Establishing a Pipeline for Personalized Therapy in Ovarian Cancer

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1 Introduction

In 2019, 7319 women in Germany received a diagnosis of ovarian cancer (Krebsregisterdaten, 2019). Ovarian cancer is the 7th most common cancer worldwide, afflicting 1-2% of women in their lifetime, and is responsible for a third of the malignancies affecting the female genital system and half of the resulting mortality (Torre et al., 2015). Despite important progress, the 5-year overall survival (OS) rate for patients with newly diagnosed advanced ovarian cancer varies between 73.2% for patients with a favorable mutational status to 32.3% for those with no targetable mutations (Ray-Coquard et al., 2023). While surgery and chemotherapy remain the cornerstones of treatment, the publication of the integrated genomic analyses of ovarian carcinoma by The Cancer Genome Atlas (TCGA) Research Group in 2011 has ushered in profound changes in the treatment landscape (Bell et al., 2011). Ovarian cancer is now sub-stratified using molecular markers of response that determine the appropriate treatment. As the ability of genetic analysis increases, the pharmacological treatment of ovarian cancer will continue to become more individualized.

Liquid Biopsy refers broadly to the analysis of biomarkers released by tumors into the bloodstream, most prominently circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). These can be isolated from the blood, cerebrospinal fluid (CSF), urine, and other body fluid compartments. Repeated sampling of tumor material through a standard blood draw is attractive due to its low invasiveness and is thereby able to provide a more dynamic assessment of tumor evolution than analysis of surgically excised tumor tissue. Used as a clinical tool, liquid biopsy can provide unique insight into tumor genomics; it is being closely studied for its potential to monitor tumor mutations, immune evasion, and resistance mechanisms as they evolve. In the age of targeted therapy, it will become increasingly important to expand our repertoire of analytic methods that allow us to react when molecular resistance develops in a subpopulation of tumor cells or to diagnose molecular recurrence before it is detected on imaging. Using liquid biopsy as the method to establish patient tumor-derived cell cultures is the next step in the evolution toward a truly personalized treatment. Cell cultures derived from circulating tumor cells harbor the potential to allow for in-vivo and in-vitro chemotherapy resistance testing before a new therapy regimen is selected for these patients.

1.1 The nature of ovarian cancer

Cancer is defined as the unchecked growth of cells due to the loss of important internal regulatory mechanisms that are imbedded on genes within the DNA of each cell's nucleus (Hanahan and Weinberg, 2011). When healthy cells divide, their entire DNA is copied and then split among daughter cells (Hustedt and Durocher, 2017). Errors in DNA replication occur at a rate of approximately 10^4-10^5 nucleotides polymerized, however, various DNA repair mechanisms are able to repair these damages to mitigate the spontaneous mutation rate in humans to approximately 10^{-10} per base pair per cell division (Drake et al., 1998, Hanahan and Weinberg, 2000, Preston et al., 2010). When these damages are not repaired and transferred to the subclone, this is termed a DNA mutation (Loeb et al., 1974). Mutations can occur anywhere in the genome but only a fraction of mutations lead to uncontrolled cell division (Moolgavkar and Knudson, 1981). The more mitotic cycles cells pass through in their lifetime, the more they will invariably acquire mutations due to an inherently imperfect DNA replication machinery. Mutations may also be passed from parents to children at conception or be induced through external factors, such as contact with radiation, or lifestyle factors, such as carcinogens in cigarette smoke. The location of the mutation in the genome is paramount. DNA mutations that occur in areas of the genome that are important for cells to divide (proto-oncogenes) or arrest division (tumor-suppressor genes) will have the effect that daughter cells increasingly leave the confines of the intricately controlled cell cycle (Tomasetti et al., 2017). When enough mutations accrue within a single cell, it will continue to divide, even though its internal regulatory machinery is damaged and DNA errors will be carried over to daughter cells. These cells may then lose their original functional role within the tissue and invade healthy tissue, disrupting its function as well. Finally, tumor cells will accrue mutations that allow it to locally enter the blood- and lymphatic system (intravasation) and exit at a distant site (extravasation) in the body to resume its growth there (metastasis) (Valastyan and Weinberg, 2011) (Figure 1).

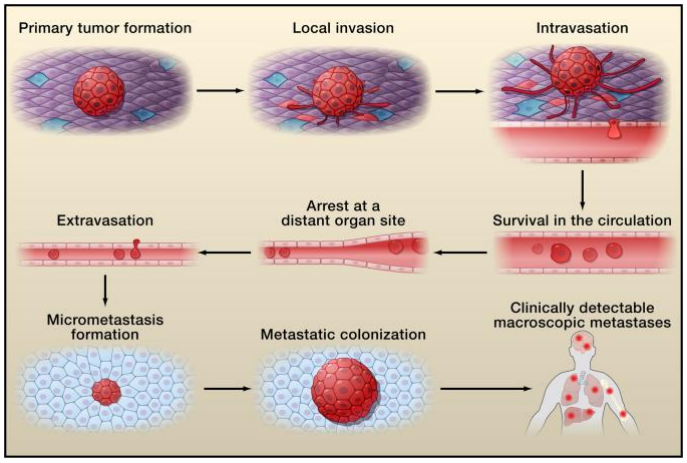

Figure 1. The Metastatic Cascade: The metastatic cascade describes the necessary steps for cancerous cells to leave their tissue of origin, enter and survive in the bloodstream, attach at a distant site in the body and finally continue their growth there. Source: https://www.cell.com/fulltext/S0092-8674%2811%2901085-3

Ovarian cancer as a whole has an incidence rate of 9.4 per 100,000 persons in the United States (Torre et al., 2018). Differentiation of ovarian tumors is based upon the cells from which they originate. Tumors arising from the epithelial cells are further differentiated into high-grade and low-grade serous, endometrioid, mucinous and clear cell ovarian cancer (Lheureux et al., 2019). Sex cord tumors arise from the stromal cells and represent a wide group of rare tumors including fibromas and Leydig cell tumors while germ cell tumors can be further differentiated into dysgerminomas, teratomas, yolk sac tumors and choriocarcinomas (Horta and Cunha, 2015, Pectasides et al., 2008). High-grade serous ovarian cancer (HGSOC) is the most frequent subtype of ovarian cancer with an incidence rate of 4.9 per 100,000 persons and is overwhelmingly malignant in nature (Lee et al., 2007, Torre et al., 2018). Distinct molecular pathways have been proposed to classify these tumors to be either low-grade or high-grade. Low-grade tumors show few mitotic figures and grow slowly, often evolving from adenofibromas or Borderline tumors. They are frequently *KRAS*, *BRAF* and *ERBB2* mutated but lack the *TP53* mutation that is ubiquitous in high-grade serous ovarian cancer (Bell et al., 2011). Their prognosis is significantly better than that of high-grade tumors. High-grade tumors have many mitotic figures, frequently metastasize to organs outside of the abdomen and their therapy remains a great challenge (Vang et al., 2009).

HGSOC is often detected late due to its relatively unspecific presentation; the most common presenting symptoms are bloating and fullness in the abdomen, lower back pain and fatigue (Olson et al., 2001). Vaginal ultrasonography of the abdomen and pelvis in conjunction with testing of the blood-based Cancer Antigen 125 (CA125) can aid in diagnosis but ultimately intraoperatively sampled tissue is needed for confirmation. The Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) staging system for ovarian, fallopian and peritoneal cancer is then used to determine the extension of these

tumors (Mutch and Prat, 2014). The FIGO system grades the tumor according to the anatomical structures that have been infiltrated (Table 1). If the tumor is detected while still localized, the cure rate is 88% (Sopik et al., 2015). However, due to the late and relatively unspecific symptoms described above, 51% of tumors are classified as FIGO III and 29% as FIGO IV at the time of diagnosis (Torre et al., 2018). At these stages, the tumor has infiltrated either the pelvis and/or peritoneum or metastasized to distant organs, respectively.

Stage I. Tumor confined to ovaries or fallopian tube(s)
T1-N0-M0 IA: tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1a-N0-M0 IB: tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1b-N0-M0 IC: tumor limited to one or both ovaries or fallopian tubes, with any of the following: IC1: surgical spill
T1c1-N0-M0 IC2: capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
T1c2-N0-M0 IC3: malignant cells in the ascites or peritoneal washings
T1c3-N0-M0
Stage II. Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer
T2-N0-M0 IIA: extension and/or implants on uterus and/or fallopian tubes and/or uterus
T2a-N0-M0 IIB: extension to other pelvic intraperitoneal tissues
T2b-N0-M0
Stage III. Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
T1/T2-N1-M0 IIIA1: positive retroperitoneal lymph nodes only (cytologically or histologically proven): IIIA1 (i) Metastasis up to 10mm in the greatest dimension IIIA1 (ii) Metastasis more than 10mm in the greatest dimension IIIA2: microscopic extrapelvic (above the pelvic rim) peritoneal involvement with or without positive retroperitoneal lymph nodes
T3a2-N0/N1-M0 IIIB: macroscopic peritoneal metastasis beyond the pelvis up to 2cm in the greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
T3b-N0/N1-M0 IIIC: macroscopic peritoneal metastasis beyond the pelvis more than 2cm in the greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
T3c-N0/N1-M0
Stage IV. Distant metastasis excluding peritoneal metastasis
Stage IVA: pleural effusion with positive cytology Stage IVB: parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)
Any T, any N, M1

Table 1. FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication (Prat, 2015).

1.2 *BRCA1/2* mutations in HGSOC

In Germany, all patients with HGSOC are routinely offered testing for somatic *BReast CAncer 1/2* (*BRCA1/2*) gene mutations. Somatic mutations, those occurring after fertilization, have been found in 8% of HGSOC patients (Hauke et al., 2019), while a further 21.8% have been found to be carriers of germline *BRCA1/2* mutations which are inherited (Manchana et al., 2019). In *BRCA1/2* mutated patients, the homologous recombination pathway is defective (Figure 2). An intact homologous recombination pathway serves to repair double-stranded DNA breaks that might otherwise lead to chromosomal instability and cell death. Patients with a germline *BRCA1/2* mutation are predisposed to developing ovarian cancer and at an earlier age because they only possess only one functional tumor suppressor gene unlike healthy adults who retain a functional copy when one is lost. The loss of *BRCA1/2* function, however, is also a positive prognostic marker for response to platinum-based chemotherapy and especially PARP-inhibitor treatment therapy in ovarian cancer. DNA damage caused by chemotherapy such as Carboplatin is not easily repaired in this subpopulation due to the defect in the homologous recombination pathway, causing higher rates of tumor cell death and improved outcomes in ovarian cancer patients with *BRCA1/2* mutations compared to wild-type patients (Moore et al., 2018).

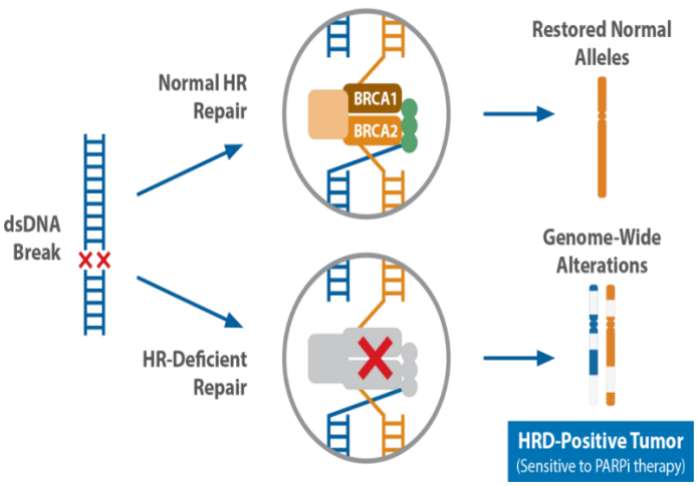

Figure 2. Homologous Repair Deficiency:
In healthy cells, double-stranded DNA breaks are repaired by the BRCA1/2 proteins that are expressed by the *BRCA1/2* genes, which act as tumor-suppressor genes. This process is impaired in patients with mutations to the BRCA1/2 genes, causing double-stranded DNA breaks to be passed onto daughter cells and increasing the likelihood of tumorigenesis.
Source: https://www.carislifesciences.com/products-and-services/molecular-profiling/profiling-technologies/whole-exome-sequencing/hrd/
dsDNA: double stranded DNA; HR; Homologous Recombination; HRD: Homologous Recombination Deficiency; BRCA1/2: BReast Cancer Gene 1/2.

Homologous recombination deficiency is not exclusive to *BRCA1* or *BRCA2* mutated cells. Several other genes, including *RAD51*, *ATM*, *BRIP1* and *CHEK2*, have been shown

to play a role in homologous recombination (Ledermann et al., 2016). Mutations in any of these genes, collectively termed *Homologous Recombination Deficiency* (*HRD*) genes, can be found in up to 50% of patients with wild-type *BRCA1/2* genes and have also been found to be a risk factor for the development of ovarian cancer while concurrently increasing the vulnerability of ovarian cancer cells towards the cytotoxic effects of pharmacotherapy (Konstantinopoulos et al., 2015).

Finally, it is not only permanent DNA mutations within the coding region of a gene that can hinder the production of a functional protein. DNA methylation refers to the reversible attachment of methyl groups to DNA bases without a change to the genetic sequence. This process is achieved by methyltransferase enzymes, which catalyze the attachment of methyl groups to CpG (5′-Cytosine-Phosphoguanine) repeats in the promoter region of genes. Their addition to the promoter region consequently hinders DNA polymerase enzymes from transcribing the gene sequence and thus from forming a functional protein (Singal and Ginder, 1999, Moore et al., 2013) (Figure 3). The role of DNA methylation in cancer, and specifically HGSOC, is the subject of intense research.

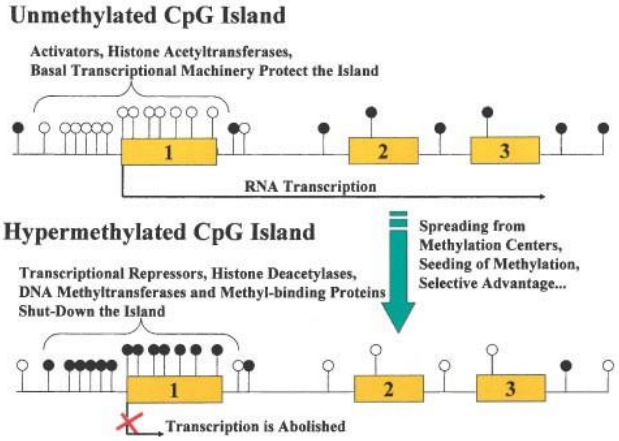

Figure 3. Hypermethylation of CpG islands in the genome blocks RNA transcription:
The covalent linkage of methyl groups to the promoter sequence of genes hinders the transcription machinery from attaching, thereby hindering the production of a functional protein.
Source: https://www.nature.com/articles/1205600
CpG : 5'—Cytosine—phosphate—Guanine—3'

1.3 The treatment landscape of HGSOC

The standard of care for newly diagnosed HGSOC is radical surgical resection followed by adjuvant chemotherapy (S3 Leitlinie Maligne Ovarialtumoren). When the cancer is detected**,** patients undergo cytoreductive surgery. This involves the surgical removal of the uterus, cervix, both ovaries and fallopian tubes, the omentum, and depending on the tumor stage also pelvic and paraaortic lymph nodes. Furthermore, if macroscopic invasion is seen intraoperatively, resection may be extended to organs such as the colon, peritoneum, appendix, or liver. Presence of residual tumor after primary cytoreductive surgery has been described as a poor prognostic factor (Polterauer et al., 2012).

