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NY-ESO-1 Expression and Clinicopathological Features in Epithelial Ovarian Cancer

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS (in alphabetical order)

ABC	Avidin/biotin complex
ACS	American Cancer Society
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
AJCC	American Joint Committee on Cancer
AP	Alkaline phosphatase
AWD	Alive with disease
AWMF	Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften
BMI	Body mass index
BRCA1 / BRCA2	Breast cancer gene 1 / Breast cancer gene 2
CA 125	Cancer antigen 125 or Carbohydrate Antigen 125
CAR / CARs	Chimeric antigen receptor / Chimeric antigen receptors
cDNA	Complementary desoxyribonucleic acid
CD19	Cluster of Differentiation 19
CT	Computed tomography
CT6.1	Cancer/testis antigen 6.1
CTAG1	Cancer/testis antigen 1
DAB	3,3'-diaminobenzidine
DNED	Dead no evidence of disease
DOD	Dead of disease
EDTA	Ethylenediaminetetraacetic acid
EEOC	Endometrioid epithelial ovarian carcinoma
EOC	Epithelial ovarian cancer
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
Fig.	Figure
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
GEKID	Gesellschaft der epidemiologischen Krebsregister in Deutschland
GOG	Gynecologic Oncology Group
H-Score	Histological score
H_2O_2	Hydrogen peroxide
HGSC / HGSOC	High-grade serous (ovarian) carcinomas/cancer
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
Hx	History
IHC	Immunohistochemistry
kDa	Kilodalton
LAGE-2	L-antigen-family-member 2
LCRI	Ludwig Institute for Cancer Research
LGSC / LGSOC	Low-grade serous carcinomas / Low-grade serous ovarian carcinomas
LION	Lymphadenectomy in ovarian neoplasms
MAGE-1	Melanoma antigen 1
MD	Medical doctor
MEOC	Mucinous epithelial ovarian cancer
MHC	Major histocompatibility complex

MRI	Magnetic resonance imaging
NCI	National Cancer Institute
NED	No evidence of disease
NLM	National Library of Medicine
n. s.	not significant
NSCLC	Non-small-cell lung carcinoma
NY-ESO-1	New York esophageal squamous cell carcinoma 1
OC / OCs	Ovarian cancer / Ovarian cancers
OCCC	Ovarian clear-cell carcinoma
PAOLA-1	Platine, Avastin, and OLAparib in 1st line
PARP	Poly (ADP-ribose) polymerase
PBLs	Peripheral blood lymphocytes
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein Ligand 1
PET	Positron emission tomography
PET/CT	Positron emission tomography- computed tomography
pH	‘potential of hydrogen’ or ‘power of hydrogen’
Ph.D.	Doctor of Philosophy
RKI	Robert-Koch-Institut
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCRS	Secondary cytoreductive surgery
SEER	Surveillance, Epidemiology, and End Results Program
SEREX	Serological analysis of expression cDNA libraries
TAA	Tumor-associated antigens
TAHBSO	Total abdominal hysterectomy, bilateral salpingo-oophorectomy
TBS	Tris-buffered saline
TCR / TCRs	T cell receptor / T cell receptors
TILs	Tumor infiltrating lymphocytes
TMA	Tissue microarray
TMN	Tumor, (lymph) nodes, metastasis
Tris/EDTA	Trisaminomethane/Ethylenediaminetetraacetic acid
UCLA	University of California, Los Angeles
UICC	Union for International Cancer Control
US / USA	United States / United States of America
WHO	World Health Organization
ZfKD	Zentrum für Krebsregisterdaten

1 BACKGROUND

1.1 Epithelial Ovarian Cancer (EOC)

Ovarian cancer is the second deadliest gynecologic malignancy behind cervical cancer in the world (Bray et al. 2018). Late onset of symptoms and lack of effective screening methods result in late diagnosis and lead to a relatively restricted prognosis (Buys et al. 2011; Jacobs et al. 2016). In the last decades, many advances have been made diagnosing and treating ovarian cancer, but the prognosis for ovarian cancer patients remains limited, especially in more advanced cases (Torre et al. 2018; Vaughan et al. 2011; ZfKD und RKI 2019; SEER at the NCI 2021). Consequently, more effort is required to identify further and research optimal therapeutic targets.

PARP (Poly (ADP-ribose) polymerase) inhibitors belong to the most promising experimental treatment approaches (Ray-Coquard et al. 2019; Gonzalez-Martin et al. 2019); other recent studies have dealt with immunotherapy as a supplement to conventional ovarian cancer treatment (Krishnan, Berek, and Dorigo 2017; Odunsi 2017; Kandalaft, Odunsi, and Coukos 2019). A few study groups have explored the presence of the cancer/testis antigen NY-ESO-1 (New York esophageal squamous cell carcinoma-1) in ovarian carcinoma rendering contradictory results (Yakirevich et al. 2003; Szender et al. 2017; Odunsi et al. 2003). This study aims to further investigate the protein NY-ESO-1 as a prognostic marker and potential target for immunotherapy for ovarian cancer.

1.1.1 Classification of Ovarian Malignancies

Ovarian malignancies encompass a heterogeneous group of tumors differentiated by the site of origin, histomorphology, and molecular pathologic findings. Epithelial cancers are the most prevalent malignant ovarian neoplasms accounting for about 90 percent of cases, with malignant Germ cell (e. g., dysgerminomas, teratomas, et cetera, 2 - 3 %) and sex cord-stromal tumors (e.g., granulosa cell tumors, et cetera, 5 - 6 %) making up the majority of non-epithelial cancers (Torre et al. 2018).

Based on their different underlying etiopathogenesis and cell origin, molecular characterization, and gene expression, the World Health Organization (WHO) (Kurman et al. 2014) further classifies epithelial ovarian cancers as Type I ovarian cancers, including low-grade serous carcinomas (LGSC, > 5 %), endometrioid epithelial ovarian carcinoma (EEOC, ~ 9 – 11 %), ovarian clear cell carcinomas (OCCC, ~ 12 – 13 %) as well as mucinous epithelial ovarian carcinomas (MEOC, ~ 3 %). High-grade serous carcinomas (HGSC, ~ 68 – 71 %) are, among the rarer types, grouped as Type II ovarian cancers (*Fig.1.1*) (Reid, Permuth, and Sellers 2017; Chen et al. 2003; McCluggage 2011). Grading of endometrioid carcinomas of the ovary still pursues the former classification from well- (G1) to poorly differentiated (G3), analogously to the grading of endometrial carcinomas. Clear cell carcinomas are always graded “poorly differentiated” or G3, respectively. Mucinous carcinomas are not being ranked according to grading (Kurman et al. 2014). The previous grading system acted on the misassumption of all ovarian cancer subtypes evolving from increasing dedifferentiation of formerly benign lesions. The currently employed binary grading system is based on biological evidence indicating that Type I and II tumors develop through different genetic pathways and show distinct differences in clinicopathological features, response to treatment, and outcome. Confined to the ovary, most Type I tumors have a relatively favorable prognosis; only advanced-stage tumors show a poor outcome due to poor chemosensitivity. Type I tumors only account for ten percent of deaths from ovarian cancer. Type II cancers that frequently show p53 gene abnormalities are characterized by acquired chemoresistance after initial response and considerably poorer prognosis (Vang, Shih, and Kurman 2009; Cho and Shih 2009; Shih and Kurman 2004; Kurman and Shih 2016).

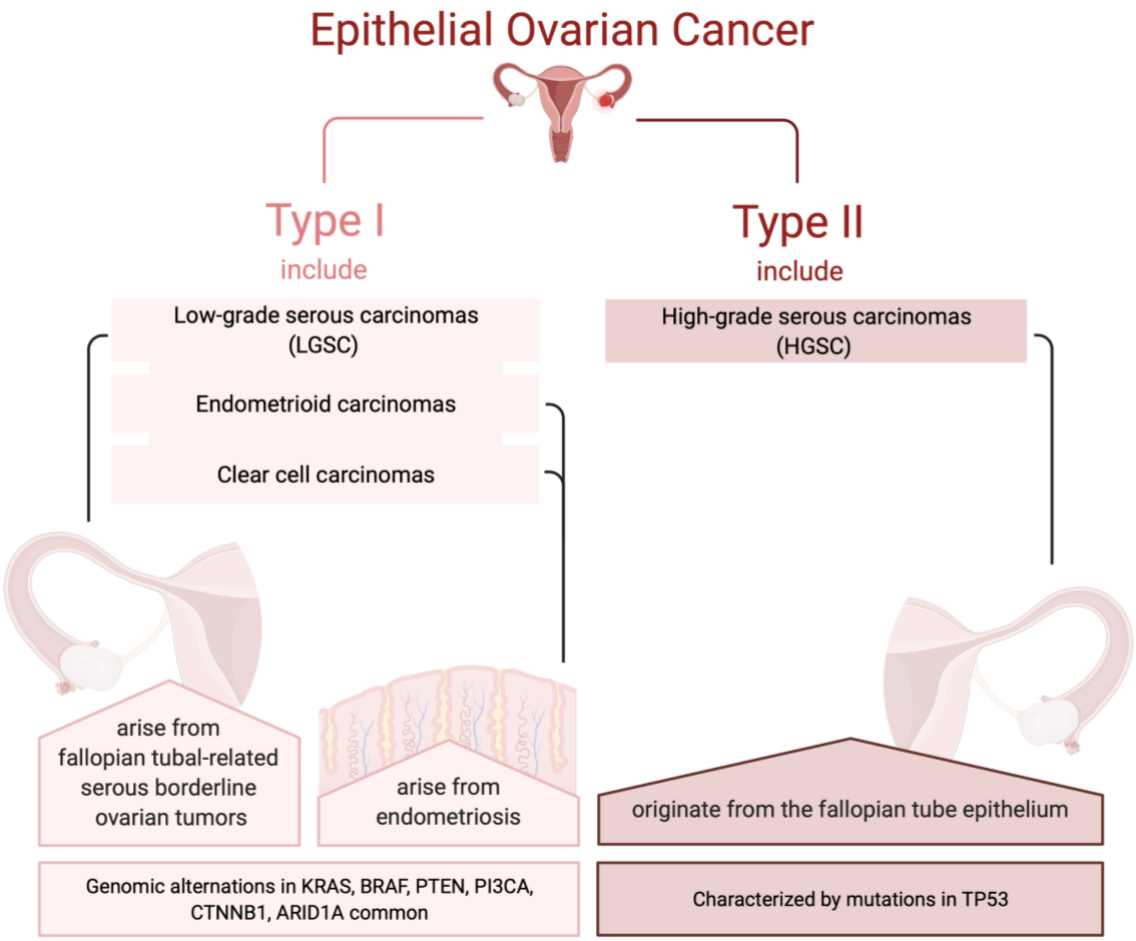

Fig. 1.1: Binary classification of epithelial ovarian cancers. The binary classification divides epithelial ovarian cancers into Type I ovarian cancers, including low-grade serous carcinomas (LGSC), endometrioid carcinomas, and clear-cell carcinomas; high-grade serous carcinomas (HGSC) are grouped as Type II ovarian cancers. Type I ovarian cancers, for the most part, arise from endometriosis or fallopian tubal-related serous borderline ovarian tumors, while Type II ovarian cancers mostly originate from the fallopian tube epithelium. The underlying genetic mutations also differ among Type I and Type II ovarian cancers.

Figure created with BioRender.com

Modified from: R.J., Shih I.-M. The Dualistic Model of Ovarian Carcinogenesis: Revisited, Revised, and Expanded. Am J Pathol. 2016;186(4):733-747.

1.1.2 Epidemiology of EOC

Ovarian cancer is the eighth most common cancer and the eighth leading cause of death from cancer in women in the world (*Fig. 1.2*) (Bray et al. 2018).

Incidence		**Mortality**	
1. Breast	24.2 %	1. Breast	15.0 %
2. Colorectum	9.5 %	2. Lung	13.8 %
3. Lung	8.4 %	3. Colorectum	9.5 %
4. Cervix uteri	6.6 %	4. Cervix uteri	7.5 %
5. Thyroid	5.1 %	5. Stomach	6.5 %
6. Corpus uteri	4.4 %	6. Liver	5.6 %
7. Stomach	5.1 %	7. Pancreas	4.9 %
8. Ovary	**3.4 %**	**8. Ovary**	**4.4 %**
9. Liver	2.9 %	9. Esophagus	3.6 %
10. NHL	2.8 %	10. Leukemia	3.1 %

Fig. 1.2: Distribution of cases and deaths for the ten most common cancers in women in 2018.

Figure created with BioRender.com

Modified from: Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.

According to the latest WHO data, in 2018, 295,414 women were diagnosed with ovarian cancer worldwide, accounting for 3.4 percent of all new cancer cases in women. Respectively, 184,799 women died of the disease in 2018, amounting to 4.4 percent of cancer-related deaths in women. Estimated new ovarian cancer cases are more than seven times higher in countries with very high human development indices compared to countries with low human development indices; mortality rates are devastating in countries with low human development indices but still fatal in countries with very high human development indices (Bray et al. 2018).

In Germany, ovarian cancer ranks eighth in estimated new cancer cases in women and is the second leading gynecological cancer behind endometrial cancer. Ovarian cancer is regarded as the fifth leading cause of death from cancer in women in Germany.

Currently, there is no nationwide cancer registry in Germany. However, the Gesellschaft der epidemiologischen Krebsregister in Deutschland (GEKID) and the Robert-Koch-Institut (RKI) estimate that in Germany in 2017, 7,350 women were diagnosed with ovarian cancer (3.1 percent of female cancers), and 5,486 women died of the disease (cancer-related mortality in women: 5.2 percent); 7,000 new cases were expected in Germany in 2020. In Germany, the incidence of ovarian cancer increases up to the age of 85; the median age of disease onset is 68 years. The lifetime risk of developing the disease is about 1.3 percent. Three-quarters of patients in Germany present at T3 (UICC TMN classification, see below) when the cancer already had metastasized (*Fig. 1.3*) (ZfKD und RKI 2019).

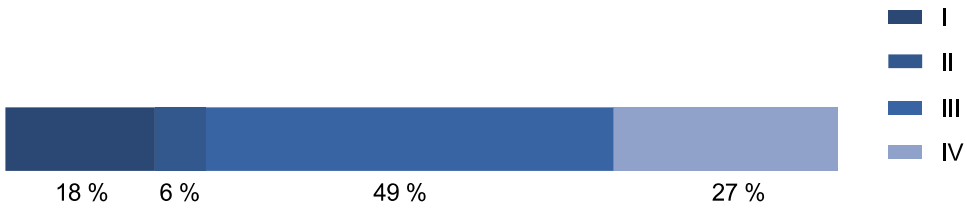

Fig. 1.3: UICC stages at the time of ovarian cancer diagnosis in Germany (2015 – 2016). Patients with not known UICC stage at the time of ovarian cancer diagnosis or patients with death certificate only information were not included.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

Modified from: Zentrum für Krebsregisterdaten (ZfKD) und Robert-Koch-Institut (RKI). Krebs in Deutschland - Eierstöcke. *https://www.krebsdaten.de/Krebs/DE/Content/Publikationen/Krebs_in_Deutschland/kid_2019/kid_2019_c56_eierstoecke.pdf?__blob=publicationFile* (Published 2019, accessed May 23rd, 2021).

While incidence and mortality rates of ovarian cancer keep decreasing, survival rates nearly remain stagnant with overall survival at five years of 43 percent, primarily driven by advanced-stage diagnoses (*Fig. 1.4 a*) (ZfKD und RKI 2019).

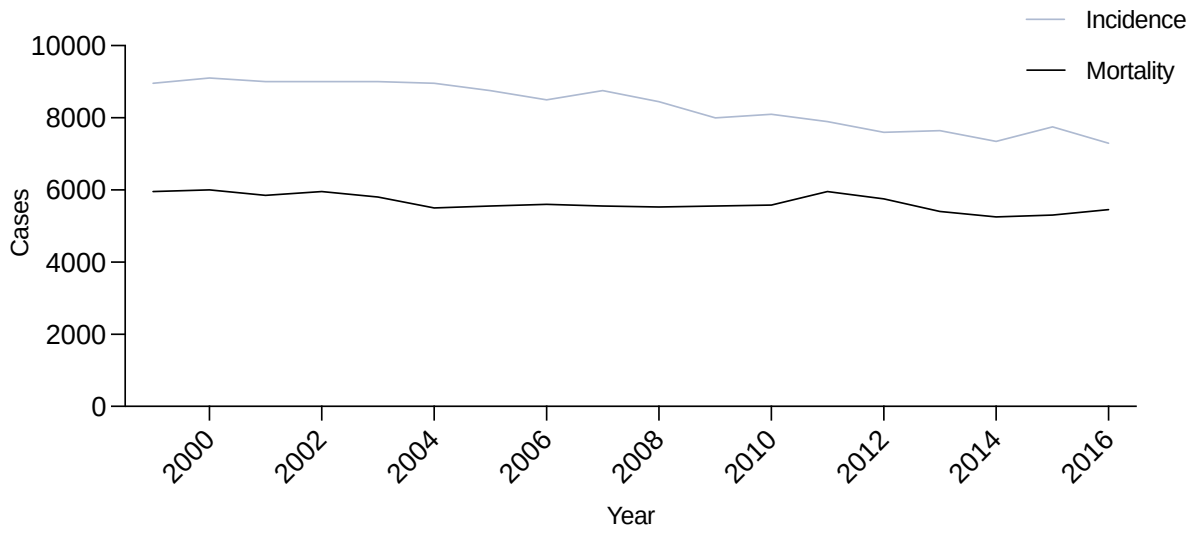

Fig. 1.4 a: Incidence and mortality rates in ovarian cancer patients 1999 – 2016 in Germany.

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GraphPad Software, San Diego, California USA, www.graphpad.com

Modified from: Zentrum für Krebsregisterdaten (ZfKD) und Robert-Koch-Institut (RKI).
Krebs in Deutschland - Eierstöcke.
https://www.krebsdaten.de/Krebs/DE/Content/Publikationen/Krebs_in_Deutschland/kid_2019/kid_2019_c56_eierstoecke.pdf?__blob=publicationFile (Published 2019, accessed May 23rd, 2021).

Respectively, ovarian cancer ranks eleventh in estimated new cancer cases and is expected to be the fifth leading cause of cancer-related deaths, and the most lethal gynecological cancer, in women in the United States of America (USA) in 2021. The American Cancer Society (ACS) estimates that 21,410 women will be diagnosed with ovarian cancer in the United States (US) in 2021, and 13,770 will succumb to the disease, accounting for 2.4 percent of cancer diagnoses and 5.4 percent of all cancer-related deaths among women. The median age at diagnosis of ovarian cancer is 63 years in the US. A women's average lifetime risk of being diagnosed with ovarian cancer in the United States is 1.2 percent.

The majority of serous ovarian carcinomas are diagnosed at FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) stage III (51 %) or IV (29 %); consequently, the survival at five years is 43 percent and 26 percent, respectively. Ovarian cancers that are less aggressive in growth are mostly diagnosed at FIGO stage I leading to a 5-year survival of 82 percent, 71 percent, and 66 percent for endometrioid, mucinous, and clear cell carcinoma, respectively (Siegel, Miller, and Jemal 2020; SEER at the NCI 2021; Torre et al. 2018; Peres et al. 2019). Despite decreasing incidence and advances in treatment and diagnosis of ovarian cancer, for newly diagnosed patients, the 5-year overall survival has also only marginally improved since the late-1970s after widely introducing platinum-based chemotherapy for the treatment of ovarian cancer (*Fig. 1.4 b*) (Lowe et al. 2013).

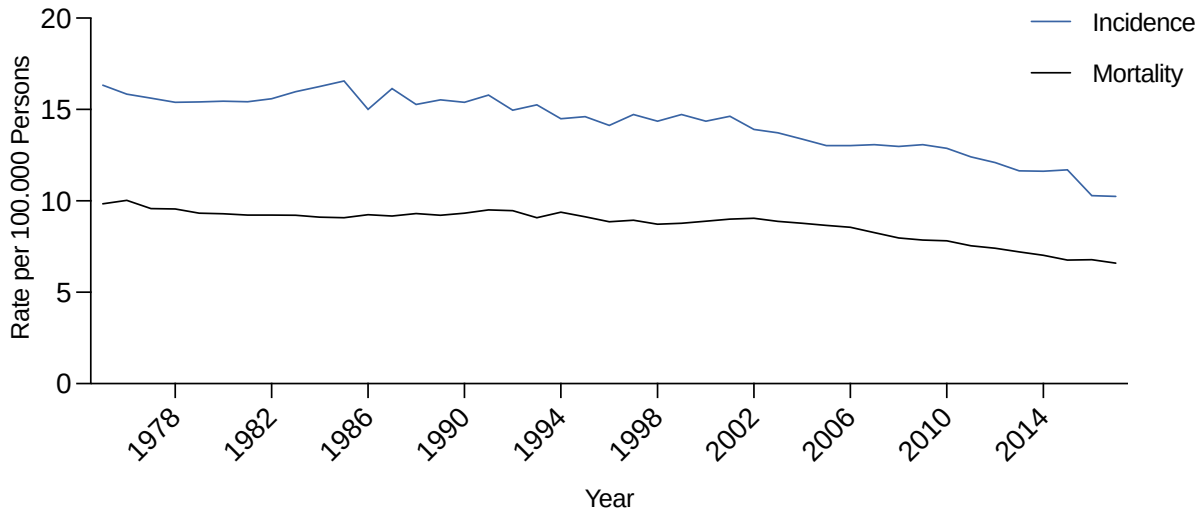

Fig. 1.4 b: Incidence and mortality rates in ovarian cancer patients 1999 – 2017 in the USA.

