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Ovarian Carcinoma - Early Detection and Prognostication

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Early Detection and Prognostication

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Abstract: The high mortality of ovarian cancer can be mainly attributed to its unspecific symptoms, which usually appear when the disease has reached an advanced stage. The most common and lethal subtype is high-grade serous carcinoma (HGSC). The recognition that early detection could improve prognosis has driven large randomized trials in the last decades. These trials, using vaginal ultrasound and the tumor marker CA125, have failed to show significant mortality reduction and population-based screening is today not recommended. Novel molecular approaches include the detection of the, in HGSC, ubiquitous *TP53* mutation appearing in cervical, uterine and plasma samples.

Study I aimed at detecting tumor derived *TP53* mutations in pre-diagnostic liquid based cervical samples (CS), taken months to years before the HGSC diagnosis. We identified 15 women with pre-diagnostic CS and found 14 somatic *TP53* mutations in their tumor tissues. Subsequently, we analyzed both pre-diagnostic and diagnostic CS with an ultrasensitive droplet digital PCR. We found the tumor corresponding mutations in six of eight diagnostic samples and in one of 20 pre-diagnostic samples, obtained 20 months prior to the HGSC diagnosis.

In **Study II** we present a novel approach exploring copy number alterations (CNAs) in diagnostic and pre-diagnostic cervical samples (CS) with the goal of developing a new screening test. A total of 204 samples from HGSC patients, controls and *BRCA* mutation carriers were analyzed with low coverage whole genome sequencing. Mathematical models were used to subgroup six different copy number (CN) features into 32 components, generate six CN signatures and cluster the CS into the six signatures. We found similar CNAs in some CS from HGSC patients and tumors. CNAs could not be found in benign tissues or CS. Further understanding of the CN features and cell types contributing to each signature, and inclusion of more cervical samples into the approach, will hopefully identify a novel tumorigenic signature for early detection of HGSC in cervical samples.

With the goal of implementing targeted treatment and improving clinical outcomes, significant research efforts have been made to identify novel prognostic and predictive biomarkers. Despite initial response rates of >80% to platinum-based chemotherapy, most patients relapse and eventually develop platinum resistance. Further, the response rates to the recently introduced checkpoint inhibitors are poor and there are no predictive markers selecting patients who could benefit from immunotherapy.

Study III investigated the tight junction protein Claudin-4 as a predictive marker for platinum resistance and as a prognostic marker. Claudin-4 expression was evaluated by immunohistochemistry in a tissue microarray of 140 cases of ovarian carcinoma. Even if Claudin-4 seemed to predict outcome, it could not predict response to platinum in our patient cohort.

In **Study IV** we mapped the presence of macrophages and lymphocytes located within the tumor epithelium, the cell type-specific expression of PD-1 and PD-L1, and their impact on survival in a cohort of 130 women diagnosed with advanced HGSC. Women with higher lymphocyte tumor infiltration and PD-1/PD-L1 expression had improved survival.

In conclusion, these studies aimed at exploring molecular methods for early detection and prognostication, which contribute to understanding this genetically complex and mortal disease.

Key words HGSC, early detection, pre-diagnostic cervical sample, *TP53*, copy number alterations and signatures, Claudin-4, immune cell infiltration, PD-1/PD-L1 pathway.

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Ovarian Carcinoma

Early Detection and Prognostication

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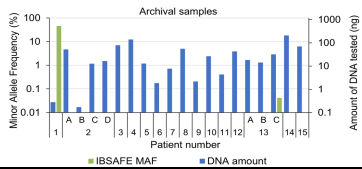
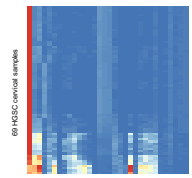
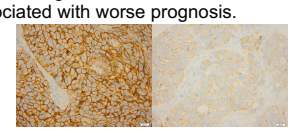
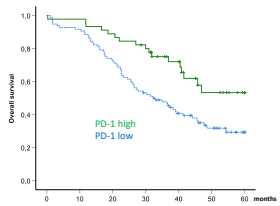
To all ovarian cancer patients

Table of Contents

Thesis at a glance

Study	Question	Methods	Results and implications
I	Can tumor-derived *TP53* mutations be found in cervical samples (CS), taken months to years prior to the HGSC diagnosis?	Targeted DNA sequencing of 15 tumors. Identification of *TP53* mutations in CS by ultra-accurate droplet digital PCR (IBSAFE).	IBSAFE achieved a sensitivity of 75% in the diagnostic CS. Among 20 pre-diagnostic CS, we detected a *TP53* mutation in a sample taken 20 months prior to diagnosis. 
II	Can copy number alterations be found in diagnostic and pre-diagnostic cervical samples (CS) from HGSC patients?	Low coverage whole genome sequencing. Bioinformatic tools for estimation of absolute copy number (CN). Mixture modelling to subgroup CN features and non-negative matrix factorization to generate six CN signatures and cluster the samples.	Some CS from HGSC patients showed different CN feature distributions compared to normal CS. Tumors and HGSC CS displayed different exposures to certain signatures compared to normal tissues and CS. 
III	Is Claudin-4 a prognostic factor in ovarian carcinoma and predictive of response to platinum-based treatment?	Immunohistochemical analysis of Claudin-4 in 140 ovarian carcinomas (TMA). Analysis of mRNA levels and survival in an external dataset.	Claudin-4 overexpression predicted shorter survival. Higher mRNA values were also associated with worse prognosis.  *complete vs. partial membrane staining*
IV	Does immune cell infiltration (CD3, CD68 and CD163) have an impact on prognosis? Does the receptor PD-1 and its ligand PD-L1, have an impact on prognosis?	IHC analysis of immune cell markers in 130 cases of advanced stage HGSC (TMA). Validation of the prognostic value of mRNA levels of PD-L1 and CD3 in an external dataset.	PD-L1 expression was mainly displayed by macrophages and PD-1 by lymphocytes. Women with higher intratumoral expression of CD3 (lymphocytes), PD-1 and PD-L1 had a favorable prognosis. 

List of original studies

This thesis is based on the following studies, which are referred to throughout the text with their roman numerals:

I. Arildsen NS, **Martin de la Fuente L**, Måsbäck A, Malander S, Forslund O, Kannisto P, Hedenfalk I. Detecting *TP53* mutations in diagnostic and archival liquid-based Pap samples from ovarian cancer patients using an ultra-sensitive ddPCR method. *Sci Rep*. 2019 Oct 29;9(1):15506.

II. **Martin de la Fuente L**, Li M X, Måsbäck A, Malander S, Kannisto P, Veerla S, Hedenfalk I. Copy number signatures for early diagnosis of high-grade serous ovarian carcinoma. *In manuscript*.

III. **Martin de la Fuente L**, Malander, S, Hartman, L, Nilbert M, Måsbäck A, Hedenfalk I. Claudin-4 expression correlates with progression free survival but not with chemotherapy response in ovarian cancer. *Int J Gynecol Pathol* 37, 101-109 (2018).

IV. **Martin de la Fuente L**, Westbom-Fremer S, Arildsen NS, Hartman L, Malander S, Kannisto P, Måsbäck A and Hedenfalk I. PD-1/PD-L1 expression and tumor-infiltrating lymphocytes are prognostically favorable in advanced high-grade serous ovarian carcinoma. *Virchows Arch*. 2020 Jul;477(1):83-91.

Study contributions

Study I

I was involved in the study design and participated in DNA extractions. I was responsible for data and sample collection. I contributed to writing and revision of the manuscript.

Study II

I was involved in the study design. I was responsible for data and sample collection. I contributed to the evaluation and interpretation of the sequencing data. I was responsible for writing of the manuscript.

Study III

I evaluated the immunohistochemical marker, and I conducted the statistical analysis. I was responsible for writing and revision of the manuscript.

Study IV

I participated in the study design and methodological considerations. I evaluated the immunohistochemical markers. I was responsible for the collection of clinical data, data analysis, and for writing and revision of the manuscript.

Abbreviations

BRCA 1/2	Breast cancer type 1/2 susceptibility gene
CA125	Cancer antigen 125
CD3	Cluster of differentiation 3
CD68	Cluster of differentiation 68
CD163	Cluster of differentiation 163
CI	Confidence interval
CNAs	Copy number alterations
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
FIGO	International Federation of Gynecology and Obstetrics
FFPE	Formalin fixed paraffin embedded
HE	Hematoxylin & Eosin
HGSC	High-grade serous carcinoma
HR	Hazard ratio
HRD	Homologous recombination deficiency
IHC	Immunohistochemistry
MAF	Mutant allele frequency
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
OS	Overall survival
p53/*TP53*	Tumor protein 53 – Protein/Gene
PARP	Poly-(ADP-ribose)-polymerase
PAX8	Paired Box 8
PFS	Progression-free survival
PD-1	Programmed death receptor 1
PD-L1	Programmed death receptor ligand 1
PR	Progesterone receptor
RMI I	Risk of malignancy index I
STIC	Serous tubal intraepithelial carcinoma
TAMs	Tumor associated macrophages
TCGA	The cancer genome atlas
TILs	Tumor infiltrating lymphocytes
TF	Tumor fraction
TMA	Tissue microarray
WGS	Whole genome sequencing
WHO	World Health Organization
WT1	Wilms' tumor protein 1

Populärvetenskaplig sammanfattning

I Sverige drabbas nästan 700 kvinnor av äggstockscancer varje år. Den vanligaste och dödligaste varianten är den så kallade ”höggradiga serösa äggstockscancern”. Trots namnet vet man idag att de flesta äggstockscancrar har sitt ursprung i äggledaren. Medelåldern vid insjuknande är cirka 65 år. Sjukdomen ger vaga symtom och upptäcks därför ofta vid avancerade stadier (III och IV), då prognosen är betydligt sämre jämfört med tidiga stadier (I och II). Behandling av äggstockscancer är i första hand kirurgisk. Därutöver ger man ofta så kallade tilläggsbehandling med tumörhämmande läkemedel (t ex cytostatika). Sjukdomen är vanligtvis känslig för cytostatika till en början. Många patienter drabbas av återfall och då uppvisar tumören oftast en nedsatt känslighet för cytostatika (förvärvad cytostatikaresistens).

Studie I

Höggradiga serösa äggstockstumörer bär på en sjukdomsalstrande mutation i *TP53*-genen (också kallad ”genomets väktare”) som uppstår tidigt i tumörutvecklingen. Tidigare studier har visat att *TP53*-mutationer kan påvisas i cellprover tagna vid diagnostillfället (när cancern upptäckts). Dessa prover tas idag rutinmässigt i syfte att upptäcka förstadier till en annan cancertyp, livmoderhalscancer. Anledningen till att man kan hitta mutationer från en tumör med sitt ursprung i äggledaren i prover tagna i livmoderhalsen, är att äggledaren har anatomisk förbindelse med livmoderhalsen genom livmoderkroppen. Med syfte att utveckla en metod för tidig diagnostik undersökte vi förekomsten av *TP53*-mutationer i cellprover tagna flera månader till år före det att patienten diagnostiserats med sin äggstockscancer. Eftersom cellproverna innehöll väldigt lite genetiskt material (DNA) och kunde vara insamlade upp till flera år före det att kvinnorna hade insjuknat i sin äggstockscancer, fick vi använda en mycket känslig metod som förutsätter att man först hittar den specifika mutationen i själva tumören och därefter bekräftar förekomsten av den i cellprovet. Vi var den första forskargruppen i världen som kunde påvisa förekomsten av en *TP53*-mutation i ett cellprov taget så tidigt som 20 månader före det faktiska diagnostillfället.

Studie II

Mutationer i *TP53*-genen är sjukdomsalstrande och orsakar omfattande avvikelser på vår arvsmassa. Gener som kodar för proteiner utgör endast 2% av arvsmassan och resten är tyst eller icke-kodande arvsmassa. För att öka sannolikheten att finna sådana avvikelser undersökte vi hela arvsmassan, alltså även den så kallade tysta delen av arvsmassan i cellprover (tagna före och vid diagnostillfället) från patienter med äggstockscancer. Vi upptäckte kromosomavvikelser typiska för tumörer i flera av cellproverna. Våra fynd verkade således vara specifika för patienter som utvecklat äggstockscancer. Med matematiska modeller, indelade vi cellproverna i olika undergrupper. I nästa steg ville vi identifiera exakt vilka avvikelser i

cellproverna som skulle kunna utskilja friska kvinnor från kvinnor med äggstockscancer. Studiens mål var att utveckla en teknik för att diagnostisera äggstockscancer tidigt.

Studie III

Claudin-4 är ett protein i cellmembranet (cellens yttersta hinna) som bland annat gör att celler kan hålla ihop med varandra. Tidigare studier har pekat på att detta protein kan vara inblandat i cancerutveckling och cytostatikaresistens. Vi undersökte förekomsten av Claudin-4 vid äggstockscancer och fann att patienter vars tumörer hade riklig förekomst av proteinet levde kortare än patienter vars tumörer hade mindre förekomst. Vår undersökning kunde inte visa något samband mellan uttryck av claudin-4 och känslighet för cytostatikabehandling.

Studie IV

Många studier har pekat på att patienter med äggstockscancer vars immunceller infiltrerar tumören har bättre överlevnad, som ett tecken på att kroppens immunförsvar bekämpar tumören. Vi undersökte detta fenomen hos 130 patienter med höggradig serös äggstockscancer och kunde bekräfta en överlevnadsvinst för patienter som hade högre tumörinfiltration av en sorts immuncell, lymfocyter. Det är känt att vissa cancertyper kan hämma immunförsvaret för att undkomma angrepp, växa och sprida sig. Hämningen sker bland annat genom att proteinet PD-L1 binder till en mottagare, PD-1, som finns på lymfocyter och agerar som broms. I vårt material visade det sig att det var en annan celltyp, makrofager, i vårt immunförsvar som uppvisade detta hämmande protein. Märkligt nog visade det sig att patienter med en högre förekomst av proteinerna PD-L1 och PD-1 hade längre överlevnad. En rimlig förklaring till detta motsägelsefulla fynd kan vara att infiltrationen av våra immunceller i sig, ger en överlevnadsvinst som är oberoende av bromssystemet.

Background

General introduction to the thesis

Ovarian cancer is the most lethal gynecologic malignancy and is a complex disease with multiple histological subtypes. The origin and pathogenesis of ovarian cancer have been poorly understood in the past. The most common subtype is high-grade serous carcinoma (HGSC) that, in most cases, arise from the tubal fimbriae epithelium, and not in the ovaries. This thesis focuses mainly on HGSCs which comprise tumors located in the fallopian tubes, ovaries, or peritoneum. HGSC are poorly differentiated, possess few common mutations except for the ubiquitous *TP53* and are genomically unstable with large regions of deletion or amplification in certain regions of the genome.

The high case-fatality ratio of ovarian cancer can be attributed in part to its vague and unspecific symptoms, which usually don't appear until the disease has reached an advanced stage. The recognition that early detection of ovarian cancer could improve prognosis has driven the development of large randomized controlled trials in the last decades. The screening tools used were vaginal ultrasound and/or a longitudinal algorithm of the tumor marker CA125. However, none of the two trials studying mortality has demonstrated significant disease-specific mortality reduction [1, 2]. According to calculation models based on serous carcinomas in *BRCA1* mutation carriers, these tumors spread when still small, making the development of a test for early detection challenging [3]. However, a several years long window of opportunity for early detection, from pre-malignant lesions to invasive cancer, has been identified by different research approaches [3, 4]. Currently, there is a lack of tests that can detect the disease early enough, and in a sufficient number of cases, to impact mortality and screening is not recommended in the general population.

Given gained insights in the disease tumorigenesis and advances in sequencing technologies, novel screening modalities are being studied. Targeted sequencing of the, in HGSC, ubiquitous *TP53* mutation appearing in cervical, uterine and plasma samples, has been a promising approach [5-8]. However, the accumulation of cancer-like age-associated somatic *TP53* mutations in normal tissues that also can be detected with ultra-accurate DNA sequencing technology has emerged as the major challenge [7, 9]. In our MaNiLa project (Mats, Nicolai, Laura, the original PhD students in the project) we prospectively recruited 187 women with suspected ovarian tumor or adnexal mass, as well as 12 *BRCA* mutation carriers who

underwent risk-reducing surgery and genomically interrogated collected tissue, plasma, endometrial and cervical samples. In a subset of patients, we even analyzed cervical samples taken months to years before the HGSC diagnosis, when patients were still asymptomatic. We used PCR (digital polymerase chain reaction)-based methods for the detection of *TP53* mutations and even explored copy number alterations as a novel biomarker for early detection.

The most powerful prognostic markers in ovarian carcinoma are volume of residual disease after cytoreductive surgery, tumor stage, histologic type and grade of differentiation. Significant research efforts have been made in the last decades to identify novel prognostic and predictive biomarkers based on genetic and/or proteomic tumor characteristics, with the goal of implementing targeted treatment and improving clinical outcomes. At present, the only markers predicting treatment response are homologous recombination deficiency (HRD), especially mutations in *BRCA1/2*, which predict response to poly (ADP-ribose) polymerase (PARP) inhibitors.

Despite initial response rates of >80% to platinum-based chemotherapy, most ovarian carcinoma patients relapse and eventually develop platinum resistance [10], which is a major clinical problem due to the lack of alternative effective treatments. Tight junction (TJ) proteins are often altered in human carcinomas [11]. We studied the TJ protein Claudin-4 as a predictive marker for platinum resistance and as a prognostic marker.

Numerous studies have confirmed the prognostic significance of tumor infiltrating lymphocytes (TILs) in HGSC [12]. However, the response rates to checkpoint inhibitors for patients with HGSC are poor and there are no predictive markers selecting patients who could benefit from immunotherapy or possibly combination of therapies. In this thesis we further explore the prognostic impact of immune cell infiltration in HGSC.

Epidemiology and risk factors

In 2020, ovarian cancer ranked as the eighth most common cancer diagnosis and cause of death in women, with estimated 314 000 cases and 207 000 deaths worldwide [13] to compare with the most common female malignancy, breast cancer, with 2.3 million cases and 685 000 deaths. Of note, the relatively higher ovarian cancer mortality compared to breast cancer, can be explained by unspecific symptoms and lack of specific biomarkers resulting in late diagnosis. There is a geographical variation in the age-adjusted incidence worldwide, with the highest incidence in northern Europe and north America and the lowest in southeast Asia and parts of Africa [14]. These differences are probably related to environmental and lifestyle factors as well as varying genetic predisposition. In Sweden, 669

women were diagnosed with ovarian cancer (including tubal carcinoma) in 2020, this is an incidence of 13/100 000 to compare with 26/100 000 in 1975 [15] (Figure 1). This difference can be explained by the increased use of oral contraceptives, known to lower ovarian cancer risk [16] and changes in the diagnostic classification criteria.

The relative 5-year survival of ovarian carcinoma is 46%. However there is a wide variation in 5-year survival depending on tumor stage, ranging from >90% in stage I to 31% in stage III-IV [17].

