and A distribution in -592 loci and associated IL-10 concentrations in serum of OC and CG*

The genotyping of the -589 locus revealed that in the control group the presence of only the C allele is associated with the highest levels of IL-10 in serum. In contrast, the ovarian cancer patients showed high expression in association with the A/C genotype, the C/C genotype being accompanied by the lowest IL-10 level (Fig. 49). There were differences in the association of allelic distribution in all three positions with IL-10 serum concentrations in the ovarian cancer group and the control group.

A significant correlation between the serum levels (p=0.019) and the allele frequency in -1082 locus was found in the ovarian cancer group. The polymorphisms seem to influence the expression of IL-10 in ascites, although this correlation reached

no statistical significance (p=0.053). The presence of A/A in -1082 position was associated with the highest expression in serum and ascites of ovarian cancer patients (Fig. 48-49).

The mean concentrations of IL-10 in serum were: 12.71 pg/ml (range 7.8-40.29 pg/ml) for the GG-1082 polymorphism, 13.92 pg/ml (range 7.8-65.51 pg/ml) for the heterozygote type and 34.08 (range 7.8-256.13 pg/ml) for the AA polymorphism. As for ascites, the mean concentrations were: 44.94 pg/ml (range 9.17-134.02 pg/ml) for GG polymorphism, 71.96 pg/ml (range 7.8-389.48 pg/ml) for the heterozygote type and 105.94 pg/ml (range 7.8-344.32 pg/ml) for the AA allele.

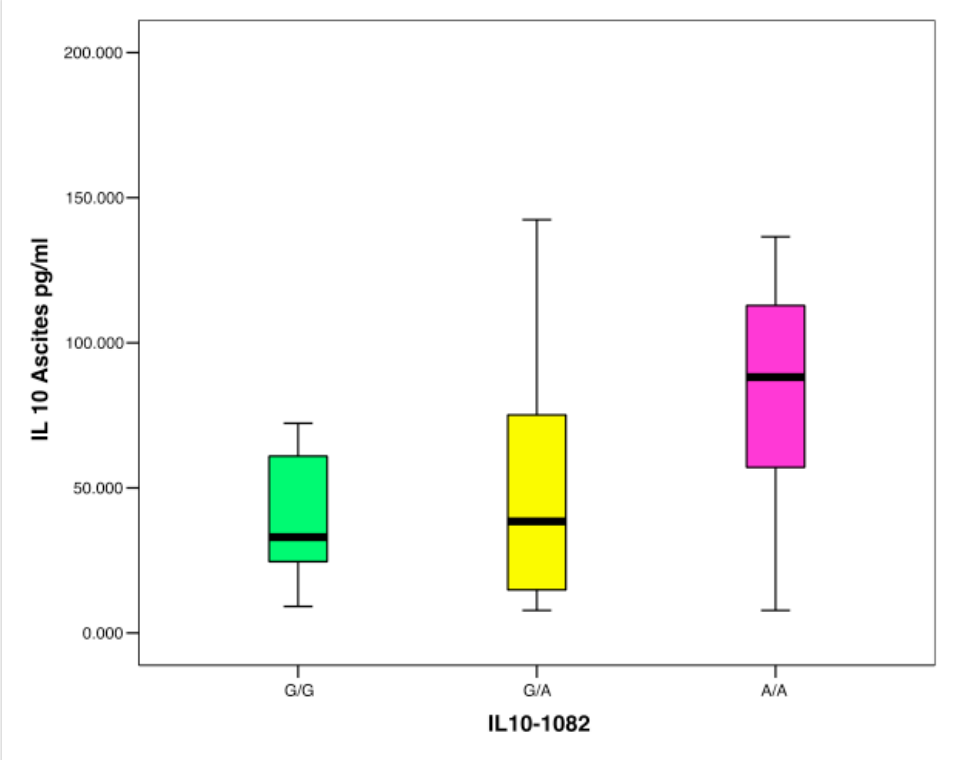


Figure 48 *The impact of IL-10 -1082 polymorphism and expression in ascites from patients with ovarian cancer (p=0.056)*

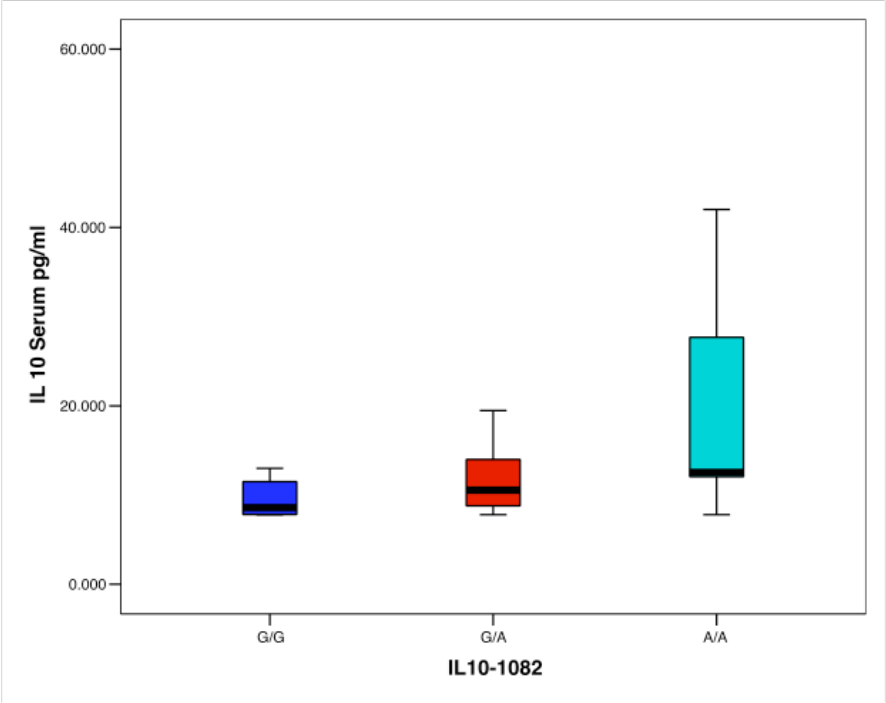


Figure 49 *The impact of IL-10 -1082 polymorphism on the expression in serum from patients with ovarian cancer (p=0.019)*

These analyses reached no statistical significance when the different tumor entities were studied. For the primary and recurrent ovarian cancer patients there were no statistically significant association between the polymorphisms and the expression of IL-10 in ascites and serum (Tab. 33).