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Modified from: https://seer.cancer.gov/statfacts/html/ovary.html (accessed May 23rd, 2021).

1.1.3 Etiopathology of EOC, including Model of Carcinogenesis and Risk Factors

Ovarian cancer is predominately a disease of the elderly. Genetic disposition, however, leads to an earlier onset of the disease. Approximately 20 percent of women with ovarian cancer show inherited genetic mutations that predisposed them to the disease; alternations in BRCA1 (Breast Cancer gene 1) or BRCA2 (Breast Cancer gene 2) tumor suppressor genes prevail (Pennington et al. 2014; Harter et al. 2017). BRCA1 carriers have a reported lifetime risk of developing high-grade serous ovarian cancer (HGSC) of over 40 percent and are diagnosed approximately ten years earlier than the median age of diagnosis (Mavaddat et al. 2013; Kuchenbaecker et al. 2017; Easton, Ford, and Bishop 1995).

Most ovarian cancers, however, are assumed to occur sporadically. Predisposing risk factors include but are not limited to: increasing age (Torre et al. 2018), overweight and obesity (Olsen et al. 2007; Reid, Permuth, and Sellers 2017), endometriosis (Pearce et al. 2012), and polycystic ovary syndrome (PCOS) (Chittenden et al. 2009), personal or family history of breast, colorectal and ovarian cancer (Stratton et al. 1998; Permuth-Wey et al. 2016; Lynch et al. 2009) and long-term hormone replacement therapy (Riman, Nilsson, and Persson 2004; Lacey et al. 2002); while, for instance, (multi)parity (Adami et al. 1994), breastfeeding (Li et al. 2014), the use of oral contraceptives (Collaborative Group on Epidemiological Studies of Ovarian Cancer et al. 2008), and tubal ligation (Narod et al. 2001) are assumed to decrease the risk of developing ovarian cancer (Reid, Permuth, and Sellers 2017).

Based on pathologic observations and supported by epidemiologic studies, it had long been assumed that a majority of ovarian cancers originate from the epithelium on the ovarian capsule. This model of carcinogenesis (“incessant ovulation”) involves epithelial ovarian cancer arising with the dedifferentiation of the epithelium covering the ovaries triggered by cell damage through ovulation. The incorporation of these cells into the ovary follows the dedifferentiation of the epithelium and subsequent local proliferation (Fathalla 2013, 1971).

The lifetime number of ovulatory cycles as an index of ovarian cancer risk through identified risk factors such as nulligravidity or PCOS and protective factors, e. g. multigravidity and oral contraception, support this theory (Scully 1995; Hunn and Rodriguez 2012; Purdie 2003). The fact that epithelial ovarian cancers are composed of structures that histologically show a strong resemblance to Müllerian-derived epithelium contradicts this theory (Dubeau 2008). Most parts of the female genital tract, including the fallopian tubes, uterus, cervix, and upper vagina, are derived from the Müllerian ducts, but the ovaries (Sadler, Drews, and Brand-Saberi 2020).

Ongoing research suggests that, for instance, most HGSC arise from dysplastic precursor lesions in the fallopian tube (Piek et al. 2001; Rebbeck 2002) and ovarian mucinous carcinomas from transitional cell nests at the tubal-mesothelial junction (Seidman and Khedmati 2008). Endometriotic cysts associated with endometriosis have already been linked to the development of clear cell and endometrioid tumors (Kurman and Shih 2010). Periodic rupture and repair through recurring ovulations might cause a local inflammatory microenvironment with chemotactic cytokines and growth factors at the ovulatory wound site that can recruit extra-ovarian pre-malignant and malignant cells (Yang-Hartwich et al. 2014). Traditionally grouped and designated as epithelial ovarian cancers, these findings suggest a multifocal origin of the disease leading to a clinically and pathologically heterogeneous disease pattern: epithelial ovarian cancer types are now hypothesized to be separate entities with distinct clinical and biological behaviors. This paradigm shift conceivably has an enormous impact on research and clinical decisions.

For the first time, the FIGO classification from 2013 and revised WHO classification from 2014 give credit to the updated perception of ovarian cancer pathogenesis (Meinhold-Heerlein et al. 2016; Prat J and Figo Committee on Gynecologic Oncology 2015; Kurman et al. 2014) (*Table 1*).

1.1.4 Symptoms, Metastatic Spread, and Diagnosis of EOC

Ovarian cancer presents with minimal, nonspecific, and vague symptoms. Many patients with ovarian cancer complain about pelvic or back pain, distended abdomen or abdominal masses, bloating, urinary symptoms, and abnormal vaginal bleedings. Paraneoplastic syndromes due to tumor-mediated factors lead to various presentations.

These symptoms being vague and widely shared in the general population makes ovarian cancer unlikely to be diagnosed at an early stage (Goff et al. 2004; Olson et al. 2001; Ebell, Culp, and Radke 2016; Bankhead et al. 2008; Bankhead, Kehoe, and Austoker 2005). Late-onset symptoms and a lack of reliable screening methods often result in a late diagnosis of ovarian cancer and a restricted prognosis.

Early diagnosis of this disease determines the outcome: in 70 percent of cases, ovarian cancer is only diagnosed in late stages, such as FIGO stage III and IV, leading to a 5-year-overall survival below 40 percent (Torre et al. 2018). Multicenter randomized prospective cohort studies did not reveal survival benefits with screening women using transvaginal sonography combined with serum CA 125 (Cancer Antigen 125), neither for patients at genetic risk nor in the general population. Quite the contrary: general screening led to an increase in morbidity and mortality in patients who received false-positive ovarian cancer diagnoses (Buys et al. 2011; Jacobs et al. 2016; AWMF 2020; Henderson, Webber, and Sawaya 2018).

Epithelial ovarian cancer typically first spreads by local extension (uterus, bladder, sigmoid colon, rectum), followed by lymphatic invasion to the retroperitoneal (para-aortic and pelvic) lymph nodes and intraperitoneal implantation. In more advanced disease, malignant pleural effusion due to transdiaphragmatic passage to the pleural cavity and hematogenic dissemination to the liver, spleen, and other tissues outside the peritoneal cavity, e. g. to the lungs and bones, is common (Lengyel 2010; Springs 2018).

Based on the preoperative results of the physical examination and imaging tests, particularly transvaginal ultrasonography and, if indicated, additional computed tomography scans (CT), positron emission tomography scans (PET), PET/CT, and magnetic resonance imaging (MRI), the tumor size and metastatic disease are estimated (Nam et al. 2010; Javadi et al. 2016; AWMF 2020; Henderson, Webber, and Sawaya 2018).

To ultimately determine the tumor dignity and involvement of lymph nodes, surgical removal of the tumor mass and nearby lymph nodes and histopathological examination of the removed tissues are mandatory. Accurate staging has a decisive impact on the optimal treatment and the prognosis (Trimbos et al. 2010).

Ovarian cancer is surgically staged using the AJCC (American Joint committee on cancer) and UICC (Union for International Cancer Control) tumor-node-metastasis (TNM) (American Joint Committee on Cancer 2017), and the International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique, FIGO) (Prat J and Figo Committee on Gynecologic Oncology 2015) classification (*Table 1*). The systems are based on operative findings to determine the extent of the primary tumor, the absence or presence of metastasis to local lymph nodes, and the absence or presence of distant metastasis. The AJCC/UICC TMN classification additionally includes the categories: TX for “Primary tumor cannot be assessed” and T0 for “No evidence of primary tumor” (American Joint Committee on Cancer 2017).

Table 1: Ovarian cancer staging (UICC and AJCC TMN 2018, FIGO 2013).

TMN (2018)	FIGO stage (2013)	Stage description	
T1	*I*	*Tumor confined to ovaries or fallopian tube(s)*	
T1a N0 M0	I A	Tumor limited to 1 ovary (capsule intact) or fallopian tube, no tumor on ovarian or fallopian tube surface, no malignant cells in ascites or peritoneal washings	
T1b N0 M0	I B	Tumor limited to both ovaries (capsule intact) or fallopian tubes, no tumor on ovarian surface or fallopian tube, no malignant cells in ascites or peritoneal washings	
T1c N0 M0	I C	Tumor limited to 1 or both ovaries fallopian tube(s) with any of the following:	
		IC 1	Surgical spill intraoperatively
		IC 2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
		IC 3	Malignant cells in ascites or peritoneal washings
T2	*II*	*Tumor involves 1 or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer*	
T2a N0 M0	II A	Extension and/or implants on uterus and/or fallopian tubes and/or fallopian tubes and/or ovaries	
T2b N0 M0	II B	Extension to other pelvic intraperitoneal tissues	
T3	*III*	*Tumor involves 1 or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes*	
T1 or T2 N1 M0	III A 1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)	
		IIIA1(i)	Metastasis up to 10 mm in greatest dimension
		IIIA1(ii)	Metastasis more than 10 mm in greatest dimension
T3a N0 or N1 M0	III A 2	Microscopic extra-pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes	
T3b N0 or N1 M0	III B	Tumor involves 1 or both ovaries or fallopian tubes, or primary peritoneal cancer, with macroscopic deposits of cancer up to 2 cm spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	
T3c N0 or N1 M0	III C	Tumor involves 1 or both ovaries or fallopian tubes, or primary peritoneal cancer, with macroscopic deposits of cancer larger than 2 cm spread to the peritoneum outside the pelvis and may be on the capsule of liver or spleen and/or metastasis to the retroperitoneal lymph nodes	
Any T Any N M1a	*IV*	*Distant metastasis excluding peritoneal metastasis*	
Any T Any N M1a	IV A	Pleural effusion with positive cytology	
Any T Any N M1b	IV B	Hepatic and/or splenic parenchymal metastasis, metastasis to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)	

1.1.5 Treatment of EOC

As ovarian cancer was perceived as one disease entity for a long time, statistically “naturally” first-line therapy for epithelial ovarian cancer has primarily been investigated based on the response behavior of high-grade serous ovarian cancer (HGSC), which constitutes by far the largest subgroup of epithelial ovarian carcinomas (Reid, Permuth, and Sellers 2017). Traditionally, treatment of ovarian cancer patients in medically good conditions with advanced disease encompasses cytoreductive surgery followed by an intravenously administered chemotherapy:

Surgery includes a total abdominal hysterectomy, bilateral salpingo-oophorectomy (TAHBSO), omentectomy, and, if indicated, sampling of the pelvic and paraaortic lymph nodes (AWMF 2020; Ledermann et al. 2013; Karam et al. 2017). Macroscopically unsuspicious appearing lymph nodes are suggested to be left in place as systematic pelvic and paraaortic lymphadenectomy in the international AGO LION trial has shown not to improve overall survival (Harter et al. 2019). Next to optimal cytoreduction, surgery as the initial treatment of choice aims to confirm the diagnosis and define the disease’s extent. Optimal or complete cytoreduction, meaning to resect all visible tumor tissue, has a determining impact on the outcomes of ovarian cancer patients (du Bois et al. 2009; Chang, Bristow, and Ryu 2012). In contrast, secondary cytoreductive surgery (SCRS) shows conflicting results in the literature and is only considered for selected patients with platinum-sensitive recurrence (Coleman et al. 2019; Du Bois et al. 2020).

Standard medicinal treatment for ovarian cancer, but ovarian carcinoma FIGO stage I A (Trimbos et al. 2003), includes an adjuvant intravenous platinum- and taxane-based combination chemotherapy. Innovations in dosing and the combination of therapeutic agents have helped improve the prognosis of eligible ovarian cancer patients.

Conventional medicinal ovarian cancer treatment can be combined with targeted treatment (Ledermann et al. 2018; AWMF 2020; Ledermann et al. 2013; Colombo et al. 2019): Approved targeted therapy for ovarian cancer includes angiogenesis inhibitors (Burger et al. 2011; Perren et al. 2011) and PARP inhibitors (poly-(ADP)-ribose polymerase) (Mirza et al. 2020; Fong et al. 2009; Franzese et al. 2019).

By blocking vascular endothelial growth factor, angiogenesis inhibitors like *bevacizumab* impede angiogenesis needed to nourish the tumor tissue. Lack of blood supply delays cancer growth and spread (Chen et al. 2004). Ovarian carcinoma FIGO stage III C and IV qualify for supplemental treatment with bevacizumab as the agent demonstrated at least modest survival benefits for these patients (Burger et al. 2011; Haunschild and Tewari 2020; Oza et al. 2015). *PARP inhibitors* interfere with tumor cell repair mechanisms: through chemotherapeutic regimens damaged or genetically predisposed cancer cells fail to self-repair and induce apoptosis under treatment with PARP inhibitors (Edwards et al. 2008). Initially, PARP inhibitors were reserved for patients with a BRCA1 or BRCA2 mutation (Fong et al. 2009) and, more recently, introduced in maintenance therapy of recurrent ovarian cancer, independent of the presence of BRCA1 or BRCA2 germline mutations (Moore et al. 2018; Mirza et al. 2016; Moore et al. 2019). Further, PARP inhibitors may also benefit patients with platinum-responsive HGSC or endometrioid carcinoma in first-line therapy: Gonzales-Martin et al. found a significantly longer median progression-free survival in the subset of patients treated in first-line with niraparib than in those who received the placebo (Gonzalez-Martin et al. 2019). Possible synergistic effects of combination therapy of PARP inhibitors and bevacizumab in first-line maintenance for advanced HGSC and endometrioid ovarian cancer – following platinum-based chemotherapy and treatment with bevacizumab – were also proven (Ray-Coquard et al. 2019). In the PAOLA-1 (Platine, Avastin, and OLAparib in 1st line) trial, investigators found a statistically significant improvement of progression-free survival in patients treated with the combination therapy compared to bevacizumab alone. For predictions on the impact on overall survival, the data of these last-mentioned trials are too immature (Ray-Coquard et al. 2019)

The role of intraperitoneal chemotherapy shows conflicting results in the literature: with the recent, well-designed GOG252 trial, which suggests no clinical advantage but severe toxic side effects for intraperitoneally administered chemotherapy over a completely intravenous regimen, it further has lost popularity (Walker et al. 2019).

After widely abandoning radiotherapy in ovarian cancer treatment due to lesser efficacy and severe toxicities for the gastrointestinal and genitourinary tract compared to systemic chemotherapy, with improved dose distribution techniques, it may be re-inaugurated in the management of some ovarian cancers (Herrera et al. 2019; Iorio et al. 2019). Also, new data indicate that coadministration of radiotherapy and PARP inhibitors or immunotherapy may potentiate the effects of eradicating or controlling ovarian cancer (Demaria, Golden, and Formenti 2015; Weichselbaum et al. 2017).

Standard second look laparoscopy or laparotomy to evaluate a patient's response to treatment and detect recurrent disease was replaced by CT or PET/CT scan and MRI imaging (Gu et al. 2009). Initiation of treatment for relapsed disease only by elevation of the tumor marker CA 125 alone was proven not to improve survival but to significantly reduce the quality of life by stoking fears (Rustin et al. 2010).

Despite good initial response to systemic treatment after optimal debulking surgery in the majority of ovarian cancer patients, particularly in those diagnosed with HGSC, only a minority of patients will obtain a sustained remission — lack of effective treatment options for patients with refractory disease results in poor long-term survival (Mullen, Kuroki, and Thaker 2019). Novel treatment strategies are urgently needed to improve survival in ovarian cancer patients.

1.2 Cancer/Testis Antigens

Some cancers are able to provoke an immune response in the human body (Sato et al. 2005; Sahin et al. 1995). Immunotherapy aims to treat diseases and infections by promoting or reinvigorating a patient's immune system (Mellman, Coukos, and Dranoff 2011; Waldman, Fritz, and Lenardo 2020). Over the last decades, immunotherapeutic therapies managing various recalcitrant cancers, primarily immune checkpoint inhibitors and oncovaccines, proved successful and were implemented in standard medicinal treatment, which will be discussed below.

In the management of ovarian cancer, so far, immunotherapeutic treatment attempts have been without striking success – still, hopes are pinned on immunotherapy as an opportunity in the treatment of ovarian cancer (Odunsi 2017; Kandalaft, Odunsi, and Coukos 2019; Ghisoni et al. 2019; Krishnan, Berek, and Dorigo 2017; Yang et al. 2020; Coukos, Tanyi, and Kandalaft 2016). Cancer/testis antigens were proven to be highly immunogenic, cancer-specific and frequently expressed in various types of cancer, making them eligible as promising targets for cancer immunotherapy (Scanlan et al. 2002; Simpson et al. 2005; Caballero and Chen 2009).

1.2.1 Neoantigens

All human somatic, nucleus-bearing cells can present peptide antigens on major histocompatibility complex (MHC) molecules that interact with T cell receptors (TCR) located on a T cell membrane. If a peptide presented on the MHC is recognized as foreign, a T cell response, aiming at causing apoptosis or inactivation of the target cell, is elicited (Huang et al. 1999). When arising from healthy cells, tumor cells develop distinctive characteristics recognizable by the immune system. Either these antigens can derive from native proteins for which T cell tolerance is incomplete (e. g., tissue or time-restricted proteins), or they can evolve from proteins that are not provided in the human genome (e. g., mutated proteins) (Schumacher and Schreiber 2015). Tumor-specific antigens can enable T cell responses (Sahin et al. 1995; van der Bruggen et al. 1991).

1.2.2 Discovery of NY-ESO-1 (New York esophageal squamous cell carcinoma-1)

NY-ESO-1 (New York esophageal squamous cell carcinoma-1), also known as CT6.1 (Cancer/testis antigen 6.1), CTAG1 (Cancer/testis antigen 1), and LAGE-2 (L-antigen-family-member 2), belongs to the growing family of human cancer/testis antigens. Tumor antigens were first discovered in 1991 when Boon and colleagues proved the immunogenicity of the cancer/testis antigen MAGE-1 (melanoma antigen 1) in a melanoma cancer patient by showing its ability to stimulate the corresponding autologous cytotoxic T-lymphocytes (van der Bruggen et al. 1991). Homology searches identified various immediate family members; the Ludwig Institute for Cancer Research (LICR) lists all identified members in an online database (LICR 2021).

The homolog NY-ESO-1 was identified in 1997 by Old and his group by applying the SEREX (serological analysis of recombinant cDNA expression libraries) technique to the serum of an esophageal cancer patient (Chen et al. 1997). NY-ESO-1 was named after the location of the research group, New York, and the patient's cancer type, esophageal cancer, whose serum was screened applying the SEREX method. The suffix "1" was added because it was the first member of a newly detected gene family (Gnjatic et al. 2006).

NY-ESO-1 is of particular research interest as it exhibits a high capacity to elicit simultaneously coordinated humoral and cell-mediated immune responses in patients with NY-ESO-1 expressing tumors (Jager et al. 1998; Scanlan et al. 2001). The cancer/testis antigen NY-ESO-1 is considered an important cancer vaccine and immunotherapeutic target because of its immunogenicity. In healthy individuals, spontaneous immune responses against NY-ESO-1 were not observed (Oshima et al. 2016).

In 2009 the NCI antigen prioritization panel ranked NY-ESO-1 in the top 10 antigens to further develop immunotherapies (Cheever et al. 2009).

1.2.3 Biology of NY-ESO-1

A characteristic of many cancer/testis antigens is that their coding genes are located on the X-chromosome: this also applies to the gene coding for NY-ESO-1, located on chromosome Xq28. The gene product NY-ESO-1 is a 189 amino acid long protein of 18 kDa with a hydrophilic Glycerin-rich N-terminal region and a strongly hydrophobic C-terminal region.
Its structure could be confused with a transmembrane protein; however, association with membranes could not be shown. Staining of NY-ESO-1 can most frequently be detected in the apical and luminal aspect cell cytoplasm (Schultz-Thater et al. 2000; Fratta et al. 2011).

Aside from aberrant expression in numerous human cancer types, the expression of cancer/testis antigens in mature tissues is restricted to immune-privileged spermatogonia and primary spermatocytes: however, lacking HLA-class I molecules, male germ cells are incapable of presenting antigens to T lymphocytes (Gnjatic et al. 2006; Jungbluth et al. 2001; Simpson et al. 2005). An immune response can be evoked by extratesticular expression of cancer/testis antigens.

NY-ESO-1 expression has been reported in a wide range of tumor types: among NY-ESO-1-expressing cancers are bladder cancer, breast cancer, cervical cancer, esophageal cancer, head and neck cancer, hepatocellular cancer, metastatic melanoma, multiple myeloma, myxoid and round cell liposarcoma, non-small cell lung cancer, ovarian cancer, prostate cancer, and synovial sarcoma (Sharma et al. 2003; Sugita et al. 2004; Sarcevic et al. 2003; Chen et al. 1997; Kienstra et al. 2003; Chen et al. 1998; Nakamura et al. 2006; van Rhee et al. 2005; Hemminger et al. 2013; Lee et al. 1999; Yakirevich et al. 2003; Odunsi et al. 2003; Szender et al. 2017; Nakada et al. 2003; Kerkar et al. 2016; Park et al. 2016).