Unfortunately, in more than 60% of cases, 1 cm or more of gross tumor is unable to be resected during surgery (Rutten et al., 2014).

The chemotherapy regimen, typically given as adjuvant to surgery but in select cases as neo-adjuvant treatment, consists of Carboplatin AUC 5 and Paclitaxel 175mg/m^2 for six cycles, lasting 3 weeks each. Carboplatin works by binding to and crosslinking strands of DNA, thereby inhibiting the process of DNA replication and eventually leading to cell apoptosis (Rabik and Dolan, 2007). When ovarian cancer cells have a defect in DNA repair, it is difficult for them to repair the damage inflicted by Carboplatin. Unfortunately, many patients who have responded to Carboplatin in the past become resistant during therapy, clinically knows as platinum resistance. These patients present a major challenge and significantly contribute to the mortality-to-incidence ratio in HGSOC (Khan et al., 2021). Paclitaxel, the second component of the primary chemotherapy regimen, stabilizes the microtubule complex in the metaphase stage of the cell cycle, preventing the formation of sister chromatids, also eventually leading to cell apoptosis (Lara-Gonzalez et al., 2012). Furthermore, in patients with FIGO IIIB-IV disease, the addition of a maintenance treatment is recommended. Bevacizumab and/or a PARP-inhibitor as can be considered (S3 Leitlinie Maligne Ovarialtumoren). Bevacizumab is a monoclonal antibody that selectively binds Vascular Endothelial Growth Factor (VEGF) and inhibits the formation of new blood vessels (neo-angiogenesis) which tumor cells, with high metabolic demand, rely on (Kazazi-Hyseni et al., 2010).

As mentioned above, the presence of a *BRCA1/2* mutation in ovarian cancer cells can be a positive predictive factor in response to treatment. Patients with somatic or germline *BRCA1/2* mutations and *HRD* mutations have been found to exhibit greater sensitivity toward Carboplatin therapy and have a longer progression free survival (PFS) and overall survival (OS) than wild-type patients (Pennington et al., 2014, Vencken et al., 2011, Yang et al., 2011). Importantly, Poly (ADP-Ribose) Polymerase (PARP)-inhibitors are approved for therapy in patients with *BRCA1/2* or other *HRD* associated mutations (Farmer et al., 2005). More recent studies have also been able to demonstrate a benefit concerning progression-free survival (PFS) and overall survival (OS) in patients without *BRCA1/2* or *HRD* mutations who responded to first-line platinum-based chemotherapy (González-Martín, 2019). The PARP enzyme is involved in multiple DNA repair pathways and plays a central role in stabilizing the DNA replication fork (Ray Chaudhuri and Nussenzweig, 2017). Recent research has shown that PARP inhibitors work by inhibiting the action of *PARP1* to resolve transcription replication conflicts between the

DNA replication fork and RNA polymerases, thereby leading to double stranded breaks (Petropoulos et al., 2024). With the homologous repair pathway disrupted by mutations and the PARP pathway pharmacologically inhibited, patients with *BRCA1/2* or *HRD* mutated HGSOC have seen a significant improvement in outcomes in recent years. The addition of adjuvant maintenance PARP inhibitor therapy has improved PFS by 70% for these patients (Moore et al., 2018). A recent study has shown patients with *BRCA*-mutated, advanced HGSOC receiving the PARP-inhibitor Olaparib achieving a PFS of 56 months while those with wild-type tumors have fared much more poorly with a PFS of 16.9 months (Banerjee et al., 2021, Kim et al., 2019a). As patients with *BRCA1/2* and *HRD* mutations have benefitted so greatly from the addition of PARP-inhibitors to their therapy regimen, it is of great significance whether wild-type patients, including those not responding to first line platinum chemotherapy, could also benefit from this class of drugs (Stefansson et al., 2012). Mutations of *BRCA1/2* or *HRD* lead to less or no production of enzymes which can repair double-stranded DNA breaks, making the cancer more susceptible to chemotherapy-induced DNA damage. However, the effects of protein transcription suppression by means of methylation of the *BRCA1/2* genes, not DNA mutation, is still being investigated in ovarian cancer. In a large meta-analysis, no difference in survival between methylated and non-methylated *BRCA1* promoter could be found in patients with HGSOC that were treated with platinum chemotherapy (Kalachand et al., 2020). *BRCA1/2* promoter hypermethylation, however, is a dynamic process in which the methyl groups suppressing the transcription of DNA-repair proteins may be both acquired and lost and acquired again throughout the course of therapy. The mammalian mechanism of active DNA demethylation was demonstrated in 2009, when the TET family of DNA dioxygenases was shown to be able to convert 5-methyl-Cytosine (5mC) to 5-hydroxymethyl-Cytosine (5hmC) and further to 5-formyl-Cytosine (5fc) and 5-carboxy-Cytosine (5caC) (Kriaucionis and Heintz, 2009, Tahiliani et al., 2009). In a prospective study of 69 ovarian cancer patients it could be demonstrated that patients with *BRCA1* promoter methylation and those with methylation conversion during the course of therapy achieving significantly longer PFS with surgery and primary chemotherapy than patients whose *BRCA1* genes remained unmethylated (Elazezy et al., 2021). We hypothesize that serially testing the *BRCA1/2* and *HRD* genes for methylation status before, during, and after therapy can identify both responders and non-responders to pharmacologic agents such as Carboplatin and PARP-inhibitors before they are given. Tumor tissue sent to pathology during the operation cannot supply the dynamic

information that is required to make these decisions. We suspect there may be susceptibility windows during which patients are likely to be responsive to certain chemotherapies and unresponsive to others. Consequently, we may be missing important patient cohorts that could stand to benefit from already existing therapies.

1.4 Potential applications of Liquid Biopsy

Liquid Biopsy refers to the analysis of tumor material found in the fluid departments of the body, including the blood, urine, cerebrospinal fluid, ascites, pleural effusions, and semen (MacGregor et al., 2021, Husain et al., 2017, Leary et al., 2020, Escudero et al., 2020). A liquid biopsy can be obtained from a routine peripheral blood draw, urine and semen sample, or CSF draw and is attractive due to its low invasiveness and ease of use. Specifically, liquid biopsy can be broken into its components, or rather, the molecular markers that are isolated in the laboratory after the patient's blood has been drawn. In recent years, various surrogate markers of tumor activity have been isolated and identified from the blood (Figure 4).

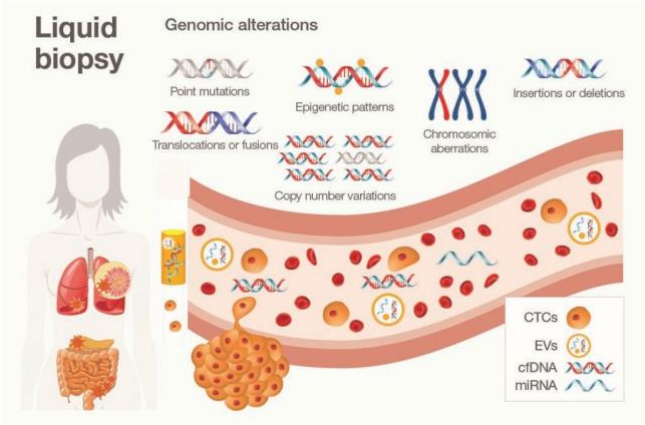

Figure 4. Components of Liquid Biopsy: CTCs, EVs, cfDNA and miRNA are just some of the surrogate markers that are shed into circulation by the tumor. Analyzing their DNA can allow for comprehensive profiling of mutations, chromosomal aberrations and epigenetic patterns.
Source: https://www.thermofisher.com/blog/behindthebench/precision-medicine-cancer-heterogeneity-liquid-biopsy/
CTCs: Circulating Tumor Cells ; EVs: Extracellular Vesicles ; cfDNA: cell-free DNA ; miRNA: microRNA

Cell-free DNA (cfDNA) can be found in the plasma of healthy individuals due to cells losing their cell and nuclear membrane due to having undergone apoptosis or necrosis or even by active secretion (Diehl et al., 2008, Hu et al., 2021). cfDNA is composed of free-floating, double-stranded fragments, ranging between approximately 100 to 100,000 base pairs in length but is most frequently isolated in the range of 100-250 base pairs (Giacona et al., 1998, Fan et al., 2010). Each cfDNA fragment contains only small regions of the genome, however, when measured in large quantities, the fragments cumulatively can relay important information about the genetic status of the origin tissue (Snyder et al., 2016). Circulating tumor DNA (ctDNA) is a subfraction of this cfDNA that is released

by tumor cells and is thus not found in healthy individuals. ctDNA has a short half-life, circulating from only between several minutes up to 2 hours before it is cleared by enzymes such as DNase I, plasma factor VII-activating protease (FSAP) and factor H (Kustanovich et al., 2019). Due to this, ctDNA can be a valuable tool by providing dynamic insights into current tumor genomics. At the time of writing, the following ctDNA-based tests have been approved by the FDA after having demonstrated reliable identification of certain actionable mutations: Cobas EGFR Mutation Test v2 test for *EGFR* mutations and deletions in NSCLC (2016), *therascreen* PIK3CA RGQ PCR for *PIK3CA* mutations breast cancer (2019), Guardant360 CDx for *EGFR* and *KRAS* mutations in NSCLC (2022) and F1 Liquid CDx for *EGFR*, *MET*, and *ALK* mutations in NSCLC, *BRCA1/2* and *ATM* mutations in prostate cancer, *BRCA1/BRCA2* mutations in ovarian cancer, and *PIK3CA* mutations in breast cancer (2023) (Vellanki et al., 2023, Malapelle et al., 2017, Narayan et al., 2021, Bauml et al., 2022, Anscher et al., 2021). The Epi proColon test can help screen for colon cancer by detecting methylation of the *SEPT9* promoter on ctDNA fragments from plasma samples of patients at risk (Molnár, 2015). Molecular markers of therapy resistance such as *KRAS* mutations in colorectal cancer patients receiving anti-*EGFR* therapy have also been reliably identified by sequencing of ctDNA fragments (van Helden et al., 2019). Increasingly, ctDNA is also being investigated as a tool to help guide clinical decision making; a recent study revealed that ctDNA levels can be used to distinguish stage 2 colon cancer patients who are subject to benefit from adjuvant chemotherapy; ctDNA-guided chemotherapy was administered less frequently than when guided by clinician's choice with no adverse effects on PFS (Tie et al., 2022). Finally, ctDNA is being investigated for its potential to help predict clinical outcomes. Quantitative measurements of ctDNA have been found to correlate with outcomes in a wide variety of cancers including lung, bladder, melanoma, and lymphoma amongst others (Raja et al., 2018, Lee et al., 2019, Sidaway, 2018). Minimal residual disease (MRD), in the form of cancer specific ctDNA being detectable in the plasma after completed treatment has the potential to predict recurrence in patients with esophageal, colorectal, breast and lung cancer before associated symptoms develop or the recurrence becomes detectable on imaging studies (Olsson et al., 2015, Abbosh et al., 2017, Tie et al., 2016, Azad et al., 2020). Finally, in ovarian cancer, detection of ctDNA has been linked to shorter PFS, while the detection rate of frequently mutated genes such as *PIC3CA* and *KRAS* was higher in patients with advanced disease and positive peritoneal cytology (Ogasawara et al., 2020). The detection of a *TP53* mutation, one of the most

frequently mutated genes in HGSOC, in plasma ctDNA samples has been proposed as an indicator of response to treatment (Kim et al., 2019b).

Circulating tumor cells (CTCs) are intact tumor cells that detach from the primary tumor or from its already seeded metastases, extravasated into the bloodstream and float individually or in clusters with other CTCs in the blood before they undergo apoptosis or form a new metastatic site. Unlike ctDNA, whose individual fragment length ranges from 150 to 200 base pairs, CTCs carry a complete tumor genome within their nucleus (Underhill et al., 2016). Isolating and sequencing the tumor genome allows for an even more comprehensive panel of molecular investigations. Like the corollary studies of quantitative measurements of ctDNA and outcome mentioned above, the detection of CTCs in peripheral blood draws has also been associated with poorer outcomes in patients with NSCLC, breast, and prostate cancer (Hanssen et al., 2018, Wang et al., 2017, Lorente et al., 2018). Even the detection of a single CTC has been shown to predict a worse PFS and OS in bladder and breast cancer compared to patients in whom none could be detected (Rink et al., 2011, Lucci et al., 2012). In a study of 216 ovarian cancer patients, the presence of just two or more CTCs per 7.5 ml of blood was correlated with a significantly reduced PFS compared to patients where just one or none could be found (Poveda et al., 2011). While CTC analysis carries great potential, there are still major obstacles that must be overcome until it can be reliably translated into clinical practice.

The CellSearch® platform relies on the positive selection of cells expressing the Epithelial Cell Adhesion Molecule (EpCAM) on their surface. EpCAM is expressed on many epithelial derived cancers, including HGSOC, and cannot be found on the surface of leukocytes which greatly outnumber the CTCs in the peripheral blood (Rao et al., 2005). Considering the high background of polymorphonuclear cells to a single CTC, detection sensitivity remains a major hurdle. Detection via EpCAM is further complicated by epithelial-to-mesenchymal transition (EMT), a process by which epithelial cells are able to change their phenotype enabling detachment from the basal membrane, bloodstream extravasation and conferring increased resistance to apoptosis (Roche, 2018). When epithelial cancer cells make this transition to a mesenchymal phenotype, the surface marker EpCAM may be downregulated in the process and thus their detection may be missed leading to false negative results (Hyun, 2016). To overcome this hurdle, another technique for CTC capture has been developed. Parsortix® is a system used to separate CTCs from other peripheral blood cells based on their significantly larger cell size. The peripheral blood is fed into the automated Parsortix® machine where it is sucked through

tubing that leads to a sorting cassette which separates the cells based on their diameter. The larger cells can thus be isolated onto a microscope slide and subsequently genetically analyzed for tumor-specific mutations.

As mentioned above, liquid biopsy is not limited to the intravascular compartment. The role of liquid biopsy has also been investigated in ovarian cancer patients presenting with ascites, with results showing a high recovery and mutational concordance between ctDNA in ascites and the tumor tissue (Han et al., 2020). It also seems compelling to use urine as source of ctDNA, since this is a routine clinical investigation even less invasive than a blood draw. To date, there are no studies evaluating the role of liquid biopsy from urine in ovarian cancer. Its application seems promising, however, as urine cfDNA analysis has successfully been used to for dynamic tracking of mutations of EGFR mutations in NSCLC and detection of recurrence in urothelial bladder cancer (Chen 2016; Togneri 2016). Circulating RNA, tumor miRNA and extracellular tumor vesicles are, among others, further potential liquid biopsy markers that are being explored. Since their role has not yet been as clearly defined as that of CTCs and ctDNA, they will not be further discussed here (Figure 4).