Hereditary predisposition is the strongest risk factor and explains up to 20% of newly diagnosed ovarian carcinomas. The major contributors to hereditary ovarian cancer are germline mutations in *BRCA1* and *BRCA2* genes, mismatch repair genes associated with Lynch syndrome, *TP53* in the Li-Fraumeni syndrome and several other genes involved in DNA double-strand repair [18].

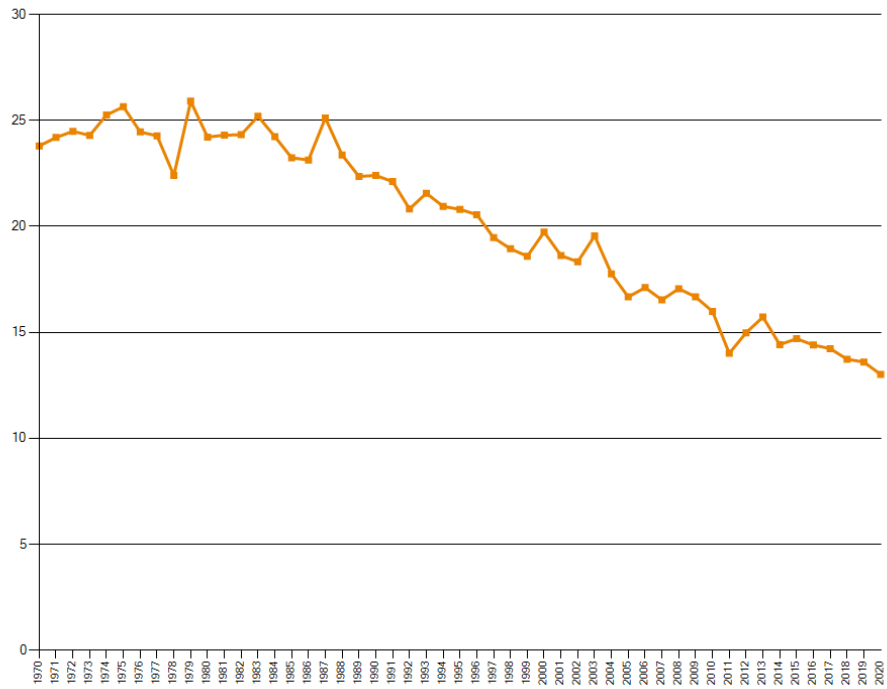


Figure 1. Number of new ovarian carcinoma cases per 100,000 women (crude rate) 1970-2020, Sweden.
The Health and Welfare Statistical Database 02/05/2022.

The lifetime risk of developing ovarian cancer for a *BRCA1* mutation carrier is 40-50% (70-80% risk for breast cancer) [19] compared to 1.3% in the general population [20]. Women with hereditary predisposition have an earlier onset of

disease and are recommended regular gynecological examinations and/or risk reducing surgery. Germline and somatic mutations in the *BRCA* genes are often associated with the most common high-grade serous histopathologic type, whereas Lynch Syndrome-associated ovarian cancers are often of endometrioid, clear cell or mucinous type.

Other risk factors for developing ovarian carcinoma include nulliparity, endometriosis and anthropometric/lifestyle factors. In 1971, Fathalla was the first to suggest a possible relation between the frequency of ovulation and the development of malignant neoplasms from the ovarian epithelium [21]. Subsequent biologic findings and epidemiologic evidence supported the so called "incessant ovulation hypothesis". Both cohort and case-control studies have shown higher risk of developing ovarian carcinoma in women who haven't undergone pregnancy and that the protection rate increases in relation to number of pregnancies [22, 23]. In line with this, oral contraceptives are protective factors [16].

Histologic subtypes

Ovarian cancer can be divided in two tumor types depending on the cell of origin: epithelial and non-epithelial ovarian cancer. Epithelial ovarian cancer, that can also be called ovarian carcinoma, accounts for 90% of all ovarian cancer cases while non-epithelial ovarian cancer, mainly germ cell and sex cord stromal cell cancers account for 10%. Henceforth, the term ovarian carcinoma refers to epithelial ovarian cancer. According to WHO classification, the five most common ovarian carcinoma histotypes are high-grade serous, low-grade serous, clear cell, endometrioid and mucinous [24].

Morphology is the basis of classification. Since histotypes arise from different cells of origin, cell lineage-specific diagnostic immunohistochemical markers and histotype-specific oncogenic alterations can confirm the morphological diagnosis. A four-marker immunohistochemical panel (WT1, p53, napsin A and PR) can distinguish the five principal histotypes with high accuracy [25]. Main diagnostic criteria and differential diagnosis of the five tumor subtypes are summarized in Table 1 [26-28].

Comprehension of the embryologic origin of the different parts of the genital system is critical when understanding the origin of ovarian, peritoneal and tubal carcinomas. Fallopian tubes, uterus, cervix and upper vagina are embryologically derived from the Mullerian duct, whereas ovarian surface mesothelium is derived from the coelom or intraembryonic body cavity. Most epithelial tumors originated in Mullerian-derived tissues express the nuclear marker PAX8. The nuclear marker WT1 is a useful marker for extrauterine HGSC, as most of these tumors express it, in contrast to serous carcinomas of endometrial origin [29]. Aberrant p53 expression

is used to confirm the diagnosis of an HGSC. Missense *TP53* mutations correlate with overexpression, the most common pattern, whereas nonsense mutations result in a truncated protein not detected by the p53 antibody, and thus complete absence of staining [30].

The WHO 2014 classification introduced the division of ovarian serous tumors into low-grade and high-grade carcinomas, instead of the previous three-tiered grading system. Low and high-grade serous carcinomas represent two completely different tumor types, with different morphology, pathogenesis, molecular events, and prognosis [26, 31].

Table 1. **Histopathological characteristics and differential diagnosis of the most common ovarian carcinomas**

	High-grade Serous	Low-grade Serous (LGSC)	Endometrioid (EC)	Clear cell (CCC)	Mucinous (MC)
Fraction of ovarian carcinoma	75-85%	<5%	10%	5-25%	3%
Common FIGO stage	III or IV	I or II	I or II	I or II	I or II
Key features	Areas with solid growth High mitotic rate	Micropapillary architecture with psammoma bodies Serous borderline component	Squamous or mucinous differentiation Rarely solid growth	Papillary and tubulocystic architecture Glycogen rich cytoplasma	Complex glandular morphology Goblet cells
Differential diagnosis	EC, but no squamous differentiation and WT1+ in HGSC	HGSC, but more uniform nuclei and lower mitotic rate in LGSC	HGSC, but WT1- in EC	HGSC with clear cells, but lower mitotic rate, ER-, WT1- in CCC LGSC, but ER- WT- in CCC	EC with mucinous differentiation, but ER- and PR- in MC Metastatic carcinoma
IHC staining					
PAX8	95%	87-100%	82%	95%	39-47%
WT1	97%	98-100%	10-14%	1%	0-1%
p53, aberrant	94-98%	0%	15%	12%	61-66%
Napsin A	1%	0%	3-8%	92%	0-3%
ER	80%	96%	86%	13%	6%
PR	37-42%	60%	81-85%	5-7%	0-4%

FIGO= International Federation of Gynecology and Obstetrics, WT1= Wilms' tumor protein 1, ER= estrogen receptor, PR= progesterone receptor, PAX8= Paired Box 8.

Origin and pathogenesis of HGSC

Before the 2000s, the assumed origin of most high-grade tumors was the ovary and thereby they were termed as ovarian. Nowadays, extrauterine HGSC refer to tumors located in the fallopian tubes, ovaries, or peritoneum, but not the uterus or cervix [32]. According to current criteria for site assignment in extrauterine HGSC, a high

proportion of cases are classified as being of tubal origin (~80%), whereas primary peritoneal HGSCs are rare.

In the traditional model of ovarian carcinogenesis, the ovarian surface mesothelium was the presumed cell of origin for serous neoplasia as a result of metaplastic changes to a tubal epithelial type, with subsequent neoplastic transformation related to mutational events occurring because of ovulatory trauma. However, no precursor lesions could be found in the ovarian surface mesothelium. For this reason, ovarian cancer was described to arise "*de novo*" and pathologists designated serous carcinomas as ovarian or peritoneal based on the extent to which these organs were involved (WHO, 2003) [33, 34].

New insights came after 2000 when pathologists started to report dysplastic changes and cancer *in situ* in prophylactically removed fallopian tubes of women predisposed to developing ovarian cancer [35, 36]. In 2003, Piek *et al*. postulated that occult tubal carcinomas shed malignant cells that implant and grow on the ovary, simulating primary ovarian cancer [37]. The new proposed cell of origin was also supported by the anatomical proximity of the fimbriae of the fallopian tube and the ovary, that allowed for cell drop or simply overgrowth. In 2007, Kindelberger et al. showed the frequent presence of cancer *in situ* in the fimbriated end of the fallopian tube in non *BRCA*-related cases of serous carcinoma presumed to be of ovarian origin. Furthermore, they identified identical *TP53* mutations in five cases classified as primary ovarian with co-existing tubal cancer *in situ* lesions, establishing the first evidence for a causal relationship between cancer *in situ* in the tuba and serous carcinoma, in not genetically predisposed women [38]. The cancer *in situ* lesions identified in the tubal fimbria have been designated serous tubal intraepithelial carcinoma (STIC).

The p53 signature and STIC

In 2006 Lee *et al.* studied the relationship between p53 signature and STIC in germline *BRCA* mutated women and controls. The p53 signature was defined as a discrete population of at least 12 secretory cells with intense nuclear p53 staining (Figure 2). Even though p53 signatures were equally common in benign tubes from *BRCA* mutation carriers and controls, p53 signatures were more frequently present in association with STIC and in one case the p53 signature harbored the same *TP53* mutation as its contiguous STIC. In 2012 Kuhn et al. studied 29 cases of STICs with concurrent HGSC and showed identical *TP53* mutations in 27 pairs [39], supporting the clonal relationship of the two lesions. Subsequent targeted sequencing analyses have shown that fallopian tube lesions harbor the same *TP53* mutation as surrounding invasive carcinomas [40, 41]. In 2017, whole exome sequencing and copy number analysis in five cases of sporadic HGSC with co-existing p53 signatures and/or STICs enabled evolutionary analysis that supported the theory of *TP53* mutations as early events driving malignant transformation and identified a

window of seven years between development of a STIC and initiation of HGSC [4]. Thus, several histopathological and immunohistochemical as well as genomic findings throughout the past 20 years have established the fallopian tube epithelium as the cell of origin of HGSC with implications for prevention, early detection, and therapeutic intervention of this disease.

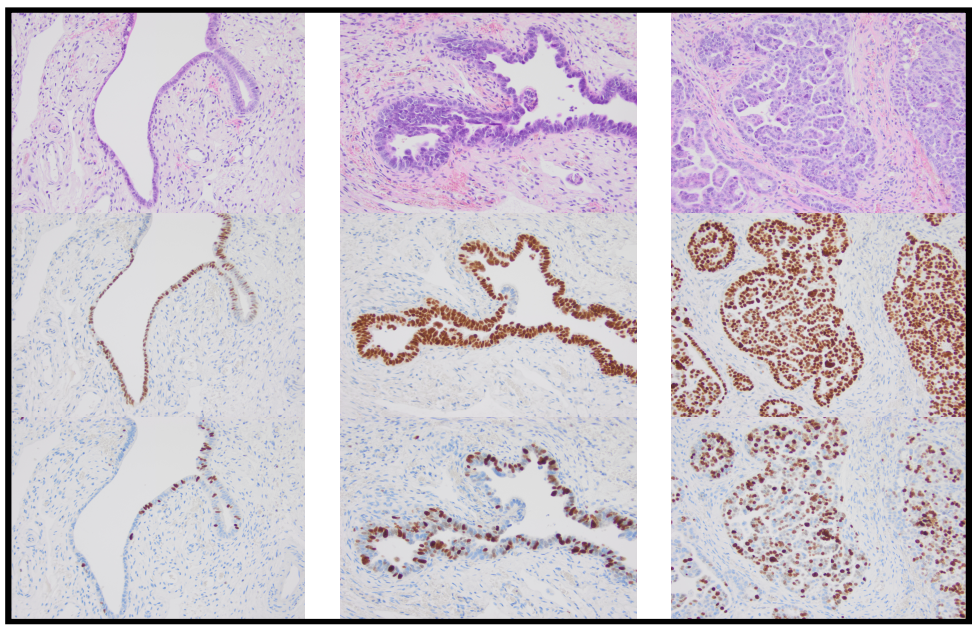


Figure 2. Tumor progression within the fallopian tube in a case of HGSC included in Study II. From left to right; p53 signature, serous tubal intraepithelial carcinoma (STIC) and invasive carcinoma. From top to bottom; HE, p53 and Ki67 IHC. Own picture. Magnification x20.

The dualistic model

In 2004 Shih et al. presented a new categorization of ovarian carcinoma in two main entities, based on gained insights in their pathogenesis (Table 2) [33]. One of the groups, designated type I tumors had a well-differentiated phenotype and was thought to arise in a stepwise manner from borderline tumors whereas type II tumors were high-grade neoplasms. Type I tumors were associated with distinct molecular changes that are rarely found in type II tumors, while type II tumors were characterized by the frequent *TP53* mutations (Table 2). Type I tumors presented generally at Stage I-II, were slow-growing and had poor response to chemotherapy, while type II tumors were more aggressive, presented more often with metastasis and their initial response to chemotherapy was good. In this early categorization, no precursor lesions for high-grade tumors had been well-established yet.

In later publications by the same authors the theory was revised and expanded by emerging knowledge. They stated that type I tumors develop from benign extra-ovarian lesions that implant on the ovary and which can subsequently undergo malignant transformation. Further, they proposed that type II tumors originate in the tubal fimbriae epithelium or less commonly in inclusion epithelium in the ovary [32, 42, 43] (Table 2). Namely, normal tubal epithelium (Mullerian-type) from the fimbria implants at the site of rupture on the ovary when ovulation occurs, forming cortical inclusion cysts that may then undergo malignant transformation, explaining the minor subset of HGSC developing from cysts in the ovary [32].

Table 2. Precursor and molecular features of most common ovarian carcinomas divided in type I and II tumors

	Tissue of origin/ Putative precursor	Most frequent mutation(s)	Level of chromosomal structural alteration
Type I tumors			
Low-grade serous carcinoma	Fallopian tube epithelium Serous borderline tumor	*KRAS, BRAF, ERBB2*	Low
Low-grade endometrioid carcinoma	Endometriosis Endometrioid tubal intraepithelial neoplasia? *	*CTNNB1, PIK3CA, PTEN, ARID1A*	Low
Clear cell carcinoma	Endometriosis	*PIK3CA, ARID1A, FBXW7*	Low
Mucinous carcinoma	Mucinous borderline tumor	*KRAS*	Low
Type II tumors			
High-grade serous carcinoma	Fallopian tube epithelium STIC	*TP53, BRCA1/2*	High
High-grade endometrioid carcinoma	Endometriosis Endometrioid tubal intraepithelial neoplasia? *	*TP53*	High

* [44, 45] both publications from 2020. STIC, serous tubal intraepithelial carcinoma.

The dualistic model has been criticized to be an oversimplification and only applicable to serous carcinomas. Further, clinical studies have failed to demonstrate its prognostic value [46].

Molecular features of HGSC

In 2011, the Cancer Genome Atlas (TCGA) project presented a large-scale multi-omics characterization of HGSC using mRNA expression, promoter methylation, DNA copy number and DNA sequencing data. Mutations in *TP53* were present in at least 96% of the tumors. With the exception of *BRCA1/2*, somatic point mutations in other driver genes occurred at a low frequency [47]. Missense or nonsense *TP53* mutations are currently the earliest known molecular events in HGSC [48], with very few recurrent mutations, as the ten most common mutations only comprise around 20% of all *TP53* mutations in HGSC data sets reported on the cBio Cancer Genomics Portal. Approximately 50% of HGSC are defective in the homologous

recombination (HR) DNA repair pathway, with *BRCA1* and/or *BRCA2* germline and somatic mutations or epigenetic alterations in 20% of the cases and smaller contributions from mutations in other genes associated with HR [47, 49]. *TP53* mutations and thus p53 dysfunction leads to genomic instability with high levels of copy number alterations, of both extended chromosome regions and smaller focal aberrations that results in gene amplifications or losses [47, 50, 51]. Using genomic data from 12 cancer types from the TCGA data set, Ciriello *et al.* classified ovarian serous carcinoma as a "C class" tumor, dominated by copy number alterations and genomic instability, in contrast to "M class" tumors characterized by recurrent mutations [50].

Early detection

Population based screening

While the incidence and mortality of cervical cancer have decreased radically since the introduction of the Papanicolaou test (cervical cytology), overall survival from ovarian cancer has not changed substantially over the past 50 years [13]. Since women detected at stage I have an over 90% survival rate [17], a lot of effort has been made to develop a screening method for early detection.

The main goal of population-based screening is to decrease mortality by finding and treating those with previously undetected disease while avoiding harm to those who do not have the disease. However, this goal has proven to be challenging. High specificity and sensitivity are important requirements for any diagnostic test. When further applied as a screening test in asymptomatic individuals in the general population, even the positive predictive value (PPV) is an important criterion. PPV is the proportion as of cases truly diagnosed as positive of the total who had a positive test result and, besides sensitivity and specificity, is dependent on disease prevalence. As the prevalence of undiagnosed ovarian cancer is very low in the general population, specificity ideally approaching 100% is necessary to reduce possible harm due to false-positive findings [52]. Even high sensitivity is necessary to enable the detection of low volume tumors. Already year 2009, Brown *et al.* defined the target for early detection of serous ovarian cancer and calculated that to achieve a 50% reduction in mortality with an annual screen, a test would need to detect tumors of only 0.5 cm in diameter [3].

The discovery of the protein biomarker CA125 in 1983 initiated the effort to identify biomarkers for the early detection of ovarian cancer [53]. In the last four decades, several trials have used combinations of the biomarker CA125 and pelvic imaging using transvaginal ultrasound scans [2, 54, 55]. The largest of these studies, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), randomized over

200,000 women to either annual multimodal screens (two-stage risk algorithm with longitudinal measurement of CA125 followed by transvaginal ultrasound), annual transvaginal ultrasound screens or no screen, in a ratio 1:1:2. The primary outcome was death due to ovarian or tubal carcinoma. For detection of primary invasive ovarian and tubal carcinoma, the multimodal screen achieved high sensitivity, specificity and PPV (89.5%, 99.8% and 35%, respectively) [56]. Furthermore, increased incidence of stage I/II (and decrease of stage III/IV) was observed in the multimodal screening group compared to the no screening group. Despite these encouraging results, long-term follow-up (median 16.3 years) reported last year a non-significant average mortality reduction between the screening and control arms [1]. The authors concluded that there is a need for a screening strategy that can detect ovarian and tubal carcinomas in asymptomatic women even earlier in its course and in a larger proportion of women than the tests used in the trial. Thus, this exemplifies the challenge of population-based screening for relatively rare diseases.

Population based ovarian cancer screening is today not recommended [57]. However, for predisposed women who have not undergone risk-reducing bilateral salpingo-oophorectomy, transvaginal ultrasound scan and CA125 every 6 months starting at age 30-35 years may be considered with the patient advised on lack of evidence [58, 59].