Table 33 *Correlation between expression of IL-10 and promoter polymorphisms*

Locus	Concentration of IL-10	Significance (p)
	Primary OC	
-1082	Ascites	0.19
	Serum	0.065
-819	Ascites	0.92
	Serum	0.22
-592	Ascites	0.80
	Serum	0.27
	Recurrent OC	
-1082	Ascites	0.11
	Serum	0.31
-819	Ascites	0.056
	Serum	0.37
-592	Ascites	0.056
	Serum	0.37

3.4 The impact of tumor debulking on the expression of IL-10

3.4.1 Patients' characteristics

From January 2003 to November 2003, blood was collected from 27 women with a histological confirmed ovarian cancer. Blood was drawn one day before surgery in the conditions mentioned before. Blood collection and IL-10 serum measurement were repeated 24 hours, four and eight days, after operation.

Median age at diagnosis among the patients was 56 years (range 19-84). The median follow-up was 30 months (range 3-225). Sixteen patients (59.3%) reached the end of the study.

Most of the patients (70.4%) had recurrence, 29.6% had primary ovarian cancer. Also advanced disease was encountered in the majority of cases. The stage FIGO III was found in 40.7% of the patients and FIGO IV in 33.3%. Only 3 patients (11.1%) were diagnosed as having a FIGO stage I carcinoma. The most common histological type was serous-papillary (77.8%) (Tab. 34).

Table 34 *Patients characteristics*

Parameters	**Value**
Age at treatment in years, median (range)	56 (19-84)
Tumor status, n (%)	
• primary	8 (29.6)
• reccurence	19 (70.4)
Histology, n (%)	
• serous-papillary	21 (77.8)
• endometroid	3 (11.1)
• mucinous	1 (3.7)
• mixed/others	2 (7.4)
Grading, n (%)	
• II	12 (44.4)
• III	15 (55.6)

FIGO Stage, n (%)	
• I-II	6 (23)
• III-IV	21 (67)
Ascites volume, n (%)	
• none	6 (23.1)
• ≤500 ml	11 (42.3)
• >500 ml	9 (34.6)
Postoperative residual tumor mass, n (%)	
• macroscopically tumor free	10 (37)
• residual mass	17 (63)
Lymph nodes status, n (%)	
• positive (N1)	8 (29.6)
• negative (N0)	18 (66.7)
• not determined (Nx)	1 (3.7)
Follow-up period months (median/range)	30 (3-225)
Tumor-free survival months (median/range)	11 (0-29)

3.4.2 ELISA

Patients showed a temporary increase in IL-10 levels one day after surgery but this was not statistical significant (p=0.58). The levels decreased significantly on the fourth postoperative day (p=0.02) and were lower on the eighth day (p=0.01). The median concentrations of IL-10 in serum prior to and one, four and eight days after surgery were: 7.91pg/ml, 10.75pg/ml, 7.85pg/ml and 7.8pg/ml, respectively (Fig. 50).

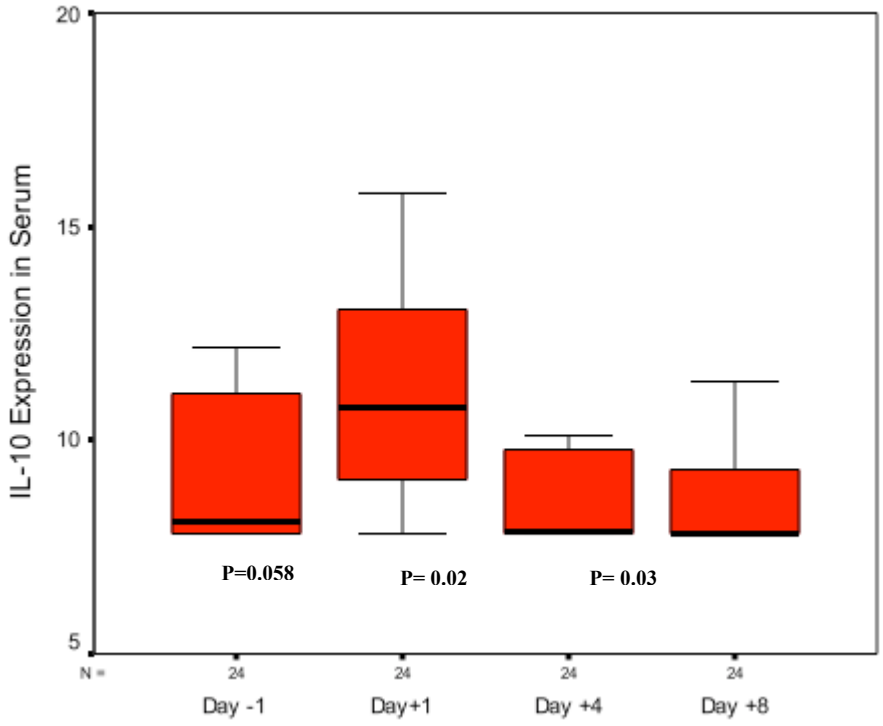


Figure 50 *Serum concentrations of IL-10 modify with surgery*

There was no significant correlation between the expression of IL-10 and the presence or absence of postoperative residual tumor mass: p=0.40 for day -1, p=0.12 for day +1, p=0.84 for day +4 and p=0.60 for day +8 (Fig. 51).

The median concentrations for patients who benefited most from a cytoreductive surgery and no residual tumor mass were: 7.8 pg/ml, 9.05 pg/ml, 7.92 pg/ml, 7.8 pg/ml, in days -1, +1, +4 and +8, respectively. For the patients presenting residual tumor mass, the median concentrations in serum were: 8.13 pg/ml, 10.8 pg/ml, 7.8 pg/ml and 7.8 pg/ml in days -1, +1, +4 and +8 respectively.