The Cancer Antigen 125 (CA-125) is a biomarker with decades of proven clinical utility in the treatment of patients with HGSOC. While its sensitivity and specificity are too limited to be of use as a screening marker for the general population, it can be used for follow-up in patients with already diagnosed HGSOC or to determine the likelihood of new HGSOC in conjunction with other clinical and sonographic findings (Grossman et al., 2018). Elevated CA-125 can predict worse outcomes and elevations following completed therapy may be used to monitor for clinically occult recurrence (Nebgen et al., 2019).

There is great potential for the use of liquid biopsy in ovarian cancer. Transabdominal ultrasound-guided biopsy or even laparoscopic biopsy of a suspected recurrence is often not possible due to adhesions after primary cytoreductive surgery. Liquid biopsy is being investigated to fill this gap as a potential early marker for recurrence, perhaps even before the tumor grows large enough to be detected on imaging.

1.5 CTCs in cell culture

In 1977, Sanger sequencing, also aptly named the chain-termination method, was developed, marking the first time DNA could be reliably sequenced. With Sanger sequencing, the DNA fragments from the region of interest are combined in a reaction tube with a DNA primer, DNA polymerase and nucleotide analogs modified to lack the 3' hydroxyl group that is required for 5' to 3' strand extension. After DNA fragment extension by the polymerase enzyme and chain termination due to the lack of an adjoining hydroxyl-group, electrophoresis separates the individual fragments based on their length. The unique fluorescent signal emitted from the four different nucleotide bases is then captured. In this way, the position of each nucleotide within the DNA region of interest can be reliably identified (Sanger et al., 1977). While Sanger sequencing remains an accurate and widely used sequencing technique, it can only sequence one DNA fragment at a time. Next Generation Sequencing (NGS) is an evolution of Sanger sequencing that also relies on the chain termination principle but can sequence millions of DNA fragments in parallel, making it much faster and less time-consuming (Margulies et al., 2005).
Coinciding with this increased availability of sophisticated genetic analysis there has been a growing effort to not only analyze the CTCs extracted from blood but to culture them and develop them into a (semi-) permanent cell culture. One problem hindering the incorporation of liquid biopsy into clinical practice has been low CTC recovery (Joosse et al., 2015). A background of 100 million peripheral blood mononuclear cells (PBMCs) to a single CTC has been described (Alix-Panabières et al., 2012). This discrepancy is even greater in a non-metastatic setting (Rink et al., 2012, Rack et al., 2014). To increase CTC yield, it would be necessary to increase the amount of blood drawn from often severely ill patients which may not be well tolerated. Establishing a permanent primary cell culture of millions of clonal cells expanded from one or more CTCs of a cancer patient has therefore been considered a potential solution to many of these problems. Establishing a CTC-based cell culture would greatly expand the repertoire of genetic analyses that can be carried out (Cayrefourcq et al., 2015, Koch et al., 2020b). NGS would allow for the tracking of mutations which are driving tumor growth at that moment in time. Furthermore, a theoretical infinite number of clonal tumor cells could be tested in vitro with various combinations of chemotherapies. Following this line of inquiry, susceptibility may then be correlated with the molecular status of the tumor. Thus, the potential information to be gained from the successful culturing of a liquid biopsy based primary cell culture of ovarian cancer cells is vast. In a proof-of-concept study involving 10 patients, ovarian cancer cells were isolated, positively identified and transiently

enriched in culture. In vitro chemosensitivity testing of the cell culture was predictive of response to hypoxic abdominal perfusion (HAP) therapy (Guadagni et al., 2020). An additional interesting facet of CTC culture is the potential to glean information about the tumor heterogeneity. Expanding different tumor cell subpopulations would allow for a better understanding of resistance to therapy and the mechanisms of recurrence. With large subpopulations of cells that are cultured separately, we may be able to sequence these cells and determine why some parts of the tumor respond well to our therapy whereas others do not and drive recurrence.

1.6 Aims of the study

In this study we aimed to show that CTCs and ctDNA derived from the plasma and ascites of HGSOC patients can be successfully and reliably isolated using the methods described in detail below, to investigate whether these liquid biopsy biomarkers can be correlated with clinical status and to determine if they can be used to predict clinical outcomes. Further, we aim to describe a novel, cost-effective and easily replicable method of establishing and maintaining a cell culture from liquid biopsy-derived CTCs from HGSOC.

2 Materials and Methods

2.1 Study population

Female patients over 18 years of age visiting the Department of Gynecology at the University Medical Centre Hamburg-Eppendorf between July 2020 and April 2022 were considered as potential subjects of this study. The initial cohort comprised two main groups: patients presenting with newly diagnosed, probable HGSOC and patients with probable recurrence of HGSOC. All patients included in the study gave written informed consent (ethical approval number: PV5392).

The patients included in the final cohort had a pathologically confirmed diagnosis of HGSOC. Patients with concurrent secondary malignancies were excluded from this study as were patients who did not receive either cytoreductive surgery and / or platin-containing chemotherapy.

2.2 Sample collection

In the cohort of patients with newly diagnosed, primary HGSOC, blood was drawn one day before and within three days after the primary cytoreductive operation. Thereafter, blood was drawn before application of the next chemotherapy dose in intervals of 3-4 months or earlier if recurrent disease was suspected.

In the group of patients with recurrent disease, blood was drawn before the initiation of a new regimen of chemotherapy and then in 3–4-month intervals or earlier if progression was suspected. Follow-up blood sampling was discontinued in patients who did not receive a platinum agent as part of their new chemotherapy regimen.

Blood was drawn from the median cubital vein, dorsal metacarpal veins, or from a central venous catheter into sterile 7.5 ml EDTA tubes after a first tube was discarded to avoid sample contamination with keratinocytes from the skin (catalog no.: 01.1605.001, Sarstedt). Each patient received a blood draw of 2 tubes with 7.5 ml of blood in each for a total of 15 ml of blood.

If a patient presented with ascites, this was obtained for cfDNA and CTC analysis either intraoperatively or in the clinic only when medically indicated.

Tumor tissue removed during surgery was obtained either directly from the surgeon or the pathology department whenever feasible.

5 patients with metastatic disease, FIGO stage IV, were considered as candidates for processing with a spiral microfluidic device (Warkiani et al., 2014). These patients received a blood draw in the range of 49 ml to 60 ml before administration of their next

chemotherapy dose. The selection of these 5 patients was based primarily on the advanced stage of their tumor it has been shown that the number of CTCs in the blood correlates with extent of disease and that CTCs are more likely to be detected in the context of metastatic disease (Bidard et al., 2014, Bidard et al., 2016). The five patients were therefore selected in order to increase the probability of capturing sufficient viable tumor cells in the blood to allow for the creation of (semi-) permanent cell cultures.

2.3 Sample processing

Blood from patients with newly diagnosed ovarian cancer was analyzed for CTC count and ctDNA. Sample processing was carried out in the laboratory within 1 hour of the blood draw.

2.3.1 Plasma Separation

One EDTA tube containing 7.5 ml of patient blood from primary patients and two EDTA tubes containing 7.5 ml of blood from recurrent patients were centrifuged for 10 minutes at 300 x g. The supernatant plasma from each tube was then centrifuged again for 10 minutes at 1800 x g to remove residual cells. The plasma was then frozen in 15 ml Falcon tubes at -80°C until further use.

2.3.1.1 cfDNA isolation

The InviGenius® Plus (Invitek Molecular) is a fully automated platform that allows for nucleic acid purification based on magnetic bead technology. Patient plasma and ascites samples of 4 ml volume were loaded into the machine and the samples were lysed. The nucleic acids are then bound to magnetic particles from rods that enter the sample and automatically transfer them through the process of extraction, purification, and elution.

2.3.1.2 cfDNA measurement

The Qubit™ dsDNA BR and HS Assay Kit (Catalog no. Q32853 and Q33231, Invitrogen, Thermo Fisher Scientific) was employed to measure the amount of cfDNA that was extracted from each 4 ml plasma sample. These assays contain target-selective dyes which emit fluorescence only when bound to their double stranded DNA target. The intensity of fluorescence is translated to dsDNA concentration using a standard calibration curve.

2.3.2 Parsortix®

The size-based enrichment of CTCs from primary patients was performed with the Parsortix® PC1 clinical system (ANGLE Biosciences). Within two hours of the blood draw, the samples were filled into 10 ml EDTA vacutainers and connected to the machine. The blood was sucked into the machine and the cells were passed through a cassette with a 6.5 µm gap size, previously validated by our laboratory on breast, lung, and gastro-intestinal cancer samples, for size-based sorting (Koch et al., 2020a). Intact CTCs were sequestered in the cassette due to their larger diameter and lesser compressibility compared to other blood cells. Once the entire sample has passed through the cassette, the tubing of the machine was flushed with PBS solution and the CTCs captured in the cassette caught on a single microscopy slide. The slide was dried at room temperature overnight, then wrapped in tinfoil and frozen at -80°C.

2.3.3 CellSearch®

Affinity-based CTC isolation for primary patients was achieved using the FDA-cleared CellSearch® system (Menarini Silicon Biosystems) which relies on the ferromagnetic interaction of CTCs with the Epithelial Cell Adhesion Molecule (EpCAM; CD326). EpCAM is a transmembrane glycoprotein which has been shown to be overexpressed in malignant ovarian cancers, thereby making it useful as a surface tumor-marker (Kim et al., 2003). At each follow-up, 7.5 ml of primary patient blood sample was filled into a CellSave® preservative tube and processed in the semi-automated CellSearch® machine within 72 hours. First, the system aspirates and discards the red blood cells and plasma. The sample is then resuspended with a ferromagnetic fluid containing antibodies targeting EpCAM. EpCAM positive cells migrate to a magnetic surface and are thereby separated from the other cells in the sample. Here, the cells are stained with keratin antibodies specific to epithelial cells, CD45, an antibody specific to leukocytes, and DAPI (4′,6-diamidino-2-phenylindole), a marker for an intact nuclear membrane. The cells are then incubated in the dark for 20 minutes during which time they align along a magnetic pole and are optically scanned. The system counts a CTC when the object shows positivity for epithelial cell-specific keratins and the nuclear marker DAPI but not the leukocyte marker CD45. Objects with a nucleus not surrounded by cytoplasm or those with borderline epithelial-specific keratin positivity are termed “suspicious objects” and are not counted.

2.3.4 Ficoll-Paque

Peripheral blood mononuclear cell (PBMC) and CTC separation for recurrent patients was achieved using a density-based cell separation technique (Joosse 2012). After removal of the plasma supernatant from the patient blood sample, as described above, the corpuscular blood components were resuspended in 30 ml 1xPBS to which 20 ml of Ficoll (catalog no. 171440, Avantor) was added. This mixture was spun at 400 x g for 30 minutes without acceleration or deceleration at 4°C. The interface layer was discarded and the supernatant, forming a thin, cloudy middle layer in the tube containing the tumor cells and leukocytes, was carefully pipetted into a 50ml Falcon tube. This tube was then filled with PBS and subsequently centrifuged at 400 x *g* for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 30ml PBS and once again centrifuged at 400 x g for 10 minutes. The supernatant was discarded and the pellet was then resuspended in 900 µl of RPMI-medium and 100 µl of DSMO before being frozen in a CryoTube at -80°C.

2.3.5 Ascites processing

Ascites was obtained from clinicians in a sterile 20 ml syringe. The ascites was then transferred to a 50 ml Falcon tube and centrifuged for 5 minutes at 300 x g. The supernatant was transferred to another 50 ml Falcon tube and re-centrifuged for 10 minutes at 2600 x g. 4 ml of the supernatant was transferred into a 20 ml Falcon tube and frozen at -80°C for later cfDNA extraction using InviGenius®, as described above.

The pellet containing potential CTCs from the first centrifugation was resuspended in 900 µl of RPMI-medium and 100 µl of DSMO before being frozen in a CryoTube at -80°C.

2.4 Liquid-Biopsy-based cell culture

Patients with advanced disease, ≥ FIGO IIIC, were deemed potential candidates for the establishment of a CTC-based primary cell culture. These patients received blood draws in the range of 49 to 60 ml to enhance the probability of CTC isolation (Table 11).

2.4.1 Sample processing for cell culture patients

The blood from these selected patients was collected in six to eight sterile 7.5 ml EDTA tubes via standard peripheral blood draw and processed in the laboratory within 1 hour.

At first, the blood was transferred from the smaller EDTA tubes into 50 ml Falcon tubes. Then, Red Blood Cell Lysis Buffer was added in a 3:1 ratio to the blood (catalog no. 158904, Qiagen). The mixture was incubated for 5 minutes before being centrifuged at 1000 x g for 5 minutes. After discarding the supernatant, the pellet was resuspended in 10 ml of RBC buffer. The process was repeated twice until there was no macroscopic trace of erythrocytes in the sample.

The remaining pellet was resuspended in 20 ml of Dulbecco's Phosphate-Buffered Saline solution (DPBS). Next, the viable cell concentration in the sample was measured using the ViCell XR (Beckman Coulter). The concentration of the cells in the sample was then titrated with DPBS to measure $5x10^6$ cells/ml.

2.4.2 Slanted Spiral Microfluidic Device

The slanted spiral microfluidic device, designed by Warkiani et al., was used to separate the larger tumor cells from the smaller PBMCs in the DPBS sample (Warkiani et al., 2014). The sample was drawn into a sterile 20 ml syringe and connected to an infusion pump set at a speed of 1.7 ml $min.^{-1}$. The sample was then continuously infused through the tubing into the slanted spiral microfluidic device where smaller cells were retained in the periphery and larger cells exited through the central pore. The cells from the central pore were collected in a 50 ml Falcon tube.

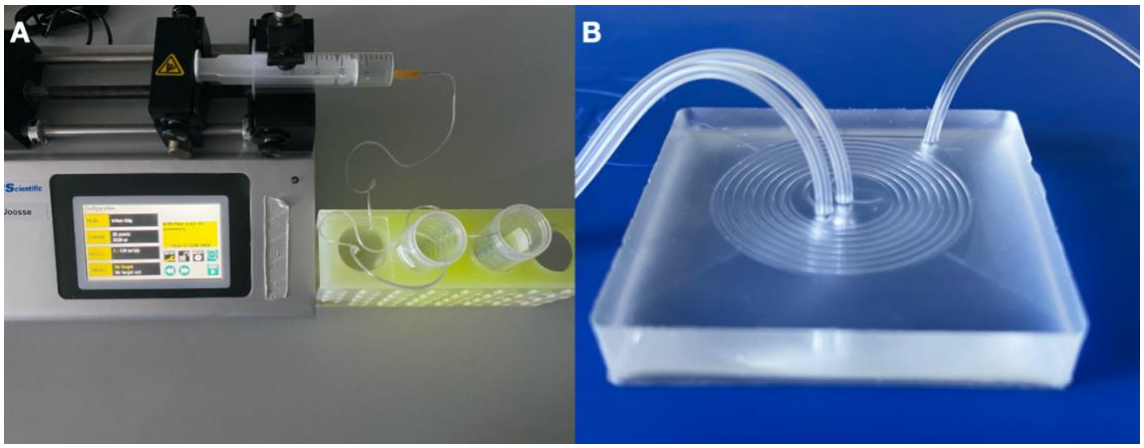

Figure 5. Experimental set-up of slanted spiral microfluidic device:
A) The patient sample, previously cleared of red blood cells and titrated with DPBS to $5x10^6$ cells/ml, is infused at a rate of 1.7 ml min-1 into the slanted spiral microfluidic device at the periphery. Large and small cells are passed through the two tubes emanating from the center and are collected in two separate 50 ml Falcon tubes. B) The right tube carries blood mononuclear cells and CTCs to the microwell. CTCs are forced to the inside wall due to inertial lift force and Dean drag force (Warkiani et al., 2014).