Novel biomarkers for early detection

There is growing evidence that earlier detection of ovarian cancer by novel molecular technologies is the most promising approach for reducing mortality in ovarian carcinoma. Numerous studies exploring **circulating tumor DNA**, with the goal of developing pan-cancer screening tests, have included small numbers of ovarian cancer patients [60]. In 2017, using the highly sensitive sequencing method TEC-Seq (targeted error correction sequencing), Phallen et al. could detect somatic mutations in 68% (19/28) of patients with stage I or II ovarian cancer [61]. A year later, the CancerSEEK test, that combines multiplex PCR-based mutational analysis in cell-free DNA (including *TP53*) and eight protein biomarkers (including CA125), achieved a higher sensitivity of 98% for ovarian cancer patients [62]. However, most of the 54 ovarian carcinoma patients included had stage III disease, which probably to a large extent explains the higher performance. Further, the CancerSEEK test, in combination with PET-CT imaging, has been evaluated as a pan-cancer screening test in a study including nearly 10,000 asymptomatic women (DETECT-A study). The reported pan-cancer sensitivity and PPV were only 27 and 19%, respectively [63]. Of note, the detection of somatic mutations in plasma coming from white blood cells in healthy individuals has emerged as a challenge for these approaches [60].

Another pan-cancer assay is the Galleri test, developed by the company GRAIL, that detects altered DNA methylation in plasma by targeted whole genome bisulfite sequencing and machine learning. The Galleri test has been investigated in a

prospective multicenter case-control study and reported an overall specificity of 99.5% (all cancer types) and sensitivity of 83% (54/65) for ovarian carcinoma patients (varying from 50% in stage I to 95% in stage IV) [64]. Active trials assessing the implementation of the Galleri test as a multi-cancer screening tool include the NHS (National Health Service)-Galleri trial in the UK and the PATHFINDER trial in the US, that apart from the general population also recruited women with genetic predisposition for ovarian carcinoma (ISRCTN91431511 and NCT04241796, respectively).

Other novel approaches have focused on **samples of cervical or uterine origin** (summarized in Table 3). A promising development occurred in 2013, when Kinde *et al*. showed that somatic mutations in DNA shed from endometrial and ovarian cancers could be detected in standard liquid-based Pap test specimens by massively parallel sequencing (Safe-SeqS). While highly sensitive for endometrial cancer, the method was not able to detect more than 41% of ovarian cancers using a panel of 12 genes commonly mutated in these tumors [5]. In a later study from 2018, this research group in collaboration with others attempted to increase the sensitivity by introducing an intra-uterine brush, by combining the Pap test with plasma sampling and by complementing the Safe-SeqS assay for mutations with an assay for aneuploidy. The highest sensitivity of 63% was achieved when combining mutational analysis of Pap brush and plasma samples [6].

Another approach trying to increase sensitivity was the lavage of the uterine cavity presented in 2015 by Maritschnegg *et al*. Mutations (mainly in *TP53*) were identified in the lavages of 18/30 patients (60% sensitivity) with targeted next-generation sequencing and a further six patients could be identified by digital droplet PCR which however requires previous analysis of the corresponding tumor tissue (together 80% sensitivity) [65]. A more recent study from 2019, used an ultra-accurate DNA sequencing technology (Duplex Sequencing) to examine 10 uterine lavages from women with ovarian carcinoma (from the previously named study by Maritschnegg *et al.*) and 11 lavages from controls. This study demonstrated an 80% sensitivity for ovarian carcinoma detection but also revealed age-related, cancer-like low-frequency *TP53* mutations in uterine lavages and other tissues from healthy women [7]. A subsequent study, that analyzed endocervical cytobrush samples with similar Duplex Sequencing showed a lower sensitivity of 37% (3/8 Pap tests) for ovarian carcinoma detection and abundant low-frequency *TP53* mutations in Pap samples from both women with and without ovarian carcinoma [9]. Thus, sensitivity remains a limiting factor and specificity has emerged as the major challenge for the ultra-sensitive next generation DNA sequencing approaches.

Table 3. Novel approaches for ovarian carcinoma detection using samples of cervical or uterine origin

Study/ year	Nr patients	Sample type	Method	Limit of detection *mutant:wildtype alleles*	Sensitivity	Comments
Kinde *et al.* [5]/ 2013	22	Endocervical brush*	Targeted NGS 1.Safe-SeqS 2.PapGene (12 genes)	*1:1,000* (PapGene)	41% (9/22) Safe-SeqS 36% (8/22) PapGene	Previous detection in tumor required with Safe-SeqS. PapGene designed for screening.
Maritschnegg *et al.* [65]/ 2015	30	Uterine lavage	1.NGS (multiplex) 2.ddPCR (singleplex)	N.D.	60% (18/30) NGS 80% (+6) ddPCR	Detection with NGS confirmed by Safe-SeqS. Previous detection in tumor required for ddPCR.
Wang *et al.* [6]/ 2018	245	Endocervical Pap brush* n=245 Intrauterine Tao brush* n=51 Plasma n=83	1.Safe-SeqS (18 genes) 2.aneuploidy (NGS)	N.D.	33% (81/245) Pap brush 45% (23/51) Tao brush 63% (52/83) Pap brush +plasma	Mutations found in some Pap and Tao brushes could not be found in matching tumors. Clonal proliferation of normal tissues proposed as main explanation.
Salk *et al.* [7]/ 2019	10	Uterine lavage	Duplex Sequencing (DS) of *TP53*	1:25,000	80% (8/10)	Low-frequency *TP53* mutations in uterine lavages and other tissues from healthy women (clonal proliferation).
Krimmel-Morrison *et al.* [9]/ 2020	9	Endocervical (Pap) brush*	CRISPR-DS of *TP53*	1:25,000	37% (3/8, 1 sample excluded due to prior tubal ligation)	Authors claim low sensitivity of Pap brush for detection. Low-frequency *TP53* mutations in Pap brush from controls.
Arildsen *et al.* [66]/ 2019	15	Diagnostic (n=9) and pre-diagnostic endocervical brush* (n=20)	ddPCR *TP53* 1.BioRad 2.IBSAFE	 1:10,000 1:100,000	IBSAFE: 75% (6/8‡) for diagnostic, 5% (1/19‡) for pre-diagnostic	*TP53* mutation in a cervical sample taken 20 months prior to diagnosis.
Paracchini *et al.* [8]/ 2020	17	Diagnostic and pre-diagnostic endocervical brush †	BioRad ddPCR *TP53*	1:10,000	64% (11/17)	Several *TP53* mutations in cervical samples taken up to 68 months prior to diagnosis.

* Liquid-based preservation of the sample. † Traditional Papanicolaou slides. ‡ One patient excluded due to germline mutation. Abbreviations: NGS, Next Generation Sequencing. ddPCR, droplet digital Polymerase Chain Reaction. CRISPR-DS, Duplex Sequencing incorporates CRISPR/Cas9 target enrichment prior to library preparation. ND, not defined.

The ongoing population based DOvEEgene Phase III Trial from Canada aims at early diagnosis of ovarian and endometrial cancers through the identification of pathogenic somatic mutations in uterine Pap samples in combination with a machine learning algorithm and plans to recruit 3,600 asymptomatic women (NCT04891029). It is important to stress that even if ongoing screening trials (NHS-Galleri, PATHFINDER and DovEEgene) are encouraging, none of them include mortality as the primary outcome.

In conclusion, the key limitation of current screening strategies is the lack of tests that can detect premalignant and early-stage disease [67]. Further, the limitation

imposed by the low positive predictive value could be circumvented by focusing on high-risk subjects, where the prevalence of occult malignancy is higher than in the general population. Thus, improved risk stratification is fundamental for defining the target population for screening. Current risk prediction algorithms are based on family history and epidemiological factors (for example BOADICEA and BRCA PRO). Interestingly, a recent study described a differential epigenetic DNA methylation of cervical cells in *BRCA1/2* germline mutation carriers and in women with ovarian carcinoma, which could be used as an indicator for cancer predisposition [68].

Our investigative approach to early detection

Previous presented investigative approaches based on cervical/uterine sampling have been applied in patients at the time of diagnosis. It was not until 2019 that our group for the first time could detect a *TP53* mutation with an ultra-sensitive droplet digital PCR method (IBSAFE, now known as SAGAsafe®) in an archival liquid-based cervical sample obtained 20 months prior to the ovarian cancer diagnosis (**Study I**). In 2020 a similar study could detect *TP53* mutations in brush-based Papanicolaou test slides up to 68 months before diagnosis [8].

In HGSC, *TP53* mutations are the ubiquitous initiating event that leads to genomic instability with high levels of copy number alterations and genomic rearrangements [51]. Copy number signatures are recurrent genomic patterns that are the imprints of mutagenic processes accumulated over the lifetime of a cancer cell [69, 70] and can be used not only as prognostic and predictive biomarkers but also in diagnostics [71]. In later years, a new genomic stratification based on copy number signatures predicting outcome has been proposed for high-grade serous carcinoma by Macintyre and colleagues [72].

In **Study II** we explored patterns of copy number alterations (CNAs) using low coverage whole genome sequencing in cervical samples taken at diagnosis and months to years before the women were diagnosed with cancer, with the goal of generating tumorigenic signatures for early detection of HGSC. This is a new approach trying to circumvent the emerging challenge of very-low frequency *TP53* mutations in healthy women/tissues.

Prognostic and predictive factors

The inherent complexity of multifactorial diseases such as cancer renders the process of patient prognosis and prediction of response to therapy extremely difficult. Clinically well-established prognostic factors for ovarian carcinoma are stage, age at diagnosis, performance status, residual disease after surgery and

histopathologic type [73, 74]. Despite many years of research there are still few prognostic and predictive markers that have translated into the clinic, and morphological classifications mainly steer prognostication and treatment decisions until date. The only reliable molecular markers predicting treatment response for HGSC are *BRCA1* and *BRCA2*, germline and somatic mutations, and homologous recombination deficiency (HRD), which predict response to PARP inhibitors.

Homologous recombination deficiency

As many as 50% of all HGSCs harbor Homologous Recombination Deficiency (HRD) [47]. Homologous recombination (HR) is the most accurate DNA repair mechanism and repairs double-strand breaks. Mutations in genes required for the HR system lead to extensive genomic damage. It is now accepted that identification of HR status via individual genetic mutations is limited, as not all genomic events leading to HRD have yet been defined. Instead, HR status can be determined by measurement of the genomic scars caused by HRD. The HRD score measures some of these scars or allelic imbalances (AI), namely loss of heterozygosity (LOH), telomeric allelic imbalance (TAI) and large-scale state transitions (LST) [75].

The most widely used assay in clinical trials is the myChoice® HRD test by Myriad Genetics, which was developed in a cohort of *BRCA1/2*-mutated triple-negative breast cancers [76]. Recently, an investigative approach has presented an optimized algorithm to detect HRD in HGSC (ovaHRDScar). It measures the same AIs with improved accuracy for HGSC by optimizing AI size selection, which is distinct in HGSC [77].

Results from the PAOLA-1 study [78] led to the approval of Olaparib (PARP inhibitor) for patients with HRD at 1st line in 2020. This actualized the necessity of HR testing for clinical use. In Sweden and at this moment, the myChoice® HRD test is used in the clinical routine, but a new HRD test developed by molecular pathologists within Genomic Medicine Sweden and based on a gene panel (560 genes) has proven enough to determine HRD status and will shortly be implemented.

Molecular classifications of HGSC

HGSCs are morphologically heterogenous and morphologic patterns are insufficient for subclassification [79]. Thereupon a lot of effort has been invested in developing a molecular subclassification to be applied in the clinical setting as a prognostic and predictive marker. In 2008, Tothill *et al*. proposed the first molecular classification of serous and endometrioid ovarian carcinomas in six clusters based on mRNA expression data [80]. In 2011 The Cancer Genome Atlas (TCGA) Research Network expanded the efforts and defined four subgroups of HGSC defined as

"immunoreactive", "differentiated", "proliferative", and "mesenchymal" [47]. In the coming years validation studies confirmed the existence of these subtypes and differences in their prognosis [81, 82]. In 2017 a larger study including 2,000 patients from 14 public databases (among them patients from the Tothill and TCGA studies) performed small modifications to the earlier version [83]. In 2020, a large validation study applied previously described algorithms for molecular classification to NanoString gene expression data on >3,800 HGSCs from the OTTA (Ovarian Tumor Tissue Analysis) consortium with the aim of establishing a validated assay applicable in trials and the clinical setting, and thus enabling the evaluation of the predictive value of the molecular subclassification of HGSC [84]. The prognostic value of the "NanoString signature" was also demonstrated [85]. A study by Schwede *et al.* has questioned these studies and pointed out the effect of stroma admixture in the subtyping and proposed microdissection or single-cell analysis as possible better tools for clinically relevant classification [86].

With the technological development of whole genome sequencing (WGS), new molecular classifications inferred from structural variation (SV) patterns have been proposed. In 2017 Wang *et al.* presented a classification of ovarian carcinoma, where HGSC was subdivided in H-FBI (foldback inversion SV signature) and H-HRD (Homologous-recombination-deficient mutation signature) [87]. In 2018, Hillman *et al.* proposed WGS-based genomic rearrangement signatures associated with prognosis [88]. In 2019, Funnel *et al.* used advanced statistical models combining Single Nucleotide Variants mutation signatures and SV signatures to define the following subtypes: HRD-Dup (tumors with *BRCA1*-associated tandem duplications), HRD-Del (tumors with *BRCA2*-associated interstitial deletions), FBI (tumors with *CCNE1* amplification-associated foldback-inversions) and TD (tumors with *CDK12*-associated tandem duplications), with FBI and TD tumors exhibiting the worst prognosis [89]. In 2018 Macintyre *et al.* proposed a new classification based on copy number aberrations obtained through shallow WGS predicting relapse and survival. They proposed seven so-called copy number signatures that defined tumors by multiple features rather than dividing them into groups and concluded that HGSC genomes are shaped by multiple mutational processes that preclude simple subtyping [72]. In 2021, Vazquéz-García *et al*. proposed a further characterization of the WGS-based mutation signatures (Funnel *et al.*) by incorporating single-cell RNA sequencing of both cancer and immune cells from different anatomic sites. This study described intra-patient tumor microenvironment heterogeneity across tumor sites, and further defined the FBI mutational subtype as immunologically inert with worse prognosis, compared with the HRD tumors which showed intrinsic immunogenicity [90]. Most recently in 2022, Funnell *et al*. further investigated cell-to-cell copy number alterations in HGSC and showed a clonal dependent level of amplifications in known oncogenes associated with phenotypic variation, which adds complexity to the previously proposed classifications [91].

Taken together, molecular classifications of HGSC have proven challenging due to its extreme genomic complexity and high degree of heterogeneity. While these classifications convey prognostic information, whether they can predict response to therapy remains to be determined. Separating prognostic information from predictive information requires controlled clinical trials and can often only be inferred from observational cohort studies [25].

Investigative biomarker Claudin-4

Claudins belong to a multigene family, with ~24 members, and are considered one of the main tight junctions (TJ) forming proteins [92, 93]. A TJ acts as a cellular barrier and is involved in paracellular permeability and cell polarity [94, 95]. Since the discovery of claudins in 1998, many studies have aimed to characterize the claudin family, revealing that the specific combination and co-expression of different claudin species determines the TJ barrier characteristics in a tissue-specific manner [96, 97]. TJ structure and function are often altered in human carcinomas, where TJ loss can contribute to cancer progression [11]. For example, claudin-1 has been found to be downregulated in breast cancer, and claudin-2 is downregulated in breast and prostate cancer [98, 99]. In contrast, claudin-4 has been reported to be upregulated in pancreatic, colorectal, gastric, breast, prostate, and ovarian cancers [99-106]. Previous studies have reported increased expression of claudins 3 and 4 in ovarian cancer compared with normal ovarian surface epithelium and benign ovarian tumors [106-108]. Claudin-4 has been proposed as a possible diagnostic and prognostic biomarker in ovarian carcinoma [109, 110]. However, there are limited and contradictory data regarding the involvement of claudins in chemotherapy resistance [111-113].

Investigative immune cell markers and prognosis

Many ovarian carcinomas demonstrate tumor infiltrating lymphocytes (TILs) and the survival advantage of high numbers of TILs in HGSC has been shown in several studies [12, 114-116]. *In vitro* studies have described an immunosuppressive environment in HGSC induced by tumor associated macrophages (TAMs) [117, 118] and regulatory T lymphocytes [119, 120]. Moreover, gene expression analyses from the TCGA Research Network have identified the immunoreactive molecular subtype correlated with higher presence of TILs and with improved survival compared to the mesenchymal subtype [81]. Taken together these data suggest that a subset of HGSC may be immunogenic, and its microenvironment could have an impact on prognosis and response to immunotherapy. In Study IV we mapped the intraepithelial (intratumoral) expression of the T-cell marker **CD3**, which measures the total number of T cells (Figure 3).

Programmed Cell Death Protein 1 (**PD-1**) plays a vital role in inhibiting immune responses through, among other mechanism, modulating the activity of T-cells. Programmed Cell Death Ligand 1 (**PD-L1**) is a trans-membrane protein that is considered to be a co-inhibitory factor of the immune response and plays an important role in various malignancies where it can attenuate the host immune response to tumor cells (Figure 3) [121]. The prognostic value of PD-1 and PD-L1 in HGSC has been studied with ambiguous results [115, 122-124]. Mapping the expression of PD-1/PD-L1 and immune cells in HGSC is clinically relevant because in addition to its prognostic value, it may provide important information for further study of their potential to predict treatment response to immunotherapy.

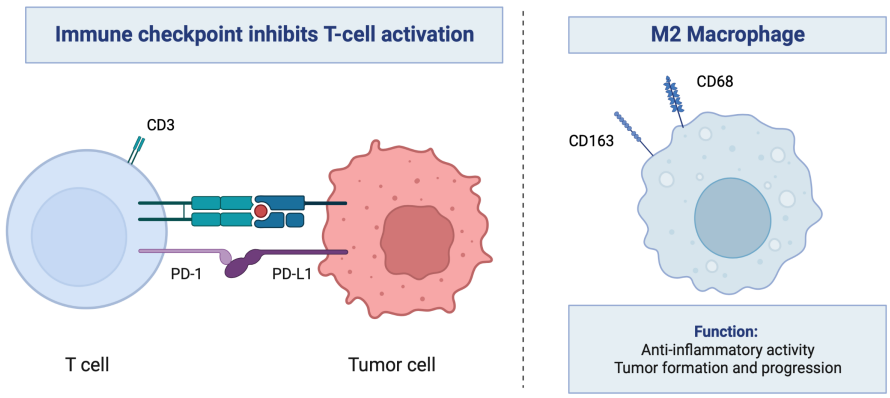


Figure 3. Simplified schematic representation of the immune cell markers investigated in Study IV.
T cell, lymphocyte. Created with BioRender.com.