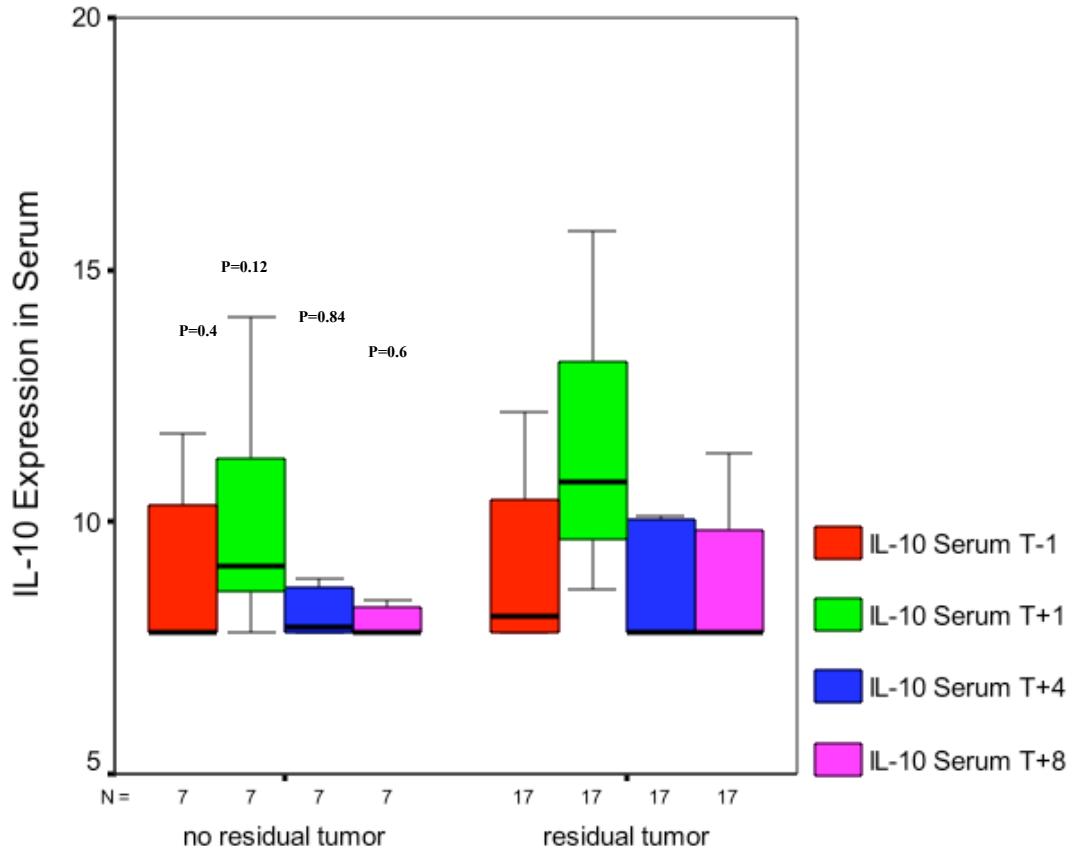


Figure 51 *IL-10 concentrations and the presence of residual tumor*

When comparing the IL-10 expression in serum in patients with primary and relapsed ovarian cancer, no significant association was found between the kinetics of circulatory IL-10 levels prior and after surgery in the two groups (Tab. 35).

Table 35 *IL-10 concentrations and recurrence status*

Day	Ovarian cancer	n	Mean pg/ml	Median pg/ml	Min.	Max.	Significance
T-1	Primary	8	12.95	7.91	7.8	43.72	1
	Recurrent	18	10.88	7.96	7.8	33.44	
T+1	Primary	8	15.99	10.89	7.8	48.07	0.58
	Recurrent	19	9.14	7.8	7.8	16.75	
T+4	Primary	7	9.09	7.8	7.8	13.38	0.73
	Recurrent	19	9.14	7.8	7.8	9.33	
T+8	Primary	6	8.09	7.8	7.8	9.33	0.41
	Recurrent	18	8.71	7.88	7.8	11.36	

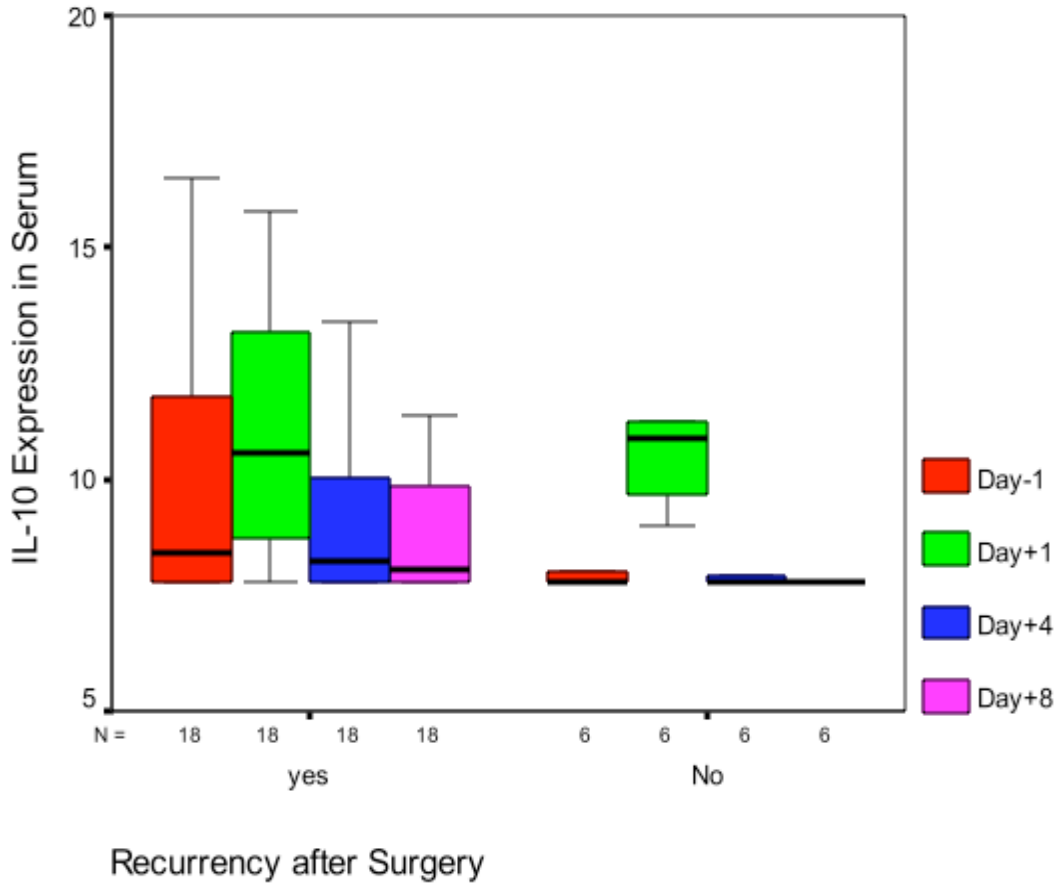


Figure 52 *IL-10 concentrations and the presence of recurrence after surgery*

No association was found between the expression of IL-10 before and after surgery and other established clinical prognostic factors such as: lymph node involvement, FIGO stage, histology, residual tumor mass and platinum resistance (Tab. 36).