2.4.3 CTC cell culture

2.4.3.1 Preparing agarose gel wells

PDMS master molds with imprinted microwells were used as templates to create agarose wells on which the CTCs could later be cultured. This technique has been demonstrated by Tu et al. to facilitate cell-to-cell contact, enabling growth signaling to take place, and aid in the formation of 3-dimensional cell clusters (Tu et al., 2014). The PDMS molds were placed in the center of microscopy slides and covered with funnel chambers that were fixed into slide carriers. A 2% agarose gel was prepared in an Erlenmeyer flask by addition of 0.2g of SeaKem® LE Agarose to 10 ml of distilled water (catalog no. 50004, Lonza, Rockland, ME USA). The mixture was heated in a microwave until a fluid state with no bubbles was observed. 600 µl of agarose gel mixture was then carefully pipetted into the space surrounding each PDMS master mold. The slide carriers were then covered with Parafilm (catalog no. 13-374-13, Bemis Company Inc PM1000) and centrifuged for 4 minutes at 500 x g before being placed in the fridge at 3°C. For each patient sample, 12 agarose wells were molded.

Figure 6. PDMS master molds to create agarose molds for CTC culturing: The master molds are each fixed in a Cytospin funnel with the microwells facing upwards. The funnel is covered with Parafilm and cooled at 4°C overnight. The agarose mold is then separated from the master mold under sterile conditions and used as a surface onto which CTCs and cell culture medium is pipetted.

2.4.3.2 Establishing the CTC cell culture

Under the hood of a sterilized cell culture bench, the agarose wells were carefully separated from the PDMS master molds. Each agarose well was then placed in a chamber of the 12-well plate. 200 µl of DPBS containing the enriched cells was then pipetted into each agarose well with the microwells facing toward the liquid. Then, 2 ml of DMEM medium with 10% fetal bovine serum, 1% glutamate, 1% streptomycin, and 1% penicillin was carefully pipetted around each of the wells until overflowing the DPBS containing CTSs.

2.4.3.3 Maintenance of the cell culture

The well plates containing the cells were maintained in a sterile incubator with 21% O_2 and 5% CO_2 at 37°C. The cell culture medium was changed on day one and subsequently every 7 days. For this, medium was carefully pipetted from the periphery, taking care not to disturb the cells in the microwells.

The 12-well plate was routinely observed under a bright light microscope (ZEISS Primovert) throughout the time of culturing and pictures were taken using ZEISS AxioVision 4.9.1 Software. The cell cultures were monitored for probable tumor cell expansion for a period of six weeks. They were discarded if no growth was observed throughout this time.

2.4.3.4 Harvesting the cell culture

In patient CTC cell cultures with suspected expansion of tumor cells, a laboratory member unaffiliated with the research was consulted for an unbiased assessment. The cells were observed until a confluency was achieved, then the cells were split and a fraction was isolated for further investigation. After removal of the medium the wells were washed with DPBS. After washing, the cell suspension was pipetted onto microscopy slides and observed under the microscope. The slides were then dried overnight and frozen for further processing. Additionally, washed DPBS, containing cultured cells, was filled into 5 ml Eppendorf tubes and frozen for later analysis.

2.4.3.5 CTC immunofluorescence detection

Cultured cells that had been placed onto microscopy slides were fixed with 0.5% Paraformaldehyde (PFA) (Merck) in DPBS for 10 minutes. They were then washed three times with DPBS. Then, for 45 minutes, the cells were left in 10% AB Serum (catalog no. 805135, Bio-Rad Laboratories) to provide growth factors, nutrients, and trace elements to ensure vitality of the cell. Immunofluorescence staining was carried out with the nuclear stain DAPI (1:700) (catalog no. D1306, Invitrogen, Thermo Fisher Scientific), the epithelial stain AE1/AE3 labeled with Alexa Fluor® 488 (1:500) (catalog no. 53-9003-80, eBioscience™, Invitrogen, Thermo Fischer Scientific) and the anti-human leukocyte CD45 antibody labeled with Alexa Fluor® 647 (1:700) (catalog no. 304018, Biolegend) overnight at 4°C (Table 2).

Table 2. Utilized primary antibodies

Specificity	Antigen	Dilution	Source
AT regions of DNA	DAPI (4′,6-diamidino-2-phenylindole)	1:700	Invitrogen, Thermo Fischer Scientific, catalog no. D1306
Anti-Pan-keratin	AE1/AE3 Alexa Fluor® 488	1:500	eBioscience, Invitrogen, Thermo Fischer Scientific, catalog no. 53-9003-80
Anti-human leukocyte CD45 (monoclonal)	CD45 (LCA, leukocyte common antigen)	1:700	Biolegend, catalog no. 304018

2.4.3.6 Single tumor cell picking

Tumor cells were manually identified under a fluorescence microscope (Zeiss Axio Observer). Using ES-Blastocyst injection pipettes with a 15µm inner diameter (BioMedical Instruments), single cells that expressed positivity for both the epithelial stain AE1/AE3 and the nuclear stain DAPI but not the leukocyte stain CD45 were picked and transferred into 0.5 ml PCR tubes. The PCR tubes were frozen at -80°C until further use.

2.5 Analyzing the culture enriched tumor cells

2.5.1 Whole Genome Amplification

The DNA of the cultured tumor cells was amplified using the AmpliOne™ Whole Genome Amplification (WGA) Set (catalog no. WG001R, Silicon Biosystems). After cell membrane lysis, the DNA is digested with a restriction enzyme and adaptors attach to the DNA fragments, preventing differential amplification of fragments and loss of alleles. Amplification takes place in the same tube using a single PCR primer for all fragments and a DNA polymerase with 3'-5' proofreading capability. Every allele and gene locus from the single tumor cells is thus amplified and represented as a library of 0.2-2 kb fragments. The total DNA in each sample after amplification was assessed using the Qubit™ dsDNA BR and HS Assay Kit (catalog no. Q32853 and Q33231, Invitrogen, Thermo Fisher Scientific).

2.5.2 Multiplex PCR

Multiplex PCR was used to determine the quality and confirm the human origin of the DNA after amplification. Before starting the PCR, remnant primers and enzymes from the WGA reaction were removed using the PCR purification kit (catalog no. 28104, Qiagen). Human female DNA (catalog no. G1521, Promega, Thermo Fisher Scientific) was used as a positive control, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as a control of DNA from human origin and our amplified single cell DNA was used as the template DNA. According to a validated protocol by van Beers et al., 100, 200, 300 and 400 basepair fragments from nonoverlapping targets in the GAPDH gene were used as the reaction primers (van Beers, 2006). 1 U *Taq* DNA-polymerase (Invitrogen cat. 18038-26), 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 75 mM KCl and 0.2 mM dNTPs were added to the reaction mixture with 0.1333µM of each of the eight forward and reverse primers. PCR was performed for 4 minutes at 94°C, 35 cycles each of 1 minutes at 94°C, 1 minute of 56°C and 3 minutes at 72°C, followed by 7 minutes at 72°C ending at 15°C (van Beers, 2006). Electrophoresis was used to determine the lengths of the PCR amplification products to assess the suitability of the samples for downstream NGS. To prepare the gel, 2 µl of ethidium bromide and 2 g of SeaKem® LE Agarose (catalog no. 50004, Lonza Bioscience) was added to 100 ml of TAE buffer (catalog no. B49, Thermo Scientific). Each chamber was filled with 10 µl of DNA and 2 µl of DNA loading dye (catolog no. R0611, Thermo Scientific). A voltage of 100 V was applied for 1 hour. The electrophoresis gel was placed under a UV light and photographed (Figure

13). DNA samples with three or more bands were deemed suitable candidates to be submitted for NGS.

2.5.3 Next Generation Sequencing

The DNA amplification products were analyzed by rapid whole genome sequencing (rWGS) using DNA Nanoball Sequencing (DNB) (BGI, Denmark). FastQ files were processed using fastp v0.20.1 (Chen 2018) to remove Ampli1 adapters and low quality reads, followed by aligning to the human genome hg38 using BWA MEM2 v2.2.1 (Vasimuddin et al., 2019). Samtools v1.13 (Danecek et al., 2021) was used for converting to BAM file, fix mate coordinates, sort, mark duplicates, and produce an index file. Copy number alterations were detected using control-FREEC v11.6 (Boeva et al., 2011).

3 Results

3.1 Patient Cohort

In the time between July 1, 2020, and June 1, 2022, 67 patients with suspected newly diagnosed and 59 patients with probable recurrent HGSOC were recruited for this study. In the cohort of patients with probable primary HGSOC, 5 patients were excluded due to not receiving surgery. Another 18 patients were excluded due to a histology other than HGSOC. Of these, the following were excluded: 6 unclear, 3 low-grade serous ovarian cancer, 2 endometrioid, 1 mucinous, 1 clear cell, 1 neoplasia appendix, 1 leiomyoma, 1 Borderline, 1 small intestine, 1 Müllerian mixed tumor. Finally, 9 patients were excluded due to problems with blood analysis or missing relevant clinical data. The final cohort of HGSOC patients with primary disease consisted of 35 patients.

In the cohort of patients with probable recurrent HGSOC, 5 were excluded due to no recurrence being detectable. One patient was excluded due to having received chemotherapy before the blood draw. One patient was excluded due to a histology other than HGSOC. 4 patients died before sufficient samples could be collected. 18 patients were excluded due to problems with blood draw analysis or missing relevant data. The final cohort of HGSOC patients with recurrent disease consisted of 31 patients (Figure 7).

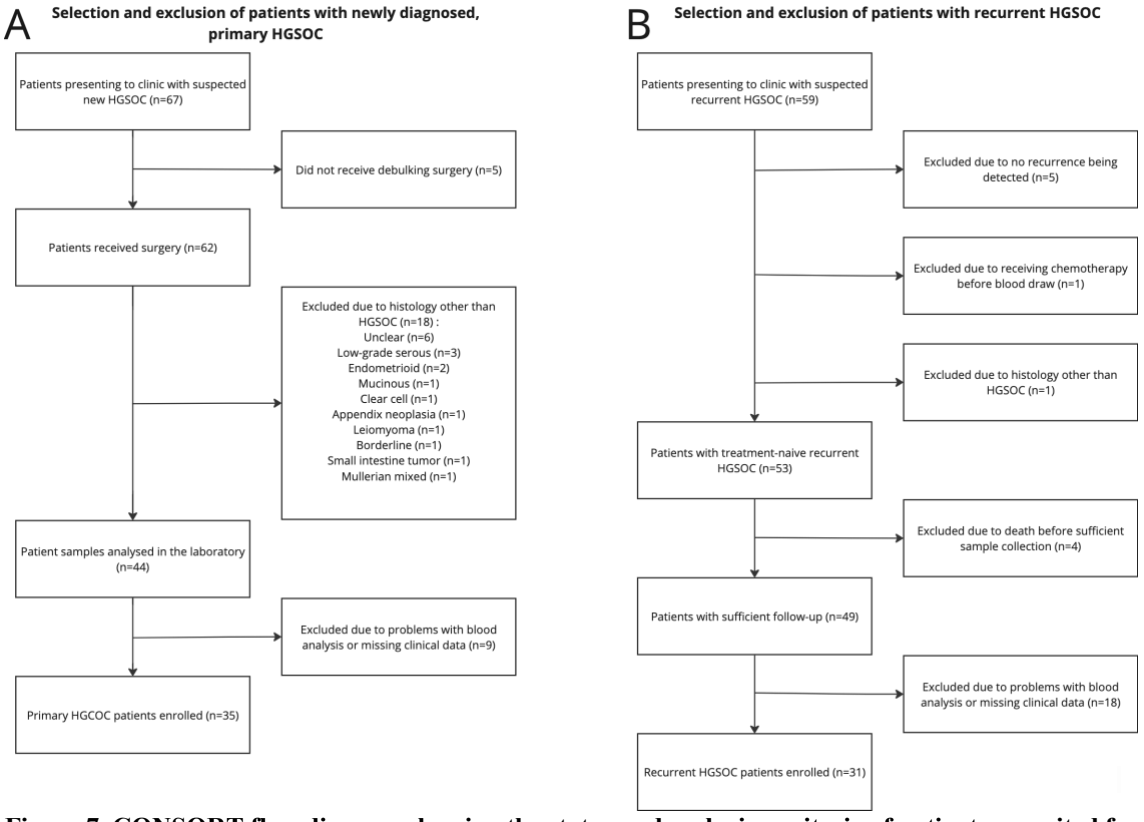

Figure 7. CONSORT flow diagram showing the status and exclusion criteria of patients recruited for this study: A) Enrollment and selection of patients presenting to the clinic with probable newly diagnosed, primary HGSOC B) Enrollment and selection of patients presenting to the clinic with probable recurrent HGSOC.

Our final primary and recurrent HGSOC cohort were of comparable ages, showing no statistically significant difference using Student's two-sample t-test ($p<0.61$) (Table 3.) 13 patients in the primary and 15 in the recurrent cohort had metastases either at time of diagnosis or during observation. The sites of metastases in the primary cohort were: liver 2, umbilical 2, skeletal 1, lymphatic 2, dermatologic 1, rectosigmoid 1. The sites of metastases in the recurrent cohort were: liver 5, pleura 3, lymphatic 3, stomach 2, kidney 1, lung 1, mediastinum 1, spleen 1. 4 primary patients experienced a recurrence during the time of observation. 2 deaths in the primary and 2 deaths in the recurrent patient cohort occurred during the defined study period. Where clinical information regarding mutational status was available, no patients were found to have concurrent *BRCA1/2* und *HRD* mutations.

The clinical characteristics of the two groups were compared using the Fisher's Exact test (Table 3). There was no significant difference in the mutational status (*BRCAwt*, *BRCAmt*, *HRDmt*) between the primary and recurrent cohort ($p=0.1$). There was no significant difference between the two groups regarding FIGO stage ($p=0.36$), T-stage ($p=0.082$), N-stage ($p=0.43$), presence of metastasis ($p=0.42$), residual tumor ($p=0.22$), lymphatic invasion ($p=0.59$), venous invasion ($p=0.085$), death ($p=1$) or PARP-inhibitor treatment ($p=0.1004$).