Even the prognostic value of TAMs has been studied with ambiguous results. A meta-analysis including nine studies with almost 800 cases of ovarian carcinoma showed no association between the macrophage markers **CD68** and **CD163** and overall survival [125]. CD68 is a transmembrane protein highly expressed by cells of the monocyte lineage including all macrophages. CD163 is a marker for alternatively activated macrophages (M2) considered to promote tumor progression (Figure 3). In Study IV, we also explored the prognostic value of TAMs in a cohort of advanced HGSC.

Clinical practice

Symptoms and diagnosis

Although ovarian cancer has been termed 'the silent killer' there is compelling evidence that over 90% of women with ovarian cancer have symptoms before diagnosis [126]. Evidence from several retrospective case control studies point out that women with ovarian cancer are more likely to experience abdominal pain, abdominal distension, urinary symptoms, abdominal mass and/or abnormal bleeding before their diagnosis, than women without ovarian cancer [127]. However, these symptoms are unspecific and can be vague. The low prevalence of ovarian cancer in women presenting with symptoms results in low positive predictive values for the tumor marker CA125 and transvaginal ultrasound. However, and based on systematic reviews from studies in secondary care and screening studies, there is a consensus that women with these symptoms should be assessed with CA125 and transvaginal ultrasound [128]. However, early detection of "ovarian carcinoma" has proven to be difficult because of the physically inaccessible location of the annexes and the lack of specific symptoms in early disease.

In women with an adnexal mass, it is important to distinguish between benign and malignant pathology before surgical treatment, as ovarian cancer surgery requires tumor-specific gynecological surgical competence [129, 130]. According to a systematic review of diagnostic studies, the risk of malignancy index I (RMI I) proposed by Jacobs *et al*. in 1990 is currently the best index in terms of sensitivity and specificity [131]. It combines three pre-surgical features: serum CA125, menopausal status and ultrasound score [132]. Women with high RMI I score should be referred to a specialist multidisciplinary team. Further clinical assessments that should be performed in women with suspected ovarian cancer are CT of the chest and abdomen, complete tumor marker panel, cytologic analysis if ascites or pleural effusion present, biopsy for histopathological examination if spread disease and evaluation of performance status and surgical risk [128].

Surgery and staging

The overall goals of surgery are to confirm diagnosis, perform staging and to remove all macroscopic tumor tissue (radical debulking surgery). The updated, revised FIGO (International Federation of Gynecology and Obstetrics) staging system from 2014 combines the classification for ovarian, fallopian tube, and peritoneal cancer and it is based on findings made mainly through surgical exploration (Figure 4).

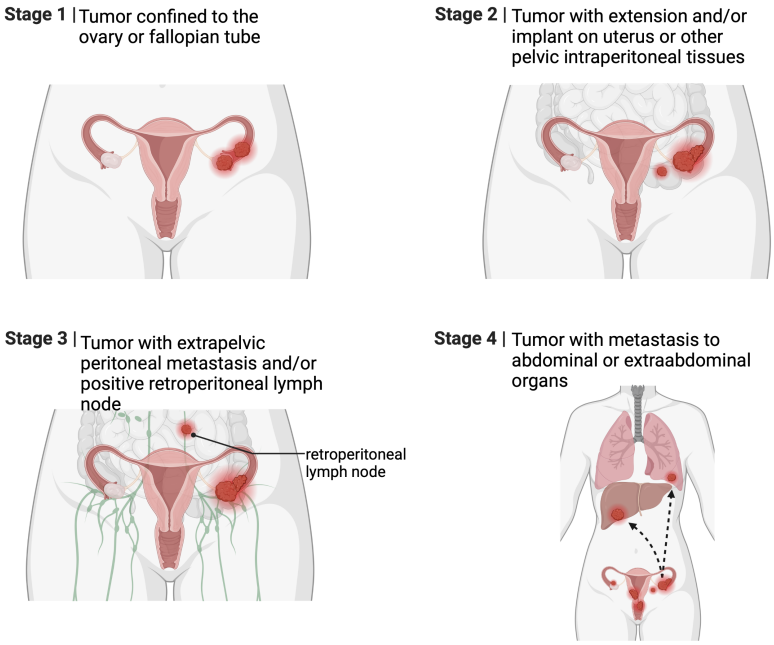


Figure 4. Simplified representation of stage classification for ovarian and fallopian tube carcinoma. Created with BioRender.com.

The surgical procedure depends on the tumor stage and in advanced stages may include intestinal resection, peritoneal stripping, diaphragmatic resection, removal of bulky para-aortic lymph nodes and splenectomy. In advanced ovarian adenocarcinoma complete cytoreduction of all macroscopically visible disease has been shown to be associated with increased overall survival (OS) in several retrospective observational studies [133-135]. Furthermore, a retrospective study has shown that women with stage IIIC and gross residual disease 1-10 mm had a survival benefit, compared to residual disease >10 mm [136], and justifies the surgical effort in stage IIIC if suboptimal debulking can be achieved [137]. The timing of surgical cytoreduction in relation to chemotherapy is still debated [135, 138] and the ongoing randomized controlled trial called “TRUST” (Trial on Radical Upfront Surgical Therapy) will hopefully answer this question.

Most commonly in Sweden, primary surgery is offered first if possible, based on the worse prognosis seen in patients receiving neoadjuvant chemotherapy in a Swedish population-based study [139]. Treatment decisions on whether primary surgery, interval debulking surgery (after neoadjuvant chemotherapy), or palliative chemotherapy is recommended should always been taken in a specialist multidisciplinary round.

Chemotherapy

Post-operative chemotherapy has the goal of eliminating remaining tumor cells, thus increasing the chance for cure or to delay disease relapse. Cisplatin and carboplatin are platinum-based compounds that form covalent bonds to DNA, thereby inducing DNA damage and subsequently apoptotic cell death. They were approved by the U.S. Food and Drug Administration (FDA) in the 1980´s and the second-generation platinum drug carboplatin with proven less nephrotoxicity is currently the pillar of ovarian carcinoma chemotherapy. In the 1990´s the taxane drug paclitaxel which inhibits cell division by disruption of microtubule function was introduced as clinical trials (GOG 111 and OV10) showed increased OS for cisplatin/paclitaxel compared to cisplatin/cyclophosphamide [140, 141].

Despite most ovarian carcinoma patients initially responding well to treatment, many patients suffer relapses with a median time to recurrence of less than two years [140]. The prognosis and probability of response to second-line therapy and subsequent lines depends in great part on the progression-free interval after the last dose of the preceding line of chemotherapy [129]. A relapse >6 months from the end of a platinum-based chemotherapy is considered as a platinum sensitive relapse and platinum-based chemotherapy should be reintroduced if possible.

Targeted treatments

Anti-angiogenic treatment with the vascular endothelial growth factor inhibitor bevacizumab has proven effective in advance stage ovarian carcinoma. The approval was based on two studies showing an increase in the median PFS of 4-8 months for patients non-radically operated [142, 143]. Year 2015, the ICON7 trial confirmed a five-months OS benefit in the same patient group [144] . In Sweden, bevacizumab is added to adjuvant chemotherapy for patients non-radically operated since 2014.

The recent introduction of PARP inhibitors into the clinical practice is transforming survival prospects for women with advanced HGSC [145, 146]. In Sweden, and based on several clinical trials, PARP inhibitors are currently indicated as maintenance treatment for advanced-stage HGSC patients with a *BRCA* mutation or HRD, who had at least partial response to 1st line platinum-based chemotherapy [78, 147-149]. In the relapse setting, PARP inhibitors can be used as maintenance after at least partial response to platinum-based chemotherapy, regardless of *BRCA* mutational status or HRD score [146, 150-152].

Aims

The aims of this thesis were to:

1. Explore the possibility of developing a DNA-based diagnostic test for HGSC through examination of cervical samples, taken at the time of diagnosis and pre-symptomatically from women who later developed HGSC.
2. Study prognosis and treatment prediction of the marker Claudin-4 in ovarian carcinoma.
3. Investigate immune cell infiltration and the prognostic impact of immune cell markers in advanced HGSC.

Methods

Patient cohorts

The MaNiLa project

Between 2015 and 2017, 187 women with suspected ovarian tumor or adnexal mass, as well as 12 *BRCA* mutation carriers who underwent prophylactic surgery, were recruited within the MaNiLa project. A total of 79 women were diagnosed with HGSC. Both liquid-based cervical samples and endometrial biopsies were collected (ethical approval Dnr 2014/717). We had access to blood, plasma, and fresh frozen tumor through the biobank for gynecological conditions in the southern Swedish healthcare region (GUNNEL biobank, RBC Syd, ethical approval Dnr 2013/735).

Since the project came to focus on pre-symptomatic cervical samples, among the 79 patients with HGSC, we initially found nine patients whose liquid-based cervical samples had previously been collected within the cervical cancer screening program at the region of Malmö (Department of Medical Microbiology, Lund, Sweden). Moreover, six patients with archival liquid-based cervical samples were identified among patients belonging to our HGSC tissue microarray (TMA) cohort, that will be presented in the next section. Instead of fresh frozen tumor tissue and blood, this cohort had only formalin fixed paraffin embedded (FFPE) tumor tissue available. Thus, a total of 15 patients were included in **Study I** (Figure 5).

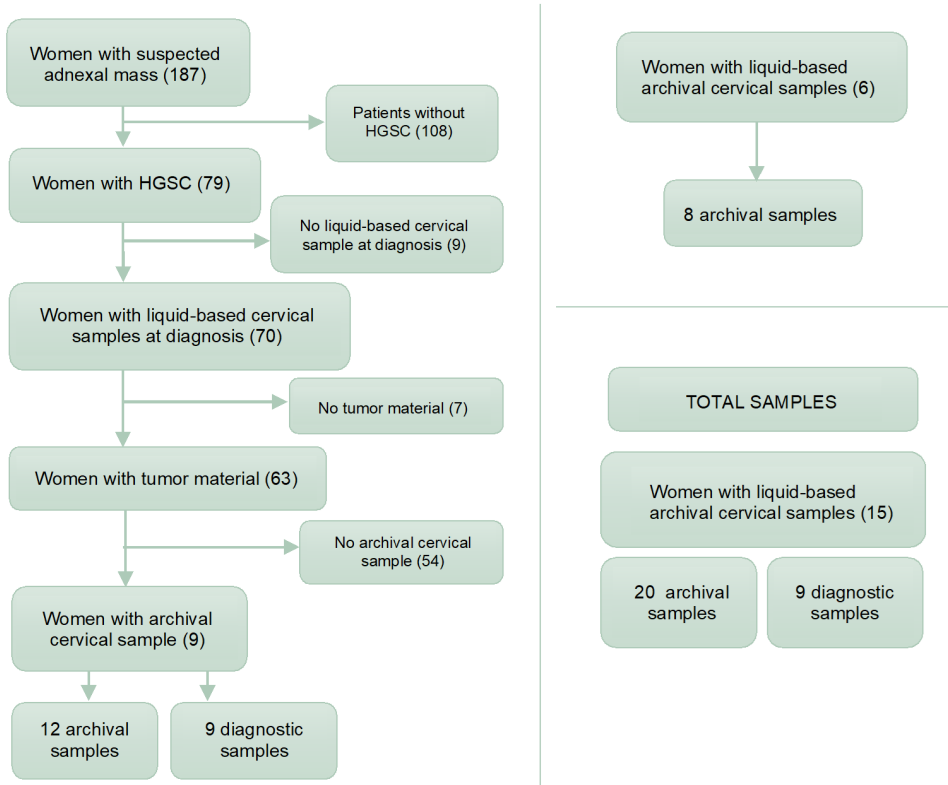


Figure 5. Flowchart of patients included in Study I. MaNiLa patients (left), HGSC tissue microarray patients (top right) and the total number of samples (bottom right). Adapted from Arildsen *et al*., 2019.

In a second phase of the MaNiLa project, we further identified 38 MaNiLa patients with HGSC who had ThinPrep® (Hologic) cervical cytology sample slides (ThinPrep slides, n=90) archived at different Pathology Departments in the southern Swedish healthcare region. Of those patients, we selected 11 patients based on the presence of STICs, as we planned to analyze those. Furthermore, we have retrospectively identified 39 patients diagnosed with HGSC in Lund between 2018-20, whose liquid based ThinPrep® cervical cytology samples (liquid ThinPrep samples) have been collected in a biobank at the Pathology Department in Lund since late 2017. Of those patients we chose seven randomly (three of them with STIC lesions) and included them in Study II (total HGSC n=18). Even four *BRCA* mutation carriers from the MaNiLa cohort were included. Fimbriae epithelium, and in one case a STIC, from these patients were analyzed. The remaining seven patients included Study II were controls from the MaNiLa cohort with benign gynecological conditions (borderline tumors excluded). Table 4 shows the patients selected for **Study II** and different tissues and cervical cytology types analyzed.

Table 4. Patients and samples included in Study II

	MaNiLa cohort 2015-17			Lund cohort 2018-20
	HGSC (n=11)	*BRCA* (n=4)	Benign gynecological conditions (n=9)	HGSC (n=7)
ff tumor	x	x	x	x
blood	x	x	x	x
endometrial biopsy	x	x	x	x
plasma	x	x	x	x
STIC (FFPE)	x (n=7)	x (n=1)		x (n= 2)
MaNiLa cervical sample*	x	x	x	
ThinPrep slide*	x	x	x	
Archival ThinPrep slide	x			
Archival liquid ThinPrep sample				x
Archival liquid SurePath sample		x (1 pat, 2 samples)		

*Samples taken at surgery. Ff, fresh frozen. STIC, serous tubal intraepithelial carcinoma. FFPE, formalin fixed paraffin embedded.

The cervical samples

The archival liquid-based cervical samples included in Study I were all preserved using the SurePath® solution (Becton Dickson, Stockholm, Sweden).

In Study II most of the MaNiLa patients had a diagnostic liquid-based cervical sample and a ThinPrep slide, both taken at surgery, plus one or several archival ThinPrep slides. Among the Lund cohort from 2018-20, no patient had diagnostic samples, but all had one or more archival liquid ThinPrep samples.

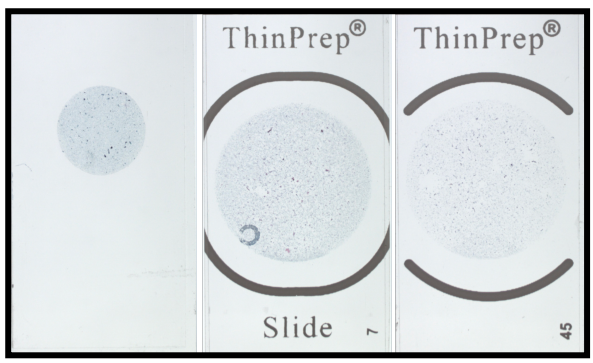


Figure 6. Different types of ThinPrep cervical cytology sample slides included in Study II.
In order: Older “small” ThinPrep slide (coverslip made of glass). ThinPrep slide with coverslip made of glass. ThinPrep slide auto-mounted with film.

The prospectively collected MaNiLa cervical samples have been preserved in DNAgard® (DNA guard solution, Sigma Stockholm, Sweden). Almost all the other cervical samples have been preserved in ThinPrep solution (Hologic Inc., Sollentuna, Sweden) which contains methanol, with the exception of two samples preserved in SurePath solution (Table 4). To further produce slides the ThinPrep samples have undergone an automated process (ThinPrep5000 Processor) which includes filtration, ethanol fixation, IHC, and film mounting. In this process blood and mucus are rinsed away and cervical epithelium is well dissociated and evenly distributed within the circle on the slide (Figure 6). Archival liquid ThinPrep samples (Lund cohort 2018-20) were extracted from the bottom of the ThinPrep jar after 30 minutes of sedimentation.

The ovarian carcinoma TMA

Between 1998 and 2000 128 women with ovarian carcinoma were consecutively recruited in the southern Swedish healthcare region. In addition, 18 patients were recruited at the oncogenetic counseling at Lund University Hospital (Sweden) between 1981 and 1997. Six patients with unknown primary tumor or missing follow-up information were excluded. Thus, a cohort of 140 ovarian carcinoma patients was assessed for CLDN4 protein expression in **Study III**.

The HGSC TMA

Between 2011 and 2015 a total of 156 consecutive cases of HGSC were collected in the southern Swedish healthcare region. After review by a gynecologic pathologist 140 cases remained and, of those, 130 with stage III and IV were included in **Study IV** (Figure 7).

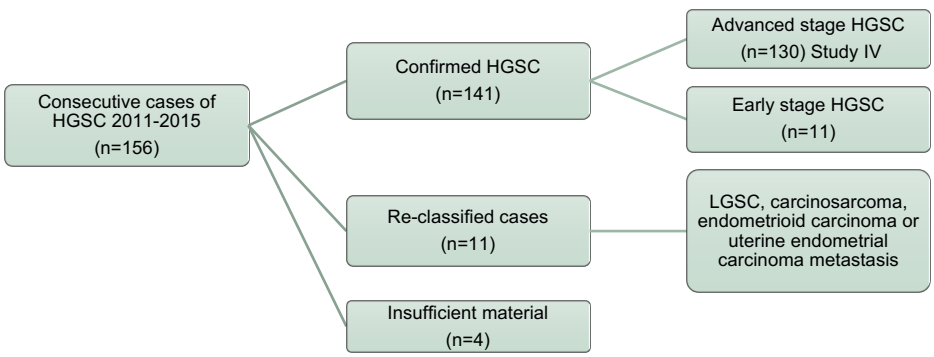


Figure 7. The HGSC TMA Construction and patient inclusion chart for Study IV.

In Silico validation cohorts

Gene expression (GEX) analyses are performed on mRNA. The amount of public gene expression databases, that allow researchers further analysis and interpretation, is constantly growing. This is the result of the decreasing cost of sequencing and that many journals encourage uploading of data to public databases.

In **Study III** and **IV** we used gene expression datasets from an online tool [153] and online repository [80], respectively. This online repository is called Gene Expression Omnibus (GEO) and is managed by the National Center for Biotechnology Information (NCBI). Within the GEO database, we used the GEX dataset (GEO - GSE9891) with GEX data derived from microarrays and contains 285 ovarian carcinoma samples [80]. Of those 285 cases, 203 patients with HGSC were selected for our validation.

Tissue Microarray (TMA) and Immunohistochemistry

This technique developed by Kononen and colleagues [154], allows the evaluation of e.g. protein expression of many tissue samples on a single slide. Another advantage is evaluating multiple markers with minimal differences in staining conditions. Two TMAs were used in **Study III** (ovarian carcinoma) resp. **IV** (HGSC). The construction technique was similar, with identification of viable tumor areas on Hematoxylin-Eosin-stained sections, retrieval of core biopsies from FFPE tumor tissue blocks and collection in a recipient block of paraffin. The main technical differences are the number of punches (3-12 in **Study III** resp. 6-8 from multiple sites in **Study IV**) and the diameter of the punches (0.6 mm in **Study III** resp. 1 mm in **Study IV**). The constructions of the TMAs are outlined by Malander *et al* [155] and in the section "the HGSC TMA" above.

One potential limitation is the correct representation of each tumor with the small tissue core, due to tumor heterogeneity. However a validation study, comparing immunostaining of Ki-67, estrogen receptor and p53 in whole sections and 1 mm cores, showed that the probability of 3 cores to correctly represent all three markers was 98%, demonstrating TMA is a reliable technique in ovarian carcinoma [156].