Tumor grading seems to influence significantly the expression of IL-10. In the group of patients with well differentiated tumor, the median circulatory levels of IL-10 were 7.8 pg/ml (range 7.8-33.46 pg/ml), 9.37 pg/ml (range 7.8-14.04 pg/ml) and 7.8 pg/ml (range 7.8-9.83 pg/ml) the day prior to surgery, and one day and eight days after surgery, respectively. For the group of women with undifferentiated tumor, mean circulatory levels of IL-10 were 8.64 pg/ml (range 7.8-43.72 pg/ml), 11.5 pg/ml (range 8.23-48.07 pg/ml) and 8.45 pg/ml (range 7.8-11.36 pg/ml) the day before surgery, and one day and eight days after surgery, respectively (Tab. 37, Fig. 53). Patients with GI-II ovarian tumors, the IL-10 levels rose significantly one day after surgery (p=0.008), later on the levels dropped off, without reaching a statistical significance. Patients with poorly

differentiated tumors, had significant higher circulatory IL-10 levels one day after surgery ($p<0.001$)

Table 36 *Expression of IL-10 in serum before and after surgery and classical prognostic factors*

Day	Lymph node	*p*	FIGO	*p*	Histology	*p*	Ovarian Cancer	*p*	Residual mass	*p*	Platinum resistance	*p*
T-1	N_0	0.31	I-II	0.13	Serous-papillary	0.08	Primary	1	None	0.4	Resp.	0.73
	N_1		III-IV		Others		Recurrent		Present		Non-resp.	
T+1	N_0	0.19	I-II	0.37	Serous-papillary	0.97	Primary	0.58	None	0.12	Resp.	0.57
	N_1		III-IV		Others		Recurrent		Present		Non-resp.	
T+4	N_0	0.62	I-II	0.70	Serous-papillary	0.22	Primary	0.73	None	0.84	Res.	0.92
	N_1		III-IV		Others		Recurrent		Present		Non-resp.	
T+8	N_0	0.22	I-II	0.45	Serous-paillary	0.97	Primary	0.41	None	0.6	Resp.	0.66
	N_1		III-IV		Others		Recurrent		Present		Non-resp.	

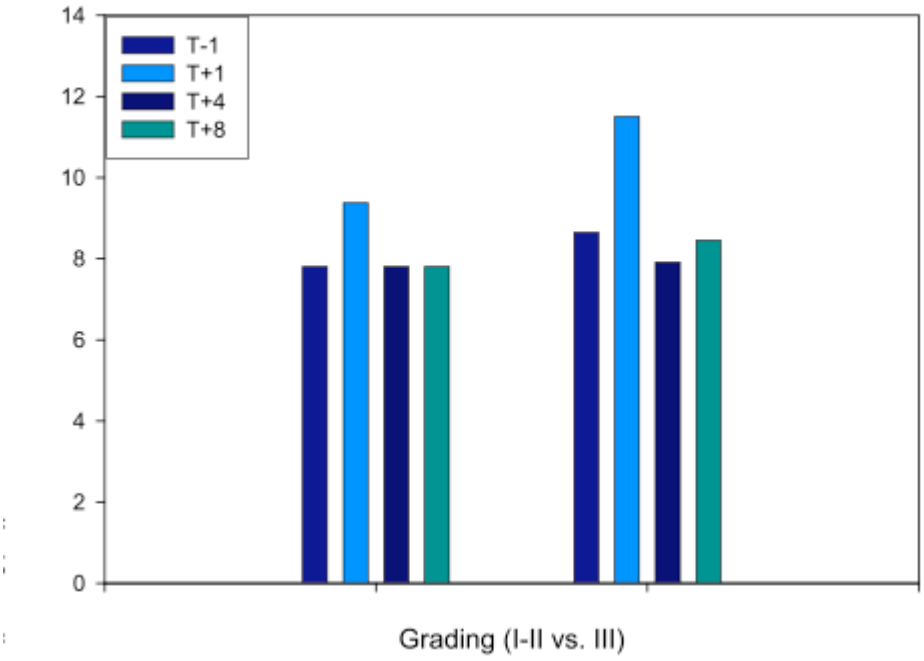


Figure 53 *IL-10 Expression in serum depending on histological grading*

Table 37 *Correlation between IL-10 concentrations and histological grading*

Day	Grading	Mean pg/ml	Median pg/ml	Min pg/ml	Max pg/ml	*p*
T-1	I-II	10.07	7.8	7.8	33.44	0.02
	III	12.76	8.64	7.8	43.72	
T+1	I-II	9.81	9.37	7.8	14.04	0.04
	III	15.15	11.5	8.23	48.07	
T+4	I-II	8.54	7.8	7.8	10.10	0.72
	III	9.56	7.91	7.8	16.75	
T+8	I-II	8.01	7.8	7.8	9.83	0.02
	III	9.01	8.45	7.8	11.36	

The IL-10 concentrations in two of the analyzed patients could be considered outliers, because they varied in a different way from the rest of the collective (Fig. 54). Their clinical and histological features could not explain these differences. When the two patients were excluded from the analyzed lot, the correlations between the expression of IL-10 before and after surgery became more significant. The levels of IL-10 were significantly higher after operation (p=0.03) and they dropped off after the fourth day (p=0.02) and the lower levels were maintained on the eighth day.

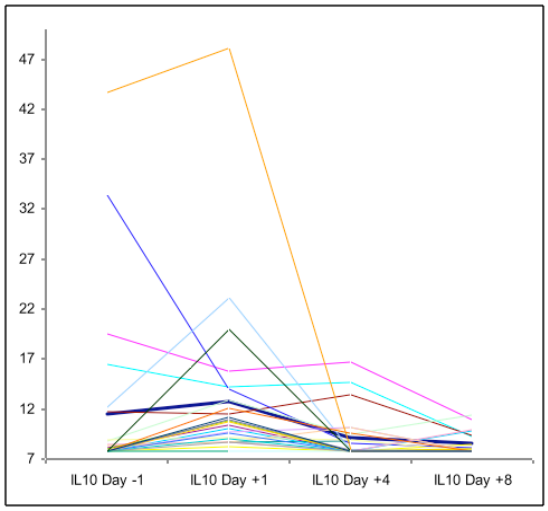


Figure 54 *Variation of IL-10 before and after surgery (n=24)*

4 Discussion

4.1 The overexpression of IL-10 in ovarian cancer patients

Different studies suggest that immunological components play a key role in the development of cancer (10, 14, 186, 192, 195, 196). Cytokines are involved in the regulation of the immune system and have been implicated in the pathogenesis of different gynecological malignancies (18, 19, 147, 155, 163, 186, 195). Several cytokines such as IL-10, IL-1, IL-6, TNF-α, VEGF, were described as being over-expressed in the ascites of patients with ovarian cancer (189, 193, 194, 196).