Only a few cancer/testis antigens have been ascribed a role yet. The exact biological function of the cancer/testis antigen NY-ESO-1 is still unknown.

In contrast to its cytoplasmatic expression in cancer cells, NY-ESO-1 shows nuclear expression in mesenchymal stem cells (Cronwright et al. 2005). In the gamete, the localization and restricted expression during early spermatogenesis suggest a role in germ cell self-renewal or differentiation. Proposed functions of cancer/testis antigens in germ cells include implication in the sperm metabolism, contribution to maintaining genomic integrity, and regulation of mRNA expression. Modulation of gene expression, regulation of tumorigenic signaling, and inference with the mitotic progression of tumor cells are among the suggested functional roles of cancer/testis antigens in tumors (Whitehurst 2014).

Several monoclonal antibodies, including ES121 (Schultz-Thater et al. 2000; Szender et al. 2017; Odunsi et al. 2003; Vaughan et al. 2004), E978 (Vaughan et al. 2004), and D8.38 (Yakirevich et al. 2003) that are believed to react with only NY-ESO-1, and not the highly homologous cancer/testis antigen to NY-ESO-1 LAGE-1 (ESO-2), are available to study the presence of NY-ESO-1 by immunohistochemistry.

1.3 Rationale

In ovarian cancer, proteins with expression patterns restricted to cancerous tissues have also been identified as potential immunotherapy targets. Based on encouraging results on anti-tumor responses, we further investigated the cancer/testis antigen NY-ESO-1. Due to its nature, NY-ESO-1 gives hope of being a promising target in antigen-specific immunotherapy: its expression in mature, targetable tissues is limited to cancerous cells, and it can be found in a wide number of different cancers, and it shows high immunogenicity in natural settings. Therefore, the current study was set to investigate the cancer/testis antigen NY-ESO-1 expression and its clinicopathological features in ovarian cancer.

2 MATERIAL AND METHODS

2.1 Tissue Microarrays

The tissue microarrays (TMA) technique is a molecular biology research method that allows simultaneous analysis of multiple specimens while requiring only a small material sample size. Kononen et al. first implemented the methodology (Kononen et al. 1998). The technique represents an enhancement of multi-tissue blocks, initially conceptualized by Battifora and colleagues in 1986 and optimized and revisited over time (Battifora 1986; Battifora and Mehta 1990).

In tissue microarrays, punched cylindrical tissue cores of morphologically representative areas in formalin-fixed, paraffin-embedded tissues are placed in prepared slots arranged in a precise pattern in the recipient paraffin block. Depending on the core diameter, usually between 0.6 mm to 2 mm, up to 1000 specimens can be arranged in one tissue microarray. Thin tissue sections (usually 4 – 10 µm) are cut using a microtome and mounted on microscope slides for further processing. Besides standard histological analyses, tissue microarrays can be used for various techniques, including immunohistochemical and immunofluorescent staining or in situ hybridization for DNA and mRNA. The method is a time- and cost-effective alternative to standard techniques by processing multiple tissue samples simultaneously. Additionally, staining the specimen following a standardized protocol can minimize experimental variability and technical artifacts (Kononen et al. 1998; Remotti 2013).

2.2 Immunohistochemistry

Immunohistochemistry constitutes a microscopy-based technique to selectively detect protein expression by visualizing antibody antigen-binding while maintaining the structure, cellular characteristics, and antigenicity of target epitopes of biological tissues. The method was first introduced by Albert Coons in 1941 (Coons, Creech, and Jones 1941) and later adopted formalin-fixed, paraffin-embedded tissues by Taylor and Burns (Taylor and Burns 1974).

It is a user-friendly, reliable, and versatile method that can be performed at a reasonable cost-benefit ratio and is now widely used in research and diagnostics. Immunohistochemical staining is based on immune reactions exploiting the principle of antibodies binding specifically to antigens. By promoting, either directly or indirectly, a color-producing reaction, these antigen-antibody complexes are detected. The direct method is characterized by a direct linkage between the specific primary antibody and the molecular marker. That molecular marker may be, for instance, an enzyme that catalyzes a color-producing reaction or a fluorophore. The indirect method requires two steps: it involves an unlabeled primary antibody that binds to the target antigen, followed by the reaction of a secondary antibody, which is conjugated to an enzyme reporter or fluorophore, to the first unlabeled antibody. Signal amplification through a labeled secondary antibody binding to the unlabeled primary antibody is reached at a lower antibody concentration with the disadvantage of an additional time-consuming process step. The indirect method is used more frequently than its single-step equivalent (Taylor, Shi, and Barr 2010).

The immunohistochemical staining protocol for this experiment was developed in the laboratory of Oliver Dorigo, M.D., Ph.D. at the University of California, Los Angeles, CA, USA (UCLA). To ensure the validity of the staining, positive and negative control tissue sections were included, and an experimental record was maintained. Before the actual staining, to visualize specific staining and minimize non-specific background noise, all IHC experiment steps must be optimized. The antibody concentration providing the strongest staining of the target antigen at the lowest background staining has to be determined by serial dilutions of the concentrated antibody. The detailed immunohistochemical staining process will be described below, and incidents that resulted in the modification of the staining protocol during the staining process will be elucidated.

2.2.1 Adjustment of the Immunostaining Protocol

2.2.1.1 Deparaffinization

Formalin-fixation and paraffin-embedding (FFPE) is one of the most widely practiced tissue sample preservation approaches to enable long-lasting stability of morphological characteristics of tissue samples. Disadvantageous is the lengthy, multi-step process of unmasking the, as a result of this preservation technique masked, epitopes. Cryopreservation of tissue samples, by contrast, is a common alternative to formalin-fixation and paraffin-embedding but is less capable in terms of long-term preserving histological morphology. Due to the retrospective nature of this study with tissue harvested over 20 years from 1989 to 2009, preservation in FFPE was chosen, which conserves tissues substantially longer than cryopreservation (Taylor, Shi, and Barr 2010; Shi et al. 2008).

Initially, using a microtome, the TMA tissue blocks were cut into 4 µm thick sections; too thick tissue sections may cause artifactually false-positive staining (*Fig. 2.1*).

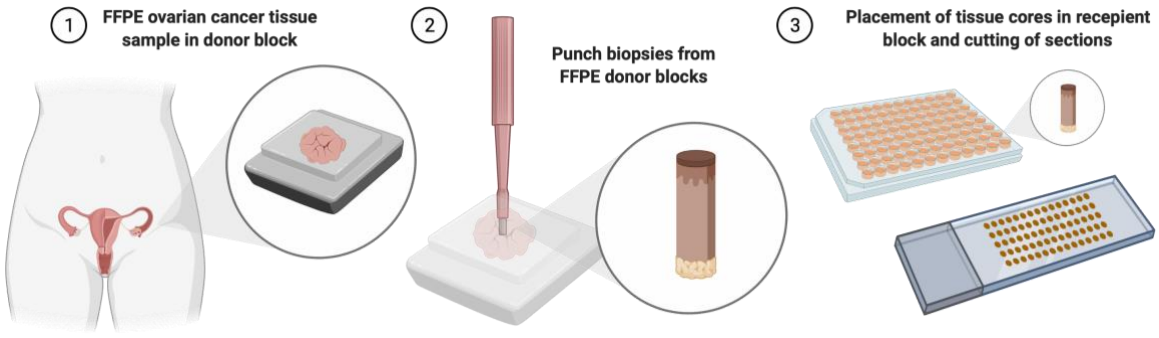

Fig. 2.1: Construction of tissue nicroarrays. Tissue samples from ovarian cancer patients were formalin-fixed and paraffin-embedded in donor blocks (①). Subsequently, cylindrical tissue samples (tissue cores) were obtained (②) and then arranged into microarray blocks that contained 150 to 180 tissue specimens. Then, the recipient block was cut into 4 µm thick sections (③) and finally adhered to the slides for immunohistochemical staining.

Figure created with BioRender.com

As noted above, in further preparation for the immunohistochemical staining, dried formalin-fixed paraffin-embedded specimens must be pretreated to unmask hidden epitopes and allow the antibodies full access to the antigens.

First, the water-repellent paraffin wax was removed from the sample, and the sample was rehydrated. In order to melt the paraffin wax, slides were heated to 55 - 60° C. Attention has to be paid to thorough deparaffinization as incomplete deparaffinization will result in uneven, blotchy dyeing and will potentially increase unwished background staining. Heating the paraffin wax to more than 60° C, in turn, might cause weak to absent staining or increased non-specific background staining (Taylor, Shi, and Barr 2010). To optimize the staining results and reduce background staining, in test runs, baking times of 30 min to 1 hour were tested; finally, a heating time of 1 hour at 60 ° C was selected.

Next, in the staining process, slides were washed multiple times in the alcohol solvent Xylene to solubilize and remove the warmed-up paraffin. Subsequently, Xylene was removed, and the specimens were rehydrated through a graded alcohol series, from pure to lower-proof ethanol. Finally, slides were rinsed in distilled water to complete the rehydration process. Tissue drying out, leading to non-specific interactions and high non-specific “background” or “off-target” staining, must also be avoided throughout the subsequent steps (Taylor, Shi, and Barr 2010). Then, endogenous peroxidase, which is physiologically present in some cell types and may also lead to false positivity, must be blocked (Radulescu and Boenisch 2007; Taylor, Shi, and Barr 2010; Streefkerk 1972). To reduce the effect of endogenous peroxidase, incubation in 3 % hydrogen peroxide (H_2O_2) is used as a standard in our laboratory to block endogenous peroxidase activity entirely.

2.2.1.2 Antigen Unmasking

Formalin is a popular preserving agent that stops autolytic processes and decomposition through the cross-linking of proteins.

Methylene bridges between proteins formed by formalin fixation can impede epitope recognition by antibodies, but the cross-linking can be reversed by exposure to heated buffer solutions of graded pH values (Fox et al. 1985; Sompuram et al. 2004). The most common method to undo the cross-linking and break the methylene bridges is heat-mediated epitope retrieval. Typically, applied solutions include heated sodium citrate (10 mM, pH 6), EDTA (1 mM, pH 8) or Tris/EDTA (pH 9) buffers. Variations of the technique primarily differ in used buffer solutions or heating modes.

Temperature, pH values, and time of incubation are crucial factors for proper antigen unmasking while causing the least possible morphological damage (Shi, Key, and Kalra 1991; Shi, Shi, and Taylor 2011; Taylor, Shi, and Barr 2010; Shi, Cote, and Taylor 2001).

In the last step to achieve unmasking, the slides were washed in Tris-Buffered Saline (TBS). TBS is used to maintain the pH level; due to a buffer capacity in a slightly alkaline range of 7,2 to 9, TBS overlaps with the physiological pH values of most living organisms. Incomplete rinsing of slides between steps might result in non-specific background staining (Taylor, Shi, and Barr 2010).

In this experiment, slides were heated for 60 minutes in a sodium citrate buffer for antigen retrieval. After cooling for 30 minutes at room temperature, slides were washed twice in TBS for 5 minutes each. A shorter incubation time of the tissue slides in antigen retrieval solution did not lead to adequate unmasking of epitopes in test runs. Extending the incubation time in antigen retrieval solution to 30 minutes led to more vigorous staining intensity with no observable morphological damage to the tissues. Alternatively, antigen retrieval can be achieved through proteases in a proteolysis-based technique by partially digesting proteins to unmask epitopes. However, enzymatic digestion carries a comparatively increased risk of altering tissue morphology (Taylor, Shi, and Barr 2010).

2.2.1.3 Immunostaining

After the sample preparation steps were completed, a blocking solution was applied to the slides before the actual staining.

Particular components in the blocking solution bind to non-specific binding sides and prevent binding of the secondary antibody leading to false-positive results. Besides self-made protein blocking buffers or commercially available pre-formulated blocking solutions, normal serum from the source species the secondary antibody was raised in is a commonly used blocking agent. Normal serum carries antibodies that bind to the non-specific reactive sites. Secondary antibodies are commonly generated by immunizing a healthy, previously unimmunized host animal, e. g., a horse, with a pooled population of immunoglobulins from the target species, e. g., a mouse, to generate highly specific reagents (Taylor, Shi, and Barr 2010).

For this experiment, we used a mouse primary antibody against the human protein NY-ESO-1. As the secondary antibody against the primary must be hosted in a third species, we utilized a biotinylated horse anti-mouse secondary antibody in this experiment. Thus, to prevent a non-specific binding of the primary antibody, normal horse serum was chosen to block all unspecific recognition sites. First, 10 percent normal horse serum was used as the blocking solution. Due to specific but weak staining intensity, we modified the protocol and diluted the blocking solution to 5 percent normal horse serum.

Next, the blocking solution was removed, and the primary antibody was added. There are two main types of antibodies, polyclonal and monoclonal. Polyclonal antibodies are more sensitive while monoclonal antibodies are more specific: polyclonal antibodies are more capable of detecting varying epitope-specificities to the disadvantage of a higher likelihood of “off-target” binding events and background staining. Monoclonal antibodies show a higher binding specificity but using a low-affinity antibody can result in false-negative errors. The staining results are dependent on the clonality of antibodies and the antibody concentration and exposure time to the antibodies. Keeping the time of exposure too long or adding too high strength antibody solution will increase the low-affinity off-target binding events once the on-target antigens are saturated with antibodies. Typical incubation times vary from one hour at room temperature to overnight at 4° C (Taylor, Shi, and Barr 2010).

Different dilutions and altered incubation times of the mouse anti-NY-ESO-1 monoclonal primary antibody were selected for test runs to optimize the staining results. Finally, the protocol was modified, and a dilution of the primary antibody of 1/200 incubation period of one night at 4° C was chosen.

Visualizing the antibody-antigen complexes is carried out through detection systems. Adding a secondary antibody conjugated to a reporter molecule, for instance a fluorophore or an enzyme that catalyzes the color-producing reaction, visualizes the antibody-target interactions. This incubation step usually takes one hour at room temperature but can be extended to overnight at 4° C. Extended incubation in the secondary antibody solution did not improve the staining intensity in test runs, so intubation time was set to 40 minutes at room temperature for the immunohistochemical staining of the final tissue microarray slides. In formalin-fixed, paraffin-embedded tissue samples, detection through color-producing enzymatic reactions is typically used: the enzyme, for example, horseradish peroxidase (HRP) or alkaline phosphatase (AP) coupled to the secondary antibody, metabolizes chromogens like 3,3' Diaminobenzidine (DAB) into brown precipitates. Chromogens tend to be very stable, which is advantageous if the stained tissue samples need to be archived (Taylor, Shi, and Barr 2010). A higher enzyme concentration at the antigenic site and, therefore, an increase in signal intensity upon adding the DAB reagent is accomplished by inserting an intermediate step: the avidin-biotin-complex (ABC) method. A biotinylated secondary antibody with specificity for the applied primary antibody is incubated with the tissue biopsies to bind to the primary antibody. Then the pre-incubated avidin-biotin-enzyme complex substrate is added to form complexes with the bound biotinylated secondary antibody. The enzyme turns the subsequently added DAB chromogen into brown precipitates, as described above. Avidin holds four binding sites and shows a high affinity to biotin. Cross-links of avidin-biotin-enzyme complexes bound to the biotinylated secondary antibody results in the accumulation of avidin-biotin-enzyme complexes at the target site, coinciding with a signal amplification upon adding the DAB substrate (*Fig. 2.2*) (Hsu, Raine, and Fanger 1981a, 1981b).

Non-specific background staining may be caused by an excessive chromogen concentration or overlong incubation time (Taylor, Shi, and Barr 2010). In this experiment, an incubation time of 30 minutes was chosen.

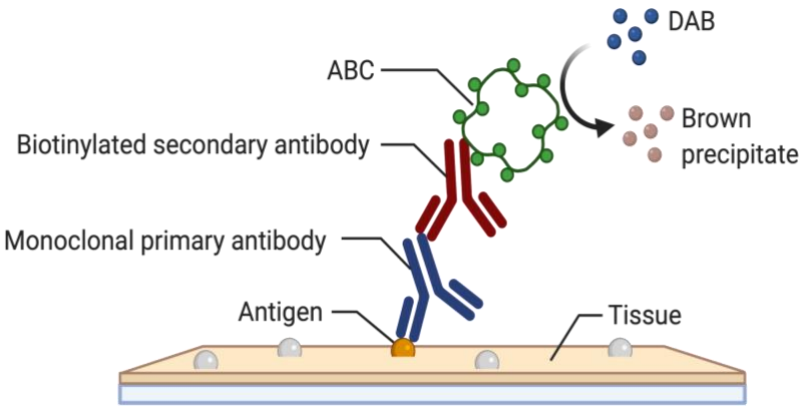

Fig. 2.2: The indirect avidin-biotin conjugate procedure. The avidin-biotin conjugate procedure can be used either as a direct or indirect technique. We employed the *indirect technique* in this experiment: First, the primary antibody was added, followed by a biotinylated secondary antibody and complexes of avidin and biotin horseradish peroxidase conjugate (ABC). The enzyme turns the subsequently added 3,3' Diaminobenzidine chromogen (DAB) into brown precipitates.

Figure created with BioRender.com

Modified from: Taylor CR, Shi S.-R., Barr, N. *Techniques of Immunohistochemistry: Principles, Pitfalls, and Standardization.* 3rd Edition ed. Philadelphia, PA: Saunders; 2010.

Counterstaining enhances the contrast and thus facilitates the interpretation of the staining results. The most common counterstaining method used for formalin-fixed, paraffin-embedded tissue samples is hematoxylin. Hematoxylin stains cell nuclei with a dark bluish color and cellular cytoplasm in a lighter bluish shade, contrasting the DAB chromogen's brown color for improved visualization of tissue morphology. Overstaining with hematoxylin is reduced by dipping the stained sections in acid alcohol for differentiation. Hematoxylin produces a red stain, and bluing can be induced by rinsing the slides with alkaline tap water or sodium bicarbonate. As residual water causes artifacts, dehydrating is essential to ensure no water remains. After dehydration is completed, coverslips are mounted onto microscope slides (Taylor, Shi, and Barr 2010).

2.2.2 Specimens on Tissue Microarrays

The study protocol was approved by the institutional review board. Specimens were collected from 242 patients who underwent cytoreductive surgery for ovarian cancer between 1989 and 2009 at UCLA (University of California, Los Angeles, CA, USA) and Kaiser Permanente Los Angeles, CA, USA. Subsequently, tissue microarrays of the archived formalin-fixed paraffin-embedded tumor specimens from ovarian cancer patients were constructed. Each case was represented by three 1.0 mm diameter tissue cores of randomly selected tumor areas on the tissue microarrays. Carcinomas were classified using the criteria of the World Health Organization Classification of Tumors of the Female Genital tract. Tumor stages were assessed following the FIGO guidelines. Histological findings were verified by a gynecologic pathologist who was not aware of patient characteristics and the disease course.

Fifty-two patients with borderline tumors and ovarian cancer diagnoses other than serous, endometrioid, and clear cell ovarian cancer were excluded from analysis because of insufficient subgroup sample sizes.

2.2.3 Investigated Patient Characteristics and Variables

All patients enrolled in the study underwent cytoreductive surgery for ovarian cancer with maximal cytoreductive effort. Their medical records were reviewed retrospectively and included information on in- and outpatient treatment, including surgery and chemotherapy. Clinically relevant information was extracted and included FIGO stage, grade, histology, age at diagnosis, race, weight, BMI, gravity, parity, preoperative CA 125 values, neoadjuvant chemotherapy, and platinum resistance. The contributing departments provided the anonymized database for analysis. Due to inconsistent assessment, the variable *residual disease* had to be excluded from the analysis.

Progression was defined as objective, image-morphological evidence of recurrent or progressive disease (Rustin et al. 2011) and *platinum resistance* as disease progression or relapse within six months after completing adjuvant first-line platinum-based chemotherapy (Wilson et al. 2017).

Progression-free survival was defined as the time from the end of initial treatment, including surgery and, if applicable adjuvant chemotherapy, to objective evidence of recurrent or progressive disease. Data were censored at the date of the last follow-up for patients with no evidence of progression, recurrence, or death. The duration of *overall survival* was the interval between diagnosis and death or last follow-up with censoring of patients who were lost to follow-up due to further treatment in an external institution.

2.3 Immunostaining Protocol

Initially, archival formalin-fixed paraffin-embedded tissues on the slides were baked for 60 min at 60° C, deparaffinized, rehydrated through graded ethanol, and treated in 3 % hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity.

The sections were then subjected to heat-induced antigen retrieval by autoclaving for 60 min in 10 mM citrate buffer, pH 6.0, and incubated overnight with the primary antibody, a mouse-anti-NY-ESO-1 E978 monoclonal antibody (Invitrogen # 18-2359). Detection was performed using the biotinylated anti-mouse IgG (BA2000) and 3,3'-diaminobenzidine (DAB) as a chromogen. Slides were then counterstained with hematoxylin and evaluated. Subsequently, the sections were dehydrated through graded ethanol and coverslipped. All cases with at least one assessable tissue core were included in the study. Figure 2.3 exemplifies strong NY-ESO-1 expression in tissue biopsy cores of clear cell, serous, and mucinous carcinomas (*Fig. 2.3*).