Immunohistochemical stainings exploit the principle of antibodies binding specifically to antigens in biological tissues and are widely used for tumor diagnosis and in basic research, to understand the distribution and localization of biomarkers. The antigen is often a protein located in one or more compartments of the cells, e.g. the membrane, the cytoplasm or the nucleus. The primary antibody is usually of IgG class and can be either monoclonal or polyclonal. Monoclonal antibodies are derived from one clone of plasma cells and react only with one epitope on the antigen, making them specific for this epitope, whereas polyclonal antibodies can

bind to several epitopes, which confers higher sensitivity [157]. Monoclonal antibodies are the most used due to their homogeneity, monospecificity and consistency. Polyclonal antibodies are produced by several B cell clones from one or several animals, whereas monoclonal antibodies are generated from so called hybridomas, where antibody producing B-cells are harvested from a mouse (or other host) and fused with immortal cancer B-cells (myeloma) to produce an hybrid antibody-producing cell line [157]. All primary antibodies used in paper III and IV were well validated, monoclonal antibodies. A summary of the primary antibodies and immunohistochemistry procedures is shown in Table 5.

Table 5. **Summary of antibodies and immunohistochemistry procedures in Study III and IV**

Antigen	Clone	Cat.No.	Supplier	Dilution	Platform	Ag retrieval (pH)	Antibody incubation (min/ºC or RT)
CLDN-4		32-9400	Invitrogen Corp	1:100	Dako Autostainer	DT 1699 (6)	30/RT
PD-L1	22C3	M3653	Agilent Dako	1:50	Dako Autostainer	DT 1699 (6)	30/RT
PD-1	NAT105	315M	Cell Marque (Sigma)	1:100	Dako Autostainer	DT 1699 (6)	30/RT
CD68	PG-M1	M0876	Agilent Dako	1:100	Dako Autostainer	DT 2367 (9)	30/RT
CD163	MRQ-26	760-4437	Ventana	RTU	Ventana Benchmark Ultra	CC1 (8.5)	32/36
CD3	Poly	A0452	Agilent Dako	1:200	Dako Autostainer	DT 2367 (9)	30/RT
Large sections							
PD-L1	22C3	M365529	Agilent Dako	1:40	Ventana Benchmark Ultra	CC1 (8.5)	64/36
PD-1	NAT105	Ab52587	Abcam	1:50	Ventana Benchmark Ultra	CC1 (8.5)	32/36
PD-1	NAT105	315M	Cell Marque (Sigma)	1:100	Dako Autostainer	DT 1699 (6)	30/RT
CD68	PG-M1	M0786	Dako	1:100	Ventana Benchmark Ultra	CC1 (8.5)	32/36
CD3	2GV6	760-4341	Ventana	RTU	Ventana Benchmark Ultra	CC1 (8.5)	32/36

CLDN-4= claudin-4, RT= room temperature, RTU= ready to use, DT 1699 or 2367= Dako Target retrieval solution 1699 or 2367, CC1= cell conditioning 1. Of note, tissue microarray and whole section immunostainings were performed on the Dako Autostainer and Ventana system, respectively, which did not seem to affect the performance of the antibodies. PD-1 immunostaining on whole sections was discordant on the Ventana system, and therefore, we performed the comparison on the same platform as the tissue microarray, the Dako Autostainer. Adapted from Martin de la Fuente *et al*., 2020.

Secondary antibodies are required for the so-called indirect detection and confer increased sensitivity due to the signal amplification from multiple secondary antibodies binding to a single primary antibody. The secondary antibody is

conjugated to a peroxidase enzyme, which reacts with a substrate (e.g. 3,3′-diaminobenzidine, DAB) producing a color-producing reaction that enables the visualization of the protein in brown color. The nuclear hematoxylin staining is often added. The blue color of the nuclei facilitates the identification of unstained cells.

Both fresh frozen tissue and FFPE blocks can be used for IHC staining but require different preparations to visualize the proteins. In FFPE tissues, used in the studies of this thesis, deparaffination, rehydration and heating of the tissues are required to retrieve the epitopes of the proteins, before the primary antibody is added.

When evaluating IHC expression it is important to have good knowledge about the studied antigen, e.g., expression in different cell compartments and tissues, as well as in normal and tumor cells. Possible pitfalls of this technique include, among others, variable staining quality depending on tissue fixation, deficient antibody performance and inter-observer variability. Furthermore, cut-off levels for high and low expression often differ in different studies which complicates comparison of results [158]. Thus, standardization of the method and immunostain interpretation are fundamental when implementing a new biomarker into the clinical practice.

In **Study III**, Claudin-4 staining was evaluated based on pattern and fraction of stained cancer cells. We found some cytoplasmic staining, but the membranous staining was more distinct and predominant. The two staining patterns were punctate and partial around the membrane or complete membrane staining (Figure 8A and B). Normal colon epithelium was the positive control, whereas ovarian stroma and follicle cells, smooth muscle and adipocytes were negative controls. We also stained whole sections with benign ovary (Figure 8C), fallopian tube, and endometrium, as well as 3 cases of serous cystadenomas.

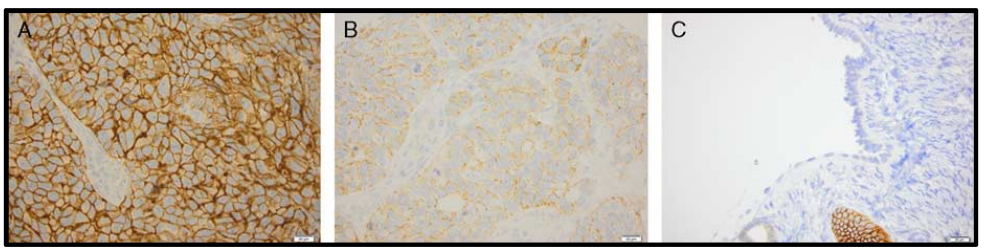


Figure 8. Immunohistochemical evaluation of claudin-4. High (**A**) versus low expression (**B**) in serous ovarian carcinoma. Negative ovarian mesothelium and positive cortical inclusion cyst (**C**). Magnification x40. Adapted from Martin de la Fuente *et al*., 2018.

In **Study IV**, we used 5 single IHC stainings (PD-L1, PD-1, CD3, CD68 and CD163) on 5 consecutive sections. We did so to achieve a "multiplex effect". At the same time, we wanted to be able to visualize each IHC marker alone, as we worked with these antibodies for the first time, and didn't want to jeopardize the evaluation

of co-localized biomarkers [159] (i.e. PD-L1 and CD68 in Figure 9, and PD-1 and CD3, were co-expressed in macrophage and lymphocyte cell membranes, respectively). We scored lymphocytes and macrophages located within the tumor epithelium, excluding areas with stroma, acute inflammation, and necrosis. Since the presence of stroma among different patients was inconsistent and necrosis was randomly present throughout the TMA cores, we decided to exclude these areas when scoring. We visually estimated the fraction of PD-L1, PD-1, CD3, CD68 and CD163 stained cells of the total cell amount in each core.

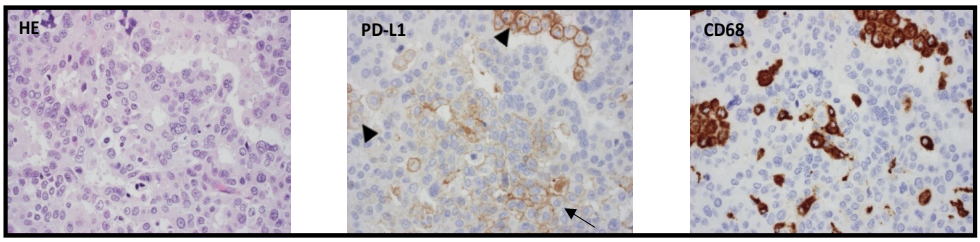


Figure 9. Pictures of corresponding tumor areas on consecutive tissue microarray sections. The macrophage marker CD68 facilitated the distinction between macrophages and cancer cells when evaluating PD-L1 expression. Arrowheads show PD-L1 expression in macrophages and arrow PD-L1 expression by cancer cells. Magnification x40. Adapted from Martin de la Fuente *et al*., 2020.

Even if TMAs have been proven a reliable technique regarding tumor markers in several studies and cancer types [156], less is known about the representability of immune cell markers in TMAs. Uneven distribution of the inflammatory cells within a tumor raises the question regarding the adequacy or inferiority of TMAs. The HGSC TMA was constructed choosing areas of viable tumor, and not areas with immune cell infiltration. Therefore, we performed large sections of the immune markers PD-L1, PD-1 and CD3 in a total of 17 pairs of cores and whole tissue sections (13 cases, 4 of them with both pairs of primary tumor and omental location) to study heterogeneity and representability of immune cell markers in our TMA (Table 5).

Statistical analysis

Two-group comparisons

When testing the null hypothesis, assumptions of the distribution of a variable in the population should be considered, as this will affect the choice of calculation test. Nonparametric tests are more usually used as no normal distribution must be assumed. In **Study III** and **IV**, the nonparametric test Pearsons Chi2-test and Mann-Whitney U-test were used, for categorical and continuous variables, respectively.

However, parametric tests should be used when possible, since these tests are more powerful. This means, they require less difference between groups and/or smaller sample size to reject the null hypothesis. In **Study IV**, the parametric t-test for the continuous variable age was used.

The *P*-value

The assumption of no difference between groups is called the null hypothesis. The probability value (*P*-value) represents the odds of finding a difference between the groups when the null hypothesis is true. A *P*-value of 0.05 is commonly used as an upper limit for statistical significance. It means that there is less than 5% probability that the null hypothesis is correct and the alternative hypothesis, namely difference between groups, is accepted. However, the magnitude or relevance of the difference cannot be interpreted from the *P*-value alone, e.g. a very small difference between two large groups can be statistically significant but may not be clinically meaningful [160].

Survival analysis

In **Study III** and **IV**, the cases were dichotomized into groups of high and low expression. There are strong arguments against dichotomizing continuous and ordinal variables for the purpose of clinical prediction. Some of these arguments are loss of information, reduced power and bias introduced by data-derived cut-offs [161]. However, in the clinical setting, a dichotomization is often needed, as a physician must know whether a result is negative or positive, to make a treatment decision.

In **Study III**, most of the cases/cores presented Claudin-4 expression and a combined fraction and pattern (partial vs. complete membrane staining) scoring was the most appropriate. Cut-off for low or high expression was identified using Kaplan-Meier plots. In **Study IV**, the situation was the opposite, as many cases/cores did not express or had very low PD-1/PD-L1 and immune cell marker expression. Here the cut-off was chosen by identifying a smaller group with higher expression.

The Kaplan-Meier (KM) method allows for analysis of time to event for patients with different start dates by setting the date of diagnosis, to day zero. Further, the KM estimate uses the exact failure and censoring time and considers the number of individuals at risk for an event, and if you lose individuals to follow up, this does not affect the estimate of survival probability [162]. The statistical relevance of the difference between groups was tested using the log-rank test, which is a nonparametric test used in survival analysis when data (events) do not follow the normal distribution.

Cox regression is the regression model most often used when working with survival data. Cox regression is interpreted using hazard ratios (HR), and with the Cox proportional hazards model, the HR estimates can be controlled for other covariates (multivariable Cox regression), making it possible to identify confounders [162]. Thus, Cox regression works on the assumption of proportional hazards, which means that the ratio of the hazards for any two individuals is constant over time. Non-proportionality can be suspected if the survival curves cross.

In **Study III** and **IV**, multivariable Cox regression was used to adjust for factors known to affect PFS and OS. Namely age, *BRCA* mutational status, stage and subtype in **Study III**, and age, stage and residual tumor after surgery in **Study IV**. Such corrections are important when assessing the strength of a potential prognostic variable. Thus, the lack of this information, e.g. residual tumor in **Study III** or *BRCA* mutational status in **Study IV**, needs to be considered when interpreting the results. All calculations and analyses were conducted using Statistical Package for the Social Sciences, Windows version 25 (IBM).

PFS and OS as end points

The prognostic value of the studied markers was investigated using Progression Free Survival (PFS) and/or Overall Survival (OS) as end points. PFS was defined as the time interval between date of diagnosis and the date of disease recurrence (pathology report or radiology) or death, whichever occurred first. Both were censored at 5 years due to the expected survival for this disease. Some patients were censored as follow-up time had not reached up to 5 years at the time of record. Many censored patients could potentially lead to misinterpretation of the KM curves. To avoid this, KM graphs were combined with a table over the number of individuals at risk at certain time points (**Study IV**).

OS is easily collected through registers and more reliable than PFS, which depends on patient and diagnostic related factors. A potential disadvantage of OS as endpoint is that it does not consider the cause of death. According to meticulous review of patients' medical charts, only 2/83 and 2/85 of the patients who died within 5 years after diagnosis in **Study III** and **IV**, respectively, died of causes not related to HGSC, making OS a suitable endpoint.

Droplet Digital Polymerase Chain Reaction (ddPCR)

The PCR method invented in 1983 by Kary Mullis [163] is the basis for multiple genetic analysis methods used today in several fields as clinical practice, molecular biology research, genetic engineering and forensic approaches. The method uses a small amount of DNA, called template, and amplifies it exponentially through DNA

synthesis. The reaction components include template DNA, a heat resistant DNA polymerase, primers, free nucleotides, and a thermal cycler. Primers are short synthetic single-stranded DNA fragments that dictate which specific segment of the template will be amplified and are required for the DNA polymerase to bind to the template and start the DNA base incorporation. The original template is amplified in exponential fashion by repeated reaction cycles. The newly synthesized DNA in each cycle is used as a template in the next cycle, thus doubling the speed of DNA synthesis per reaction cycle. The three steps in a reaction cycle are denaturation, annealing and extension, and shifting between them is regulated by changes in temperature (thermal cycler) [164]. Detection of the PCR products can be done with fluorescence dyes and agarose gel electrophoresis, but the introduction of sequence-specific fluorescent probes has significantly improved the specificity of the technology [165].

Based on regular end point PCR, quantitative PCR (qPCR) adds the measurement of the fluorescence signal (PCR products) at the end of each cycle, allowing for relative quantification. In qPCR absolute quantification requires standard curves and reference samples with known DNA amounts [166]. Digital PCR (dPCR) allows calculations of absolute concentrations with the partitioning of the PCR sample into multiple wells and subsequent use of a Poisson distribution. The technology got its name "digital" because of the discrete and binary nature of its results, as each compartment is scored as 1 (positive) or 0 (negative) [167].

The improvement of the dPCR assay led to the introduction of Droplet dPCR (ddPCR). Whereas dPCR requires the partition of the sample into multiple wells, in ddPCR the sample is spliced into multiple droplets. A PCR reaction occurs in each droplet and is analyzed separately, which confers a higher sensitivity to this assay [168]. A schematic representation of ddPCR principles is shown in Figure 10.

PCR is commonly used to enrich low DNA amounts when building sequencing libraries. Sequencing library enrichment by PCR was performed in **Study I** and **II**. Another common application is the detection of tumor-derived mutant sequences present in very small amounts in liquid biopsies. In **Study I**, we analyzed both diagnostic and archival cervical samples using an ultra-sensitive ddPCR-based method, IBSAFE (SAGA Diagnostics, Lund, Sweden) with custom-made primers. We had enough input DNA to analyze three diagnostic samples with conventional predesigned BioRad ddPCR primers also (BioRad, Solna, Sweden).

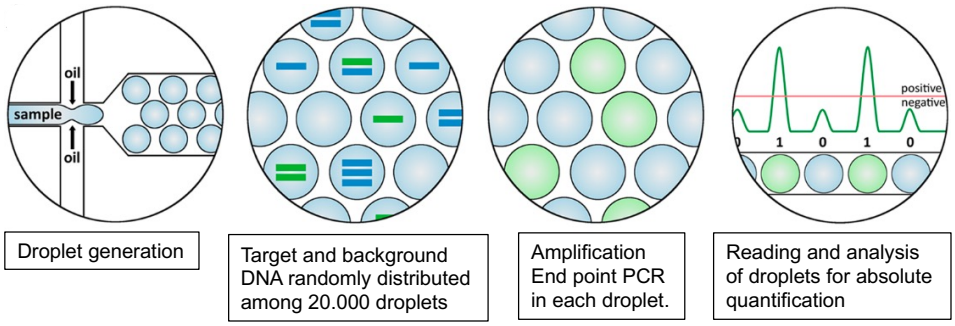


Figure 10. Principles of ddPCR. Figure adapted from Verheu *et al*., 2016 [169].

A constraint of ddPCR is that a limited number of mutant alleles can be targeted in each reaction. In our approach with the BioRad-based ddPCR assay, choosing primers for the 10 most common *TP53* mutations in HGSC (according to COSMIC), only covered 15-20% of all *TP53* mutations in HGSC. In **Study I**, *TP53* mutations were first identified by targeted NGS of the tumors and thus appropriate primers could be selected or designed for the Bio-Rad and IBSAFE assays, respectively. In the IBSAFE technology the sensitivity has been enhanced through customized primer design and modified thermocycling to reduce the negative consequence of polymerase errors. The primers are designed with asymmetrical biophysical attributes so that only correctly copied PCR product (in the 1st cycle) is enriched during a "linear" phase of 64 cycles, followed by an "exponential" phase of 27 cycles when probes bind. IBSAFE has a greatly increased signal to noise ratio, allowing for an increased specificity for accurate estimation of mutant allele frequencies (MAF) at exceedingly low concentrations (~0.001% MAF), whereas the Bio-Rad ddPCR assay suffers from false-positive noise below 0.1% MAF.

Another limitation of PCR is that the sensitivity is directly linked to the amount of DNA. The very low amount of DNA in many of the archival samples (11/20 samples <20 ng) was a limitation in **Study I**. For example, in a sample with only 20 ng DNA, corresponding to 5,600 DNA molecules, IBSAFE can never achieve detection of 1 mutant allele/molecule in 100,000 wildtype alleles, as sensitivity can never be higher than the number of DNA molecules analyzed.

High throughput methods

The first-generation sequencing by synthesis technology was developed by Sanger et al. in 1977 and is thus called Sanger sequencing [170]. In the mid-nineties new sequencing by synthesis technologies emerged. They got many common names as next generation sequencing (NGS), second-generation sequencing, massively parallel sequencing, and high-throughput sequencing. The common principles of all

sequencing by synthesis technologies are a DNA polymerase that adds fluorescent nucleotides one by one onto a growing DNA template strand, where each incorporated nucleotide is identified by its fluorescent tag. The basic sequencing process involves fragmenting DNA/RNA into multiple pieces, adding adapters, amplification by PCR, sequencing libraries, and reassembling them to form a genomic or transcriptomic sequence. All sequencing processes can be divided in the hardware step, where the sequencing machines generate the initial genomic data and the software step, where bioinformatics enable data processing and analysis.