The most common route of dissemination for ovarian cancer is by the exfoliation of cells form the primary tumor into the peritoneal cavity (4). Typically, the tumor cells remain confined to the abdominal cavity, both as free-floating cellular aggregates and peritoneal implants. Although little is known about the molecular aspects of peritoneal dissemination of ovarian carcinoma cells, tumor metastasis appears to be regulated by various gene products. Recent studies indicate that changes in surface and stroma of peritoneum might contribute to the lymphatic or hematogenous spread of the disease (14, 16,194). Two changes in peritoneum were reported during the development of the ovarian cancer: the reorganisation of the collagen-based matrix and an inflammatory process. Ovarian cells and peritoneal mesothelial cells might be able to produce cytokines (194). The analysis of the peritoneal surface revealed growth stimulation, proinflammatory, angiogenic and extracellular matrix remodeling effects (194).

Tumors could use several mechanisms to coexist with the host‘s immune system or to protect themselves from an immune response. Different modalities by which the tumor might induce a state of tolerance are: insufficient expression of cell surface molecules on tumor cells, which are important for T cell recognition, or activation or stimulation of the synthesis of cytokines that would inhibit the immune response and allow tumor progression. The abilities of IL-10 to inhibit T-helper cell proliferation and functions and to hamper the maturation of the dendritic cells and the expression of co-stimulatory molecules could make from IL-10 a potential candidate for the immune suppression state created by the tumors. The role of IL-10 remains to be elucidated, knowing that studies showed both tumor enhancing and suppressing properties (151).

In the study performed by Zeimet and co-workers 72 patients with ovarian cancer were analyzed for the expression of IL-10, IL-12, TNFalpha, neopterin in the ascites and serum. IL-12 together with FIGO staging and residual mass were the only independent prognostic factors. The levels of IL-10 were more elevated in the ascites than in the serum of patients with ovarian cancer ($p<0.01$), but there was no significant association between the levels of IL-10 and overall and disease-free survival (16).

Santin et al, analyzed also the plasma and ascites concentration of IL-10 in 28 patients with ovarian cancer and in 10 patients without any malignancy (control group). Elevated levels of IL-10 were detected in serum (mean 12 (5) pg/ml; range 8 to 23 pg/ml) and in ascites (mean 165 (137) pg/ml; range 50 to 556 pg/ml) of ovarian cancer patients, while in normal control serum samples IL-10 was not elevated. In all patients IL-10 levels were significantly higher in ascites than in serum ($p< 0.001$) (186).

In another study published by Gotlieb and co-workers the ascitic fluid from patients with ovarian or other intra-abdominal cancers contained significantly elevated levels of IL-10 (542 +/- 77 pg/ml, n = 35), compared with ascites from patients with benign gynaecological conditions (34.2 +/- 7.5 pg/ml, n = 63) ($p < 0.001$) (183). IL-10 expression in ascites did also not correlate with histology, FIGO stage, grading or disease free or overall survival (197).

Lambeck et al, included in their study 187 ovarian cancer patients and 95 controls. They determined the expression of 14 cytokines such as: IL-6, IL-7, IL-8, IL-10, monocyte chemotactic protein-1 (MCP-1), and IP-10 in the sera of the entire collective (195). Their results showed a higher circulatory levels of IL-6, IL-7, IL-10 in patients with ovarian cancer comparing with the control group. Also IL-6, IL-7, IL-8, IL-10, MCP-1, and IP-10 levels seemed to influence the disease-free and overall survival, whereas only IL-7 and IP-10 remained independent predictors of overall survival in the multivariate analysis. Using the expression if IL-7 together with the levels of CA-125 a difference between the malignant and benign ovarian tumors could be possible, the combination of the two serological markers could accurately predict 69% of the ovarian cancer patients (195).

In our study, the expression of IL-10 in serum and ascites of patients with ovarian cancer was analyzed. IL-10 was present in all the serum and ascites samples. A significant higher concentration of IL-10 in serum and ascites was observed in patients with malignant disease in comparison with patients in control group ($p=0.029$ and $p=0.002$, respectively).

The expression of IL-10 in ascites was higher than in serum in patients with ovarian cancer, independent from the recurrence status. This observation supports the hypothesis that IL-10 synthesis occurs mostly in the tumor microenvironment.

Furthermore, the histological type of tumor seems also to play a specific role in IL-10 production, although this observation reached no significant value (p=0.063). In this study the patients with serous-papillary tumor have significantly elevated IL-10 in serum. It is known that different histological types of ovarian cancer, e.g. clear cell carcinoma or mucinous cancer, are show different biological behaviour (191).

The ascitic concentrations of IL-10 were higher in patients with more than 500 ml ascites than in the ones with a smaller amount of ascites ($p<0.001$).

The postoperative tumor load is one of the most relevant prognostic factors. This potential predictive factor could be relevant to identify the patients who will benefit more from radical surgery.

This study showed that optimal cytoreductive surgery outcome was influenced by the expression of IL-10 in serum. IL-10 levels correlated with the presence or the absence of the residual tumor mass (p=0.035).

CA-125 levels prior to surgery correlated significantly with the expression of IL-10 in serum and ascites in patients with ovarian cancer (p=0.046 and p=0.02, respectively).

In the present study, the patients' characteristics can be compared with those in the studies mentioned before. In our collective, the only independent prognostic factors were residual tumor mass and the expression of IL-10 in the serum. The levels of IL-10 in ascites did not influence the overall or disease-free survival in the univariate or multivariate analysis.

IL-10 production by the ovarian tumor cells was reported by various authors (166, 185). Also it seems that IL-10 is produced predominantly at the tumoral site but also systemically. The levels of IL-10 in serum are higher than in patients with no malignancy. The present study showed the same results. Studies, including this one, showed that expression of IL-10 is much higher in ascites than in the serum of patients with ovarian cancer. This could be explained by the susceptibility that major quantity of IL-10 in ovarian cancer patients must be produced at the site of the tumor.