Table 3. Demographic and clinical characteristics of the patient cohort

	Primary	**Recurrent**	**p-value**
Number	35	31	
Mean age (years)	63.0	64.2	0.61
FIGO stage			0.36
I-IIIB	11% (4/35)	19% (6/31)	
IIIC	57% (20/35)	58% (18/31)	
IV	31% (11/35)	16% (5/31)	
Unknown	0% (0/35)	6% (2/31)	
T-stage			0.82
T1	3% (1/35)	0% (0/31)	
T2	11% (4/35)	6% (2/31)	
T3	86% (30/35)	84% (26/31)	
Unknown	0% (0/35)	10% (3/31)	
N-stage			0.43
N0	34% (12/35)	19% (6/31)	
N1	43% (15/35)	45% (14/31)	
Nx	23% (8/35)	10% (3/31)	

Unknown	0% (0/35)	26% (8/31)	
Metastases			0.42
None	63% (22/35)	52% (16/31)	
At diagnosis	9% (3/35)	19% (6/31)	
During observation	29% (10/35)	29% (9/31)	
Residual tumor			0.22
No (macroscopic complete resection)	40% (14/35)	58% (18/31)	
Re-laparotomy			
Yes		36% (16/31)	
Lymphatic invasion			0.59
L0	43% (15/35)	26% (8/31)	
L1	57% (20/35)	52% (16/31)	
Unknown	0% (0/35)	22% (7/31)	
Venous invasion			0.085
V0	89% (31/35)	58% (18/31)	
V1	3% (1/35)	13% (4/31)	
Vx	9% (3/35)	0% (0/31)	
Unknown	0% (0/35)	29% (9/31)	
Recurrence/ Progression			
Yes	11% (4/35)		
Death			1
Yes	6% (2/35)	6% (2/31)	
PARP inhibitor treatment			0.1
Yes	46% (16/35)	77% (24/31)	
No	37% (13/35)	33% (7/31)	
Unknown	17% (6/35)	0% (0/31)	
Mutational status			0.1
BRCA Mutated	14% (5/35)	23% (7/31)	
HRD Mutated	17% (6/35)	0% (0/31)	
Wild type	29% (10/35)	45% (14/31)	
Unknown	40% (14/35)	32% (10/31)	

In the primary cohort, tissue and ascites samples could be obtained for 38 and 33 patients respectively. Preoperative blood samples for later cfDNA processing were obtained from 44 patients, postoperatively from 34 patients, then 15 and 7 samples and first and second outpatient follow-up. In the recurrent cohort, only 1 tissue sample and 6 ascites samples could be obtained, largely because these patients had either been treated externally or

their primary treatment had taken place many years before. 55 blood samples were collected at first visit to our clinic before administration of the first cycle of a new chemotherapy regimen. 34 blood samples were taken at second follow-up, 23 at third, 9 at fourth 5 at fifth and one sample at sixth, seventh and eighth follow-up (Table 4). Of these patients, some were intentionally not followed up for reasons described above (Table 3). Some patients had more samples drawn than others, owing largely to the fact that their chemotherapy regimens necessitated more visits to the clinic than others or they experienced recurrence/progression within our observational period.

Table 4. Liquid biopsy samples collected from patients with primary and recurrent HGSOC

Sample Type	Samples from primary patients	Samples from recurrent patients
Tissue	38	1
Ascites	33	6
Blood (preoperative)	44	
Blood (postoperative)		
1. Follow-up	34	55
2. Follow-up	15	34
3. Follow-up	7	23
4. Follow-up	0	9
5. Follow-up	0	5
6. Follow-up	0	1
7. Follow-up	0	1
8. Follow-up	0	1

3.2 cfDNA in the ascites fluid and plasma of patients with primary disease

Ascites fluid was obtained and the cfDNA concentration was measured from 29 HGSOC patients, of whom 23 were patients with primary and 6 were patients with recurrent disease. The cfDNA of the ascites samples was matched and compared to that of plasma samples from the same patients. The mean cfDNA in primary patient ascites was 285.9 ng/ml of plasma. For primary patient plasma, the mean cfDNA pre-operatively, post-operatively, and at 1st, 2nd, and 3rd follow-up was 5.2, 11.2, 5.8, 6.4 and 2.4 ng/ml, respectively (Table 5). Statistical analysis was carried out using a paired t-test. A significantly higher concentration of cfDNA per ml of plasma could be recovered from primary patient ascites than from plasma taken preoperatively, postoperatively, at 1st and 2nd follow-up ($p<0.0001$) and at 3rd follow-up ($p<0.0005$). The concentration of cfDNA was also significantly higher in postoperative compared to preoperative plasma

($p<0.0185$) (Figure 8). These results show a higher concentration of cfDNA in the ascites than in the plasma of patients with HGSOC and suggest ascites fluid may be a more optimal source for downstream anaylsis of cfDNA alterations.

Table 5. cfDNA concentrations in the ascites fluid and plasma of patients with primary HGSOC

Patient	Ascites cfDNA (ng/ml ascites)	cfDNA preoperatively (ng/ml plasma)	cfDNA postoperatively (ng/ml plasma)	cfDNA at 1st follow-up (ng/ml plasma)	cfDNA at 2nd follow-up (ng/ml plasma)	cfDNA at 3rd follow-up (ng/ml plasma)
1	67	2.6	3.7	3.4	3.4	
2	1466.7	8.5	16	5.6	5.7	
3	163.4	11.5	7.6	5.9	6.7	3.1
4	74.6	4	4.4	3.6	5.3	
5	168.4	2.2	9.4			
6	42.1	2.4		5.2	4.9	
7	110.5	1.6	1.7	8	7.6	2.9
8	162	6.3	13.3	7.2	2.8	1.3
9	42.7	4	9.7	9.3	13.5	
10	138	1.5	2.4	5	2.4	2.1
11	46.1	1.6	2.1	7.2		
12	130.3	3.3	15	1.1	11.9	
13	310	4.5	9.2	4		
14	980.6	8.3	11.8	5.1	6.6	
15	718	3.9		16.9		
16	141	TLTD*	9			
17	19.9	2.7	8.6	5.2		
18	65.8	8.9	25.5			
19	133	5.5	17.4	3		
20	252	TLTD	8.7			
21	510.8	4	13.8	4.7		
22	174.9	21.9	31.8	4.8		
23	636	9.5	19.6			
Mean	285.9	5.2	11.5	5.8	6.4	2.4

*TLTD (too low to determine) values were counted as 0.

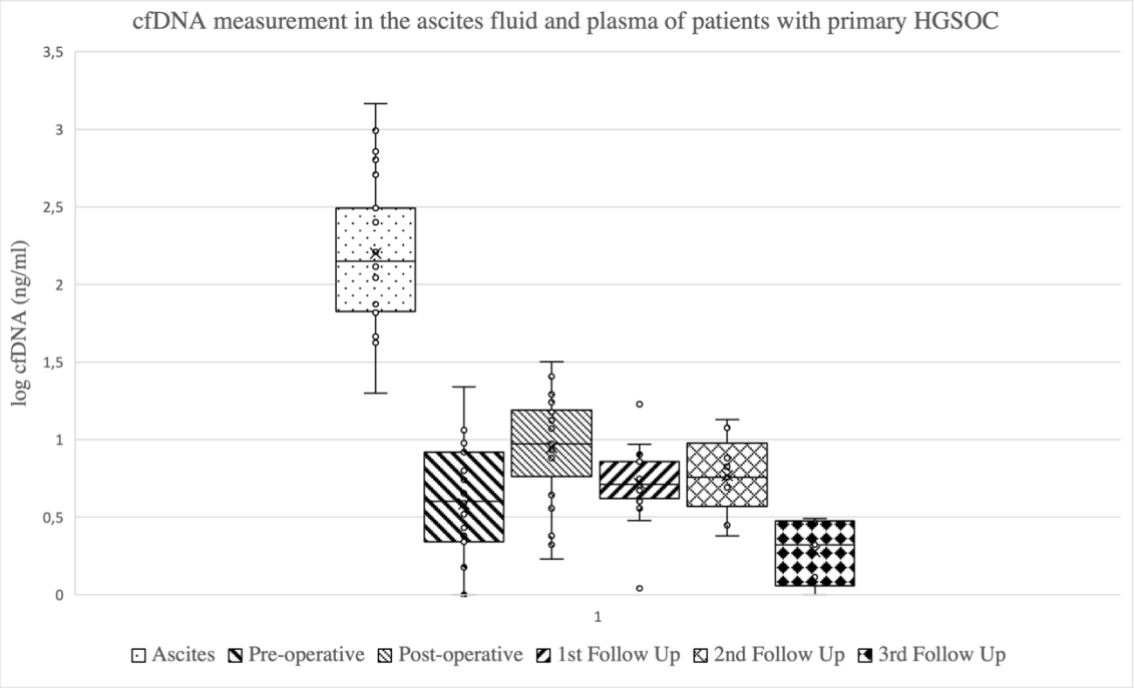

Figure 8. cfDNA in the ascites fluid and plasma of patients with primary HGSOC: A significantly higher concentration of cfDNA per ml of plasma could be recovered from primary patient ascites than from plasma taken preoperatively, postoperatively, at 1st and 2nd follow-up ($p<0.0001$) and at 3rd follow-up ($p<0.0005$). The concentration of cfDNA was also significantly higher in postoperative compared to preoperative plasma ($p<0.0185$)

3.3 cfDNA in the ascites fluid and plasma of patients with recurrent disease

The mean cfDNA concentration measured in the ascites of patients with recurrent disease was 127.8 ng/ml of plasma. In plasma samples from the same patients with recurrent disease, the mean cfDNA measurement at 1st, 2nd and 3rd follow up was 6.7, 5.4 and 2.8 ng/ml, respectively (Table 6).

Statistical analysis was carried out using a paired t-test. There was a statistically significant difference in the amount of cfDNA measured from ascites versus from plasma at 1st follow-up ($p<0.0019$) and 3rd follow-up ($p<0.0152$). There was no difference in the amount of cfDNA at 1st versus at 2nd follow-up ($p<0.3752$), 2nd versus 3rd follow-up ($p<0.3513$) or 1st versus 3rd follow up ($p<0.8426$) (Figure 9). Here we show, albeit in a smaller cohort, that the concentration of cfDNA in the ascites of patients with recurrent HGSOC is higher than that in the plasma, concurrent with the results in the primary disease cohort.

Table 6. cfDNA measurement in the ascites and plasma of HGSOC patients with recurrent disease

Patient	Ascites cfDNA (ng/ml ascites)	cfDNA at 1st follow up (ng/ml plasma)	cfDNA at 2nd follow up (ng/ml plasma)	cfDNA at 3rd follow up (ng/ml plasma)
1	63.6	1.6	3.9	3.4
2	79.8	3.5	2.8	3.8
3	83.6	3.5	8	2
4	372	3.9	12.4	1.3
5	143	25.6		
6	25	2.2	TLTD	3.5
Mean	127.8	6.7	5.4	2.8

*TLTD (too low to determine) values were counted as 0.

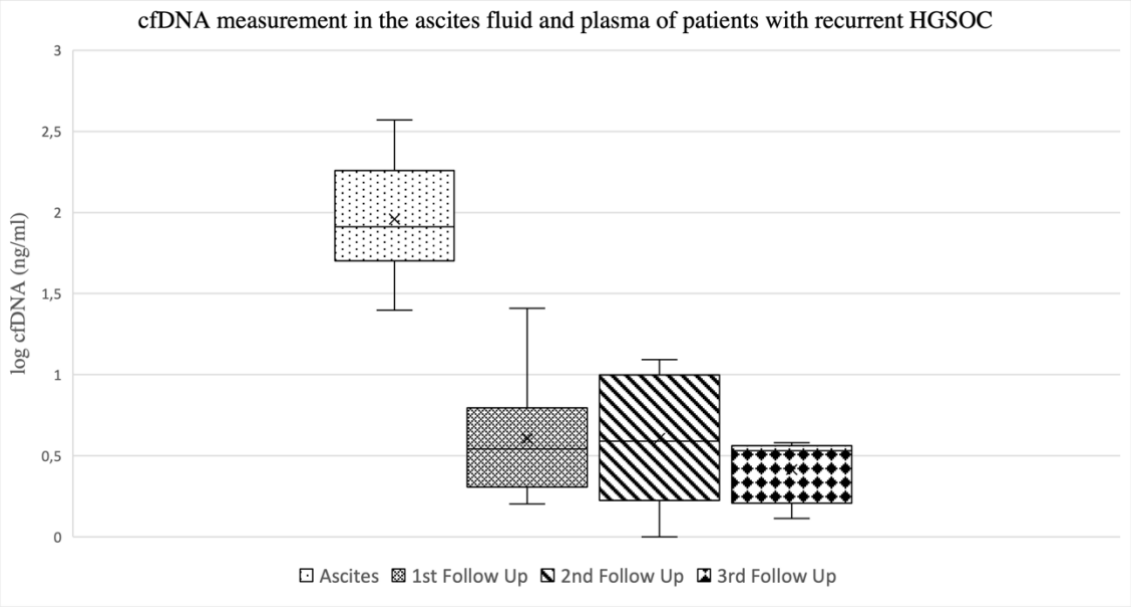

Figure 9. cfDNA in the ascites fluid and plasma of patients with recurrent HGSOC: A statistically significant difference in the amount of cfDNA measured from ascites versus from plasma at 1st follow-up ($p<0.0019$) and 3rd follow-up ($p<0.0152$). There was no difference in the amount of cfDNA at 1st versus at 2nd follow-up ($p<0.3752$), 2nd versus 3rd follow-up ($p<0.35138$) or 1st versus 3rd follow up ($p<0.8426$).

3.4 CTC count in primary patients using CellSearch®

102 CellSearch® analyses were carried out during the time of observation on patients with primary HGSOC. Pre-operative, post-operative, and follow-up samples were collected throughout the course of treatment (Figure 10). 31 pre- and post-operative samples were matched and evaluated (Table 7). Follow-up samples are available for 25 of these patients (Figure 10). At least one CTC could be found in 15 of 31 preoperative (48%) samples, 11 of 31 postoperative (35%) samples, 6 of 25 (24%) samples at first follow-up, and 2 of 13 (15%) samples at second follow-up. The median CTC count was 0 for each group.

Figure 10: Longitudinal analysis of CTC count throughout the course of treatment

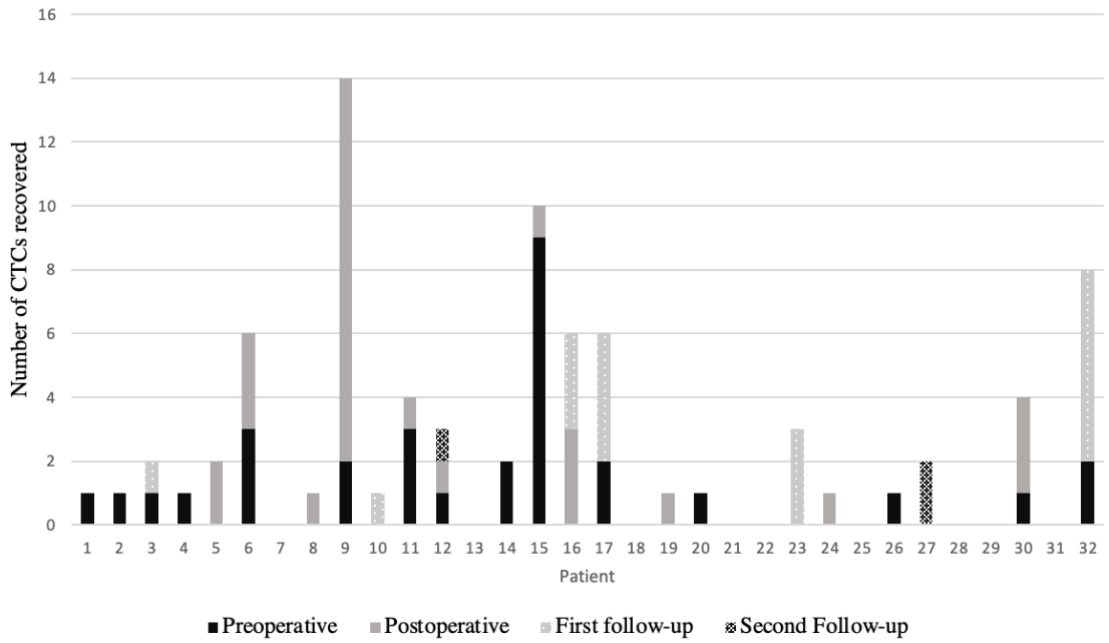

Figure 10. The number of CTCs detected in primary HGSOC patients was measured using Parsortix® pre-operatively, post-operatively and at first and second follow-up.