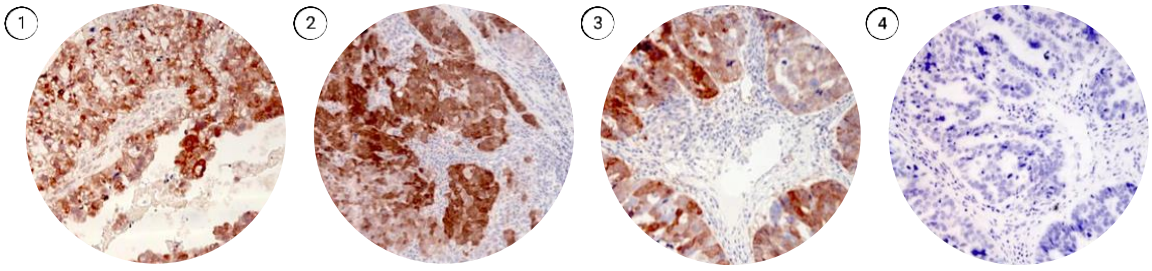

Fig. 2.3: Expression of NY-ESO-1 in various histological subtypes of ovarian cancer. NY-ESO-1 expression in clear cell carcinoma (①), serous carcinoma (②) and mucinous carcinoma (③); and a negative control (no primary antibody) (④).

Tissues from UCLA Department of Pathology
IHC with mouse anti-NY-ESO-1 monoclonal antibody (Invitrogen #18-2359) 1/200 (Dorigo Laboratory)

2.4 Analysis of Specimens on Tissue Microarrays

Stained tissue microarray slides were digitized using the bright field scanner Aperio ScanScope XT® (Aperio, Vista, CA) and imported into DEFINIENS Tissue Studio® software (DEFINIENS, Munich, Germany). The Aperio ScanScope XT® system transforms glass slides into high-resolution digital images. DEFINIENS Tissue Studio® software enables semi-quantitative automated biomarker detection. DEFINIENS Tissue Studio software was configured to quantify NY-ESO-1 staining in the tissue microarray specimens. Although automated tissue analysis of tissue microarrays in multiple studies was consistent with visual scoring by experienced pathologists, pathologist-generated interpretation and scoring remain the gold standard. Therefore, additionally to the automated assessment, a gynecologic pathologist assigned H-scores to the individual tissue cores on the tissue microarrays included in our analysis. A correlation between automated marker detection and interpretation of the experienced pathologist was seen; however, only the pathologist-assigned H-scores were included in this study's analysis.

As protein expression may not be homogenous within a tissue core leading to variable immunohistochemical staining patterns, histological scores (H-scores) were computed based on the intensity of NY-ESO-1 staining and percent positive cells at the same magnification to take the heterogeneous staining patterns of NY-ESO-1 into account (McCarty et al. 1986).

The H-score is a summation of the percentage of negative cells within a tissue core multiplied by zero, the percentage of positive cells with weak staining multiplied by one, the percentage of positive cells with moderate staining multiplied by two, and the percentage of positive cells with intense staining multiplied by three, resulting in an individual H-score for each core on a continuous scale from zero to 300 (McCarty et al. 1986). The mean of the triplicate samples for the individual patients was then calculated and included in our data analysis. A mean H-score of < 1, equivalent to no evidence of NY-ESO-1 expression in our study, was considered “negative”; in turn, any proof of NY-ESO-1 (mean H-score ≧ 1) was interpreted as “positive”.

2.5 Statistical Methods

All statistical analyses were carried out using GraphPad Prism 9.0.0 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com) and SPSS version 25.0 (SAS Institute Inc., Cary, NC). Categorical data were presented as absolute and relative frequencies. Metric data were displayed as mean with standard deviation (SD). Descriptive statistics were reported for patient characteristics and clinical variables. Inferential statistics were used to reach conclusions about the variables' associations; analyses were performed for all patients in the data set and independently for the platinum-resistant and -sensitive patient subgroups, respectively. Non-normally distribution of the parameter NY-ESO-1 in the one-sample Kolmogorov-Smirnov Goodness of fit test required non-parametric tests for inferential statistics. Comparing two independent, non-normally distributed samples, a Kruskal-Wallis one-way analysis of variance was applied; two related, non-normally distributed samples were tested using the Wilcoxon signed-rank test. The correlation of NY-ESO-1 with categorical variables (FIGO stage, grade, histology, SCRS, preoperative CA 125, platinum response) was evaluated using Pearson's chi-squared test and Fisher's exact test. Kaplan Meier curves and log-rank tests were used to estimate overall and progression-free survival. Multivariate Cox's regression analysis was performed to examine the effects of various variables on overall and progression-free survival. A p-value of less than 0.05 was considered statistically significant.

The graphical presentation was performed using Microsoft Excel 16.34 (Microsoft, Redmond, WA) for tables and GraphPad Prism 9.0.0 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com) for graphs and survival curves. Observed absolute or relative frequencies of occurrence for categorical data were displayed visually as column and circle diagrams; survival curves were visualized as Kaplan-Meier plots.

3 RESULTS

3.1 Descriptive Analysis of Patient Characteristics and Clinical Variables

The dataset includes 190 patients who underwent cytoreductive surgery for serous, endometroid, or clear cell ovarian cancer between 1989 and 2009 at UCLA and Kaiser Permanente Los Angeles. The median patient age at the time of diagnosis was 58.9 years. The duration of follow-up ranged from 0 months to 14.83 years (0 to 178.1 months; median 30.6 months). Clinicopathological parameters are specified below and summarized in Table 3.1 (*Table 3.1*).

Table 3.1: Patient characteristics

Characteristics	
Evaluable patients	190
Age at diagnosis [median (range)], years	59 (26 – 92)
Follow-up [median (range)], months	31 (0 – 178)
	n (%)
Race	
Caucasion	125 (66.5 %)
Black	10 (5.3 %)
Hispanic	17 (9.0 %)
Asian	21 (11.2 %)
Other	15 (8.0 %)
Histology	
Serous	112 (58.9 %)
Endometroid	41 (21.6 %)
Clear cell	37 (19.5 %)
FIGO stage	
I	26 (13.8 %)
II	22 (11.6 %)
III	114 (60.3 %)
III A / B*	7 (6.1 %)
III C*	107 (93.9 %)
IV	27 (14.3 %)
Grade	
1	14 (7.4 %)
2	31 (16.5 %)
3	143 (76.1 %)
BRCA1/2 mutation or Hx of breast cancer**	
BRCA1/2 mutation	4 (2.1 %)
Personal Hx of breast cancer	17 (8.9 %)
None of the above or unknown	169 (88.9 %)
Preoperative CA 125	
< 500 U/ml	83 (53.4 %)
≧ 500 U/ml	81 (46.6 %)
Secondary cytoreductive surgery	
Yes	19 (10.2 %)
No	168 (89.9 %)
Platinum response	
Platinum-sensitive	108 (72.0 %)
Platinum-resistant	42 (28.0 %)
Current disease status	
Alive, no evidence of disease (NED)	49 (27.4 %)
Dead from other causes (DNED)	4 (2.2 %)
Alive with disease (AWD)	21 (11.7 %)
Dead of disease (DOD)	105 (58.7 %)
IHC H-score NY-ESO-1	
< 1	124 (69.3 %)
≧ 1	55 (30.7 %)

**Distribution of III A/B and III C within the subgroup of stage III OCs

**limited validity; begin of patient enrollment for the study before routine assessment of BRCA-status

Due to insufficient tissue quality or lack of cancerous cells in the tissue biopsies, eleven of the 190 enrolled patients could not be assigned an H-score. Of the remaining evaluable 179 patients, 55 patients showed NY-ESO-1 expression (30.9 percent, *Fig. 3.1a*) with varying H-scores between 2 and 300 (*Fig. 3.1b*).

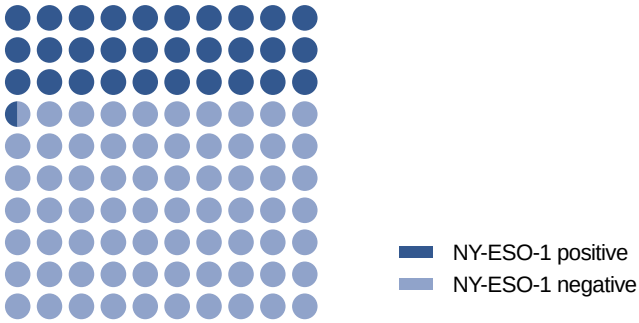

Fig. 3.1a: **Percentage of patients with NY-ESO-1 positive serous, endometrioid or clear cell ovarian carcinomas in our study cohort.** 31 % of patients in our dataset were found to show NY-ESO-1 expression (H-score ≧ 1) in their tumor biopsies.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

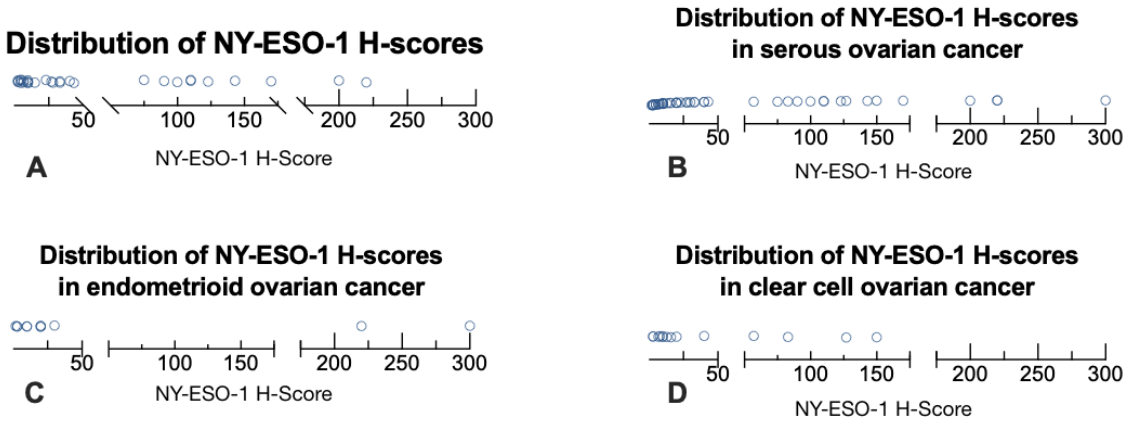

Fig. 3.1b: **Distribution of NY-ESO-1 H-scores in our study cohort.**
A The majority of the 55 tumors, that showed NY-ESO-1 expression, were assigned H-scores of 50 or less (n = 39, 70.9 %). Thirteen tumors (23.6 %) were assigned H-scores of 51 to 200 and only 3 tumors (5.5 %) showed a homogenous expression pattern with H-scores of 201 up to 300. **B – D** Distributions of NY-ESO-1 H-scores stratified by histological subtype of serous, endometrioid and clear cell ovarian cancer.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.1.1 Race

In 188 of 190 cases, the ethnic origin was known: 66.5 percent of patients were Caucasian (n = 125), 5.3 percent of patients were black (n = 10), 9.0 percent of patients were Hispanic (n = 17), and 11.2 percent of patients of Asian origin (n = 21). The remaining 15 patients (8.0 %), classified as “other”, originated from various regions (*Fig. 3.2*).

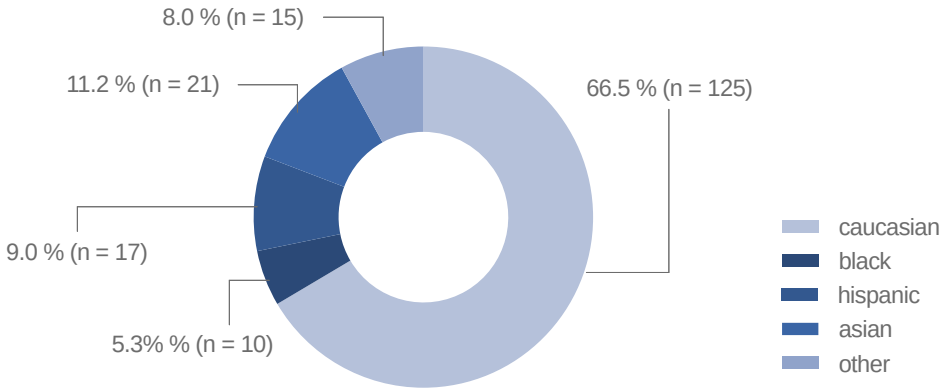

Fig. 3.2: **Percental distribution of races among patients in the dataset.**

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3.1.2 Histology

The histologic diagnosis was known in all the 190 patients enrolled in our research study. Forty-one patients (21.6 %) were diagnosed with endometroid carcinoma of the ovary, and 37 patients (19.5 %) were diagnosed with clear cell ovarian carcinoma. The majority of 112 patients (58.9 %) received the diagnosis of serous ovarian carcinoma (Fig. 3.3).

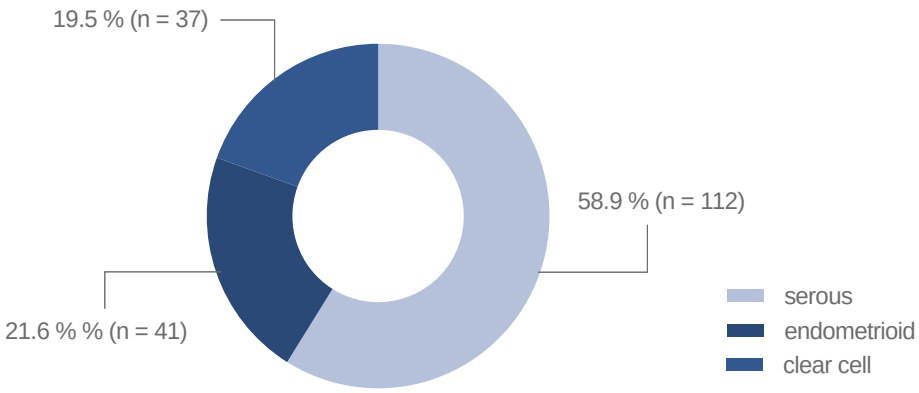

Fig. 3.3: **Percental distribution of histology among patients in the dataset.**

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3.1.3 FIGO Stage

The FIGO stage at the time of the first cytoreductive surgery was known in 189 of 190 cases. Almost three-quarters of patients (n = 141, 74.6 %) were diagnosed with ovarian cancer at FIGO stage III or IV, while 25.4 percent (n = 48) of patients received their diagnosis of ovarian cancer at FIGO stage I or II. When breaking the data down to the individual FIGO stages, 114 patients were diagnosed with ovarian cancer at FIGO stage III, accounting for 60.3 percent – among them were seven patients with FIGO stage III A or III B disease (n = 7, 6.1 %) and 107 patients with FIGO stage III C ovarian cancer (n = 107, 93.1 %). Twenty-six patients were diagnosed at FIGO stage I (13.8 %). 11.6 percent (n = 22) at FIGO stage II, and 14.3 percent (n = 27) of patients were diagnosed with ovarian cancer at FIGO stage IV (*Fig. 3.4*).

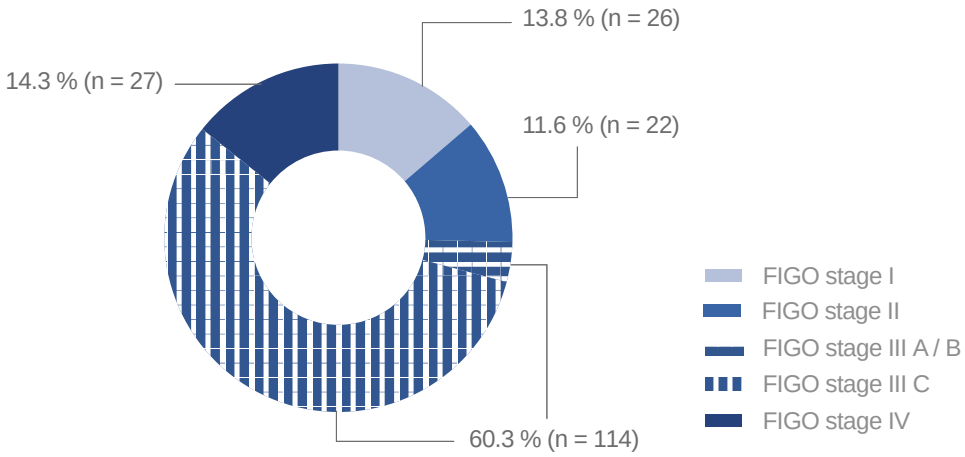

Fig. 3.4: **Percental distribution of FIGO stages among patients in the dataset.**

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3.1.4 Grade

At the time of the first cytoreductive surgery, the grade was known in 188 of 190 cases. More than three-quarters of patients (n = 143, 76.1 %) were diagnosed with ovarian cancer grade 3. 14 patients (7.4 %) had ovarian cancers grade 1, and 31 patients (16.5 %) ovarian cancers grade 2 (*Fig. 3.5*).

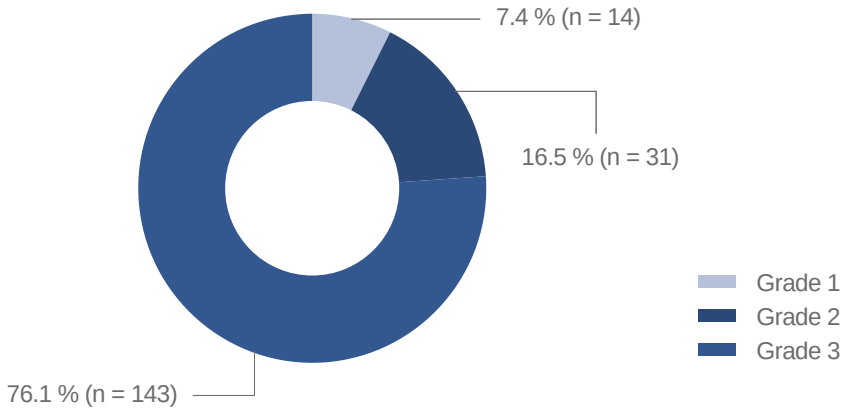

Fig. 3.5: **Percental distribution of grades among patients in the dataset.**

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.1.5 BRCA1/2 Mutation and/or Personal History of Breast Cancer

The validity of the data on BRCA1/2 germline gene mutations is limited due to the beginning of patient enrollment for the study before routine assessment of the BRCA-status. Four patients (2.1 %) in the patient cohort had a verifiable mutation in the BRCA1 or BRCA2 gene. Seventeen (9.0 %) other patients reported a personal history of breast cancer, not aware of a potential BRCA1/2 mutation. The remaining 169 patients (88.9 %) in the cohort did not have a personal history of breast cancer, were negative for BRCA1/2, or their BRCA mutational status was unknown.

3.1.6 Preoperative CA 125 and Secondary Cytoreductive Surgery

In 174 of 190 patients, CA 125 was taken preoperatively or documented. Ninety-three patients showed a preoperative CA 125 of < 500 U/ml (53.4 %) and 81 patients of ≥ 500 U/ml (46.6 %) (*Fig. 3.6)*. In 187 of 190 patients, the status of secondary cytoreductive surgery was known. One hundred sixty-eight patients (89.9 %) did not undergo secondary cytoreductive surgery, while 19 patients (10.2 %) had another cytoreductive surgery (*Fig. 3.7*).

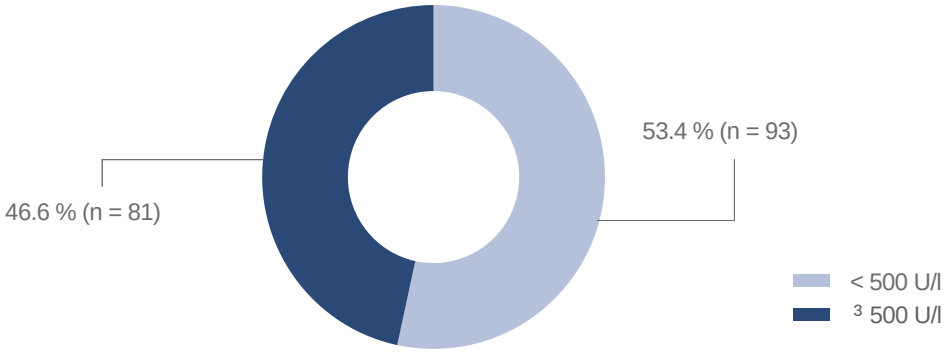

Fig. 3.6: **Percental distribution of higher and lower preoperative CA 125 measures among patients in the dataset.**

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

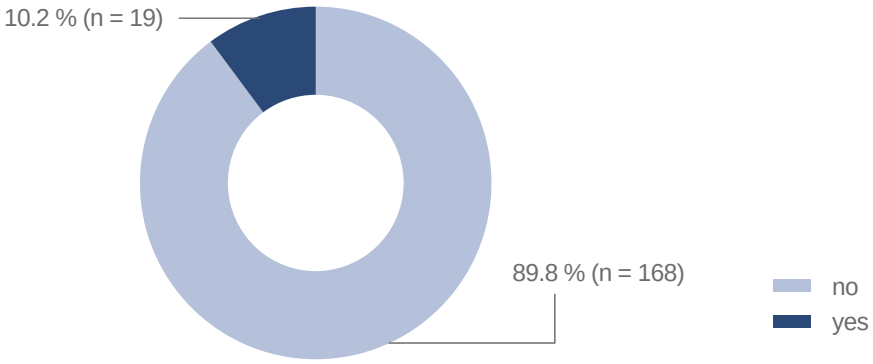

Fig. 3.7: **Percental distribution of necessary secondary cytoreductive surgery** (SCRS) **among patients in the dataset.**

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.1.7 Platinum Response

All patients reportedly underwent staging laparotomy, including radical surgical debulking. Nineteen patients, whom all were diagnosed with FIGO stage III C or IV disease, received neoadjuvant chemotherapy. In total, 163 received platinum-based chemotherapy at the participating institutions, including the 17 patients who had neoadjuvant chemotherapy before surgery. Information on medicinal treatment for patients treated postoperatively at other facilities was not available. Neither was information on supplemental targeted treatment obtained. None of the patients received radiation therapy before or after surgery.