By studying the kinetics of IL-10 concentrations before and after surgery, this study tries to reveal once more the ability of the tumor microenvironment to produce IL-10.

Only two similar studies were performed until now in the field of gastroenterology. Gianotti et al. included 45 patients diagnosed with stomach, pancreas and colorectal cancer in their study. Their results showed significantly lower (p=0.022) IL-10 levels after radical surgery and no change after palliative surgery. The conclusion of their study was that IL-10 might be produced at the tumor site beforehand in order to escape the immune surveillance (115).

Another study performed by Galizia et al. on 50 patients with histologically proven colorectal cancer and 25 patients in the control group showed a significant association between IL-10 circulatory levels and the possibility of performing curative surgery. They determined the concentrations of IL-10 in serum one day before the operation, and one day, four days, eight days and sixteen days after surgery. The expression of IL-10 in serum dropped on the first day after operation in all patients, regardless of whether they underwent curative or palliative surgery. The difference was that in patients who did benefit from curative surgery the circulatory levels of this cytokine remained lower even on the sixteenth day. All patients undergoing curative surgery but still having high IL-10 levels experienced recurrence. These data support the theory that this cytokine could be used as a tumor marker in following the therapeutical results, and that its circulatory levels could indicate even a microscopical residual mass (116).

The results of our study are in concordance with those published until now. The levels of IL-10 rose temporary on day one after surgery, but then they dropped significantly on day 4 and day 8 after the operation.

In the study, no significant association between the residual mass and the circulatory levels of IL-10 was found. Also no significant correlation was found between cytokine's expression and lymph node involvement, tumor type and the presence of recurrence after operation. These differences might be explained by the small number of patients analyzed in this study (24 vs 45 Gianotti and 50 Galizia).

The observation that IL-10 levels increase immediately after surgery but after that they drop, suggests that IL-10 is produced prior at the site of the tumor and that the tissue removal is associated first with increased circulatory levels, explained by the IL-10 mobilization from the malignant tissue depot. This finding also supports the hypothesis that serum levels of IL-10 are correlated with tumor proliferation. Future trials should investigate other markers for tumor proliferation, such as KI-67/MIB1, MCM-2 and hCG-1 to confirm our observation.

Local and systemic secretion of immunosuppressive cytokines may play an important role in the impaired anti-tumor immune function commonly observed in advanced ovarian cancer (193).

Secretion of different cytokines e.g. IL-10 directly by tumor cells or by tumor-associated macrophages or lymphocytes was suggested as a possible mechanism for modulating the anti-tumour immune response of the host (166).

IL-10 has been demonstrated to have powerful immunosuppressive activities affecting the afferent components of immune response-pathways. IL-10 decreases the maturation of dendritic cells (the most potent professional antigen presenting cells known in humans for triggering the induction of an antigen-specific immune response). The main source of IL-10 detected in ovarian cancer patients may be derived from lymphoid or myeloid cells infiltrating or associated with ovarian tumors (166).

Thus only limited data are available on the circulating levels of IL-10 in patients with advanced ovarian cancer, these results are comparable with previous reports showing that a large percentage of such patients have abnormal serum and ascites levels of IL-10. Levels of IL-10 in ascites were significantly higher than in serum samples. That suggests that cells in the abdominal cavity are the major source of IL-10 in ovarian cancer patients.

The presence of cytokines such as IL-10 in the peritoneal cavity of ovarian cancer patients could be important for the growth and development of cancer, more specifically, in relation to host immune responsiveness.

In conclusion, the results of this study confirm and strengthen the hypothesis that the presence of IL-10 in serum and ascites which, is a tumor-induced immunosuppressive cytokine, may play an important causal role in progressively decreasing the immune function.

4.2 The IL-10 promoter gene polymorphism

Our findings suggest that IL-10 polymorphism might play an important role in the surgical outcome. In our report, the C/C alleles were most frequently described in the group of ovarian cancer patients without any postoperative tumor residuals. The heterozygote genotypes (C/T and C/A) in the positions -819 and -592 respectively are more frequently encountered within the group of patients with postoperative tumor mass. Furthermore, the presence of A allele in the -1082 locus seems to influence the results of the surgical act. The lacking of the statistical significance may be based on the limited number of patients in this study, so bigger prospective trials should focus on this aspect.

A number of authors investigated the relationship between specific cytokines and the surgical outcome (115, 116). Gianotti et al analyzed the influence of surgery on the circulatory levels of IL-10 in 45 patients with stomach, colorectal and pancreatic cancer and in 40 patients without any malignancy (115). The same objective was followed by Galizia et al., who investigated 50 patients with colorectal cancer (116). The results in both studies showed a significant association between IL-10 levels and the possibility of optimal debulking surgery (p=0.0005). The IL-10 concentration dropped significantly on the day after the optimal debulking surgery in comparison to patients with only palliative surgery and high tumor volume in gastric cancer. Patients with optimal radical surgery but still elevated IL-10 levels developed a tumor relapse in all cases (6 patients). This result supports the hypothesis that the tumoral microenvironment represents an important IL-10 source (115, 116) and the potential role of IL-10 as a monitoring tool, even in patients with minimal (microscopic) tumor residuals.

Our results suggest that patients with the C allele in -819 and -592 positions of IL-10 gene have a better chance to achieve an optimal surgical outcome. The polymorphism of IL-10 promoter gene at the two specified positions significantly correlated with the residual tumor mass and the disease-free survival in univariate analysis but not in the multivariate model. Further trials with a larger cohort are needed.

The meta-analysis of Bristow and co-workers demonstrated that the maximal cytoreduction is associated with a better overall survival in patients with FIGO stage III and IV. At the moment, no valid factor is available to predict optimal surgery (85, 86).

The allelic distribution within the IL-10 gene cluster at -819 and -592 loci showed significant correlation with the overall survival. The presence of the heterozygote genotype (C/T or C/A) was associated with a decreased survival rate.

The results published by Bushley et al, in 2004, regarding the role of IL-10 polymorphism in the pathogenesis of ovarian cancer revealed no significant influence on the disease free and overall survival (164). The discordance between these two studies might be explained by the differences in the patients' characteristics. Bushley and co-workers included 182 cases and 223 controls from Hawaii. In the OC group only 22% were Caucasian, but 38% were Asian and 48% from other ethnicity; in our study all patients were Caucasian (164).