The critical difference between Sanger sequencing and NGS is sequencing volume. NGS has revolutionized genomic and transcriptomic research thanks to the ability of sequencing of millions of fragments simultaneously per run, that allows sequencing of hundreds to thousands of genes or transcripts at one time. Furthermore, NGS enables powerful analysis of epigenomes and protein-DNA interactions as for example small RNA sequencing or sequencing aimed at identifying histone modifications, DNA-methylation, or chromatin accessibility. As high-throughput methods generate large amounts of data, analysis of the obtained data requires high-performance computing and interpretation is still a major constriction. And not least, a good study design predefines the downstream analyses and will lead to relevant associations between the data and biology.

Under current development are the third-generation or long-read sequencing, with the advantage of producing longer reads that alleviate the computational challenges of genome assembly or transcript reconstruction. However, these technologies are more error prone which complicates the analysis of the resulting data.

Targeted Next Generation Sequencing

Targeted sequencing introduces an enrichment step where specific genes or gene regions are selectively amplified by a PCR reaction to study them with more resolution. Targeted gene sequencing produces smaller data sets, compared to whole exome and genome sequencing, making computational analysis easier.

In **Study I** we used targeted NGS to analyze tumor DNA from either fresh frozen tumors or FFPE tumors and paired blood samples. In the INVIEW ONCOPANEL ALL-IN-ONE by GATC Biotech (Germany) genomic aberrations in 600 genes frequently mutated in cancer can be detected, with a sensitivity level of 1% allele frequency, which means it can detect mutations present in one among 100 normal alleles. Choosing this targeted panel was more than sufficient, as we in fact were primarily interested in sequencing *TP53* only.

Whole genome sequencing

In contrast to whole exome sequencing which aims at sequencing the protein-coding regions of the genome (21,000 genes) that constitute 1.1% of the total genome, whole genome sequencing (WGS) aims at covering even introns in the genome: up to 3,000,000,000 base pairs (6 billion letters!). WGS sequencing can detect single nucleotide variants, insertions/deletions, copy number changes, and large structural variants.

Coverage refers to the number of times the sequencing machine will sequence a genome and is an important concept when understanding the different applications. Given that the genome has 3 billion bp, even an ultra-accurate sequencing machine with only 0.01% error rate, will generate 600,000 errors. To reduce error rate the sequencing machine must read a genome many times. For example, when the entire genome is sequenced 30 times (30x WGS) the data obtained is considered accurate enough for health-related purposes (clinical-grade WGS). The most common approach to circumvent the costly WGS is genotyping arrays or targeted/panel sequencing that can obtain genotypes for fixed panels of common single-nucleotide polymorphisms (SNPs).

Shallow or low-coverage WGS (LG-WGS) have become popular approaches in research. A typical LG-WGS (0.1-1X) will cover the entire genome less than once. Well-established applications of LG-WGS are discovery or detection of copy number variations (CNVs), that are gains or losses of large segments of genomic DNA.

The human reference genome

The Human Genome Project launched by the Genome Reference Consortium (GRC) in 1990 is an international research project with the goal of determining the base pairs that make up the human DNA. The original human reference genome was derived from thirteen anonymous volunteers (Buffalo, New York) and the first build, completed in 2003, included only about 85% of the genome [171]. The most recent assembly is the so called GRCh38 from 2009 [172] that has been later patched and improved [173, 174]. Between 2020 to 2022, and thanks to third-generation sequencing technology, all the gaps have been filled, including telomeres, ribosomal DNA and the Y chromosome [175]. The human reference genome is routinely used to reassemble sequencing reads and fill the genomic gaps through statistical imputation in high throughput sequencing (e.g. targeted NGS, genotyping arrays, LG-WGS). Thus, the human reference genome is assembled from a limited number of individuals and its utility comes from the fact that most of the human genome is the same in all humans. However, it lacks information about the variations in genomes across the human population. The 1000 Genomes Project Consortium collected more than 2,600 samples from 26 populations between 2008 and 2013,

and is creating a database of human genomic variation which in turn can be used for association studies relating genetic variation to disease [176, 177].

In **Study I**, the Genome Reference Consortium Human Built 37 (GRCh37, also called hg19) was used. In **Study II**, the sequenced reads were first aligned to the GRCh37 and for copy number analysis with QDNA a new control set with 58 human genomes was obtained from the 1000 Genomes Project Phase 3.

DNA extraction and library preparation

For ThinPrep® cervical cytology slides the covering film or glass was first demounted with xylene or acetone, respectively [178]. DNA from fresh-frozen/FFPE tissues, blood, plasma, endometrial biopsies, liquid-based cervical samples, and cervical cytology slides was extracted by column or magnetic bead-based methods based on the sample type and performance (the different kits used are detailed in **Study I** and **II**). DNA was measured by Qubit fluorometric assays and quality checked (ThermoFisher). Even if DNA quality from the slides was poorer compared to the liquid-based cervical samples [179, 180], the results of the library preparation and sequencing were satisfactory.

In **Study II**, 1-10 ng DNA/sample was used to prepare NGS libraries. Genomic DNA was first fragmented. In **Study II** DNA was fragmented to a median length of 350 bp. The fragmented ends were then repaired. The 5′end was phosphorylated and the addition of adenosine residues at the 3′end facilitates adapter ligation. Adapters are oligonucleotides that serve as a connection for the DNA strands to attach to the flow cell (Figure 11) during the sequencing reaction and contain "barcodes" that allow for pooling of multiple samples into the same sequencing reaction. Finally, the DNA libraries were amplified with qPCR. In **Study II**, 6-12 cycles were used.

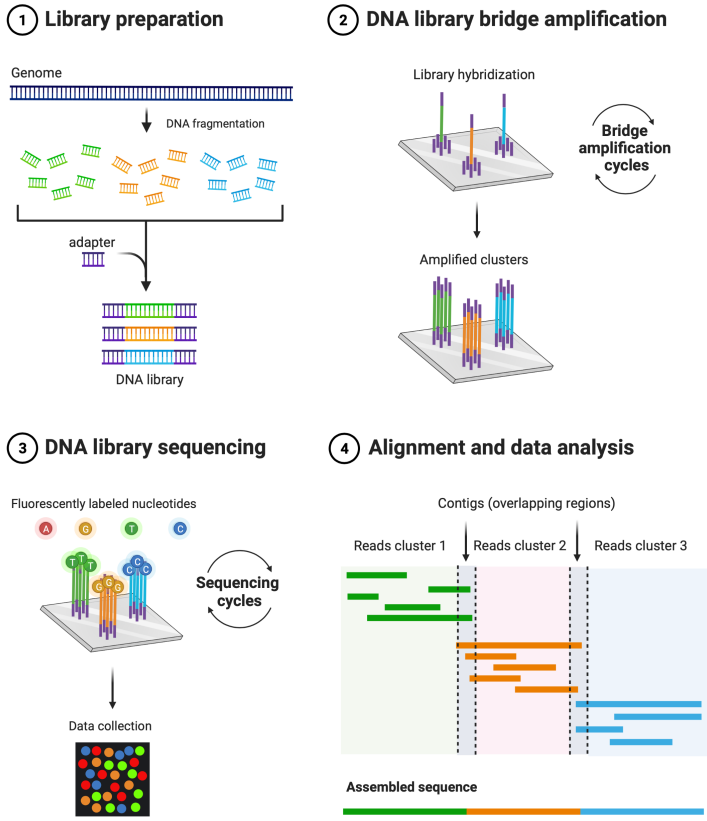


Figure 11. Sequencing by Illumina. Created with BioRender.com

The prepared libraries were loaded onto a flow cell, wherein primers complementary to the library adaptors are attached to the surface. Steps of clonal amplification and cluster generation are shown in Figure 11. Clusters of clonal DNA strands were then sequenced by adding fluorescently labelled reversible terminator nucleotides in cycles. For every cycle, the clusters are visualized as a spot of color corresponding to the last added base [181]. In **Study II** LG-WGS (0.4-2.2X) was performed by NovaSeq 6000 System (Illumina).

WGS data analysis

The raw files generated from WGS were demultiplexed using Illumina's bcl2fastq software to generate FASTQ files. These files were used to align the sequenced reads to the reference genome GRCh37 by Burrows-Wheeler Aligner software [182]. The sequencing reads from each sample were assembled in a binary

alignment map file. Normalization, removal of systematic biases by GC-content, and exclusion of duplicate reads were performed with the tools SAM and Picard [183].

The copy number analysis was performed by modified QDNAseq [184]. The bioinformatic tools ACE (Absolute Copy number Estimation), Rascal (Relative to absolute copy number scaling) and ichorCNA were used to calculate tumor fraction, ploidy and thereby absolute copy number. In brief, ACE was developed for calculation of absolute copy number in solid tumors [185]. Rascal was developed based on concepts of ACE and further adapted to the biological nature of HGSC samples [186]. Both methods perform analysis on the output of QDNAseq. In contrast, the **ichorCNA** algorithm was developed for detection of tumor circulating DNA and uses a Hidden Markov Model for the probabilistic modelling instead of using output from QDNAseq [187]. Detailed information about these four bioinformatic tools and how we applied them can be found in **Study II**.

Copy number signatures

As previously explained, the original HGSC CNsig (Macintyre et al.) were constructed from 91 HGSCs with high-quality copy number profiles [72]. In our approach we interrogated cervical samples from patients diagnosed with HGSC to construct our copy number signatures (CerCNsig).

The first step was to identify the following six **fundamental copy number features** for each cervical sample: breakpoint count per 10Mb, copy number of the segments, copy number change point, breakpoint count per chromosome arm, lengths of the oscillating copy number segment chains and segment size. All these features have been previously reported as genomic aberrations in solid tumors. Thereafter the sum of features in the whole data set was calculated and Gaussian or Poisson mixture models were applied to determine the optimal number of components for each feature. **Mixture modelling** was performed in the FlexMix V2 package in R [188]. This resulted in a total of 32 mixture components.

Next, for each copy number event, the probability of belonging to a component was calculated. For each sample, these event vectors were summed and represented in a sample-by-component sum-of-posteriors probabilities matrix (Figure 12 left).

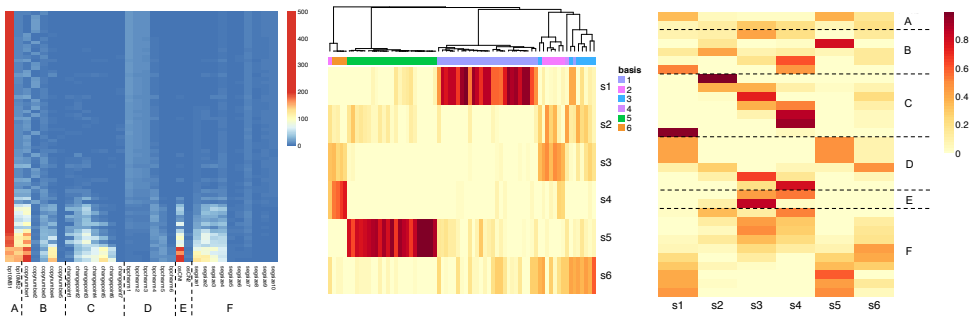


Figure 12. Constructing copy number signatures with cervical samples from HGSC patients (CerCNsig).
Left, sample-by-component matrix. 32 components grouped by the six fundamental features (A-F). A= breakpoint count per 10Mb, B= segment copy number, C= copy number change point, D= breakpoint count per chromosome arm, E= length of segments with oscillating copy number and F= segment size. **Middle**, sample-by-signature matrix. Light yellow to dark red scale representing 0-100% of a signature in each sample. **Right**, signature-by-component matrix. Light yellow to dark red scale representing 0-100% of a component for the six signatures.

In the next step, **non-negative matrix factorization** (NMF package in R [189]) was used to deconvolute the sample-by-component matrix into the sample-by-signature matrix (Figure 12 middle) and the signature-by-component matrix (Figure 12 right). NMF is a low-rank approximation method that aims at minimizing the complexity of a model that fits the data. Thus, the sample-by-signature and signature by-component matrices are approximating matrices with reduced rank/complexity. Our 32 components were regrouped and clustered in 6 different signatures. The optimal number of signatures was chosen using four model selection measures in the NMF package (mathematical coefficients, details in Suppl Fig 2 in Study II).

Ethical considerations

All PhD students and supervisors in the research group were responsible for the content and writing of the ethics application. The ethical approval (Dnr 2014/717) comprises the HGSC TMA and the MaNiLa project in this thesis. Patients in the MaNiLa project gave informed written consent for participation. The approval allowed for our investigative mutational analysis not reporting back to the patients.

In our published study from the MaNiLa project, we analyzed mutations in *TP53*. Germline mutations of *TP53* are linked to Li-Fraumeni syndrome, a rare hereditary disorder that pre-disposes carriers to cancer development. Unexpectedly, we found a patient with a *TP53* germline mutation among the 15 patients included in Study I. This mutation has been reported in COSMIC (Catalogue Of Somatic Mutations In Cancer) but scored as neutral (not pathogenic) in the Functional Analysis through Hidden Markov Models (FATHMM) [190] scoring by GEMINI (GEnome

MINIng). Thus, we followed our decision of not informing the patient, as this allele variant has not been linked to disease.

Whitin the MaNiLa project we planned to collect archival cervical samples, both cervical cytology slides and liquid-based cervical samples, from patients with diagnosed HGSOC, *BRCA*-mutation carriers and controls. The routine is to apply to the regional biobank center in Southern Sweden which is responsible for evaluating and giving the approval. At the same time, the healthcare principal at the Pathology Department also has to approve the application.

What makes borrowing cervical samples different from borrowing tumor blocks, is the fact that cytology slides cannot been returned, as all DNA in the slide is extracted. This originated an administrative and ethical issue, as the pathology department was initially reticent to provide cytology slides, but even liquid-based cervical samples. The main reason was that the review of previous archival samples was part of the diagnostic quality assurance in case of finding a case of cervical cancer. Eventually, the healthcare principal decided that cervical samples from deceased patients and from patients that had performed hysterectomy could be handed over, as the latter group could not suffer from cervical cancer anymore. Thus, we could not get archival cervical samples from controls and *BRCA* mutation carriers that had not performed a hysterectomy. Thus, the diagnostic quality assurance was respected, and the potential violation of patient interests was avoided.

Results and discussion

Detection of *TP53* mutations in cervical samples, Study I

As described above most of the previous studies evaluating early markers of disease originate in samples collected at the time of cancer diagnosis. Within the MaNiLa project, a subset of 15 patients were identified to have archival liquid based cervical samples available from the cervical cancer screening program. Two patients had more than one archival sample. Thus, a total of 20 archival samples were taken 20 to 95 months prior to the HGSC diagnosis.

Paired tumor and blood samples from each patient were analyzed with targeted sequencing (NGS) and at least one *TP53* mutation was identified for each patient using GEMINI and a hard filter minor allele frequency (MAF) cut-off of 5%. Ten missense mutations, two nonsense mutations, and three frameshift deletions were identified. Mutations were dispersed across the *TP53* gene, with no overlap between patients. All mutations (but one) were previously reported in COSMIC (Catalogue Of Somatic Mutations In Cancer). MAFs in the tumors ranged between 8–85% with a median of 64% (Figure 13A). Tumor samples from the 15 patients were also analyzed with IBSAFE and in three cases even with Bio-Rad for comparison with ddPCR using IBSAFE and NGS. MAFs obtained with the three approaches were similar (Figure 13A).

DNA concentrations in archival samples were lower than in diagnostic samples, and not linked to disease stage. All 20 archival samples were analyzed using the ultra-sensitive ddPCR-based method IBSAFE and Bio-Rad ddPCR, whereas only nine available diagnostic samples were analyzed with IBSAFE (3/9 even with BioRad). The IBSAFE method was able to detect tumor-identified mutations in seven of nine diagnostic samples, however one mutation was determined to be a germline event; hence, true somatic mutations were detected in six of eight diagnostic samples. Despite the age and the low abundance of DNA in the archival samples, the IBSAFE method was successful in all the individual assays and in detecting a somatic tumor corresponding mutation in one archival sample (Patient 13, Figure 13C) obtained 20 months prior to diagnosis. This patient had samples collected even 46 and 32 months prior to diagnosis, but the mutation was not detectable in these earlier samples. No mutations were detected in the remaining archival samples (Figure 13C). Of note, the high MAF of patient nr 1 in both diagnostic and archival Pap samples, representing a *TP53* germline mutation (Patient 1, Figure 13B and C).

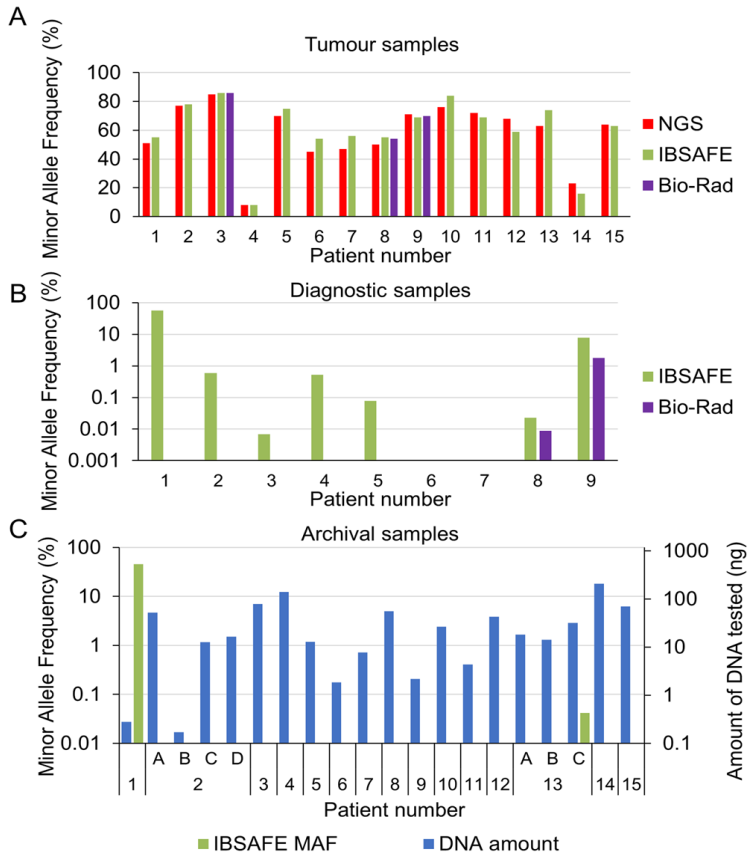


Figure 13. *TP53* mutations in diagnostic and archival Pap samples.

Minor allele frequencies (MAFs) (primary y-axis) for tumors (**A**), diagnostic samples (**B**) and archival samples (**C**). Amount of DNA tested (secondary y-axis) from archival samples (C). Note the log scale on the y-axis. Red, next-generation sequencing; green, IBSAFE assays; purple, Bio-Rad assays; blue: amount of DNA in ng. Adapted from Arildsen *et al*., 2019.