Platinum resistance was defined as ovarian cancers refractory to platinum-based chemotherapy or recurrence or progress of the disease within six months after initial debulking surgery. One hundred eight patients responded to platinum-based chemotherapy (72.0 %), while 42 patients (28.0 %) showed platinum resistance. The follow-up of 13 patients was too short (0.0 to 5.2 months) to categorize them as platinum-resistant or platinum-sensitive (*Fig. 3.8*).

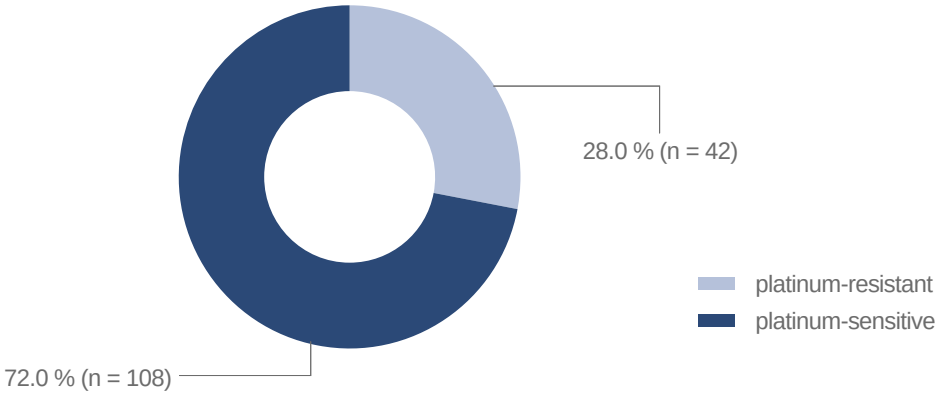

Fig. 3.8: Percental distribution of platinum-resistant and -sensitive patients among patients in the dataset.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.1.8 Disease Status

In 179 of 190 cases, the current disease status was known. One hundred five patients had died from ovarian cancer (DOD, 58.7 %), and four patients had passed away without evidence of disease (DNED, 2.2 %). Twenty-one patients were alive, still struggling with ovarian cancer (AWD, 11.7 %), while 49 patients were alive without evidence of disease (NED, 27.4 %) (*Fig. 3.9*).

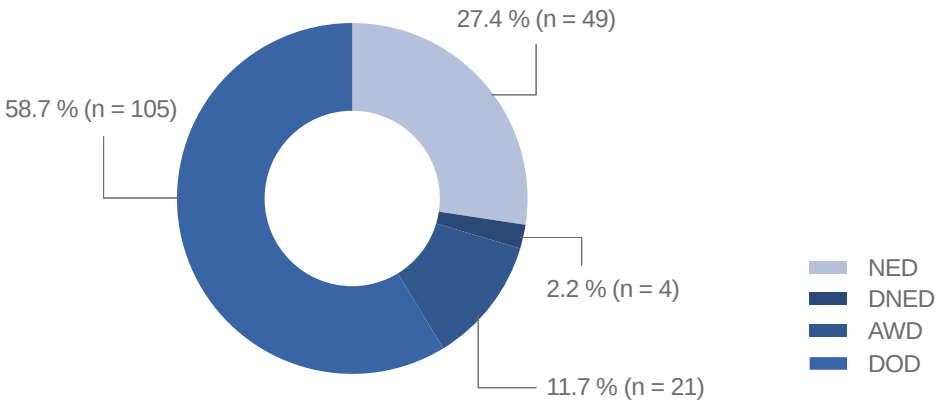

Fig. 3.9: **Percental distribution of disease status** (NED, alive with no evidence of disease; DNED, dead no evidence of disease; AWD, alive with disease; DOD, dead of disease) **among patients in the dataset.**

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.2 Stratification according to Platinum Response

Chi-square test of independence, or Fisher's exact test, respectively, were conducted to examine the relations between platinum response and clinical variables. The distribution of disease status (*Fig. 3.10*) was significantly different among the subsets of platinum-resistant and platinum-sensitive patients ($p < 0.0001$). Distributions of other important clinical variables, such as race, histology, FIGO stage, grade, SCRS, and preoperative CA 125, did not differ significantly ($p > 0.05$) between the two subgroups of platinum-resistant versus platinum-sensitive patients. Neither was the expression of NY-ESO-1 significantly different in platinum-resistant and platinum-sensitive patients ($p = 0.4686$) (*Fig. 3.12*).

The results are summarized in Table 3.2; the statistically significant distributions of disease status and histology as well as the statistically not significant distribution of NY-ESO-1 expression in platinum-resistant and -sensitive patients are shown in graphs Fig. 3.10 and 3.11, respectively (*Table 3.2, Figures 3.10 and 3.11*).

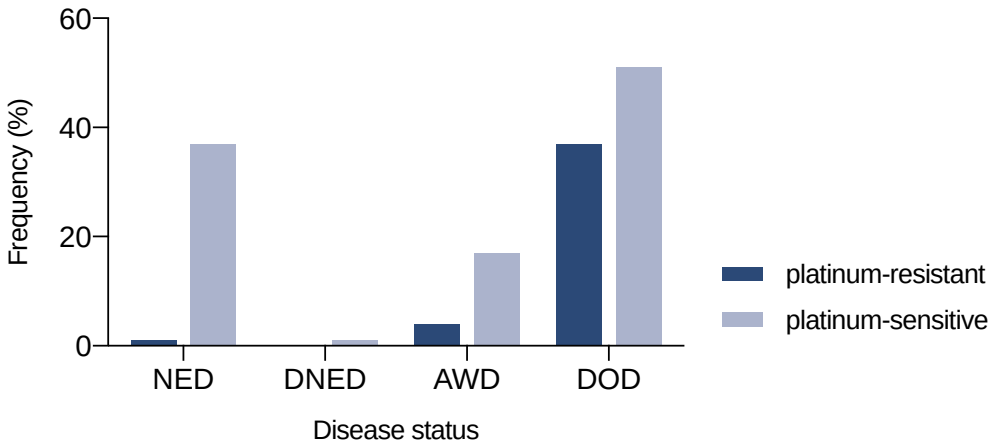

Fig. 3.10: Disease status in platinum-resistant and -sensitive patients. The distribution of **disease status** (NED, alive with no evidence of disease; DNED, dead no evidence of disease; AWD, alive with disease; DOD, dead of disease) was found to be significantly different among the subsets of platinum-resistant and platinum-sensitive patients ($p < 0.0001$).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

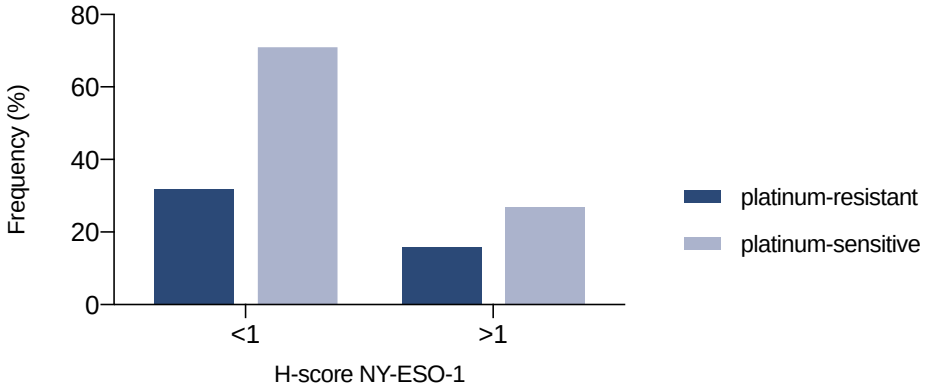

Fig. 3.11: NY-ESO-1 expression in platinum-resistant and -sensitive patients. The distribution of **NY-ESO-1 positivity** was not significantly different among the subsets of platinum-resistant and platinum-sensitive patients (p = 0.4686).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

Table 3.2: Summary of patient characteristics and clinical variables stratified by platinum response.

Characteristics	*Platinum-resistant n (%)*	*Platinum-sensitive n (%)*	*p-value*
Race			
Caucasion	28 (29.8 %)	66 (70.2 %)	0.160
Black	4 (44.4 %)	5 (66.6 %)	
Hispanic	5 (33.3 %)	10 (77.7 %)	
Asian	1 (5.2 %)	18 (94.7 %)	
Other	4 (33.3 %)	8 (77.7 %)	
Histology			
Serous	22 (22.4 %)	76 (77.6 %)	0.076
Endometroid	7 (31.8 %)	15 (68.2 %)	
Clear cell	13 (43.3 %)	17 (56.7 %)	
FIGO stage			
I	2 (15.4 %)	11 (84.6 %)	0.153
II	3 (17.6 %)	14 (82.4 %)	
III	27 (27.6 %)	71 (72.4 %)	
III A / B*	2 (33.3 %)	4 (66.6 %)	
III C*	25 (27.2 %)	67 (72.8 %)	
IV	10 (45.5 %)	12 (54.5 %)	
Grade			
1	2 (22.2 %)	7 (77.8 %)	0.249
2	3 (13.6 %)	19 (86.4 %)	
3	36 (30.5 %)	82 (69.5 %)	
Preoperative CA 125			
< 500 U/ml	24 (33.8 %)	47 (66.2 %)	0.213
≧ 500 U/ml	17 (24.3 %)	53 (75.7 %)	
Secondary cytoreductive surgery			
Yes	37 (28.2 %)	94 (71.8 %)	0.967
No	5 (27.8 %)	13 (72.2 %)	
Current disease status			
ANED	1 (2.6 %)	37 (97.4 %)	0.0001
DNED	0 (0 %)	1 (100 %)	
AWD	4 (19.0 %)	17 (81.0 %)	
DOD	37 (42.0 %)	51 (58.0 %)	
IHC H-score NY-ESO-1			
< 1	28 (28.0 %)	72 (72.0 %)	0.469
≧ 1	14 (34.1 %)	27 (65.9 %)	

*Distribution of III A/B and III C within the subgroup of FIGO stage III ovarian cancers

3.3 Overall and Progression-free Survival

The Kaplan-Meier estimator was used to estimate the overall and progression-free survival. Patients with loss to follow-up were censored. The duration of follow-up ranged from 0 months to 14.83 years (178.1 months); the median time of follow-up was 30.6 months. One year after the initial diagnosis of ovarian cancer, 24 patients in the dataset had died, leading to a one-year survival rate of 86.4 percent. After five years, 46.2 percent of patients were still alive (median = 49.4 months). The Kaplan-Meier curve below gives a visual representation of overall survival for all patients in the cohort (*Fig. 3.12*).

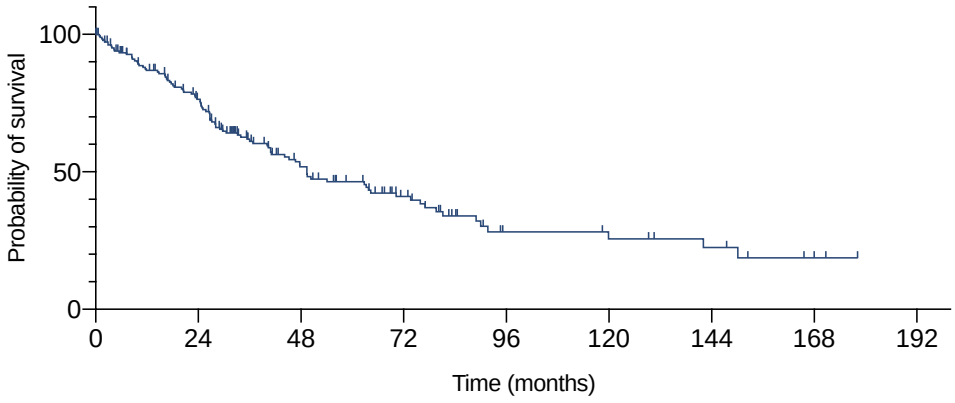

Fig. 3.12: Kaplan-Meier estimator for overall survival. Overall survival curve for all patients in the dataset.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

The subset of patients treated with adjuvant platinum-based chemotherapy was stratified by their platinum response with, again, *platinum resistance* defined as progression or recurrence of disease within the first six months after completion of platinum-based chemotherapy. Patients resistant to platinum-based chemotherapy showed a one-year survival of 61.6 percent and five-year survival of 8.4 percent (median = 18.3 months).

In contrast, all patients sensitive to platinum-based chemotherapy were still alive one year after the initial diagnosis of ovarian cancer and showed a survival rate of 62.3 percent at five years (median = 75.93 months).

The results of the Kaplan-Meier analysis suggest that the 1- and 5-year mortality probability was higher in platinum-resistant than in platinum-sensitive patients; according to the log-rank test, this difference was statistically significant ($p < 0.0001$) (*Fig. 3.13*).

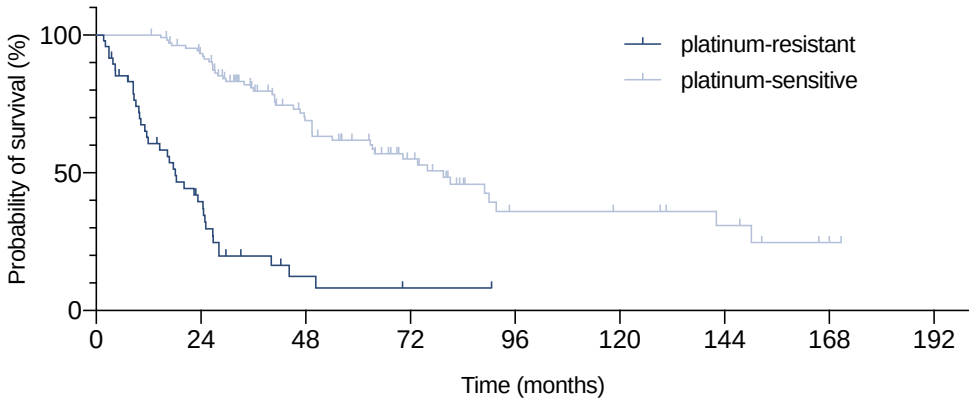

Fig. 3.13: Kaplan-Meier estimator for overall survival in platinum-resistant and -sensitive patients. Overall survival curves for patients based on their platinum response ($p < 0.0001$).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

By definition, all patients resistant to platinum showed progression of the disease within six months of completion of platinum-based chemotherapy. Conversely, patients sensitive to platinum-based chemotherapy showed a one-year progression-free survival chance of 73.8 percent and a five-year progression-free survival chance of 23.1 percent. In terms of progression-free survival, the log-rank test proved distinguishable progression-free survival probability, comparing the subgroups of platinum-resistant and platinum-sensitive patients ($p < 0.0001$). The Kaplan-Meier curve below gives a visual representation of progression-free survival for platinum-sensitive and -resistant patients (*Fig. 3.14*).

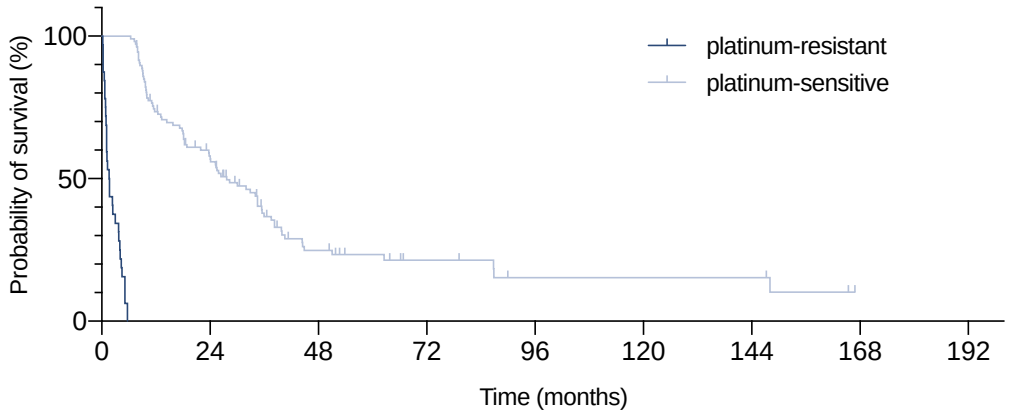

Fig. 3.14: Kaplan-Meier estimator for progression-free survival in platinum-resistant and -sensitive patients. Progression-free survival curves for patients based on their platinum response ($p < 0.0001$).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

Irrespective of their platinum response, patients had a one-year progression-free survival chance of 53.7 percent and a five-year progression-free survival chance of 17.6 percent (median = 17.7 months). The Kaplan-Meier curve below gives a visual representation of progression-free survival for all patients in the cohort (*Fig. 3.15*).

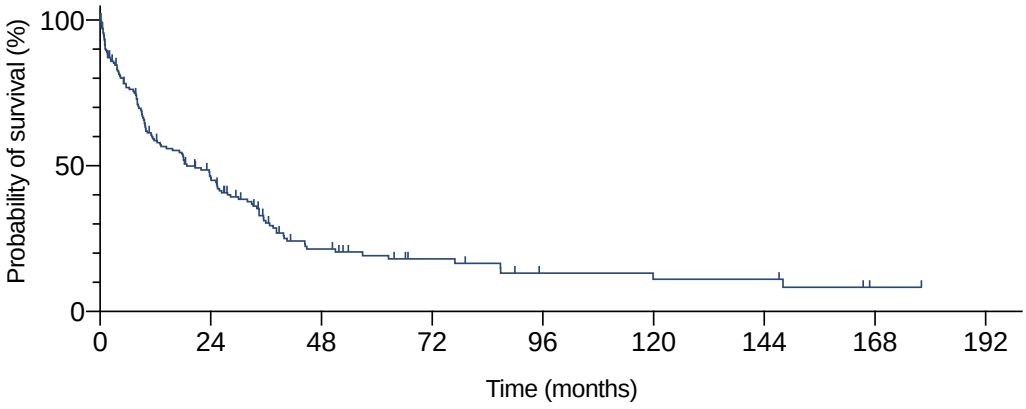

Fig. 3.15: Kaplan-Meier estimator for progression-free survival. Progression-free survival curve for all patients in the dataset.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.4 Association of NY-ESO-1 Expression, Clinicopathological Variables and Survival

3.4.1 NY-ESO-1 Expression and Clinicopathological Variables

Further, NY-ESO-1 expression H-scores and their distribution across clinical variables among all patients and solely for the platinum-resistant and platinum-sensitive patient subgroups, respectively, were examined. Testing three or more alternatives, the independent-samples Kruskal-Wallis test was used, and comparing only two different alternatives, the independent-samples Mann-Whitney U test was applied.

NY-ESO-1 H-scores did not correlate significantly with any of the tested clinical variables of histology, FIGO stage, grade, preoperative CA 125, SCRS, and platinum response when taking into account all patients in the dataset ($p > 0.05$). With increasing FIGO stage, increasing mean H-scores were documented, but not at a significant level. (*Table 3.3a*).

Neither were significant differences found among the subgroups of platinum-sensitive or platinum-resistant patients regarding NY-ESO-1 expression and the examined clinical variables (disease status, histology, FIGO stage, grade, SCRS, and preoperative CA 125) ($p > 0.05$) (*Table 3.3b and 3.3c*).

Table 3.3a: Correlation analysis of NY-ESO-1 expression levels with clinical variables.

Characteristics			
	n	*NY-ESO-1 H-score mean (SD)*	*p-value*
Histology			
Serous	107	15.5 (41.6)	0.392
Endometrioid	38	16.8 (59.3)	
Clear cell	34	16.1 (36.0)	
FIGO stage			
I	24	3.8 (8.3)	0.871
II	19	9.9 (28.9)	
III	108	18.6 (50.1)	
III A / B*	7	12.3 (11.8)	
III C*	101	19.0 (51.3)	
IV	27	20.6 (50.0)	
Grade			
1	14	1.0 (2.7)	0.336
2	31	15.7 (44.1)	
3	143	17.7 (47.3)	
Preoperative CA 125			
< 500 U/ml	93	16.2 (43.1)	0.693
≧ 500 U/ml	81	16.5 (49.0)	
SCRS			
Yes	168	15.5 (41.3)	0.893
No	19	23.3 (15.4)	
Platinum response			
Platinum-resistant	42	8.7 (22.2)	0.697
Platinum-sensitive	99	17.4 (49.9)	

*Distribution of III A/B and III C within the subgroup of FIGO stage III ovarian cancers

Table 3.3b: Correlation analysis of NY-ESO-1 expression levels with clinical variables for platinum-resistant patients.