It is known, that the variety in polymorphism depends on ethnicity, therefore special attention must be paid when interpreting studies from different geographic regions.

Bushley and co-workers determined the polymorphism of IL-10 promoter gene in the -1082 and -819 positions. The distribution of the allele was as follows: at the -1082 position, the AA, GA and GG genotypes were encountered in 31.11%, 47.22% and 21.66% respectively; at the -819 position the TT, TC and CC genotypes were present in 71.11%, 23.33% and 6.11%, respectively (164). In our study the presence of the homozygote genotype for C allele in the -819 position was the most frequent one among the patients from the OC group (51%). Additionally, there are some differences in the distribution of the tumor stages. In the study of Bushley et al FIGO III and FIGO IV was encountered in only 40-50% (40 women) of the patients in comparison to our study where 76.4% were FIGO stage III and IV. These differences regarding the ethnicity, the allelic distribution and the clinical features of the patient collective, might explain the differences between the two results (164).

4.3 Does the allele frequency have an impact on the expression of IL-10?

The variability of cytokine secretion was found to be determined by genetic factors in about 50-75% of the cases (165). Also other factors have impact on the circulatory levels of IL-10 (166).

The expression of IL10 seems to be determined primarly by the polymorphism in the -1082 position. Studies showed the association of -1082 alleles G and A with a low (AA), medium (AG), high (GG) IL 10 production (165, 198).

The data in a study published by Alamartine and co-workers on 40 patients with skin squamous cell malignancy and a control group indicated a strong relationship between gene polymorphisms and IL-10 secretion in individuals. It must be taken in account that all patients were renal transplant patients (198).

The present study involved 62 patients with histologically confirmed ovarian cancer and 18 patients with no history of malignancy. The circulatory levels of IL-10 were highly correlated with its promoter polymorphism within the two groups (p=0.001). The highest expression was associated with the -1082 A/A, -819 C/T, -592 A/C genotypes in the cancer group and with the -1082 G/A, -819C/C , -592 C/C genotype in the control group.

The data from the literature showed that the high producer IL-10 genotype is the GCC, which means the presence of G/G, C/C and C/C polymorphisms in the -1082, -819 and -592 loci, respectively (198). The polymorphism in -1082 position seems to have the most important impact on the expression of the cytokine (140, 165). The G/G polymorphism, as mentioned previously, determines the highest expression, the G/A the medium concentration and the A/A the lowest expression. In this study this was significant only for the patients in the control group, exactly the opposite being the case for the patients with a malignant tumor. This might reflect that IL-10 levels in the malignant patients might be influenced by other factors and not only by the genotype. It is known that other proteins such as, IL-2, IL-6, TNFα may enhance the production of IL-10. It is also known that cytokines form a complex network, and they interact with each other, changing their actions and their synthesis.

Further prospective multicentric studies involving more patients with ovarian cancer and control groups should be performed.

5 Conclusions

The property of ovarian cancer to remain confined to the peritoneal cavity even in very advanced stages, raised the suspicion that local immunosuppressive factors could be involved in the incapacity of the immune system to fight against tumors. One of these possible key factors could be IL-10, considering its capacity to hamper the maturation of antigen presenting cells and the expression of co-stimulatory molecules.

In this study, the results revealed a higher cytokine expression in ascites and serum in patients with ovarian cancer compared with those from the control group. Also the so called “high producer genotype” did not correlate with high circulatory levels in the cancer group, showing that tumor cells might produce IL-10 themselves or could induce its synthesis in the tumor infiltrating or associated lymphocytes in order to help the tumor to escape the immune surveillance.

A higher expression of IL-10 in ascites was associated with the presence of residual mass after surgery. In contrast, the lower IL-10 concentrations in ascites are associated with an optimal surgical outcome. The influence on the ability to perform a curative surgical treatment was valid also for the IL-10 polymorphism. According to these results, the present study could propose a new predictive marker for ovarian cancer patients.

The circulatory levels of IL-10, besides the residual tumor mass, was the only independent prognostic factor, and they remained so even when the analysis was performed separately for primary and recurrent ovarian cancer.

In conclusion, IL-10 promoter polymorphism may be related to the ability to achieve optimal tumor debulking. Polymorphism in IL-10 gene seems also to influence the overall and disease free survival rate. Subsequent multi-institutional studies with large number of patients are warranted to confirm these results.

6 Abstract

Background: Epithelial ovarian cancer is one of the most frequent causes of death attributable to gynecologic malignancies, 75% of all cases being detected in advanced stages. Late detection is due to an absence of specific symptoms while the disease is still localized, as well as the lack of effective prevention, screening and early detection strategies. Despite the current improvement in surgical and medical treatment, the five-year survival rate is low. The development of new tumor marker and screening tests is one of the major current goals on this field of research.

Various preclinical and early clinical studies suggest a strong involvement of immunological factors in the tumorigenesis of ovarian cancer. Cytokines are well described and supposed to play an important role in the carcinogenesis of various malignant solid tumors, including ovarian cancer. Cytokines might also contribute to tumor development by enhancing the angiogenesis and tumor cells adhesions or by interfering with the antitumoral mechanisms of the immune system, such as the down-regulation of the dendritic cells. B and T lymphocytes are able to produce IL-10 under various stimuli. IL-10 synthesis by ovarian cancer cells was also reported. Only limited data are available about the clinical and prognostic value of the expression of IL-10 in serum and ascites, and of polymorphisms of IL-10 promoter gene in patients with advanced ovarian cancer.

Methods: In the present study, 199 patients with histological proven ovarian cancer and a control group of 159 patients with no malignancy were included.

Expression of IL-10 in serum and ascites

We analyzed 114 ovarian cancer patients and 30 women without malignancy. Blood drawing and ascites collection were performed at the time of the surgical procedure.

IL-10 serum and ascites concentrations were measured with an enzyme-linked immunosorbent assay kit (ELISA) according to the manufacturer's protocol (Quantikine, R&D Systems Inc., Minneapolis, MN, USA). All samples were measured in duplicate.

The polymorphism of IL-10 promoter gene

Further on we analyzed the polymorphism of IL-10 promoter gene in 147 patients with ovarian cancer and 129 patients with no malignancies. Polymorphism of IL-10 promoter gene (G/A at -1082, C/T at -819, and C/A at -592) was detected using the Pyrosequencer PSQ 96 and PSQ 96 SNP Reagents Kit (Uppsala Sweden). The oligonucleotides primers used for genotyping the -1082 (G/A) polymorphism were: forward, 5'-AACCCAACTGGCTCTCCTTA-3' and reverse, 5'BIOTIN-GCTGGATAGGAGGTCCCTTA-3'.