To our knowledge this is the first publication where a tumor corresponding mutation has been found in an archival sample collected 20 months before diagnosis from a patient subsequently diagnosed with a stage IIIB tumor, while we did not detect any mutations in the two other samples collected at time points earlier than 20 months prior to diagnosis. The limited number of patients in the present study precluded the possibility of further exploring the timeframe for detecting *TP53* mutations with this technology. Another limitation is the very low amount of DNA in many of the archival samples (11/20 samples <20 ng), as sensitivity of the method is directly linked to the DNA quantity. Furthermore, IBSAFE is a targeted assay designed to find a known *TP53* mutation previously found in the patient's tumor, which is an absolute limitation in the screening situation, when the mutations are not known in

advance. Nevertheless, we provided proof-of-concept that current methods are able to detect HGSC-derived mutations in samples prior to the onset of symptoms.

After this publication, another group succeeded in finding several *TP53* mutations in pre-diagnostic traditional Papanicolaou smears, but also with a targeted assay of a previously identified *TP53* mutation in the patient's tumor (Bio-Rad) [8]. Several approaches, to highlight the one using a highly sensitive targeted sequencing called duplex sequencing, have shown the challenge of *TP53* mutations in healthy women and tissues [7, 9]. The presence of cancer-like mutations in healthy tissue highlights the need for increased specificity.

Copy number signatures in cervical samples, Study II

Estimations of absolute copy number (CN)

Estimation of the tumor fraction (TF) by a pathologist is helpful, although not flawless. The TF of our fresh-frozen tumors was unknown and other sample types required a different approach. We instead applied the probabilistic bioinformatic tools ACE, Rascal and ichorCNA for estimation of ploidy and cellularity in all sample types for comparison.

As expected, we found a very strong Spearman correlation between the ACE squaremodel and Rascal on estimation of ploidy and tumor fraction in tumor samples (R=1, p=3e^{-14} and R=0.97, p=2.2e^{-08}). However, when comparing ACE and ichorCNA we found no correlations for ploidy estimations and cellularity estimations were weaker when comparing across all sample types (R=0.54, p=2.2e^{-16}) than tumor samples only (R=0.86, p=4.6e^{-06}). The disagreement in cellularity estimations between the ACE and ichorCNA methods was mainly found among samples with low cellularity. The most plausible reason for this discrepancy is that these methods have been developed for different sample types, namely tumor and plasma, respectively.

Thus, for downstream analyses we used ACE estimations for tumors and a manually curated estimation of cellularity based on ACE and ichorCNA for the rest of the samples. The mean estimation of cellularity in HGSC cervical samples (CS) was higher than in CS from controls and *BRCA* mutation carriers (0.07 versus 0.02 and 0.04).

HGSC CNsig components in different sample sets

As a first step, we wanted to validate our approach by applying the original CN signatures (Macintyre *et al*.) to our sample sets: HGSCs (n=14), Cervical Samples (CS) from 18 HGSC patients (n=69), tissue+endometrial biopsy (EB)+CS from seven controls (n=24) and tissue+EB+CS from four *BRCA* mutation carriers (n=27).

One tissue and one CS from a control were excluded due to poor quality in copy number profile. Further, a benign sample was excluded (mucinous cystadenoma because amplification of chromosome 1). FFPE tissues (and one STIC) from *BRCA* mutation carriers were not included because of technical/analytical problems.

We first visually compared the sample-by-component matrix from all the datasets as they represent the sum of events for each component in each sample and observed similarities between the discovery cohort (n=91, Macintyre *et al*), our tumor dataset and even some similarities with the dataset with CS from HGSC patients. However, we could not find the same patterns in the datasets from controls and *BRCA* mutation carriers (Figure 14). The only component with high counts of events in all data sets was the so-called "breakpoint count per 10 Mb nr 1" that represents <1 break per 10 Mb of the genome and concluded that this component is not characteristic of samples with tumor content (red column to the left in the four datasets, Figure 14).

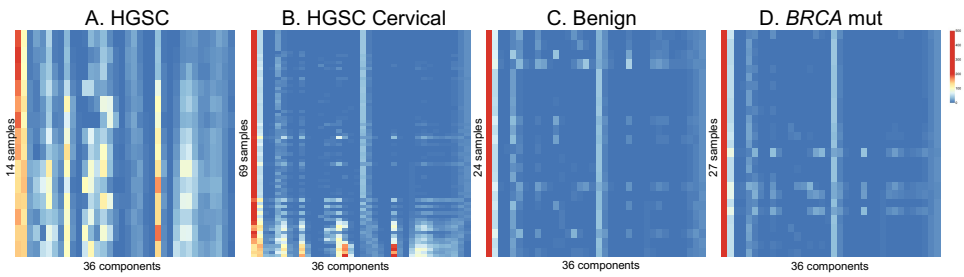


Figure 14. Sample-by-component matrix in different sample sets with the 36 original HGSC CNsig components represented.

A, 14 HGSCs (four tumors excluded due to no TF). **B**, 69 cervical samples from 18 HGSC patients. **C**, 24 samples from seven controls (13 CS, six EB and five tissues). **D**, 27 samples from four *BRCA* mutation carriers (20 CS, four EB and three tissues).

The fact that we found a subgroup of CS displaying higher counts of the same feature components as tumors (and absent in benign samples) made the investigation of copy number signatures in CS appealing.

Constructing CN signatures with cervical samples

As previously explained, we interrogated all 69 diagnostic and pre-diagnostic cervical samples (CS), from all 18 patients diagnosed with HGSC with the aim of identifying copy number signatures in CS related to HGSC. NMF clustered the identified 32 components into six copy number signatures. The sample-by-signature revealed the imprint of the six signatures in each sample and clustered them, while the signature-by-component matrix represented the component weights for the six different signatures (Figure 12). Representation of component weights for copy number signature 1 is shown in Figure 15, as an example. Henceforth, we will refer

to our copy number signatures constructed with CS from HGSC patients as CerCNsig.

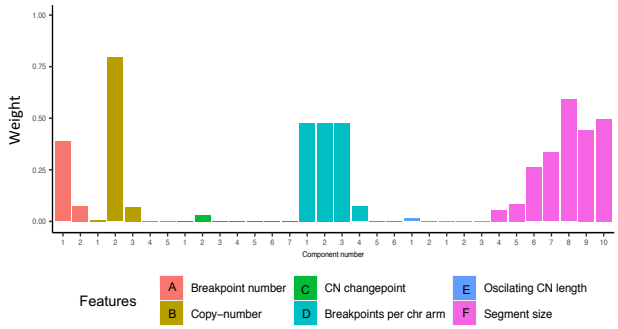


Figure 15. Component weights for copy number signature number 1.
32 components grouped by the six fundamental features (A-F).

Comparisons of cellularity and CN features in cervical samples

First, we wanted to find out if our cellularity estimations were associated with events of the 32 feature components of the CerCNsig. We observed that all benign samples displayed lower counts for 31 components, compared to a subgroup of HGSC CS (not shown), and decided to define a cut-off with blue color representing low counts and any other color (yellowish to red) high counts.

Based on the distributions of cellularity calculations, we applied comparisons with 10 and 15% as cut-offs, and observed that high cellularity estimation was associated with high counts of CN events in CS. No CS from controls or *BRCA* mutation carriers showed high count of CN events or cellularity >15% (Table 6).

When we further looked at the different types of cervical samples, we found that 6/11 liquid-based CS samples (ThinPrep solution) showed high counts of CN events. Among these, 4/6 samples were taken 5-21 months before diagnosis, when patients were still asymptomatic (according to the patients' medical reports) and the other two samples were obtained within one month before surgery. Surprisingly, none (0/8) of the liquid-based CS preserved in DNAgard, taken at surgery, showed high counts of CN events. All these eight liquid-based CS had a slide replicate (also taken at surgery), and none of them showed high counts of CN events either.

Table 6. Comparisons of cellularity and copy number events in cervical samples from the three data sets

CS sample set	CN events	Cellularity<10%	Cellularity≥10%	Cellularity<15%	Cellularity≥15%
HGSC n=69	Low	**46**	**6**	50	2
	High	**2**	**15**	7	10
BRCA n=20	low	17	3	19	1
	high	0	0	0	0
Benign n=14	low	13	1	14	0
	high	0	0	0	0

In bold the strong association between cellularity and copy number events.

Among all 103 CS in the study, we identified 11 CS (from 3 patients with HGSC) with atypical cervical cells (mild to severe); only 1/11 samples displayed high counts of CN events and 3/11 had an estimated cellularity ≥10%.

Feature distributions and signature exposures

Whit the goal of understanding our CerCN signatures we compared the distribution (histogram or density) of the six copy number features in our datasets (Figure 16). While the x-axis represents the values of each feature (e.g. 0-8 breakpoints per 10 Mb), the y-axis represents the sum of counts in a data set and differ depending on both the number of samples and the sample type in the data set represented. Tumors and HGSC cervical samples showed similar distributions for all features but segment size, with bigger segments in the CS group (red arrows). Bigger segments were even more common among benign CS, suggesting that bigger segment size is a feature not typical for tumors. As expected, distributions for the rest of the features were also different in benign CS compared with tumors and HGSC CS.

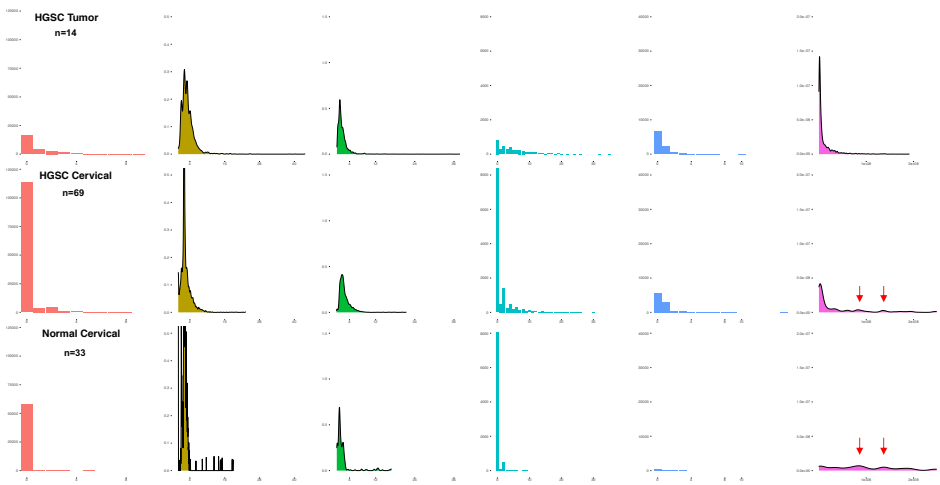


Figure 16. Feature distributions in tumors and cervical samples. "Normal Cervical" include 13 CS from controls and 20 CS from *BRCA* mutation carriers. Red arrows show larger segment sizes in cervical samples.

We further examined signature exposures across different data sets and sample types. When observing tissue samples from tumors, *BRCA* mutation carriers and controls, we saw significant higher exposures to signature 2 and 3 in tumors, compared to the other tissues (red arrows, Figure 17A). In contrast, signature 1 displayed significant lower exposure in tumors, compared to the other tissues. When comparing exposures for the signatures in HGSC CS and benign CS, differences were overall less evident (Figure 17B). The significant higher exposure for signature 3 in HGSC CS compared to benign CS became more obvious when selecting CS with cellularity ≥10% (red arrows, Figure 17B and C), which suggest signature 3 may be tumorigenic. Even exposure for signature 2 was significantly higher in HGSC CS compared to benign CS when selecting CS with cellularity ≥10%. Further, the lower exposure for signature 5 in HGSC CS became more evident after selection of CS with higher cellularity.

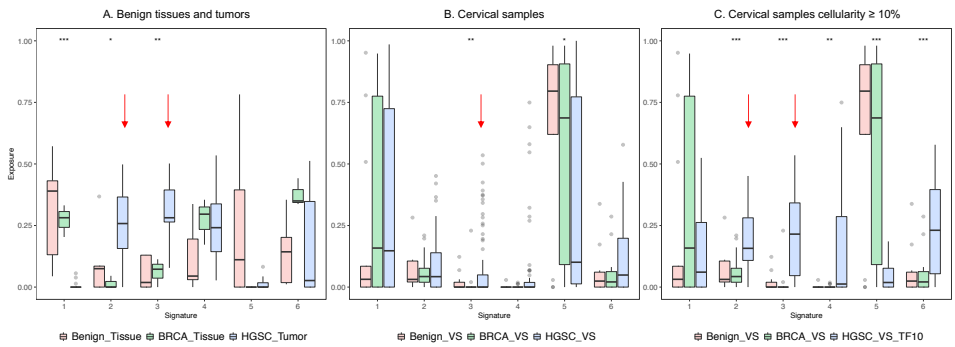


Figure 17. Signature exposures in different data sets.

A, Tissue from controls (n=11), *BRCA* mutation carriers (n=7) and tumors (n=14). **B**, Cervical samples from controls (n=13), *BRCA* mutation carriers (n=20) and HGCS patients (n=69). **C**, CS from controls, *BRCA* mutation carriers and HGSC patients with cellularity ≥ 10% (n=17). Red arrows point out higher exposure to Signature 2 and 3 in tumors and cervical samples from HGSC patients. The lower and upper hinges represent the first and third quartiles, the line in the middle is the median and dots are outliers. Significant differences are highlighted using asterices (* P<0.05, ** P<0.01, ***P<0.001).

Defining the CerCN signatures

Based on previous comparisons and component weights in the different signatures, we aimed at further defining the six CerCNsig. Component weights for the six signatures are shown in Figure 18. Signature number 1 displayed few breakpoints and low copy number states. Together with the observation that tumors showed no or very low exposure to this signature, suggested that signature 1 is likely not tumorigenic. This was further confirmed by the larger segment sizes, displayed by cervical samples.

Signature number 2 showed higher number of breaks and higher copy number states, which are features typical for tumors. Further, we confirmed high exposure for this

signature in tumors and cervical samples with cellularity ≥10%. Thus, signature 2 is likely a tumorigenic signature.

Signature number 3 with significantly higher exposure in tumors and cervical samples from HGSC patients and breaks distributed across the genome, may be a tumorigenic signature also.

At the moment of writing, we are trying to further define and validate our signatures. Thus far we speculate that several signatures capture some tumorigenic features, as the method was originally developed for clustering tumor samples.

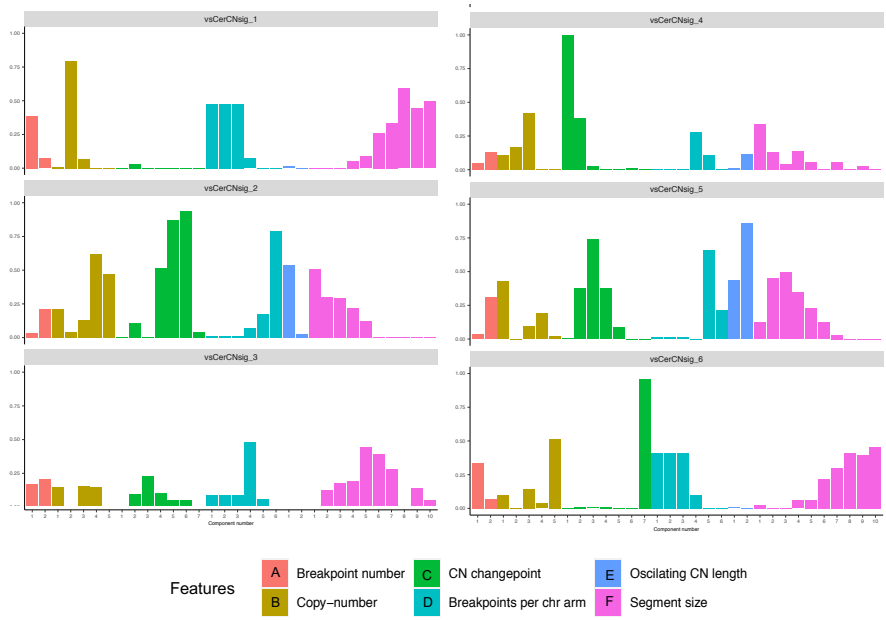


Figure 18. The six copy number signatures in cervical samples from HGSC patients.

Conclusions

Calculation of cellularity and absolute copy number in samples other than tumors was challenging. Whereas two of the methods were developed for determination of tumor fraction in tumor samples, the third method was developed for detection of tumor circulating DNA and its estimations differed from the first two. However, our tool optimization and manual curation gave us the best possible performance for absolute copy number profiles in our low cellularity samples. The next step is to establish a limit of detection for the method with *in silico* and *in vivo* approaches.

We found a subgroup of cervical samples from HGSC patients with CN features typically found in tumors and absent in controls and *BRCA* mutations carriers. Further, we generated new copy number signatures from 69 diagnostic and pre-

diagnostic cervical samples from 18 women diagnosed with HGSC. By comparing CN feature distributions and signature exposures in the different data sets, we are trying to define and characterize our signatures. The next step is further validation of our signatures with the final goal of understanding the contribution of different cell types to the different signatures and identifying which components should contribute to a novel tumorigenic signature, able to detect tumorigenic signals in cervical samples with high sensitivity and specificity.

Our results indicated that atypical cervical cells do not display the same CN features as HGSC and thus are not a confounder for our approach. Why we found CN events in 6/11 pre-diagnostic ThinPrep liquid-based CS but in none of the diagnostic CS remains unanswered. Whether ThinPrep slide production may affect cell free DNA also remains unclear. The fact that many cervical samples were retrospectively collected, at different time points and with different techniques is a limitation in this study. Hopefully, the inclusion of remaining cervical samples in the project (>300) into the signature construction will add robustness of both the methodology and the copy number features.

At the time of writing, we are analyzing synchronous STICs from nine HGSC patients (and a *BRCA* mutation carrier), with the aim of understanding CN features in these cancer *in situ* lesions. Recognizing CN alterations and signatures typical in early HGSC development may add sensitivity to our approach.

In this study we proved the concept of using patterns of copy number aberrations from low coverage whole genome sequencing data from cervical samples taken up to five years before the women were diagnosed with HGSC, with the final goal of finding a biomarker for early diagnosis. This is a new approach trying to circumvent the emerging challenge of very-low frequency cancer-like *TP53* mutations in healthy women/tissues. Whether copy number signatures generated from cervical samples from patients diagnosed with HGSC can ultimately be used for early detection of HGSC should be validated in larger and prospective studies.

Claudin-4 as prognostic factor in ovarian carcinoma, Study III

As previous studies reported increased expression of claudin-4 in ovarian carcinoma compared with normal ovarian surface epithelium and benign ovarian tumors, we performed claudin-4 immunohistochemical staining on relevant control tissues. Whereas ovarian surface mesothelium showed no staining, cortical serous inclusion cysts (Figure 8C) and 3 cases of serous cystadenomas were strongly positive. Furthermore, epithelium from normal fallopian tube and normal endometrium displayed high claudin-4 expression, in line with the Human Protein Atlas [191].

These findings differ from previous studies and are fundamental when making conclusions on claudin-4 overexpression and malignant transformation. Considering the cell of origin of the different ovarian carcinoma subtypes, we could not conclude that claudin-4 may be involved in malignant transformation, as the "cells of origin" studied were strongly positive for claudin-4.