Characteristics

	n	***NY-ESO-1 H-score mean (SD)***	*p-value*
Histology			
Serous	22	2.8 (8.0)	0.125
Endometrioid	7	16.3 (22.9)	
Clear cell	13	14.7 (34.2)	
FIGO stage			
I	2	1.5 (2.1)	0.998
II	3	6.7 (11.6)	
III	27	6.1 (14.1)	
III A / B*	2	5.5 (6.4)	
III C*	25	14.2 (20.0)	
IV	10	18.0 (38.8)	
Grade			
1	2	6.5 (5.0)	0.158
2	3	0.0 (0.0)	
3	36	9.8 (23.8)	
Preoperative CA 125			
< 500 U/ml	24	4.7 (10.8)	0.333
≧ 500 U/ml	17	15.0 (31.9)	
SCRS			
Yes	5	12.6 (16.1)	0.235
No	37	8.2 (23.0)	

*Distribution of III A/B and III C within the subgroup of FIGO stage III ovarian cancers

Table 3.3c: Correlation analysis of NY-ESO-1 expression levels with clinical variables for platinum-sensitive patients.

Characteristics

	n	***NY-ESO-1 H-score mean (SD)***	*p-value*
Histology			
Serous	76	14.6 (40.1)	0.361
Endometrioid	15	40.9 (94.4)	
Clear cell	17	8.3 (22.0)	
FIGO stage			
I	9	5.9 (11.0)	0.938
II	11	2.5 (4.7)	
III	67	20.5 (54.4)	
III A / B*	4	20.8 (41.5)	
III C*	63	20.4 (55.4)	
IV	12	22.9 (62.8)	
Grade			
1	7	0.0 (0.0)	0.106
2	17	7.8 (11.5)	
3	75	21.3 (56.7)	
Preoperative CA 125			
< 500 U/ml	42	16.6 (44.0)	0.740
≧ 500 U/ml	49	20.7 (58.2)	
SCRS			
Yes	11	28.2 (90.2)	0.574
No	87	16.3 (43.3)	

*Distribution of III A/B and III C within the subgroup of FIGO stage III ovarian cancers

3.4.2 NY-ESO-1 Positivity and Clinicopathological Variables

Further, patients with NY-ESO-1 scores ≥ 1 and patients with NY-ESO-1 scores < 1 were grouped as NY-ESO-1 positive and NY-ESO-1 negative, respectively. Chi-square test of independence, or Fisher's exact test, respectively, then were conducted to examine the relations of NY-ESO-1 positivity and clinical variables. NY-ESO-1 positivity did not correlate significantly with any of the tested clinical variables of histology, FIGO stage, grade, preoperative CA 125, and SCRS, when taking into account all patients in the dataset ($p > 0.05$) (*Table 3.4*). Clear cell ovarian carcinomas were documented to be NY-ESO-1 positive in 41.2 %, endometrioid and serous carcinomas in 28.9 % and 28.0 % of cases; however, this finding was not significantly different. Neither did age nor BMI correlate significantly with NY-ESO-1 positivity ($p > 0.99$, $p > 0.09$, respectively).

Raising the cut-off for NY-ESO-1 positivity to H-scores of 5, 10, 20, and 50, respectively, did not reveal any significant correlations between NY-ESO-1 expression and any of the stated clinicopathological variables. No significant correlations with NY-ESO-1 positivity or expression levels were uncovered when grouping FIGO stages in I/II and II/III or I-IIA and IIIB-IV; neither were significant correlations revealed grouping grades in 1/2 and 3 or 1 and 2/3 – neither for all patients nor for the subgroups of platinum-resistant and -sensitive patients.

Table 3.4 Correlation analysis of NY-ESO-1 positivity and clinical variables.

Characteristics	*n NY-ESO-1 negative*	*n NY-ESO-1* positive	*p-value*
Histology			
Serous	77 (72.0 %)	30 (28.0 %)	0.339
Endometrioid	27 (71.1 %)	11 (28.9 %)	
Clear cell	20 (58.8 %)	14 (41.2 %)	
FIGO stage			
I	18 (75.0 %)	6 (25.0 %)	0.948
II	12 (63.2 %)	7 (36.8 %)	
III	75 (69.4 %)	33 (30.6 %)	
III A / B*	5 (71.4 %)	2 (28.6 %)	
III C*	70 (69.3 %)	31 (30.7 %)	
IV	19 (70.4 %)	8 (29.6 %)	
Grade			
1	12 (85.7 %)	2 (14.3 %)	0.288
2	18 (62.1 %)	11 (37.9 %)	
3	93 (69.4 %)	41 (30.6 %)	
Preoperative CA 125			
< 500 U/ml	59 (67.0 %)	29 (33.0 %)	0.499
≧ 500 U/ml	55 (72.4 %)	21 (27.6 %)	
SCRS			
Yes	110 (78.6 %)	30 (21.4 %)	>0.999
No	11 (84.6 %)	2 (15.4 %)	
Age			
≥ 60	61 (69.3 %)	27 (30.7 %)	>0.999
< 60	63 (69.2%)	28 (30.8 %)	
BMI			
≥ 25	46 (82.1 %)	10 (17.9 %)	0.095
< 25	44 (67.7 %)	21 (32.3 %)	
Platinum response			
Platinum-resistant	28 (77.7 %)	14 (33.3 %)	0.469
Platinum-sensitive	72 (72.7 %)	27 (27.3 %)	

3.4.3 Impact of NY-ESO-1 Expression on Survival

Patients with NY-ESO-1 positive (NY-ESO-1 H-score ≥ 1) tumors showed a one-year overall survival of 79.6 percent and five-year overall survival of 36.6 percent (median = 40.9 months). In contrast, 88.7 percent of patients with NY-ESO-1 negative (NY-ESO-1 H-score < 1) tumors were still alive one year after the initial diagnosis of ovarian cancer and showed a survival rate of 45.8 percent after five years (median = 49.4 months). However, according to the log-rank test, the difference in Kaplan-Meier survival curves of patients with NY-ESO-1 and without NY-ESO-1 expression was not statistically significant (p = 0.437). The Kaplan-Meier curve below gives a visual representation of overall survival, comparing NY-ESO-1 negative and positive patients (*Fig. 3.16*).

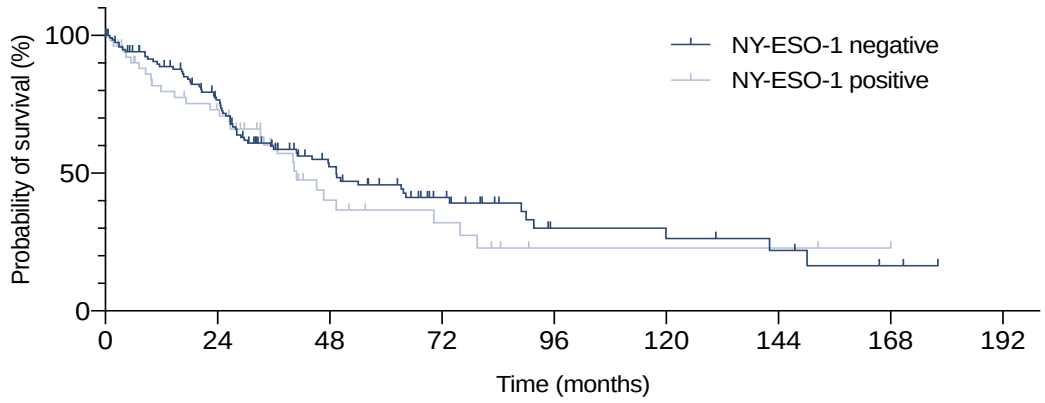

Fig. 3.16: Kaplan-Meier estimator for overall survival in patients with NY-ESO-1-positive and -negative ovarian cancers. Overall survival curves for patients based on their NY-ESO-1 expression status (p = 0.437).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

In terms of progression-free survival, patients with NY-ESO-1 expressing tumors had a one-year progression-free survival chance of 55.9 percent and a five-year progression-free survival chance of 7.5 percent (median = 17.8 months); patients with NY-ESO-1 negative tumors showed a one-year progression-free survival of 57.3 percent and five-year progression-free survival of 21.7 percent (median = 18.8 months). Log-rank test disproved distinguishable progression-free survival probability comparing the subgroups of patients with NY-ESO-1 negative and positive cancers (p = 0.550). The Kaplan-Meier curve below gives a visual representation of progression-free survival, comparing NY-ESO-1 negative and positive patients (*Fig. 3.17*).

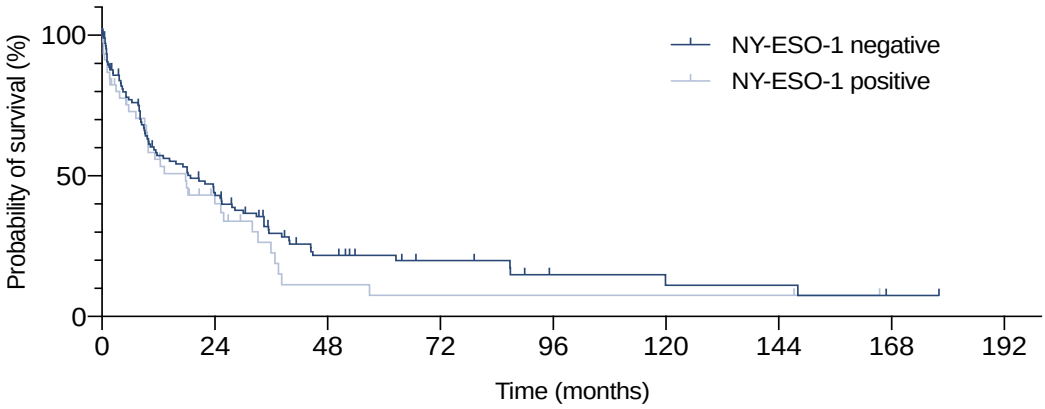

Fig. 3.17: Kaplan-Meier estimator for progression-free survival in patients with NY-ESO-1-positive and -negative ovarian cancers. Progression-free survival curves for patients based on their NY-ESO-1 expression status (p = 0.550).

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

Though the Kaplan-Meier estimator rules out NY-ESO-1 positivity as a predictor for overall survival, it suggests the FIGO stage (p = 0.006) and platinum response (p < 0.0001) influence the overall outcome (*Fig. 3.18*). To identify independent predictors of overall survival, we subsequently conducted a multivariate Cox regression analysis.

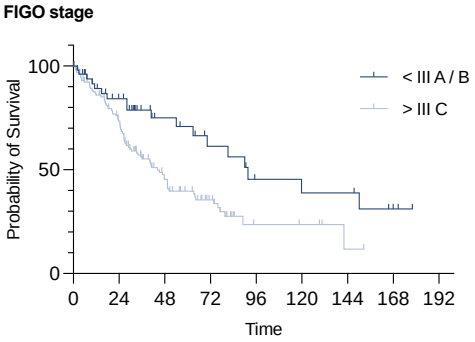

Fig. A: Kaplan-Meier-Estimator for overall survival for patients based on their FIGO stages.

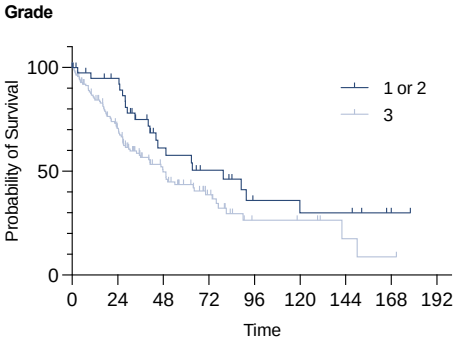

Fig. B: Kaplan-Meier-Estimator for overall survival for patients based on their FIGO grades.

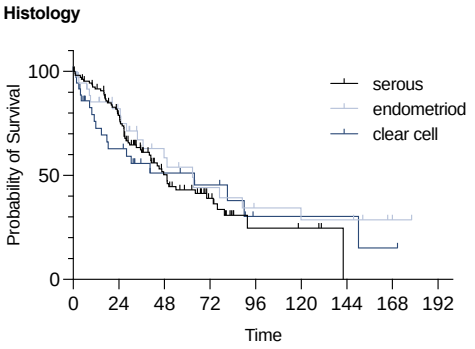

Fig. C: Kaplan-Meier-Estimator for overall survival for patients based on their ovarian cancer histologies.

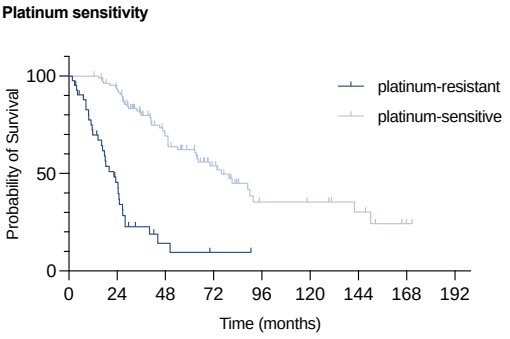

Fig. D: Kaplan-Meier-Estimator for overall survival for patients based on their ovarian cancer histologies.

Fig. 3.18: Kaplan-Meier estimators for overall survival for patients based on their A: FIGO stage (p = 0.006)**, B: grade** (p = 0.0528)**, C: histology** (p = 0.6064)**, and D: platinum-sensitivity** (p < 0.0001)**, respectively.**

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

3.4.4 Multivariate Cox Regression Analysis

Multivariate Cox regression analysis revealed platinum response, distinguished by platinum-sensitive and -resistant, and FIGO stage, classified in early (FIGO stage I and II) and late (FIGO stage III/IV), as independent predictors of overall ($p < 0.0001$ and $p = 0.011$, respectively) survival. FIGO stage was shown to also influence progression-free survival independently ($p < 0.0001$). For instance, the Hazard's ratio prognosticates a 6.4-fold increased risk of dying from ovarian cancer with higher stage disease at first diagnosis (Hazard ratio: 6.409; 95 percent confidence interval: 1.228 to 5.035) and an almost 5-fold increased risk of disease progression (Hazard ratio: 4.623; 95 % confidence interval: 2.324 to 9.198). Expression of NY-ESO-1 did not independently influence overall and progression-free survival. In the subgroup of platinum-sensitive patients, multivariate Cox regression analysis suggested that higher FIGO stage at the time of diagnosis was negatively correlated with overall and progression-free survival ($p = 0.012$, Hazard ratio: 3.335; 95 % confidence interval: 1.327 to 9.418 and $p < 0.001$, Hazard ratio: 5.951; 95 % confidence interval: 2.153 to 16.442, respectively). In the subgroup of platinum-resistant patients, multivariate Cox regression analysis did not identify an independent covariate for overall nor progression-free survival.

3.5 Correlation of Automated Tissue Analysis and the Pathologist's Rating

According to the Shapiro-Wilk test for normality, NY-ESO-1 H-scores were found to be not normally distributed. Consequently, the Spearman r test for correlation was used to examine the relation of H-scores assigned to the individual tissue cores assigned by an experienced pathologist and by the automated tissue analysis program DEFINIENS TissueStudio®. A statistically significant relation was found ($p < 0.0001$). However, in this study's analysis, only the pathologist-assigned H-scores were included as this represents the gold standard technique (*Fig. 3.19*).

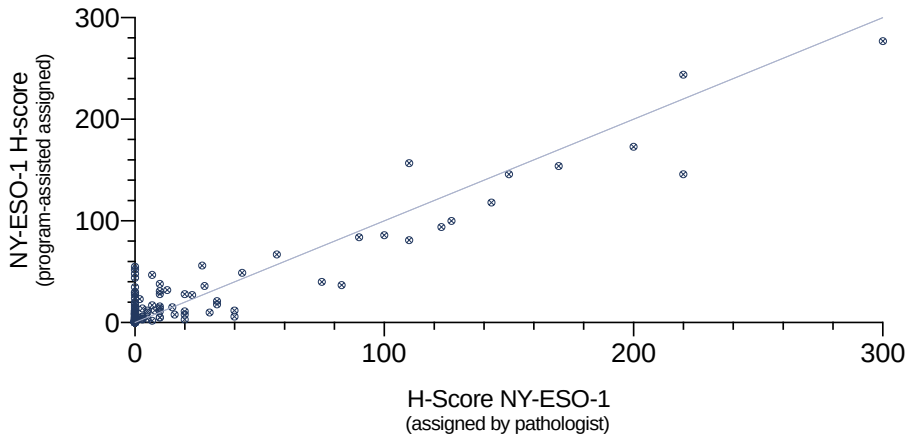

Fig. 3.19: Correlation of NY-ESO-1 H-scores assigned by an experienced gynecologic pathologist and computer-generated NY-ESO-1 H-scores.

Figure created with GraphPad Prism version 9.0.0 for MacOS, GraphPad Software, San Diego, California USA, www.graphpad.com

4 DISCUSSION

To date, aggressive cytoreductive surgery followed by primary platinum-based combination chemotherapy is considered the gold standard in managing ovarian cancer patients. Over the last decade, the introduction of anti-angiogenetic therapies and PARP inhibitors as supplements to conventional cancer treatment has led to modest improvements in the long-term survival of affected patients (SEER at the NCI 2021; ZfKD und RKI 2019).
Despite a good initial response to systemic treatment after optimal debulking surgery, remission to first-line therapy is rarely enduring in ovarian cancer patients: most patients will develop recurrent disease, or disease will progress. Lack of effective treatment options for patients with refractory disease results in poor long-term survival (Christie and Bowtell 2017).

Given the lack of effective treatment modalities and the poor prognosis of advanced disease, novel treatment strategies are urgently needed to improve survival in ovarian cancer patients. Immunotherapeutic treatment approaches have gained attention in recent years, with cancer/testis antigens being considered especially favorable targets. Cancer/testis antigens are characterized by restricted expression in healthy somatic tissues concomitant with re-expression in solid epithelial cancers, possibly being pinpoint targets for immunotherapeutic agents while causing less off-target toxicities. NY-ESO-1 has been identified as one of the most immunogenic among the family members eliciting a spontaneous immune response (Thomas et al. 2018; Raza et al. 2020; Esfandiary and Ghafouri-Fard 2015). Also, recently, Li et al. showed impaired proliferation, migration, and invasion of human multiple myeloma cells by knock-out of NY-ESO-1 (Li et al. 2020).

There are 45 recruiting, active, or proposed clinical trials targeting NY-ESO-1 using various immune-based treatment approaches (NLM at the NIH 2021b); among them are ten recruiting or active clinical trials for NY-ESO-1 targeted interventions for ovarian cancer alone (NLM at the NIH 2021a).

4.1 Comparability of Patient Cohort with General Ovarian Cancer Patient Population

The cohort selected for this study appears to be representative of the larger population of ovarian cancer patients in countries with high or very high human development indices in terms of relevant clinicopathological characteristics such as median patient age at the time of diagnosis, diagnosis at advanced FIGO stage disease, frequency of diagnosed ovarian cancer histological subtypes, and distribution frequency of platinum response.

The five-year overall survival rates of slightly over 40 percent are comparable between the study cohort and the general ovarian cancer population in countries with high or very high human development indices (Torre et al. 2018; Peres et al. 2019; SEER at the NCI 2021). The median patient's age at disease onset was 59 years in our patient population; in the United States, the median age at the time of ovarian cancer diagnosis statistically is 63 years (Torre et al. 2018). Ovarian cancer patients frequently only become symptomatic late in the course of the disease, like the patients in our cohort, who, for the most part, presented at an advanced stage (Peres et al. 2019): approximately three-quarters of patients in our dataset were diagnosed at FIGO stage III C or IV. Type I ovarian cancers, including clear cell and endometroid carcinomas or low-grade serous carcinomas, are characterized by a less aggressive growth rate, enabling an earlier stage diagnosis of the disease associated with a better prognosis. With an advanced-stage diagnosis of Type I ovarian carcinomas, the tide turns as primary platinum-resistant cancers are usually seen in Type I, rather than in the more common and more aggressive in growth Type II ovarian cancers (Kurman and Shih 2016). However, the development of secondary resistance to platinum-based chemotherapy in initially platinum-sensitive patients is rather the rule than the exception (Christie and Bowtell 2017).

In our patient cohort, the longest documented follow-ups of more than 13 years had, as expected, three patients with FIGO stage I endometrioid ovarian cancer and one patient diagnosed with clear cell carcinoma FIGO stage I.

Surprisingly, among the ten patients with the longest follow-up periods, namely 129.3 – 178.1 months, were three patients diagnosed with FIGO stage III C or IV serous ovarian cancer and one patient with stage IV endometrioid ovarian cancer. Similar observations have been reported before (Cress et al. 2015).

Before the introduction of maintenance therapies with anti-angiogenetic drugs and PARP inhibitors, *platinum sensitivity*, defined as no recurrence or disease progression within six months after completion of primary adjuvant chemotherapy, was about 60 – 80 percent among ovarian cancer patients; the patients recruited for our study showed a comparable platinum sensitivity of 72.0 percent (Torre et al. 2018; Ozols 1999). Consistent with incidence and mortality rates in the general ovarian cancer population, almost 60 percent of patients in the study cohort had died of ovarian cancer within five years of diagnosis. Also, as is the case in the general ovarian cancer population, more than half of the patients in the dataset were diagnosed with serous ovarian cancer rather than endometrioid or clear cell ovarian cancer (Torre et al. 2018).