Using a Chi-square test, we found a statistically significant decrease in the number of CTCs recovered at second follow up compared to pre-operatively ($p<0.018$). We did not find a difference in the number of CTCs pre- and postoperatively ($p<0.31$). Furthermore, no difference was found between the number of CTCs postoperatively and at first follow up ($p<0.23$) and at second follow-up ($p<0.15$) or pre-operatively versus at first follow up ($p<0.42$).

Table 7. Longitudinal analysis of CTC count throughout the course of treatment

Patient	Pre-operative CTC number	Post-operative CTC number	1st follow-up visit	2nd follow-up visit
1	1	0	0	
2	1	0	0	
3	1	0	1	
4	1	0		
5	0	2	0	0
6	3	3	0	0
7	0	0	0	0
8	0	1	0	0
9	2	12		
10	0	0	1	0
11	3	1		

12	1	1	0	1
13	0	0	0	0
14	2	0	0	0
15	9	1	0	0
15	0	3	3	0
16	2	0	4	
17	0	0	0	
18	0	1	0	0
19	1	0	0	
20	0	0	0	
21	0	0		
22	0	0	3	
23	0	1	0	
24	0	0	0	0
25	1	0	0	
26	0	0	0	2
27	0	0		
28	0	0		
29	1	3	0	
30	0	0		
31	2	0	6	
Mean	0.97	0.91	0.72	0.23
Median	0	0	0	0

3.5 Correlating CTC count with clinical outcome in patients with primary disease

The influence of residual tumor after cytoreductive surgery, presence of metastasis, recurrence or progression and death during the observation period were correlated with the CTC count. Using Fisher's Exact Test, no statistically significant difference regarding the presence of residual tumor (p=028), presence of metastasis, (p=0.71) recurrence or progression (p=0.09), and death (p=0.21) could be found between patients with no CTCs at baseline and patients with one or more CTCs at baseline (Table 8).

Table 8. Correlating the finding of CTCs (n>=1) compared to no CTCs (n=0) at baseline with patient clinical outcome

	CTC 0 at baseline	**CTC ≥1 at baseline**	**p-value**
Residual Tumor			
R0	53% (8/15)	31% (4/13)	0.28
R1	47% (7/15)	69% (9/13)	

Metastasis			
M0	60% (9/15)	69% (9/13)	0.71
M1	40% (6/15)	31% (4/13)	
Recurrence/ Progression			
Yes	0% (0/15)	23% (3/13)	0.09
No	100% (15/15)	77% (10/13)	
Death			
Yes	0% (0/15)	15% (2/13)	0.21
No	100% (15/15)	85% (11/13)	

Using Fisher's Exact Test no significant difference could be found between patients where no CTCs were recovered at any time during the observation period and patients where one 1 or more CTCs were recovered at any point after chemotherapy regarding the presence of residual tumor (p=0.28), presence of metastasis (p=0.58), recurrence or progression (p>0.99), and death (p>0.99). The clinical data from two patients was not available and they were excluded from this analysis (Table 9).

Table 9. Correlating the finding of constantly low (n=0) or increased (>=1) CTC count after chemotherapy on patient clinical outcome

	CTC constantly 0	**CTC>=1 after chemotherapy**	**p-value**
Residual Tumor			
R0	67% (4/6)	25% (2/8)	0.28
R1	33% (2/6)	75% (6/8)	
Metastasis			
M0	50% (3/6)	75% (6/8)	0.58
M1	50% (3/6)	25% (2/8)	
Recurrence/ Progression			
Yes	0% (0/6)	0% (0/8)	>0.99
No	100% (6/6)	100% (8/8)	
Death			
Yes	0% (0/6)	0% (0/8)	>0.99
No	100% (6/6)	100% (8/8)	

Further, there was no significant difference between patients whose CTC count decreased after cytoreductive surgery and patients whose CTC count increased after surgery regarding the presence of residual tumor (p=0.61), presence of metastasis (p>0.99),

recurrence or progression (p=0.47), and death (p>0.99). The clinical data from two patients was not available and they were excluded from this analysis (Table 10).

Table 10. Correlating the effect of decreased or increased CTC count after surgery on patient clinical outcome

	CTC decreased after surgery	CTC increased after surgery	p-value
Residual Tumor			
R0	25% (2/8)	43% (3/7)	0.61
R1	75% (6/8)	57% (4/7)	
Metastasis			
M0	62.5% (5/8)	57% (4/7)	>0.99
M1	37.5% (3/8)	43% (3/7)	
Recurrence / Progression			
Yes	25% (2/8)	0% (0/7)	0.47
No	75% (6/8)	100% (7/7)	
Death			
Yes	12.5% (1/8)	14% (1/7)	>0.99
No	87.5% (7/8)	86% (6/7)	

3.6 Selection of patients for the spiral microfluidic device

The blood of 5 HGSOC patients with advanced disease who exhibited metastasis was used for processing in the slanted spiral and then in cell culture. The clinical characteristics of the 5 patients chosen for CTC cell culture are listed below (Table 11.)

Table 11. Clinical data and experimental results from spiral patients

Patient ID	1	2	3	4	5
Age (years)	54	62	71	58	55
Histology	HGSOC	HGSOC	HGSOC	HGSOC	HGSOC
Disease status	Recurrent	Recurrent	Recurrent	Primary	Primary
Volume of blood draw (ml)	56	52.5	60	49	56
First expansion noted	Day 37	Day 26	None	None	None
FIGO stage	Unknown	FIGO IIIC	unknown	FIGO IVA	FIGO IIIC
Metastatic site	Hepatic	Peritoneal	Peritoneal	Pleura	Peritoneal
Initial staging	pT2a N0 cM0 G3	pT3c pN1b L1 V0 Pn0	pT3c L0 V0 G3	pT3c L1 V0 Pn0 G3	pT3c pN1b L1 V0 Pn0
BRCA1/2 and *HRD* Mutation Status	unknown	*BRCA1*	negative	*BRCA1*	negative
Prior Chemotherapy	Carboplatin/ Paclitaxel; Carboplatin/ Doxorubicin; Bevacizumab	Carboplatin/ Paclitaxel; Bevacizumab; Carboplatin/ Doxorubicin; Olaparib; Carboplatin/ Doxorubicin	Carboplatin/ Paclitaxel; Bevacizumab; Carboplatin/ Doxorubicin	Carboplatin/ Paclitaxel; Bevacizumab	Carboplatin/ Paclitaxel; Bevacizumab
Residual Tumor	Macroscopic tumor free	Less than 1cm	Macroscopic tumor free	Less than 1cm	Macroscopic tumor free
Prior medical history	Diabetes mellitus II Arterial Hypertension Heart Failure	Sinustachy-cardia Hypercholesterinemia	Atrial fibrillation Hypothyroi-dism Neuroborre-liosis Arterial Hypertension	Arthrosis, Hypothyroi-dism Arterial Hypertension	none

Expansion of a subpopulation of large cells distinct from the surrounding leukocytes was observed in the cell culture of patients 1 and 2 on days 37 and 26, respectively.

3.7 Establishment of a semi-permanent CTC cell culture from two HGSOC patients

Blood from patient 1 was determined to hold promise for further culturing as she had extensive primary disease that had progressed after primary treatment in the form of a single liver metastasis. She was initially staged as G3 pT2a N0 cM0 and was intraoperatively determined to be free of any macroscopic tumor. Her prior treatment included Carboplatin and Paclitaxel following primary resection followed by Carboplatin in conjunction with pegylated liposomal Doxorubicin plus four applications of Bevacizumab as treatment for her recurrence, which presented with liver metastasis.

56 ml of blood was drawn on day 0, before a new chemotherapy regimen was to be initiated. The blood sample was processed and cultured according to the protocol described in the methods. On day 37, rapid expansion of cells was seen under the microscope in 2/12 microwells (Figure 11). On day 38, these two agarose microwells were turned with the pore facing down. This was done to facilitate adhesion of the cells to the floor of the 12-well plate. On day 44, the cells adherent to the floor of the 12-well plate were lysed and transferred to a 6-well plate and a T25 flask. The cells were further monitored for confluency. On day 62, a fraction of the cells were harvested. The medium from all agarose wells in which there had been growth was pipetted into a 20 ml Falcon tube. The tube was centrifuged and the supernatant was discarded. The pellet was then washed with 2 ml PBS and transferred to cytospin funnels. These were centrifuged and the microscopy slide was left to dry overnight.

The remaining cells were left in culture for further expansion. On day 139 it was decided that the growth of the cells had stalled therefore the cell culture was discarded.

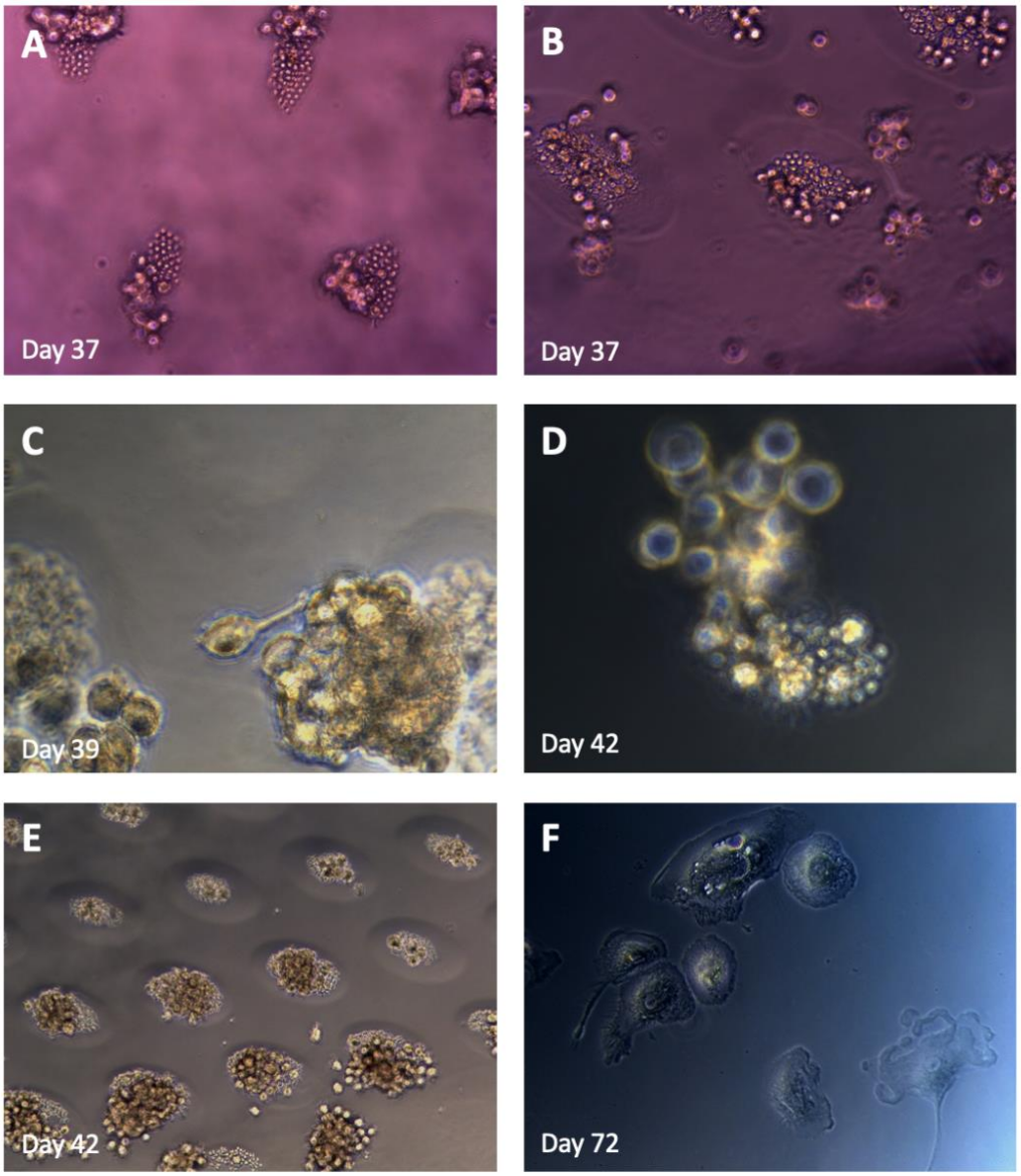

Figure 11. CTCs derived from patient 1 visualized under the microscope on agarose molds containing indentations to promote cell growth and cell-to-cell signaling: A-B) On day 37 in cell culture, proliferation of a population of large cells was noted for the first time C) On day 39, formation of microtubules was observed D-E) On day 42 large cells completely filled the agarose microwells and were visualized migrating to other microwells. F) A subset of cells was transferred from agarose wells to the floor of a 12-well plate on day 71. Cells are adherent and extensions are visible.

Blood from patient 2 was determined to hold promise for further culturing as the patient had not achieved complete macroscopic resection during primary cytoreductive surgery and had a second recurrence following treatment for extensive primary disease involving the peritoneum (FIGO IIIC). Further, a somatic *BRCA1* mutation was identified in this patient. She was staged as pT3c, pN1b, L1, V0, Pn0. Her prior treatment included Carboplatin and Paclitaxel in combination with Bevacizumab for primary disease,

Carboplatin and pegylated liposomal Doxorubicin for the first recurrence and adjuvant maintenance treatment with Olaparib, a PARP-inhibitor, thereafter.

52.5 ml of blood was drawn on day 0 before the initiation of chemotherapy for the second recurrence. The blood sample was processed and cultured according to the protocol described in the methods. On day 26, rapid expansion of cells was seen under the microscope in 2/12 microwells (Figure 12). On day 27, the agarose microwells were turned with the pore facing down. This was done to facilitate adhesion of the cells to the floor of the 12-well plate. On day 35, the cells adherent to the floor of the 12-well plate were lysed using trypsin enzyme and transferred to a 6-well plate and a T25 flask. The cells were further monitored for confluency. On day 46, the decision was made to harvest a fraction of the cells. The medium from all agarose wells in which there had been growth was pipetted into a 20 ml Falcon tube. The tube was centrifuged and the supernatant was discarded. The pellet was then washed with 2 ml PBS and transferred to Cytospin funnels. These were centrifuged and the microscopy slide was left to dry overnight.

The remaining cells were left in culture for further expansion. On day 116, it was decided that the growth of the cells had stalled. The medium was switched to RPMI complete medium with Hydrocortisone and Choleratoxin in hopes of salvaging the cells. On day 123, the cell culture was abandoned.