A C to T transition in the -819 position of the IL-10 gene was searched using the following oligonucleotides primers: forward, 5'BIOTIN-TTCTCAGTTGGCACTGGTGT-3'; reverse, 5'-AACTGTGCTTGGGGGAAGT-3' .

For analyzing the -592 position of IL-10 gene, we used the following primers: forward, 5'BIOTIN-TGGAAACATGTGCCTGAGAA-3'; reverse, 5'-CAAGCAGCCCTTCCATTTTA-3'.

Surgery impact on IL-10 expression in serum in ovarian cancer patients

Blood was drawn from the cubital vein of 27 patients with histologically confirmed ovarian cancer one day before surgery. Blood collection and IL-10 serum measurements were repeated 24 hours, four and eight days, after the surgery. We used ELISA technique for obtaining the IL-10 concentrations.

Aim of the study: The main aim of the study was to determine the predictive and prognostic role of IL-10 in ovarian cancer patients. As secondary aims, we analyzed differences in IL-10 expression and IL-10 polymorphism pattern between oncological patients and control group, the correlation of IL-10 expression and polymorphism with classical clinical prognostic factors, the impact of polymorphism on the expression of IL-10 in serum and ascites and the impact of surgery on the circulatory levels of IL-10.

Results: The median age among patients with ovarian cancer was 57 years (range 19-88 years). The distribution of FIGO tumor stage of ovarian cancer was as follows: FIGO I-II 39 (19.6%) patients and advanced disease (FIGO stage III-IV), 159 patients (80.4%).

Expression of IL-10 in serum and ascites

The results of the ELISA analysis showed that concentrations of IL-10 in ascites and serum were significantly increased in patients with ovarian cancer in comparison to the control group (p=0.029 and p=0.002, respectively). When analyzing the ovarian cancer patients only, the expression of IL-10 was higher in the ascites when compared with the one in serum, this remain significant even when the statistical assessment was performed separately for the primary and recurrent ovarian cancer. These results confirm the role of tumoral environment in producing several cytokines. In our study, IL-10 expression correlated with the residual tumor mass after surgery. Patients having lower IL-10 circulatory level benefit most from the cytoreductive surgery. In the multivariate analysis, expression of IL-10 in serum and the postoperative residual tumor mass were the only independent prognostic factors for overall survival.

The polymorphism of IL-10 promoter gene

In the second part of this study, the polymorphism of IL-10 promoter gene was analyzed. The results revealed its importance in predicting the optimal surgical outcome. The allelic distribution within IL-10 gene cluster at -819 and -592 loci showed to be significantly associated with the surgical outcome and to influence the overall survival. Our results suggest that patients with the C allele in -819 and -592 loci of IL-10 promoter gene have a better chance to achieve optimal surgical reduction. The presence of the heterozygote genotype (C/T or C/A) was associated with a decreased overall survival. The presence of C/C genotype in -819 and -592 loci was significantly associated with a better overall and disease free survival.

Surgery impact on IL-10 expression in serum in ovarian cancer patients

The last part of the study analyzes the impact of surgery on the circulatory levels of Il-10. The levels of IL-10 rose temporarily on day one after surgery, but then they dropped significantly on day 4 and day 8 after the surgery.

The observation that IL-10 levels increase immediately after surgery and drop after that, suggests that IL-10 is produced beforehand at the site of the tumor. The kinetics of IL-10 levels show first increased circulatory levels, explained by the IL-10 mobilization from the malignant tissue depot, followed by decreased levels of IL-10. Local and systemic secretion of immunosuppressive cytokines may play an important

role in the impaired anti-tumor immune function commonly observed in advanced ovarian cancer.

Conclusions: In the present study we could demonstrate that the cytokine expression in serum and ascites from patients with ovarian cancer is significantly higher than in those from the control group. The increased levels of IL-10 in ascites indicate that IL-10 is primarily produced by the direct tumor environment. Serum levels correlate significantly with the postoperative residual tumor mass and the IL-10 concentration in the ascites correlate with the volume of ascites. In the multivariate analysis, lower serum levels and the absence of macroscopically residual mass were associated with better overall survival. The present data demonstrate that IL-10 promoter gene could represent a predictive factor for optimal surgical outcome. Therefore, a confirmation of these results in a prospective multicenter trial is warranted.

7 Literature

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8 Summary of the tables and figures

8.1 Tables

8.2 Figures

9 List of abbreviations

Fig.	Figure
Tab.	Table
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
APC	Antigen Presenting Cells
ASCO	American Society of Clinical Oncology
CAP	Cyclophosphamid, Adriamycin, Cisplatin
CASA	Cancer Associated Serum Antigen
CG	Control Group
CI	Confidence Interval
CSF	Colony Stimulating Factor
DC	Dendritic Cells
ELISA	Enzyme-Linked-Immunosorbent Assay
FIGO	Fédération Internationale de Gynécologie et d´Obstétrique
GCIG	Gynaecological Cancer Intergroup
GINECO	Groupe Investigation Nationale Etude Cancer Ovaire
GOG	Gynecologic Oncology Group
HR	Hazard Ratio
ICON	International Collaborative Ovarian Neoplasm Group
IL-10	Interleukin 10
IMO	Intraoperatives Mapping des Ovarialkarzinoms
MHC	Major Histocompatibility Complex
NK	Natural Killer
NOGGO	Nord-Ostdeutsche Gesellschaft für Gynäkologische Onkologie
OC	Ovarian cancer
OCA	Ovarian cancer associated Antigen
OR	Odds Ratio
PAI	Plasminogen Activator Inhibitor
PCR	Polymerase Chain Reaction
SCOTROC	Scottish Gynaecological Cancer Trials Group
SWOG	Southwest Oncology Group
TAL	Tumor Associated Lymphocytes
TGF	Tumor Growth Factor
TIL	Tumor Infiltrating Lymphocytes
UICC	Union Internationale Contre le Cancer
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

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11 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

12 List of publications

12.1 Book Chapters

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13 Erklärung der Eigenständigkeit

„Ich, Elena Ioana Braicu, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Polymorphism and expression of IL-10 in serum and ascites from patients with advanced ovarian cancer, selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Unterschrift