According to the most current knowledge of ovarian cancer pathogenesis, high- and low-grade serous carcinomas differ distinctively in terms of the course of disease and survival (Kurman and Shih 2016). At the time of data acquisition, high- and low-grade serous carcinomas were not considered different tumor entities but a morphological spectrum in terms of advanced dedifferentiation. The statistical relevance should be trivial with a reasonable sample size in the study cohort of 190 patients and a statistically marginal fraction of low-grade serous carcinomas of less than 5 percent among patients diagnosed with serous ovarian cancer. Interim amendments of the FIGO and WHO classifications with incorporating the fallopian tube and primary peritoneal cancers should not affect statistical calculations (Prat J and Figo Committee on Gynecologic Oncology 2015).

4.2 Comparison of the Findings in this Study with Other Studies

4.2.1 NY-ESO-1 Expression in other Tumors

The expression of NY-ESO-1 was detectable in surgically removed primary or metastatic cancer, both in its RNA by reverse transcriptase PCR (RT-PCR) and on protein level by immunohistochemistry. A few studies have investigated both RNA and protein expression in the same patient cohorts. Discrepancies between evidence of RNA and protein expression levels are observed frequently, for instance, by Sugita et al. in breast cancer or by Szender et al. in ovarian cancer (Szender et al. 2017; Sugita et al. 2004); this may contribute to the wide variety of reported expression levels, between different types of cancer but also within cancer entities.

Confirmed by immunohistochemistry, NY-ESO-1 expression has been reported to be present in numerous solid tumors at some stage during the illness. Patients in disease remission show variable patterns, with some patients experiencing antigen loss over time while others show continuous expression. Tumors with reported NY-ESO-1 expression include, among others, bladder cancer, breast cancer, cervical cancer, esophageal cancer, head, and neck cancer, hepatocellular cancer, metastatic melanoma, multiple myeloma, myxoid and round cell liposarcoma, non-small cell lung cancer, ovarian cancer, prostate cancer, and synovial sarcoma (Sharma et al. 2003; Sugita et al. 2004; Sarcevic et al. 2003; Chen et al. 1997; Kienstra et al. 2003; Chen et al. 1998; Nakamura et al. 2006; van Rhee et al. 2005; Hemminger et al. 2013; Lee et al. 1999; Yakirevich et al. 2003; Odunsi et al. 2003; Szender et al. 2017; Nakada et al. 2003; Kerkar et al. 2016; Park et al. 2016). Among these tumor types, the frequency of expression of NY-ESO-1 detected by immunohistochemistry differs considerably. Myxoid and round cell liposarcomas show NY-ESO-1 expression most frequently (89 % - 100 %) (Pollack et al. 2012), followed by melanomas (45 %) (Barrow et al. 2006) and ovarian cancer (up to 43 %) (Odunsi et al. 2003; Szender et al. 2017; Yakirevich et al. 2003). In the majority of these tumors, NY-ESO-1 is frequently expressed in metastatic, advanced-stage tumors and is associated with an unfavorable prognosis.

4.2.2 NY-ESO-1 Expression in Ovarian Cancer

The presented study results show the expression of NY-ESO-1 in 30.9 percent of ovarian cancers: amongst the researched subentities, clear cell ovarian cancers were found to express NY-ESO-1 in a remarkable 41.2 % of cases. While the effectiveness of platinum-based chemotherapy in this entity is especially limited, recent studies suggest particular responsiveness of the subentity of clear cell ovarian cancers in experimental treatment approaches with immune checkpoint inhibitors (Bronger 2021).

The prevalence of NY-ESO-1 expression of 28.0 % and 28.9 % in serous and endometrioid ovarian cancers is also higher than in many other solid tumors and is comparable to the NY-ESO-1 expression rates detected through IHC as reported in two earlier publications:

Yakirevich et al. described positive staining for NY-ESO-1 (clone D8.38) in 10 of 53 (18.9 %) archival, formalin-fixed, paraffin-embedded serous ovarian cancer samples (Yakirevich et al. 2003) and Old's group Odunsi et al. observed positive staining for NY-ESO-1 (clone ES121) in 62 of 143 (43.4 %) archival, formalin-fixed, paraffin-embedded tissue biopsies spanning various types of ovarian cancer (Odunsi et al. 2003).

Alongside the 53 patients diagnosed with serous ovarian cancer, the study cohort of Yakirevich et al. included ten patients with serous cystadenomas and eleven patients with serous ovarian tumors of borderline malignancy; in serous cystadenomas and serous ovarian tumors of borderline malignancy, NY-ESO-1 expression was not detectable. Neither was an expression of NY-ESO-1 described in non-cancerous tumors of other tissues in multiple studies (Yakirevich et al. 2003).

The study population of Odunsi et al. was composed of 111 patients with serous ovarian cancer, five patients with clear cell and endometrioid ovarian cancer each, four patients with mucinous, and four patients with undifferentiated ovarian cancer; 13 patients were diagnosed with tumors classified as "others" (Odunsi et al. 2003). The majority of patients in both cohorts, Yakirevich et al. and Odunsi et al., presented at stage III C. Complete response to frontline therapy was achieved in more than half of the patients; differences in NY-ESO-1 expression between platinum-sensitive and platinum-resistant patients were not examined in detail.

The detailed cohort characteristics and results of previous analyses by other groups compared to our study are summarized below in Table 4.2.

Table 4.1: Overview on studies on NY-ESO-1 expression in ovarian cancer.

Variables	Odunsi et al. 2003	Yakirevich et al. 2003	Szender et al. 2017	Our study
Evaluable patients, n	190	84	1002	190
Age [median (range)], years	61 (22 – 89)	63 (40 – 82)	61 (13 – 91)	59 (26 – 92)
Follow-up [median (range)], years	25 (0.7 – 126)	42 (4 – 104)	-	31 (0 – 178)
Histology [n (%)]				
serous	143 (79 %)	53 (63 %)	676 (68 %)	112 (59 %)
endometroid	8 (4 %)	-	45 (4 %	41 (22 %)
clear cell	8 (4 %)	-	43 (4 %)	37 (19 %)
mucinous	4 (2 %)	-	40 (4 %)	-
undifferentiated	6 (3 %)	-	-	-
others	13 (7 %)	31 (37 %)	198 (20 %)	-
FIGO stage [n (%)]				
I	10 (5 %)	5 (9 %)	89 (10 %)	26 (14 %)
II	11 (6 %)	9 (17 %)	91 (10 %)	22 (12 %)
III A/B	5 (3 %)	37 (70 %)	35 (4 %)	7 (4 %)
III C	149 (78 %)		565 (63 %)	107 (56 %)
IV	15 (8 %)	2 (2 %)	119 (13 %)	27 (14 %
Grade [n (%)]				
1	11 (6 %)	6 (11 %)	81 (8 %)	14 (7 %)
2	10 (5 %)	26 (49 %)	133 (14%)	31 (17 %)
3	169 (88 %9	21 (40 %)	754 (78 %)	143 (76 %)
Debulking				
optimal	-	44 (83 %)	604 (70 %)	-
suboptimal	-	9 (17 %)	261 (30 %)	-
Clinical response				
complete	97 (51 %)	31 (58 %)	397 (50 %)	78 (48 %)
partial	81 (42 %)		8 (1%)	27 (17 %)
persis./stable disease	-	22 (42 %)	199 (25 %)	42 (26 %)
progression	3 (2 %)		31 (4 %)	
death on treatment	-	-	152 (19%)	7 (4 %)
unknown	9 (5 %)	-	-	9 (6 %)
NY-ESO-1 [n (%)]				
RNA negative	75 (70 %)	-	742 (74 %)	-
RNA positive	32 (30 %)	-	260 (26 %)	-
IHC negative	81 (57 %)	43 (81 %)	642 (73 %)	124 (69 %)
IHC positive	62 (43 %)	10 (19 %)	232 (27 %)	55 (31 %)
RNA + IHC combined	82 (43 %)*		408 (41 %)**	

Table 4.1: Overview on studies on NY-ESO-1 expression in ovarian cancer (continuation):

Analyses	Pearson's r, X^2 test or Fisher's exact, univariate analysis (Kaplan-Meier), log-rank-test	X^2 test or Fisher's exact, univariate analysis (Kaplan-Meier), log-rank-test, Cox proportional Hazard model	Student's t-test, X^2 test or Fisher's exact, univariate analysis (Kaplan-Meier), log-rank-test, Cox proportional Hazard model	Pearson's r, X^2 test or Fisher's exact, univariate analysis (Kaplan-Meier), log-rank-test, Cox proportional Hazard model
Method of quantification	negative, focal, +, ++, +++, ++++	negative, focal, +, ++, +++, ++++	negative, focal, +, ++, +++, ++++	H-score
NY-ESO-1 positivity	any staining from focal to ++++	staining of > 5 % of cells	any staining from focal to ++++	H-score ≥ 1
Extent of staining	mostly heterogeneous (with 66 %: focal, +, ++; 34 %: +++ or ++++)	70 % heterogeneous (with < 50 % of malignant cells)		mostly heterogeneous (70.9 %: H-scores 2 – 50; 23.6 %: H-scores 51 – 200; 5.5 %: H-scores > 201)
Significant associations	**NY-ESO-1 pos. patients:** • more likely to have higher stage disease		**NY-ESO-1 pos. patients:** • 3 years older • more likely to be diagnosed with stage IIIC or IV • more likely to be diagnosed with higher grade • more likely to be diagnosed with serous histology • less likely to have complete response to initial therapy • shorter OS and PFS	

* "significant correlation" (Odunsi et al. 2003)
** "concordance moderate" (Szender et al. 2017)

Odunsi et al. additionally analyzed fresh-frozen ovarian cancer tissue samples from patients of the same cohort, if available, by RT-PCR.

As mentioned above, reported RNA expression data might differ considerably from protein expression levels determined by immunohistochemistry. Odunsi et al. demonstrated NY-ESO-1 expression in 43 percent of available tumor specimens by IHC. In this study, the investigators found a significant correlation between proof of RNA and NY-ESO-1 expression using both RT-PCR and IHC in ovarian cancers. In their report, NY-ESO-1 positivity was defined as the detection of NY-ESO-1 either by RT-PCR or IHC, so in total 82 of 190 specimens (43.2 %) were found NY-ESO-1 positive. Due to the significant correlation, the different methods of NY-ESO-1 detection did not impact the overall detection rates (Odunsi et al. 2003).

4.2.3 Possible Clinical Implication of RNA Positivity versus Antigen Positivity

Diverging detection rates of RNA and protein expression levels are observed frequently. Crick's central dogma of molecular biology "DNA makes RNA makes proteins" (Crick 1958) fools to assume a direct correlation between RNA and protein levels; however, on the one hand, the existence of RNA may not habitually induce protein expression; on the other hand, the number of proteins per transcript may vary widely.

Quantification RT-PCR provides information on the absolute amount of gene expression; the analysis of protein expression by IHC has the advantage of revealing its intratumoral expression heterogeneity. In ovarian cancer, Szender et al. showed deviant detection rates of NY-ESO-1 positive tumors by RT-PCR and IHC, respectively (Szender et al. 2017). Further details of the study conducted by Szender et al. will be discussed later. Different detection methods limit the comparability of studies.

Current treatment approaches address not the RNA directly, but only the de facto expressed proteins; this diminishes the group of patients that qualifies for exploratory NY-ESO-1 based immunotherapies.

However, as the expression of NY-ESO-1 can be induced by radiotherapy and the application of demethylation agents, the actual number of patients that may eventually be eligible for NY-ESO-1 addressing treatments might be closer to the number of patients with RNA-positive tumors rather than the count of patients with tumors showing NY-ESO-1 expression by immunohistochemistry (Lu et al. 2007; Gry et al. 2009).

4.2.4 NY-ESO-1 Positivity, Heterogeneous Staining, and H-scoring

In most cancers, immunohistochemical NY-ESO-1 expression is found in patches and with high intratumoral heterogenicity, whereas sarcomas were reported to show the most homogenous expression patterns (Hemminger et al. 2013; Pollack et al. 2012). Heterogeneous staining may be explicable by not all tumor cells within a tumor expressing NY-ESO-1 on a protein level. This finding explains the importance of taking multiple samples of a patient's tumor when evaluating the frequencies of NY-ESO-1 expression in tissue microarrays (TMAs). In terms of treatment, heterogeneous expression with a lower count of potentially attackable cells might limit the treatment response in targeted therapy. In turn, the homogenous expression pattern in sarcomas might contribute to the promising results that have been achieved in adoptive cellular immunotherapy trials (Baldi et al. 2019; D'Angelo et al. 2018; Robbins et al. 2015; Robbins et al. 2011). Not only inconsistent expression but also immunological tolerance or low binding affinity of the TCR to the MHC complex might negatively affect effective tumor cell eradication – despite proof of tumor-associated antigen (TAA) expression (Ilyas and Yang 2015).

Most successful adoptive cell transfer approaches have been shown in hematologic cancers: CD19 targeting CAR T cell therapy is available for subtypes of lymphomas and individual patients with relapsed leukemia (Locke et al. 2017; FDA 2018). Effective immunotherapy in solid cancers might additionally be hampered by the complex, dynamic immunosuppressive microenvironment that modulates the anti-tumor response (Rodriguez et al. 2018).

The definition of NY-ESO-1 positivity in immunohistochemistry is not standardized: NY-ESO-1 positivity in publications ranges from focal NY-ESO-1 expression (Odunsi et al. 2003; Szender et al. 2017) to only defining strong and extensive staining (++ to ++++ > 50 %) as NY-ESO-1 positive (Endo et al. 2015). NY-ESO-1 immunotherapy clinical trials generally enroll patients whose tumor samples stain strongly for NY-ESO-1, for instance, ++ to ++++ in staining intensity and > 50 percent in area (Robbins et al. 2011); thereby, extent and degree of target antigen expression as determining factors for failure or success of treatment approaches can be minimized. The actual correlation between NY-ESO-1 expression and possible treatment response is unknown, and the optimal method to determine NY-ESO-1 expression in tumors for that purpose is undefined.

Yakirevich et al. and Odunsi et al. reported heterogeneous staining patterns in most ovarian cancer specimens (Odunsi et al. 2003; Yakirevich et al. 2003). We also noticed broad heterogeneous immunohistochemical reactivity in our ovarian cancer biopsies, with focal to homogeneous staining patterns and varying staining intensities. Both, Yakirevich et al. and Odunsi et al. rated the level of NY-ESO-1 expression semi-quantitatively using a five grade scoring system (negative, focal, < 5 %; +, < 25 %; + +, 25 – 49 %; + + +, 50 – 75 %; + + + +, > 75 % of cells stained), irrespective of the staining intensity (Odunsi et al. 2003; Yakirevich et al. 2003). However, staining intensity might provide information on the number of intact, immunogenic antigens carried by an individual tumor cell and might also influence the possible destructive power of targeted therapy.

In hormone receptor-positive breast cancer patients, the degree of positivity, numerically expressed by an individually assigned H-score, corresponds significantly with the response to hormonal therapy and time to recurrence, and overall mortality reduction (Stendahl et al. 2006; Brouckaert et al. 2013). Assuming a similar biological behavior in NY-ESO-1 expressing tumors and NY-ESO-1 targeted therapy, higher NY-ESO-1 expression in immunohistochemical staining might also imply more potential targets for immunotherapeutic approaches and, consequently, more effective response to therapy.

To capture the heterogeneous immunohistochemical reactivity most accurately, our group chose to take three different biopsies from morphologically representative areas in the available formalin-fixed, paraffin-embedded ovarian cancers and arrange them in tissue microarrays (TMA) as described above. The tissue microarray technique allows simultaneous staining of multiple specimens, reducing intra-experimental variations in staining intensity. After immunohistochemical staining, we assigned H-scores to the individual tissue microarray cores in collaboration with an experienced gynecologic pathologist. The H-score was estimated as described above. The mean of the H-scores assigned to the triplicate samples was calculated for each patient and included in our data analysis.

As a multiplicative score, the H-score provides a wide dynamic range of scores ranging from 0 (no staining) to 300 (homogenous, intense staining of the tumor specimen) in a continuous fashion, factoring variations in staining intensity among tumor cells and the percentage of positive cells within each TMA tissue core. It also allows the assignment of blank values to unstained tissues. A standardized scoring system for immunohistochemistry is not established yet. Among combined scoring systems, the H-score, commonly used for IHC evaluation of hormone receptors, is one of the most widely accepted. In immunohistochemical testing for estrogen and progesterone receptors in breast cancer, routinely <1 percent stained cells are considered negative (Hammond et al. 2010). Factoring both staining intensity and extent of staining, H-scoring has advantages but poses challenges in pathologist training and selective visual perception. Automated tissue analysis systems have been suggested as an alternative to human scoring.

4.2.5 Automated Tissue Analysis vs. Pathologist's Rating

In addition to manual assessment, we digitized our stained TMA slides with the Aperio ScanScope XT and analyzed images using DEFINIENS TissueStudio software to compare manual assessment and automated tissue analysis.

The image analysis systems currently used for analyzing immunohistochemical slides are, to our knowledge, incapable of reporting H-score results, so H-scores based on the intensity of NY-ESO-1 staining and percent positive cells were then manually calculated for all specimens. Although automated tissue analysis of tissue microarrays has been proven to be consistent with visual scoring by experienced pathologists in multiple studies, pathologist-generated interpretation and scoring remain the „gold standard“. We confirmed a strong correlation between automated marker detection and interpretation by an experienced pathologist; however, in the analysis for this study, as it is defined as the gold standard, only the pathologist-assigned H-scores were included in further analysis (Turbin et al. 2008; Cohen et al. 2012; Bolton et al. 2010; Howat et al. 2015; Konsti et al. 2011; Irshad et al. 2017; Gustavson et al. 2009).

4.2.6 NY-ESO-1 as a Prognostic Marker

In our analysis, NY-ESO-1 expression – both as positivity, meaning detection of staining if any, and intensity plus the extent of NY-ESO-1 expression expressed as H-score – was identified as a neutral prognostic factor lacking association with examined clinicopathological characteristics. Clinicopathological characteristics included disease status and histology, FIGO stage, the previously applied grading system with three grades from well to poorly differentiated, platinum response, the necessity for secondary cytoreductive surgery, preoperative CA 125 (grouped in < 500 U/ml and ≥ 500 U/ml), age and BMI (grouped in < 25 and ≥ 25).

In most other cancer types, for instance in melanoma, non-small cell lung cancer, and myxoid liposarcoma, NY-ESO-1 expression was reported to be associated with advanced-stage and -grade disease and a rather unfavorable prognosis (Wang et al. 2019; Velazquez et al. 2007; Gure et al. 2005; Szender et al. 2017; Barrow et al. 2006; Iura et al. 2015); but in a subset of breast cancer patients: Lee et al. identified NY-ESO-1 as an independent prognostic factor in breast cancer patients (Lee et al. 2015).

The highest calculated NY-ESO-1 H-scores we observed were among individuals with more advanced-stage disease; however, with the lion's share of patients in our dataset tested negative for NY-ESO-1 and large variance of NY-ESO-1 H-scores from 1 to 300 among patients with NY-ESO-1 positive tumors, statistical significance could not be reached in our patient cohort. Additionally, the contrast to the findings in other tumors could be explained by the majority of ovarian cancers being diagnosed at an advanced stage and by underpowered sample size to identify differences in clinicopathological characteristics and survival in our study group. However, if our initial suspicion of more intense NY-ESO-1 expression in carcinomas of patients with more advanced disease can be substantiated by further studies, especially patients diagnosed with higher stage ovarian cancer and associated statistically reduced life expectancy could benefit from a NY-ESO-1 targeted therapy.

Traditionally, ovarian cancer was perceived as a single disease entity. In most publications, all ovarian cancer subtypes are still lumped together for analyses. As high-grade serous ovarian cancer (HGSC) constitutes the largest subgroup of epithelial ovarian carcinomas, it has the most decisive statistical implications in analyses, including all histological subtypes. Inevitably, the currently employed first-line therapy with platinum-based chemotherapy for epithelial ovarian cancer has primarily been investigated based on this histological subtype's response behavior. NY-ESO-1 expression might be explicitly present in one or another histological subgroup and, thus, particularly worthy of further detailed research about treatment options. In our study, despite higher NY-ESO-1 expression prevalence in clear cell (40.2 %) compared to serous and endometrioid carcinomas (28.0 % and 28.9 %, respectively), statistically significant diverging expression patterns of NY-ESO-1 regarding different histological subtypes did not become apparent.

Further, in our analysis, patients with NY-ESO-1 positive ovarian cancers appeared to have worse outcomes, with median survival approaching 40.9 months, compared to 49.4 months in patients with ovarian cancers without evidence of NY-ESO-1 expression.

One-year overall survival was 79.6 percent for patients with NY-ESO-1 positive tumors compared to 88.7 percent for patients with tumors not expressing NY-ESO-1. Five-year overall survival was 36.6 percent for patients with NY-ESO-1 positive tumors compared to 45.8 percent for patients with NY-ESO-1 negative tumors. However, lacking statistical significance, we must assume statistical coincidence. The same applies to progression-free survival, which was not significantly different between patients with NY-ESO-1 positive and negative tumors, exhibiting a numerical difference in one-year progression-free survival of 55.9 percent and 57.3 percent and five-year progression-free survival of 7.5 percent and 21.7 percent, respectively. However, subtle differences may have been masked due to the sample size, as curve progressions could indicate a role of NY-ESO-1 expression in the advanced course of the disease.