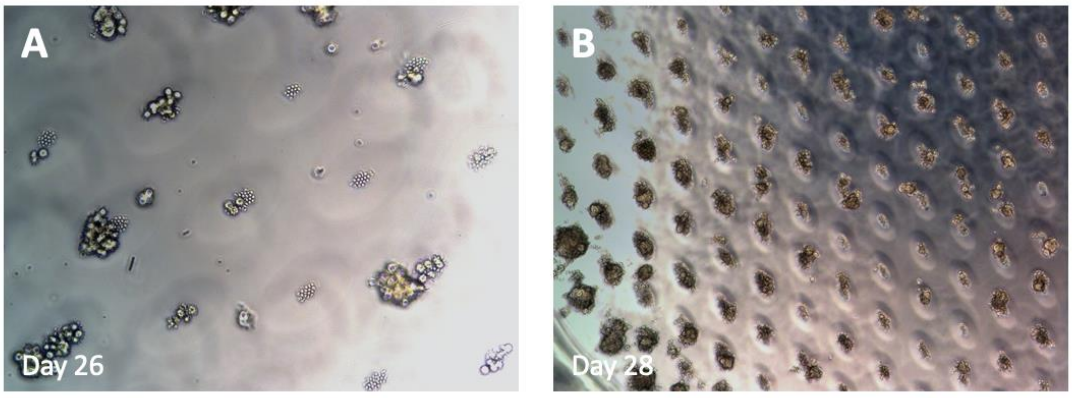

Figure 12. CTCs derived from patient 2 visualized under the microscope on agarose molds containing indentations to promote cell growth and cell-to-cell signaling: A) On day 26, large cells can be visualized inside the agarose microwells and distinguished from the smaller PBMSs B) On day 28, the cells can be seen filling and migrating beyond the borders of the agarose microwells.

Single cells and cell culture medium from patients 1 and 2 were isolated and the DNA was amplified as described in the methods. 3, 5 and 10 single cells were picked from each patient and one tube for each was filled with cell culture medium. 5 of the picked samples had sufficient a sufficient DNA concentration after amplification for further analysis with NGS (Table 12). Gel electrophoresis was used to ensure the quality of the PCR products. Samples 1, 4, 5, 7 and 8 all showed a positive result at the 500 and 200 base pair (bp) marker. Samples 5 and 7 had additional bands at the 100 bp marker while sample 8 had additional bands at the 400 and 300 bp marker (Figure 13). These results suggested that the amplified DNA from tubes 1, 4, 5, 7 and 8 was of sufficient quality for further downstream analysis.

Table 12. Selection of PCR products from patients 1 and 2 with recurrent HGOSC for further NGS analysis

PCR tube	Patient	Number of cells picked	DNA conc. after amplification (ng/µl)	Total DNA sent for NGS (µg)
1	2	3	16.9	0.51
2	2	5	Not used	
3	2	10	Not used	
4	1	10	18.9	0.57
5	1	5	16.0	0.48
6	1	3	Not used	
7	1	Flushed medium	12.4	0.37
8	2	Flushed medium	18.5	0.56

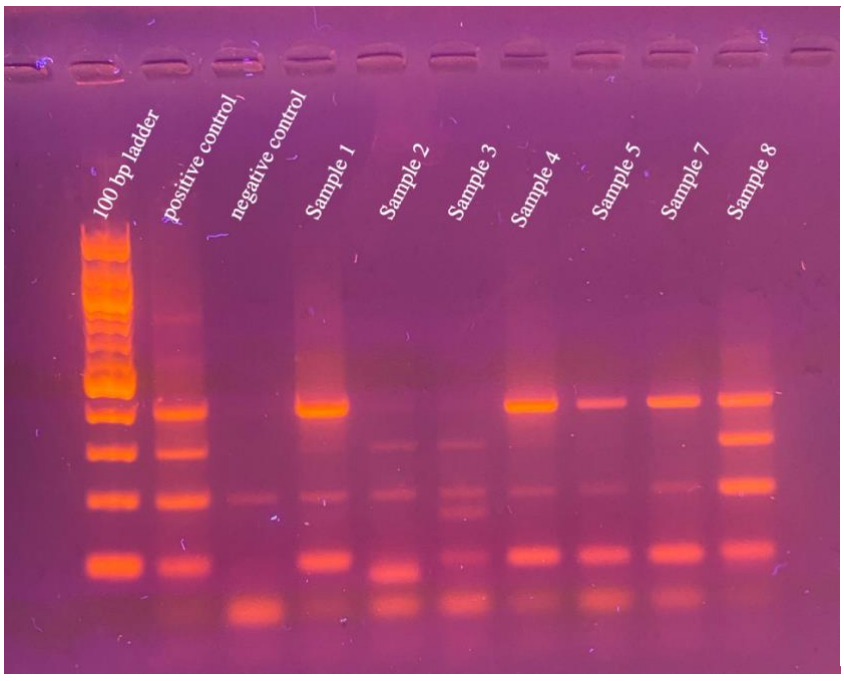

Figure 13. Agarose gel electrophoresis of PCR products from patients 1 and 2 with HGSOC: Samples 1, 4, 5, 7 and 8 all showed a positive result at the 500 and 200 bp marker. Samples 5 and 7 had additional bands at the 100 bp marker while sample 8 had additional bands at the 400 and 300 bp marker.

3.8 NGS of amplified DNA from CTCs picked from cell culture

NGS was performed on DNA from cultured single cells that had previously stained positive for both DAPI and AE1/AE3 and negative for CD45. The DNA from ten single cells from patient 1 showed chromosomal losses in 1, 2, 8, 10, 11 and 16, and gains in chromosomes 4, 7, 9, 10, 14, 17, 18, 20, 21 and X (Figure 14A). The DNA from five single cells from patient 1 showed chromosomal losses in 6, 10, 17, 18 and X (Figure 14B). The DNA found in cell culture medium from patient 1 showed chromosomal losses in 2, 3, 13, 16 and 22, and gains in 1, 2, 4, 5, 8, 9, 10, 11, 12, 17, 19 and 20 (Figure 14C). The DNA from three single cells from patient 2 showed gains in chromosomes 3, 4, 7, 9, 18 and 20 and no chromosomal losses (Figure 15A). The DNA found in cell culture medium from patient 2 did not show any gain or loss of heterozygosity (Figure 15B). These results suggest that the sequenced cells showed genetic alterations consistent with cancerous cells and that these alterations could in one sample be recovered from DNA outside the plasma membrane.

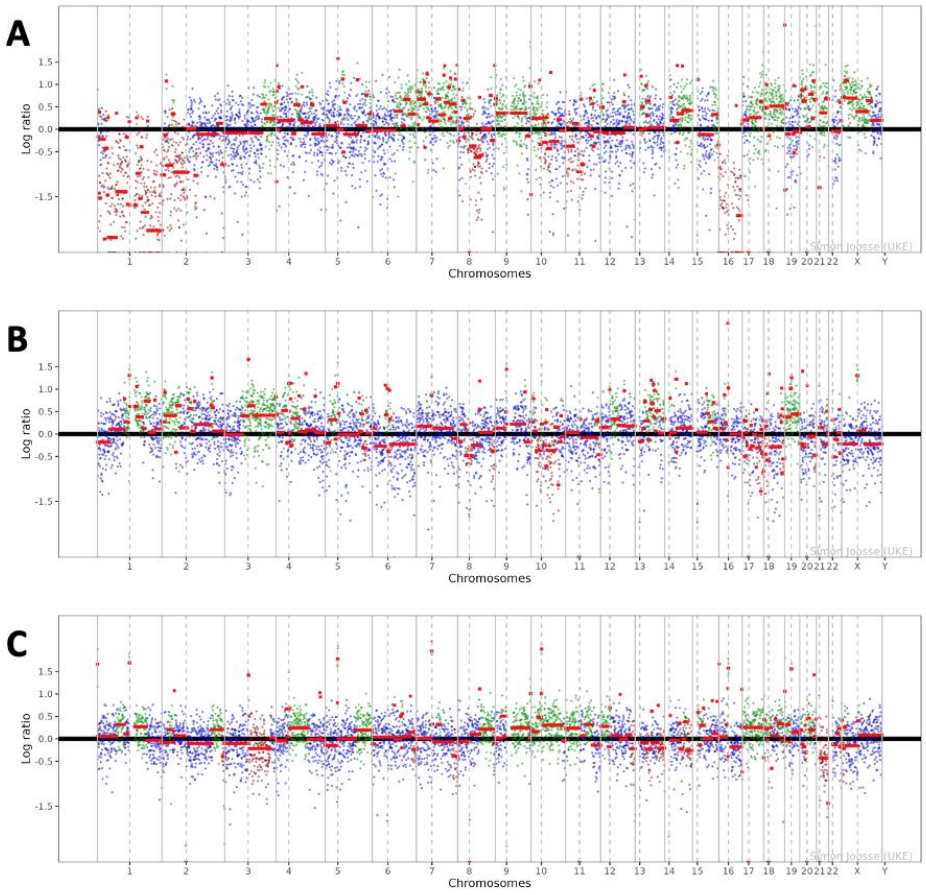

Figure 14. NGS performed on DNA from cultured single cells from patient 1 with recurrent histopathologically confirmed HGSOC. The x-axes represent the 23 pairs of chromosomes and the y-axes the log ratio of the copy number alterations. Blue color denotes chromosome regions without significant alterations: A) DNA from ten single cells that stained DAPI+, AE1/AE3+, and CD45- B) DNA from five single cells that stained DAPI+, AE1/AE3+, and CD45- C) Cell-free DNA found in cell culture medium.

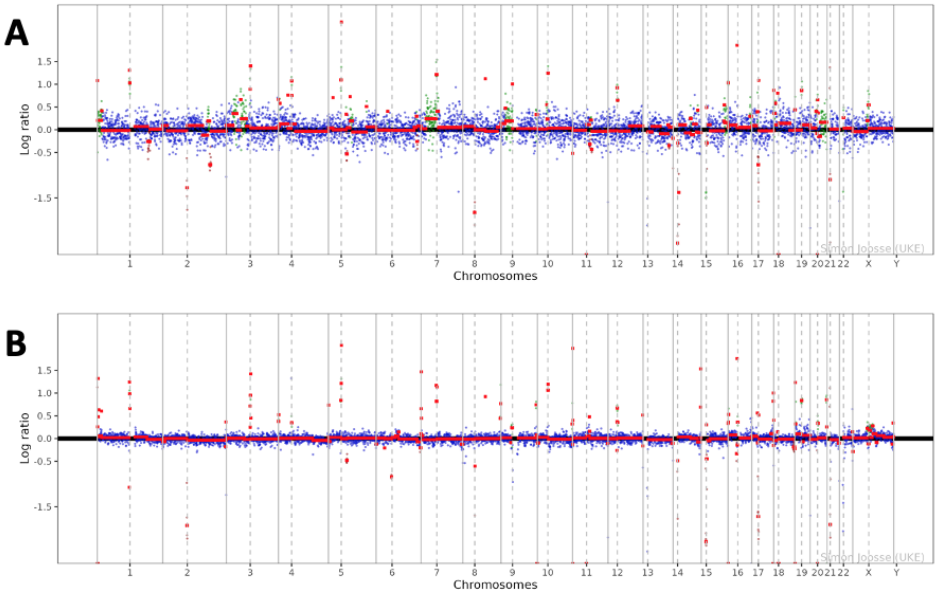

Figure 15. NGS was performed on DNA from cultured single cells from patient 2 with recurrent histopathologically confirmed HGSOC. The x-axes represent the 23 pairs of chromosomes and the y-axes the log ratio of the copy number alterations. Blue color denotes chromosome regions without significant alterations A) DNA from three single cells that stained DAPI+, AE1/AE3+, and CD45- B) Free-floating DNA from the cell culture medium

4 Discussion

The results of this study demonstrate for the first time that using the methodology described above, it is possible in high-grade serous ovarian cancer to isolate CTCs from the blood, expand them in cell culture and then sequence their genomes. The method employed is fast, cost-effective and allows for the processing of large blood volumes. Furthermore, this study sets the groundwork for analysis of the genome, transcriptome, proteome, methylome and secretome of ovarian cancer at any time point during therapy. In-vitro chemotherapeutic drug testing on patient CTC populations may become possible by expanding on the groundwork laid in this research and by overcoming obstacles that have precluded long-term culturing in this study. Further refining these methods could allow for the comprehensive profiling of ovarian tumors on a patient-by-patient basis and ultimately more targeted and effective therapies. To date, the cell lines that are commonly used in laboratories around the world are derived from solid tumor tissue. However, these cell lines might not be the best surrogate for modelling actual tumor behavior. In a study of breast cancer patients, Zhao et al. demonstrated that CTCs isolated and maintained in cell culture showed more aggressive growth, more resistance against chemotherapy and higher expression levels of epithelial-to-mesenchymal transition (EMT) than the commonly used MCF-7 cell line (Zhao et al., 2019). In another study, the human, primary tumor-derived pancreatic ductal adenocarcinoma (PDAC) cell line Panc-1 was xenografted into mice. When CTCs were subsequently isolated from the mouse blood and their phenotype was compared to the solid-tissue cell line, the CTC cell line was found to show greater capability to migrate and invade tissue with a higher expression of the *TGFBI* gene likely being a key driver of this increased aggressiveness (Sato et al., 2018). Studying a cell line derived from a solid tumor may therefore not be the ideal model when trying to investigate aggressive tumors with metastatic potential. Likewise, in ovarian cancer, a cell line derived from CTCs may better reflect genetic and proteomic changes that are required for a tumor cell to achieve metastatic potential. As with the identification of increased expression of *TGFBI* in PDAC CTCs, transcriptomic analysis of CTCs may provide an avenue for the identification of drug targets in ovarian cancer CTCs that can be inhibited to prevent the formation of metastases.

Although there have been no studies published to date that achieved successful permanent culturing of CTCs from a patient with HGSOC, a small number have been published regarding other cancer entities. In a study by Cayrefourcq et al., a long-term CTC cell culture from 2 patients with metastatic colon cancer was successfully established using a

methodology different from the one described in this thesis. Here, plasma CTC levels were measured using CellSearch® before patients were determined to be promising candidates for culturing. The 2 patients that were selected and for whom permanent CTC culturing was achieved were selected from a cohort of 71 patients and had CTC counts of 516 and 302 cells per 10 ml of blood (Cayrefourcq et al., 2015). Our study enrolled 32 HGSOC patients with primary disease, on which 102 CellSearch® analyses were carried out. Although at least one CTC could be identified in 48% of preoperative and 35% of postoperative samples, the median pre- and postoperative CTC count was 0 (range: 0-12 CTCs) per 7.5 ml of blood (Table 6). This finding is consistent with a study by Poveda et al. in which only 31 of 216 patients (14.4%) with a recent progression of advanced ovarian cancer following platin-based chemotherapy had a baseline CTC count of 2 or more per 7.5 ml of blood at baseline (Poveda et al., 2011). As mentioned before, CTC identification in this study was based on cell enumeration using the epithelial cell adhesion molecule, EpCAM, and negative selection using CD45 to distinguish leukocytes. Epithelial cells that have undergone epithelial-to-mesenchymal transition (EMT) may therefore be missed, resulting in a false negative result. In a study of 22 patients with ovarian cancer, all morphologically identified CTCs stained positive for the EMT marker vimentin but only 44% for the epithelial marker EpCAM (Usman, 2021, Jie, 2022). Further studies are required to evaluate whether co-staining with vimentin can increase the yield of positively identified CTCs in patients with HGSOC. Our findings highlight that the method employed in this paper can be successful with low numbers of CTCs and may therefore be applicable to other cancers where CTC counts in peripheral blood have been found to be low such as locally-invasive pancreatic adenocarcinoma and non-metastatic colon cancer (Bidard et al., 2013, Bork et al., 2015).