In the ovarian carcinoma TMA with 140 cases only 7 displayed no staining, and the distribution in subgroups 1–4 was as follows: 1: n=32, 2: n=30, 3: n=18, 4: n=53. For analysis, groups 3 and 4 (71 cases, 51%) were designated to the high expression group. Among the 132 patients who had received platinum-based chemotherapy, a patient with high claudin-4 expression had a 1.2 (95% CI=0.7–2.0, P=0.3) times higher risk of being platinum resistant compared with a patient with low claudin-4 expression. Thus, claudin-4 was not a good predictor of platinum responsiveness, either in the whole cohort or in any other subgroup (type I or II tumors, serous subtype, early or late-stage and *BRCA1/2* wild-type subgroup).

The patients whose tumors expressed high levels of claudin-4 displayed shorter PFS [HR=1.7 (1.1–2.6), P=0.020] (Table 7). Hazard of relapse was similar [HR=1.5 (1.0–2.4)] after adjustment for age, stage, type, and *BRCA1/2* status in a multivariable analysis, but the evidence was weaker (P=0.076, Table 6). A comparable association was observed for OS in the univariable and multivariable analyses [HR=1.6 (1.0–2.4), P=0.041 and HR=1.5 (0.9–2.3), P=0.1].

Table 7. **Univariable and multivariable analyses of progression-free survival**

			5-yr PFS univariable Cox		5-yr PFS multivariable Cox	
		N (events)	HR (95% CI)	*P*	HR (95% CI)	*P*
Claudin-4	High vs. low	70 (55) vs. 64 (38)	1.7 (1.1-2.6)	**0.020**	1.5 (1.0-2.3)	0.076
Age	>70 vs. <70	27 (22) vs. 107 (71)	1.5 (0.9-2.4)	0.1	1.1.(0.6-1.8)	0.7
BRCA1/2	Wildtype vs. mutant	105 (75) vs. 29 (18)	1.7 (1.0-3.1)	0.052	2.6 (1.5-4.6)	**0.001**
Stage	III/IV vs. I/II	94 (82) vs. 40 (11)	6.2 (3.3-11.7)	**<0.001**	4.4 (2.2-8.8)	**<0.001**
Type	II vs. I	82 (68) vs. 47 (20)	2.9 (1.8-4.8)	**<0.001**	2.2 (1.2-3.8)	**0.023**

Bold values are statistically significant *P*-value <0.05. CI= confidence interval, HR= hazard ratio. Adapted from Martin de la Fuente *et al*., 2018.

To extend our findings, we investigated the role of *CLDN4* mRNA levels in a cohort of 1,582 OC patients available through the online tool Kaplan-Meier Plotter [153]. The online tool comprises gene expression data and survival information from 13 independent public data sets (Gene Expression Omnibus and The Cancer Genome Atlas, n=28-565, 2015 version). The best performing threshold was used as cut off, where 1,105 patients (70%) were classified as *CLDN4* low and 477 (30%) as *CLDN4* high. These results were in line with our findings, as *CLDN4* high patients displayed worse 5-yr OS in a univariable analysis [HR=1.3 (1.1–1.5), P<0.001] compared with *CLDN4* low patients.

In May 2018, a letter to the editor about our article was published. It argued about the inconsistency of claudin-4 predicting outcome but not response to chemotherapy [192]. We agree on the appreciation that mixed histologies may partly explain our findings and that further analysis of the high-grade serous subgroup would be interesting to clarify the prognostic and treatment predictive role of Claudin-4 overexpression in this subtype. Thus, we analyzed the subgroup of 80 patients with HGSC (n=80, stage I/II=10, stage III/IV=70) and could find similar hazards of survival but the evidence was weaker, probably due to the smaller sample size (PFS, HR=1.4 (0.8–2.2), P=0.217 and OS, HR=1.6 (0.97–2.7), P=0.065). Of note, when calculating the risk ratio for prediction of platinum resistance 10 cases were excluded (seven did not receive platinum-based therapy due to early stage, one received other therapy and in two cases information was missing), which may have affected the results. Further, the wide CI may indicate small sample size affecting this calculation.

Moreover, we tried to validate these findings in our more contemporary HGSC TMA cohort with 141 consecutive cases collected 2011-15 (stage I/II=11, stage III/IV=130). We evaluated claudin-4 expression in this cohort and could not find any associations with survival or platinum sensitivity (data not shown). Noteworthy is a different distribution of low/high claudin-4 expression in the two HGSC cohorts, with a higher proportion of cases with low expression of claudin-4 in the validation cohort (60% vs. 45% in the first study). There was even a higher proportion of stage I-II vs. III-IV among HGSC cases in the first study compared with the validation cohort (12.5% in the first study compared with 7.8% in the validation cohort). Whether stage distribution may have affected our results remains unclear as survival calculation of advanced stage HGSC in the first study lacked power. In conclusion, we were not able to validate our results from the first study.

Immune cell markers and prognosis in advanced stage HGSC, Study IV

In this study, we mapped the presence of macrophages and lymphocytes located within the tumor epithelium, the cell type–specific expression of PD-L1 and PD-1 and their impact on prognosis in a well-characterized, contemporary, and consecutive cohort of 130 patients diagnosed with advanced HGSC.

We found significant positive associations between all markers studied, except PD-1 and CD163, which implies a division in tumors rich in immune cell infiltration versus poor or absent immune cell infiltration (“hot” and “cold” tumors, respectively).

PD-L1 was expressed mainly by macrophages, and to a far lesser extent by tumor cells and lymphocytes (Figure 19). We evaluated the membranous expression that was predominantly partial and weak, but also observed granular cytoplasmic expression. Using the same cut-off as for PD-1 (≥50% cores with ≥1% PD-L1 expressing macrophages), we identified only 15/130 patients as positive. The survival benefit of this small group was high and statistically significant but uncertain (HR 0.20 [0.07–0.66], *P*= 0.007). Thus, we considered cases with ≥2 cores with ≥1% PD-L1 expressing macrophages as positive (26/130) and found a significant association with improved OS also within this group (HR 0.47 [0.25–0.89], *P*= 0.02; Figure 20). Because of scarce PD-L1 expression in tumor cells and lymphocytes, no relevant cut-off could be determined, precluding further analyses.

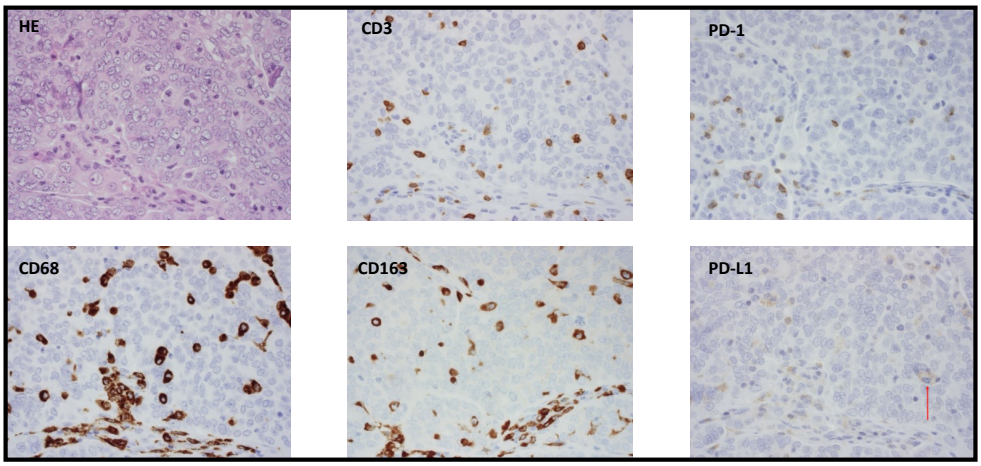


Figure 19. Example of a positive case for all immunohistochemical stainings. Pictures of corresponding tumor areas on consecutive tissue microarray sections. The red arrow shows a PD-L1 positive macrophage. Stroma seen at the bottom of the pictures excluded from scoring. Magnification x20. Adapted from Martin de la Fuente *et al*., 2020.

PD-1 was almost exclusively expressed by lymphocytes (membrane, Figure 19) and patients with ≥50% cores with PD-1 expression ≥1% (39/130) were considered to have high expression. These patients had longer OS (Figure 20), even when adjusting for well-known prognostic factors as age at diagnosis, stage and residual tumor (HR 0.55 [0.32-0.94], *P*= 0.03). Interestingly, over 2/3 of patients with high intra-epithelial CD3 expression displayed high PD-1 expression in intra-epithelial lymphocytes and had a stronger survival benefit compared to CD3 high/ PD-1 low cases (HR 0.33 [0.14–0.77], *P*= 0.01).

Furthermore, we explored the survival benefit of the subgroup with higher expression of both PD-1 and PD-L1 and found lower hazards of death compared with each marker alone (19 cases PD-1/PD-L1 high vs. 84 cases PD-1/PD-L1 low, HR 0.36 [0.17–0.79], P= 0.01; Figure 20).

Patients with ≥50% cores with ≥2% intra-tumoral lymphocytes (CD3 high, 43/130) had longer OS compared with patients with lower expression (HR 0.58 [0.35–0.94], P=0.03; Figure 20), even when adjusting for well-known prognostic factors (P= 0.04). Using the same cut-off, no significant OS difference was found between patients with high (70/130) versus low expression of intra-epithelial TAMs (CD68) or high (51/130) versus low expression of intra-epithelial M2 TAMs (CD163).

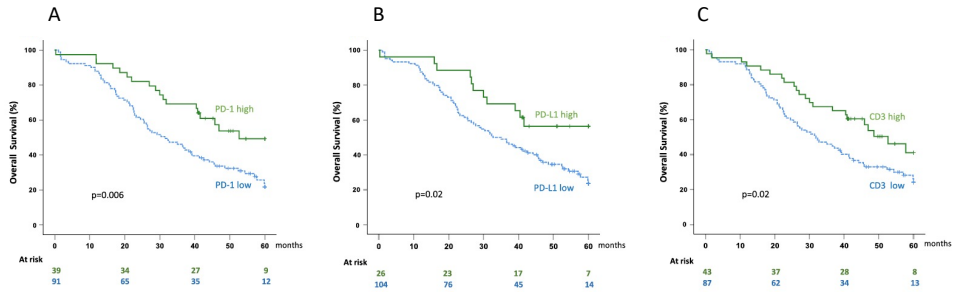


Figure 20. Association between OS and the expression of PD-1 in lymphocytes, PD-L1 in macrophages, and CD3 expression, within the tumor epithelium. A, Patients with high PD-1 expression had longer survival compared with patients with low PD-1 expression. **B**, Patients with high PD-L1 expression had longer survival compared with patients with low PD-L1 expression. **C**, Patients with high CD3 expression had longer survival compared with patients with low CD3 expression. P values were calculated using the log-rank test. Adapted from Martin de la Fuente *et al*., 2020.

Thus, in our cohort of 130 consecutive cases of advanced HGSC, higher expression of intra-epithelial lymphocytes (CD3), PD-1 (in lymphocytes), and PD-L1 (in macrophages) was a predictor of better outcome with similar hazards of death, even after adjusting for age at diagnosis, stage, and residual tumor after primary surgery. In agreement with our results, previous studies have reported a survival benefit for patients with higher intra-epithelial CD3 expression (two meta-analyses) [12, 116] and higher PD-1 expression [123, 193], in ovarian carcinoma. Of note, most previous studies included different ovarian carcinoma histologies and stages, in contrast to our advanced HGSC cohort. Furthermore, in one of the studies, PD-1 expression was described in tumor cells in most of the cases, in addition to lymphocytes [123], but we did not observe any PD-1 staining in tumor cells.

In this study, we found that PD-L1 was expressed mainly by macrophages, which has previously been reported [193-195] and not by tumor cells as some previous studies suggest [115, 123, 124, 196]. Remarkably, macrophage staining, which was necessary for the correct mapping of PD-L1 expression on macrophages, was not performed in the previous studies where PD-L1 expression in macrophages was not described. Moreover, the specificity and sensitivity of anti-PD-L1 antibodies have been debated and based on results from The Blueprint Project [197] and Brunnström et al. [198], we decided to use the FDA-approved 22C3 clone in the present study.

Taken as a whole, in this study we tried to address ambiguous findings in previous studies. Namely and according to our results, the main cells expressing PD-L1 are macrophages (and not tumor cells) and PD-1 is almost exclusively expressed by lymphocytes (and not by tumor cells).

Moreover, and trying to circumvent possible bias when evaluating immune cell markers in a TMA, we performed whole tissue sections for the immune markers PD-1, PD-L1 and CD3. We observed concordance between evaluations performed on TMA and whole tissue sections in the majority of the 17 pairs investigated (13 cases, four of which had paired samples from adnexa and metastatic site). Comparing the average staining of the cores and whole tissue sections, the highest concordance was observed for PD-1, where only 1/17 pairs was discordant. Regarding PD-L1 in macrophages, scoring on TMA underestimated whole tissue section scoring in 3/17 pairs and for CD3 2/17 pairs were discordant. Of note, TMA and whole section immunostainings were performed on the Dako Autostainer and Ventana system, respectively, which did not seem to affect the performance of the antibodies. PD-1 immunostaining on whole sections was discordant on the Ventana system, and therefore, we performed the comparison on the same platform as the tissue microarray, the Dako Autostainer.

Eventually, we did not observe a prognostic value of intra-epithelial macrophages (CD68) or alternatively activated macrophages (CD163) in the present study. Previous studies are difficult to compare with our study due to differences in the patients included (mixed histological types [199, 200]) or evaluation methodology (e. g. stromal CD163 expression [201]).

An important limitation of our study is that only 25 patients were tested for *BRCA1/2* mutations, precluding the possibility of performing survival analyses in these subgroups. However and interestingly, we noted a strong positive correlation between the expression of the immune marker that best predicted prognosis, PD-1, and platinum sensitivity. The group of platinum sensitive tumors is enriched with tumors showing homologous recombination deficiency [202], and this may imply a relationship between tumor immunogenicity and homologous recombination deficiency, as recently suggested [203].

The PD-1/PD-L1 pathway is a negative regulator of T cell activation and PD-L1 upregulation by tumor cells suppresses T cells, thereby promoting tumor growth. Supporting this theory, higher PD-L1 expression predicts inferior survival in several cancer forms [204]. Thus, the survival benefit of higher PD-L1 expression by macrophages in HGSC shown in our study, including validation in an external data set (TCGA cohort), and previous studies [193-195] stands as an unanswered paradox. As a possible explanation, a previous study referred to adaptive immune resistance, wherein activated T cells may trigger negative feedback mechanisms, resulting in an immunological stalemate [193]. The sparse expression of PD-L1 in HGSC tumor cells, in contrast to other malignancies [197], also remains an

unresolved issue. Nevertheless, the observed survival benefit suggests that the potential of tumor immunity may be harnessed in subsets of HGSC.

Conclusions and Future Perspectives

Early detection

In **Study I**, we analyzed 20 archival cervical samples, taken months to years before the HGSC diagnosis, using the ultra-sensitive ddPCR-based method IBSAFE and detected a somatic tumor corresponding mutation in one archival sample obtained 20 months prior to diagnosis. The main limitations in this study are the low amounts of DNA in archival samples and the long interval between many of the archival and diagnostic samples (10/20 samples >5 years). Furthermore, the ddPCR method, even using multiplexing, cannot target all HGSC associated *TP53* mutations, which is a constraint in the screening situation, when the mutations are not known *a priori*.

Specificity has emerged as the major challenge for the targeted ultra-sensitive NGS approaches as non-cancer derived mutations constitute a significant cause of false positivity. Whether algorithms that account for clonal hematopoiesis (in blood) and regenerative defects (tissues) in targeted NGS can be achieved remains an unresolved issue.

In **Study II**, we studied Copy Number Alterations (CNAs) in cervical samples from patients diagnosed with HGSC, healthy *BRCA* mutation carriers and patients with benign gynecological conditions. To our knowledge, our study is the first approach towards studying CNAs in cervical samples. In our opinion studying CNAs is an appealing approach as the ubiquitous *TP53* mutations in HGSC lead to broad copy number alterations. These broad CNAs are hopefully more easily identifiable than rare mutant alleles among thousands of normal ones, that is "finding a needle in a haystack". In fact, we identified a subgroup of cervical samples from patients diagnosed with HGSC that showed CNAs typically found in HGSC. No CNAs were found in benign tissues or cervical samples.

A common limitation for the methods in **Study I** and **II** is the potential absence of tumor-derived DNA in the samples. Further limitations in our retrospective approach relate to technique and time points for sampling, and sample preservation and processing.

In summary, detection of CNAs in cervical samples is a novel promising tool for early detection. However, our study is in early stage and further analyses including more patients and controls, as well as large prospective studies, are needed to determine the clinical utility of CNAs in cervical samples for early diagnosis of HGSC. The use of multi-omics as proteomics, mutational, epigenetic DNA methylation and copy number analysis, among others, together with novel

radiological approaches applied in a longitudinal manner may be the way to detect this complex disease.

Prognostic and predictive biomarkers

Previous studies have reported upregulated Claudin-4 expression in ovarian carcinoma compared to benign ovarian conditions and worse prognosis in patients with higher expression, concluding that Claudin-4 may be involved in malignant transformation. In **Study III** we also found that higher expression of Claudin-4 was a predictor of shorter survival. However, we found strong expression in normal fallopian tube epithelium and other benign epithelial lesions in the ovary. Thus, no conclusion regarding the involvement of Clauidn-4 in malignant transformation can be done, accordingly to our results. Further, why patients whose tumors downregulate claudin-4 had a survival benefit remains unanswered. We tried to validate our results in our more contemporary TMA with 141 cases of HGSC. However, we could not show a survival benefit for patients with lower Claudin-4 expression in the validation cohort.

Ovarian cancer response to immunotherapy in clinical trials is limited and checkpoint inhibitors (ICI) have currently no FDA approved indication for ovarian cancer. The use of biomarkers to select patients could increase the response rates and survival for a subgroup of patients, and spare potential side effects to unselected patient groups. The proposed markers include tumor mutation burden, PD-L1, tumor-infiltrating lymphocytes, homologous recombination deficiency, and neoantigen intratumoral heterogeneity [205]. With the goal of understanding the presence and role of immune cells in HGSC, in **Study IV,** we mapped the presence of macrophages and lymphocytes located within the tumor epithelium and the cell type-specific expression of PD-1/PD-L1, and their prognostic value in a cohort of 130 cases with advanced HGSC. In summary, we could confirm better outcome for patients whose tumor displayed higher expression of tumor infiltrating lymphocytes, whereas macrophage infiltration did not have a prognostic impact in our cohort. Further, higher expression of PD-1 by lymphocytes and PD-L1 by macrophages predicted longer survival. Similar results for PD-1/PD-L1 expression have been reported for ovarian carcinoma. This is apparently a paradox as the PD-1/PD-L1 pathway is a negative regulator of T cell activation promoting tumor growth and higher PD-L1 expression predicts inferior survival in several cancer forms [204]. However, we speculate that in HGSC, lymphocyte tumor infiltration is a prognostic marker, regardless of their PD-1/PD-L1 expression. We studied cases of newly diagnosed HGSC but studying the immune microenvironment in patients responding to ICIs in ongoing clinical trials, will hopefully be the key to refine the selection of patients eligible for therapy [206-211].

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Ovarian Carcinoma

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