We also conducted a stepwise multivariate Cox regression analysis to investigate the influence of several predictors on survival; predictors of interest included: NY-ESO-1 positivity, FIGO stage (I or II vs. III or IV), and platinum susceptibility status. The previously used grading system is obsolete for most ovarian cancer subtypes, so tumor grades were not included in the stepwise multivariate cox regression analysis. Gross residual disease after debulking surgery also is assumed to be a negative predictor of survival (Chang, Bristow, and Ryu 2012); due to inconsistent assessment, the variable *residual disease* could also not be included in the analysis. The first step included all putative independent predictors of survival. To identify independent predictors of survival and enhance the informative value of the Cox regression model, the most unlikely predictors were excluded at each step for further analysis. Multivariate Cox regression analysis revealed platinum response, distinguished by platinum-sensitive and -resistant, and FIGO stage, classified in early (FIGO stage I and II) and late (FIGO stage III/IV), as independent predictors of overall ($p < 0.0001$ and $p = 0.013$, respectively). In terms of progression-free survival, independent of the response to platinum-based chemotherapy, only the FIGO stage ($p < 0.001$) was shown to be independently predictive. Expression of NY-ESO-1 did not independently influence overall and progression-free survival.

In our study, NY-ESO-1 expression appears to be statistically irrelevant in terms of overall and progression-free survival. These findings are consistent with the publication by Yakirevich et al. from 2003, who also found lower overall survival rates in patients with NY-ESO-1 positive serous ovarian cancers than in patients diagnosed with NY-ESO-1 negative cancers. However, neither did this study prove a statistically significant difference in their survival data, nor did this study identify an association of NY-ESO-1 expression with the FIGO stage, grade, age, and response to chemotherapy in their study cohort. Multivariate analysis of their data revealed only the FIGO stage as an independent predictor of survival (Yakirevich et al. 2003).
In the group's 2003 study, Odunsi et al. found a statistically significant association between the increasing extent of NY-ESO-1 expression and an advanced FIGO stage at the time of diagnosis. Histological grade was not associated with increased NY-ESO-1 expression. Like in our study and the research by Yakirevich et al., a difference in median progression-free and median overall survival with a progression-free and overall survival benefit for patients with NY-ESO-1 negative patients became apparent, but not on a statistically significant level. No statistically significant survival differences were displayed when patients were grouped based on their tissue staining extent in higher or lower NY-ESO-1 expression (Odunsi et al. 2003).

Assuming their first 2003 study was underpowered to identify subtle differences in survival, Odunsi and colleagues, in the following referred to as Szender et al., repeated their research in 2017. Applying the same approach to a patient population five times larger than the original, they identified NY-ESO-1 as a biomarker for a more aggressive clinical phenotype: patients with NY-ESO-1 expressing tumors were diagnosed with higher stage and higher-grade ovarian cancers. Furthermore, patients with NY-ESO-1 positive tumors were, on average, three years older than patients with NY-ESO-1 negative cancers and were more likely to be diagnosed with serous ovarian cancer. Patients with NY-ESO-1 expressing tumors showed fewer complete responses to primary platinum-based chemotherapeutic treatment; association with optimal cytoreductive surgery was not detected. Patients with NY-ESO-1 expressing tumors were found to have profoundly worse outcomes.

Differences in median progression-free survival of 22 months (range, 19 – 25) versus 25 months (range, 22 – 28) and median overall survival of 43 months (range, 39 – 50) versus 50 months (range, 45 – 59) for NY-ESO-1 positive and negative patients, respectively, were statistically significant (Szender et al. 2017). The difference in the definition of progression-free survival might not be crucial for the gist of the publications on NY-ESO-1 expression in ovarian cancer; it should be noted, however, that Szender et al. "computed [progression-free survival] from the date of diagnosis to the date of initial recurrence" rather than from the date of completed primary treatment to the date of recurrence. In the group's previous publication on NY-ESO-1 expression in ovarian cancer by Odunsi et al. from 2003, they measured study outcomes from the time of definite surgery; Yakirevich et al. do not define overall and progression-free survival (Odunsi et al. 2003; Szender et al. 2017; Yakirevich et al. 2003).

A relatively large study population was investigated by Szender et al., including 676 patients with serous ovarian cancer, 43 patients with clear cell ovarian cancer, 45 with endometrioid ovarian cancer, and 40 patients with mucinous ovarian cancer; the tumors of the remaining 198 patients were classified as "other". Most patients in their cohort were diagnosed with stage III C or IV ovarian cancer and serous histology. Information on the duration of follow-up was not provided. Further, it should be noted that aside from tumors with immunohistochemical detection of NY-ESO-1, ovarian cancers with evidence of NY-ESO-1 only on RNA level were also counted as NY-ESO-1 positive (Szender et al. 2017).

In their 2003 analysis, Odunsi's group used a similar approach, but then, due to a high concordance between proof of NY-ESO-1 on RNA and protein level, the different NY-ESO-1 detection procedures did not carry weight in their outcome and association analyses (Odunsi et al. 2003). In the recent analysis, the agreement was only moderate: NY-ESO-1 was expressed by 25.9 percent of specimens measured by RT-PCR for RNA, and 26.5 percent of ovarian cancer samples stained positive for NY-ESO-1 by IHC – any case scored positive by either method was referred to as NY-ESO-1 positive, so in total, they counted 40.7 percent of tumors NY-ESO-1 positive (Szender et al. 2017).

Given the different definitions of NY-ESO-1 positivity between the previously conducted studies, including ours, and this one, the outcome data's comparability remains rather vague. Based on the studies cited above, the application of NY-ESO-1 expression as a prognostic biomarker seems to be limited; however, expression of NY-ESO-1 in 19 to 43 percent of ovarian cancer patients still makes it a promising target for targeted therapy (Odunsi et al. 2003; Szender et al. 2017; Yakirevich et al. 2003).

Demethylating agents were shown to induce NY-ESO-1 protein expression (Weiser et al. 2001). Pretreatment with these agents might raise the number of patients with NY-ESO-1 expressing tumors or make patients with lower NY-ESO-1 protein expression more susceptible to NY-ESO-1 targeted therapy; therefore, the maximum count of patients with ovarian cancers that are or could be eligible for NY-ESO-1 targeted immunotherapy might be close to the 40 percent patients with NY-ESO-1 expressing ovarian cancers reported by Odunsi's group (Szender et al. 2017).

Particularly promising concerning further research finding treatment options for ovarian cancer is that the research group among Szender et al. found the negative impacts of NY-ESO-1 positivity reversed when patients with NY-ESO-1 positive ovarian cancers received antigen-specific immunotherapy (Szender et al. 2017).

4.2.7 NY-ESO-1 in Platinum-resistant Disease

Following optimal surgical cytoreduction, standard medicinal treatment for ovarian cancer treatment includes an adjuvant platinum- and taxane-based combination intravenous chemotherapy. As mentioned above, platinum sensitivity was about 60 – 80 percent among ovarian cancer patients (Ozols 1999).

In this study, we classified patients by the platinum-free interval definition specified at the fourth Ovarian Cancer Consensus Conference (OCCC): patients who showed relapse or progression within six months after completion of initial chemotherapy we grouped as platinum-resistant. Patients with recurrence after six months or those not diagnosed with recurrent disease were classified as platinum-sensitive (Friedlander et al. 2011).

Alternatively, a more detailed classification is also used. Patients who show recurrence between six and twelve months after completion of chemotherapy are termed partially platinum-sensitive; only patients who are relapse-free for more than twelve months are completely platinum-sensitive according to this definition (Pujade-Lauraine and Combe 2016). After introducing supplemental molecular targeted therapy, criticism has been raised about the relevance of categorizing patients according to the length of progression-free survival after completion of platinum-based chemotherapy; replacement by the term treatment-free survival was proposed. Also, varying frequencies and modalities (CA 125, computed tomography or positron emission tomography scan, and clinical assessment or combinations) of investigations during follow-up care determine the diagnosis of cancer recurrence and subsequently affect the categorization in platinum-sensitive or -resistant ovarian cancer. Standardization of follow-up care is pursued (Wilson et al. 2017). As the classification quoted continues to be used in clinical practice and trials, it was also applied for patient categorization in platinum-sensitive and -resistant in our study cohort. The statistically significant difference in the distribution of platinum response between the different current disease statuses with a distinct shifting of platinum-sensitive patients to the disease status “NED” (no evidence of disease) and the platinum-resistant patients to “DOD” (dead of disease) only proves the known effectiveness of platinum-based chemotherapy. The Kaplan-Meier curves and significant corresponding log-rank tests comparing overall and progression-free survival of patients with platinum-sensitive and -resistant ovarian cancers indicate an overall and progression-free survival benefit for patients responding to first-line treatment with platinum-based chemotherapy.

In an additional analysis, we compared NY-ESO-1 expression rates in platinum-resistant and platinum-sensitive patients. Due to limited alternative medicinal treatment options, treatment of especially platinum-resistant patients still poses a particular challenge. Consequently, higher NY-ESO-1 expression prevalence in platinum-resistant ovarian cancer patients would be particularly promising for treating this subset of ovarian cancer patients.

However, neither the subpopulation of ovarian cancer patients who failed first-line platinum-based chemotherapy nor patients with platinum-sensitive tumors showed significantly higher NY-ESO-1 expression rates.

4.3 Limitations of the Study

Although all testing was performed at a single institution, the present study did include tumor specimens from one university and one multi-specialty teaching hospital in Los Angeles, CA, USA. We acknowledge the possibility of selection bias because the cohort included patients referred to these institutions for additional therapeutic options. However, comparability with other studies on NY-ESO-1 expression in ovarian cancer is given as the clinicopathological parameters of the patient cohorts are comparable.

Contrary to previous studies on NY-ESO-1 expression in ovarian cancer, we used tissue microarrays to lower the probability of interexperimental variations in immunohistochemical staining. We agree that this experimental design was chosen at the expense of analyses of the whole horizontal tissue section. According to the guidelines introduced by the College of American Pathologists Pathology and Laboratory Quality, “strength of evidence” [is] “adequate to support TMA usage” (Fitzgibbons et al. 2014). Other pre-analytic, analytic, and post-analytic issues in immunohistochemistry also tremendously impact staining, reported results, and drawn conclusions (Mengel et al. 2002). For instance, even the supposedly negligible preparatory step of blocking endogenous peroxidase with diluted H_2O_2 when using peroxidase-based detection methods may impact the staining results and, eventually, the conclusions from analyses (Bussolati and Radulescu 2011; Radulescu and Boenisch 2007).

Also, inter- and intra-observer variability is a well-known issue in estimating immunohistochemical staining as it is based chiefly on a pathologist’s qualitative assessment. Involving more experienced gynecological pathologists in scoring may have led to improved reproducibility.

On behalf of the College of American Pathologists Pathology and Laboratory Quality, in 2014, Fitzgibbons et al. published the guidelines mentioned above to standardize the analytic validation of immunohistochemical assays; the efforts were already proven to have a positive impact on laboratory performance (Fitzgibbons et al. 2014; Fitzgibbons et al. 2017).

Furthermore, the need to recognize ovarian cancer as a set of distinct diseases was highlighted. We refrained from subgroup analyses due to the lack of adequate patient counts in the individual subgroups of ovarian cancer histotypes. Research on the different ovarian cancer subtypes is indispensable to effectively target each ovarian cancer histotype's unique features (Vaughan et al. 2011).

4.4 Immunotherapy and NY-ESO-1 as a Therapeutic Target

Ovarian cancer is considered an immunogenic tumor that can be recognized by the host's immune system. Spontaneous anti-tumor immune response was found in approximately half of ovarian cancer patients. Tumor-infiltrating T cells in ovarian cancer are associated with a favorable prognosis in terms of progression-free and overall survival (Hwang et al. 2012; Zhang et al. 2003; Sato et al. 2005). While immunotherapy was already implemented as a supplement in treating other solid and hematological malignancies, no immunotherapeutic agent has yet been approved to treat ovarian cancer (Cancer Research Institute 2021). Numerous ongoing trials evaluate immunotherapeutic approaches in the treatment of ovarian cancer. The majority of trials deal with potentially synergistic combination therapy of immune checkpoint inhibitors and other therapeutic agents, including PARP inhibitors and bevacizumab, as the presence of suppressive factors in the ovarian cancer tumor microenvironment may explain the limited potency observed with the to-date researched immune-based therapies (NLM at the NIH 2021a).

Chen and Mellmann suggested the idea of a cancer immunity cycle. The model includes priming and activation of effector T cells after antigen-presenting cells presented tumor-associated antigens on MHC complexes, a process that is assumed to primarily take place in pelvic and para-aortic lymph nodes (Chen and Mellman 2013).

Lymphadenectomy as part of standard primary debulking surgery for ovarian cancer before medicinal therapy might impair the immunotherapy efficacy in the treatment of ovarian cancer. Also, tumor-specific antigens are thought to be exposed through radiation-induced tumor cell damage; antigen exposure will allow recognition by the immune system (Punnanitinont et al. 2020). Unlike in treatment for other malignancies, radiation plays a lesser role in ovarian cancer therapy.

Immune-based interventions that gained regulatory approval during the last decade include immune checkpoint blockade, cancer vaccines, and adoptive cell therapy (Cancer Research Institute 2021).

4.4.1 Immune Checkpoint Inhibitors

Immune checkpoint inhibitors block binding sites of inhibitory immune checkpoints that down-regulate the T cell-based immune response. In healthy individuals, inhibitory immune checkpoints protect from autoimmune diseases; tumor cells use them to evade destruction by the immune system (Pardoll 2012). In contrast to other malignancies, in treatment attempts for ovarian cancer, the use of antibodies impeding the immune checkpoint programmed cell death PD-1 or its ligand PD-L1 fell short of expectations (Marinelli et al. 2019). In 2019, Matulonis et al. reported an objective response rate of 8.0 percent in the so-far largest clinical trial, with 376 enrolled ovarian cancer patients (Matulonis et al. 2019). Ongoing clinical trials adjusted their approaches from single agent to combination therapy (NLM at the NIH 2021c).

Immune checkpoint inhibitors were proven to induce durable tumor regression and prolonged stabilization of disease in the treatment of advanced cancers, including melanoma, non-small-cell lung cancer (NSCLC), and renal cell carcinomas; immune checkpoint blockade gained FDA approval for treatment of these cancers, amongst others (Brahmer et al. 2012; Cancer Research Institute 2021).

4.4.2 Therapeutic Oncovaccines

Therapeutic cancer vaccines stimulate an immune response directed against specific antigens expressed by malignant cells (Chow, Berek, and Dorigo 2020). Cancer testis antigens, including NY-ESO-1, are considered suitable candidates for cancer vaccination (Sabbatini et al. 2012; Jager et al. 1999). NY-ESO-1 targeting vaccines in trials included long peptides (Odunsi et al. 2007; Pavlick et al. 2020; Tsuji et al. 2013), recombinant poxviruses expressing NY-ESO-1 (Odunsi et al. 2012), and NY-ESO-1 protein in combination with epigenetic modification inhibiting DNA methylation (Odunsi et al. 2014). A meta-analysis of trials demonstrated an overall survival advantage of two years for patients with NY-ESO-1 expressing tumors who received the vaccination, compared with patients with NY-ESO-1 positive tumors who were not treated with NY-ESO-1 directed vaccination (Szender et al. 2017).

Oncovaccines for, for instance, early-stage bladder cancer (FDA 1990), metastatic castration-resistant prostate cancer (FDA 2010), and advanced melanoma (FDA 2015) are FDA approved; NY-ESO-1 directed vaccines did not reach regulatory approval yet.

However, it should be pointed out that treatment attempts targeting CA 125 / MUC 16 using the murine monoclonal anti-idiotypic antibody Abagovomab and the mouse monoclonal antibody Oregovomab directed against CA 125 fell short of expectations in phase III studies (Sabbatini et al. 2013; Berek et al. 2009): MUC 16 glycoprotein houses the epitope CA 125 and is, in contrast to NY-ESO-1, expressed by nearly all advanced-stage ovarian cancers (Bast et al. 1983). Similarly to the immunogenicity of NY-ESO-1, the ability of CA 125 to raise a humoral and cellular immune response was observed in preclinical studies; promising phase I/II data raised hope suggesting a prolonged survival in ovarian cancer patients who responded to the vaccination (Sabbatini et al. 2006; Pfisterer et al. 2006).

The subsequent randomized phase III trials did not show benefit from vaccination treatment for patients concerning recurrence-free (RFS) and overall survival in maintenance therapy (Sabbatini et al. 2013; Berek et al. 2009).

4.4.3 Adoptive T cell transfer

Adoptive transfer of autologous, ex vivo expanded, naturally occurring, antigen-specific tumor-infiltrating lymphocytes (TILs) mediated substantial objective clinical regressions in patients with different types of advanced cancer in clinical trials. This approach seems to be particularly auspicious in patients with advanced-stage melanoma (Rosenberg et al. 1988; Rosenberg et al. 2011; Dudley et al. 2005). Tumor antigen-specific T cells are re-infused upon isolation and expansion into the lymphodepleted patients. Ex vivo expanding antigen-specific T cells to a therapeutically effective count is a technical challenge.

Another option in adoptive T cell transfer is to genetically engineer lymphocytes to produce T cell receptors (TCRs) or chimeric antigen receptors (CARs) for specific tumor antigen recognition. Tumor antigen-specificity can be accomplished by either the engineering of tumor antigen-specific TCRs with specificity for tumor-restricted peptides expressed on human leukocyte antigen (HLA) molecules or by modification of CARs encoding transmembrane proteins comprising the tumor antigen-binding site of immunoglobulins linked to T cell costimulatory molecules (Rosenberg et al. 2008; Rosenberg and Restifo 2015; Waldman, Fritz, and Lenardo 2020).

In clinical trials, immunotherapy with T cells expressing CARs was demonstrated to be an effective treatment option for hematological malignancies (Braendstrup, Levine, and Ruella 2020; Neelapu et al. 2017; Maude et al. 2018). T cell-based immunotherapies of solid tumors pose particular challenges by force of the complex, dynamic tumor microenvironment (Rodriguez et al. 2018; Hegde and Chen 2020). In solid tumors the adoptive transfer of lymphocytes engineered to express TCRs directed against melanoma-specific antigens, such as NY-ESO-1, showed promising anti-tumor responses in patients with metastatic melanoma (Robbins et al. 2011; Robbins et al. 2015).

Even though the severe side effects of TCR / CAR gene therapies are not mastered yet (Brudno and Kochenderfer 2019, 2016), genetically engineered T cells have emerged as a promising treatment modality for other malignancies, including ovarian cancer. In trials investigating new ovarian cancer treatment modalities are among the most promising those that recognize HLA-A2 restricted epitopes such as NY-ESO-1 (NLM at the NIH 2021b). So far, in ovarian cancer patients, initially promising clinical responses were not sustained, eventually leading to tumor recurrence. Hypothesized explanations for these non-satisfying results are the relatively limited long-term survival and potentially impaired effector function due to suppression or exhaustion of infused engineered T cells within the ovarian cancer microenvironment (Kershaw et al. 2006).

CD19 targeting CAR T cell therapy is available for subtypes of lymphomas and individual patients with relapsed leukemia (Braendstrup, Levine, and Ruella 2020; Neelapu et al. 2017; Maude et al. 2018); NY-ESO-1 directed adoptive cell transfer was not granted regulatory approval yet.

5 SUMMARY

In the last decades, many advances have been made diagnosing and treating ovarian cancer; however, the prognosis for ovarian cancer patients remains dismal. Therefore, immunotherapeutic treatment approaches are being researched as a supplement to conventional ovarian cancer treatment. NY-ESO-1 is a shared cancer/testis antigen with tissue-restricted expression in various cancer types and the immune-privileged testis. Its absent expression in healthy somatic tissues yields hopes for NY-ESO-1 being a pinpoint therapeutic target with limited off-target toxicities. A few study groups have explored the presence of NY-ESO-1 in ovarian carcinoma rendering contradictory results (Yakirevich et al. 2003; Szender et al. 2017; Odunsi et al. 2003); this study aimed to further investigate the protein NY-ESO-1 as a prognostic marker and potential target for immunotherapy for ovarian cancer.

In our study, based on the intensity of NY-ESO-1 immunohistochemical staining and percent positive cells, we calculated histological scores (H-scores) for all ovarian cancer specimens on the constructed tissue microarrays; an H-score of ≧ 1 was considered positive. Statistical analysis was performed on all patients in the dataset as well as on the subgroups of platinum-resistant and -sensitive patients. We found that mean H-scores increased with increasing FIGO stage, but not at a significant level. Also, we observed higher mean NY-ESO-1 H-scores in platinum-sensitive patients compared to platinum-resistant patients; however, this finding was also not significant. Further, H-scores did neither correlate statistically significantly with overall nor with progression-free survival. Although our study did not reveal a statistically significant correlation with any of the investigated clinicopathological parameters, we found a considerable NY-ESO-1 expression of 30.9 % in ovarian cancers in our dataset: among them, 28.0 % in serous, 28.9 % in endometrioid, and 41.2 % in clear cell ovarian cancers. Interestingly, recent studies suggest particular responsiveness of the subentity of clear cell ovarian cancers in experimental immunotherapeutic treatment approaches with checkpoint inhibitors. Interest remains in considering NY-ESO-1 as a viable target for future studies

researching immunotherapeutic approaches, if necessary, in combination therapy with other agents.

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7 FIGURE AND TABLE LEGEND

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