To validate whether the cell culture of peripheral blood from the two serous ovarian cancer patients in which growth was observed had indeed cultivated CTCs from ovarian cancer, NGS was performed to search for chromosomal aberrations known to be common in this cancer type. Our sequencing results showed considerable copy number alterations in five samples (Figure 14A-C, 15A). The sixth sample showed no significant chromosomal aberrations, as was to be expected, as this sample that did not contain intact tumor cells but instead the fluid in which tumor cells had been previously suspended. We speculate that this DNA had already been degraded by the time of sequencing. These findings, taken together with the human cell origin validated by GAPDH-multiplex PCR and immunofluorescent staining for epithelial markers, makes credible that the cells we

harvested from cell culture represent HGSOC cells. Future steps could include xenografting CTCs from ovarian cancer cell culture into live animals. This will provide valuable information about the tumorigenic capacity of the different subclones.

In culturing the patient derived and previously isolated CTCs, further proliferation stalled after 116 and 139 days in culture in both patient samples. The reasons for this are not clear. After rapid proliferation of cells was observed following first signs of growth after 26 and 37 days in the two samples, further expansion of the cell culture stalled and it was not possible to transfer the cells to successive T75 flasks as had been envisaged. Changing the medium to one that had been successful with a cell culture derived from breast cancer CTCs did not reverse the steady decline of the cell population. The cells that had proliferated on the agarose wells were successfully transferred to the floor of the 12-well plates when flipped, evidenced by their unfettered growth on this new surface. However, when these cells were designated for splitting to a new container, they were found to be particularly adhesive. It is possible that the cell membrane integrity was damaged when trypsin enzyme was applied repeatedly and for longer periods than is typical for splitting protocols. When the cells were transferred to larger 6-well plates and T25 flasks they did not adhere and there was no further expansion observable. This lack of adhesion may support the theory that the long exposure to trypsin enzyme irrevocably damaged the cells. Future studies will be needed to determine the optimal culturing medium for ovarian cancer CTCs and the appropriate expansion protocol.

In our cohort of newly diagnosed HGSOC patients, the plasma cfDNA was found to be significantly higher up to 3 days post-operatively than when measured pre-operatively. A study by Henriksen et al. of colorectal and muscle-invasive bladder cancer showed three- and eightfold increases, respectively, of cfDNA after surgery that persisted for up to four weeks (Henriksen et al., 2020). Our study suggests that trauma-induced cfDNA release also likely occurs in ovarian cancer. This finding should be taken into consideration when making clinical decisions based on cfDNA status. To determine whether negative molecular status has been achieved or recurrence is likely, post-operative cfDNA can be sequenced for mutations commonly found HGSOC, such as TP53, BRCA1/2, *RB1, NF1, FAT3, CSMD3, GABRA6*, and *CDK12* after four weeks when trauma-induced cfDNA levels return to baseline (Bell et al., 2011). This study also demonstrates the diagnostic potential of liquid biopsy from ascites. The amount of cfDNA found in the ascites of primary and recurrent disease patients was higher than in the plasma preoperatively, postoperatively and during follow-up measurements (Figure 8-9). These findings imply

the possibility of a greater analytical sensitivity in ascites for the detection of key genomic aberrations. In serous ovarian cancer, where there is still much debate on which patients may benefit from therapy with PARP-inhibitors, genomic aberrations found in the ascites may help make this distinction. Since *BRCA* promoter hypermethylation has been shown to be a dynamic process, an abundance of cfDNA may help to quantify to what extent *BRCA* is contributing to cancer DNA repair at a given time (Prieske et al., 2017). Furthermore, new driver mutations occurring in an initially very small percentage of cfDNA may be detectable with higher sensitivity and warn of impending resistance to platin chemotherapy. Once more targeted therapies become available for HGSOC patients, the analysis of the more abundant ascites cfDNA may be preferrable to plasma ctDNA when feasible.

In this study, there was no significant decline of CTCs recovered from peripheral blood after surgery. This finding was not surprising. It is presumed that similarly to cfDNA, CTC release can be induced by trauma to the primary tumor. In 115 men who received a transrectal ultrasound-guided biopsy (TRUS) for suspected prostate cancer, the number of CTCs was significantly increased in those that were biopsied and were cancer positive (Joosse et al., 2020). While not statistically significant pending further enrollment, CTC recovery continually decreased with increased time from surgery. In correlating the CTC counts of patients with their clinical status we found that patients who were CTC positive at baseline had a higher risk of developing recurrence or having disease progression, though this was not statistically significant, likely due to too short follow-up. No significant difference regarding residual tumor, presence of metastasis or death could be found between patients whose CTC count decreased after cytoreductive surgery and patients whose CTC count increased after surgery. These results would also seem to bolster the argument for trauma-induced CTC release, where CTCs released from invasive measures do not necessarily correlate with clinical outcomes. In a large study of breast cancer patients, positive CTC status before surgery and after 1 and 2 years was associated with worse OS and PFS but was not 1 week after surgery (van Dalum et al., 2015). However, the CTC count when measured at first follow-up, after the patients had received chemotherapy, was diminished. Only 19% (4/21) patients had a CTC count ≥1 and of these, two patients had CTC counts of 3 and 4.

As has been discussed above, CTC recovery yields in serous ovarian cancer patients are very low in comparison with other cancers, even in the metastatic setting. In this cohort, ≥1 CTCs could be recovered in just 47%, 34% and 19% of pre-operative, post-operative,

and follow-up samples, respectively. The method employed here will need further refining to increase CTC yield and ensure the reliable recovery of enough CTCs for successful cell culture in patients whose cancers are still localized. Since ovarian cancer occurs predominately in patients over 50 who are then heavily treated, blood draws exceeding 60 ml are both difficult to perform and justify in an experimental setting. Diagnostic leukapheresis (DLA), the continuous extracorporeal extraction of mononuclear blood and cancer cells from circulation, has been suggested by some as a potential remedy to this problem (Stoecklein et al., 2016). In a study of non-metastatic breast cancer patients, CTCs, which are extracted along with leukocytes, could be detected in 90% of patients when 25 liters of blood were sampled. The median CTC count was 7500 and the number was positively correlated with extent of disease (Fischer Johannes et al., 2013). In another study of breast cancer patients, the DLA product yielded 205 times more CTCs than a 7.5 ml blood draw using CellSearch® in patients with metastatic disease. Importantly, no adverse treatments effects were observed in any of the 40 patients that underwent DLA (Fehm et al., 2018). These findings suggest a potential role for DLA in the culturing of ovarian cancer CTCs. DLA has the potential to significantly increase the probability of CTC proliferation in cell culture and thereby increase sensitivity to a threshold required for therapeutic decisions. Larger yields will also aid in a better understanding of the heterogeneity of the tumor. With DLA, CTC cell culture could become reliable and valuable information about tumor mutational burden and resistance mechanisms evolving throughout therapy may be elucidated. Since DLA is an expensive, time-consuming procedure, available only at large university clinics, it is possible that its clinical application would be limited to situations when the cancer has recurred, and the best treatment option cannot be determined based on clinical findings alone.

There are several limiting factors that need to be accounted for regarding this study. To begin, the results presented in this study are preliminary and the full data will only be available at the conclusion of the prespecified data collection period of five years that may yet be extended if prespecified patient enrollment is not met during this time. As mentioned above, with the advent of PARP inhibitor treatment for *BRCA1/2* and *HRD* mutated patients, time to recurrence and progression has been significantly extended. Due to the relatively short observation time, any conclusions drawn from this data regarding outcome are bound to be incomplete. Longer follow-up and greater patient recruitment will be needed to verify, for example, if an increase in CTC count can predict earlier

recurrence. Further experiments using the patient samples included in this study will sequence the cfDNA and CTCs at various time points in the therapeutic regimen to ascertain the changing genomic landscape of HGSOC. We hope that methylomic analysis of cfDNA collected from our cohort will serve as the basis for extending targeted molecular therapies to a greater share of HGSOC during susceptibility windows.

5 Summary

Background: Despite important progress, ovarian cancer remains a major cause of morbidity and mortality worldwide. As we become better at understanding and targeting the molecular mechanisms that drive cancer growth in an individual patient, more sensitive diagnostic tools will be key to translating research into a meaningful benefit for patients.

Methods: Blood and ascites were drawn from HGSOC patients in the Department of Gynecology at the University Medical Centre Hamburg-Eppendorf between July 2020 and April 2022. Liquid biopsy analysis was performed using CellSearch®, Parsortix®, Next Generation Sequencing and a novel slanted microfluidic device to enumerate patient derived CTCs for cell culture.

Results: The concentration of cfDNA in the ascites of patients with primary and recurrent disease was found to be significantly higher than in their plasma. In patients with primary disease, the concentration of cfDNA in postoperative plasma was significantly higher than preoperatively. At least one CTC could be found in 48% of preoperative samples, 35% of postoperative samples, 24% of samples at first follow-up, and 15% of samples at second follow-up. The median CTC count was 0 for each group. No statistically significant difference regarding the presence of residual tumor, presence of metastasis, recurrence or progression, and death could be found between patients with no CTCs at baseline and patients with one or more CTCs at baseline, patients where CTCs were not observable throughout the duration of the study, or for patients whose CTC count either increased or decreased following cytoreductive surgery. In two of five experiments where CTC cell culturing was attempted, there was expansion of a distinct group of large cells which were maintained for a maximum of 139 days. Sequencing of these cells showed genomic alterations consistent with those found in HGSOC.

Conclusion: This study demonstrates a role for liquid biopsy and its derived applications in detecting, monitoring and molecularly characterizing high-grade serous ovarian cancer. We envisage that CTC and cfDNA analysis will be used to aid clinicians in detecting recurrence and choosing the optimal therapy in the future. CTC cell culture may have applications in cases where the cancer has relapsed and is not sensitive to platinum chemotherapy.

5 Zusammenfassung

Hintergrund: Trotz wissenschaftlichen Fortschrittes ist das high-grade seröse Ovarialkarzinom eine Erkrankung mit hoher Morbidität und Mortalität. Neue diagnostische Methoden sind gefordert um gezielt patientenindividuelle molekulare Veränderungen in der jeweiligen Therapie berücksichtigen zu können.

Methoden: Blut und Aszites wurde Patientinnen mit high-grade serösem Ovarialkarzinom in dem Zeitraum zwischen Juli 2020 und April 2022 an der Klinik für Gynäkologie am Universitätskrankenhaus Hamburg-Eppendorf entnommen. Eine Liquid Biopsy Analyse mittels CellSearch®, Parsortix®, Next Generation Sequencing und eines neuartigen Gerätes zur Sortierung von CTCs mittels Mikroflüssigskeitsgradienten wurde durchgeführt.

Ergebnisse: Die cfDNA Konzentration bei Patientinnen mit Erstdiagnose und im Rezidiv war im Aszites signifikant höher messbar als im Plasma. Bei Patientinnen mit Erstdiagnose war die cfDNA Konzentration postoperativ höher als präoperativ. Mindestens eine CTC konnte in 48% der präoperativen Proben, 35% der postoperativen Proben, 24% der Proben bei der ersten und 15% bei der zweiten Nachsorge nachgewiesen werden. Die mediane Anzahl an CTCs betrug 0. Es zeigten sich keine signifikanten Unterschiede hinsichtlich des Nachweises eines residualen Tumors, Vorliegen von Metastasen, Rezidiv oder Tumorprogress, und Tod zwischen Patientinnen wo keine oder ≥ 1 CTC präoperativ nachzuweisen waren, bei Patientinnen bei denen CTCs sich die CTC-Zahl postoperativ veränderte oder Patientinnen wo in der gesamten Studiendauer keine CTCs nachzuweisen waren. In zwei von fünf Experimenten wobei CTCs in einer Zellkultur angelegt wurden war eine Expansion von Zellen zu beobachten die bis maximal 139 Tage in der Zellkultur überlebten. Die isolierte DNA dieser Zellen zeigte charakteristische genomische Veränderungen vereinbar mit solchen die beim high-grade serösen Ovarialkarzinom bekannt sind.

Schlussfolgerungen: Diese Resultate demonstrieren einen wichtigen Beitrag der Liquid Biopsy und dessen abgeleiteten Anwendungen in der Detektion, des Monitorings und der molekularen Charakterisierung von dem high-grade serösem Ovarialkarzinom. Wir prognostizieren in Zukunft die Anwendung von CTC und cfDNA Analysen zur zusätzlichen Unterstützung von Klinikern in der Detektion von Rezidiven und in der Auswahl einer patientenindividuellen Therapie. Die kulturelle Anreicherung von CTCs hat womöglich eine Anwendung bei Patientinnen im Rezidiv wo eine Platin-Resistenz vorliegt.

6 Literature

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7 Appendix

7.1 Abbreviations

bp base pair

BRCA1/2 BReast CAncer Gene 1 und 2

CA125 Cancer Antigen 125

CpG 5’-Cytosine-phosphoguanine

CSF cerebrospinal fluid

CTC circulating tumor cell

cfDNA cell-free DNA

ctDNA circulating tumor DNA

DAPI 4′,6-diamidino-2-phenylindole

DLA diagnostic leukapheresis

DMEM Dulbecco's Modified Eagle's Medium

DPBS Dulbecco’s phosphate-buffered saline

DSMO dimethylsulfoxide

EDTA ethylenediaminetetraacetic acid

EMT epithelial-to-mesenchymal transition

FBS fetal bovine serum

FIGO Fédération Internationale de Gynéctologie et d’Obstétrique

GAPDH Glyceraldehyde-3-phosphate-dehydrogenase

HAP hypoxic abdominal perfusion

HGSOC high-grade serous ovarian cancer

HRD homologous recombination deficiency

LOH loss of heterozygosity

miRNA micro RNA

MRD minimal residual disease

NGS next generation sequencing

NSCLC Non Small Cell Lung Cancer

OS overall survival

PARP Poly (ADP-Ribose) Polymerase

PBMC peripheral blood mononuclear cells

PBS phosphate-buffered saline

PCR polymerase chain reaction

Appendix

PDAC pancreatic ductal adenocarcinoma

PDMS polydimethylsiloxane

PFA paraformaldehyde

PFS progression free survival

RPMI Roswell Park Memorial Institute Medium

rWGS rapid whole genome sequencing

TCGA The Cancer Genome Atlas

TRUS transrectal ultrasound-guided biopsy

VEGF Vascular Endothelial Growth Factor

7.2 List of Tables

7.3 List of Figures

8 List of Publications

BEUKER, C., WANKNER, M. C., THOMAS, C., STRECKER, J.-K., SCHMIDT-POGODA, A., SCHWINDT, W., SCHULTE-MECKLENBECK, A., GROSS, C., WIENDL, H., BARTH, P. J., ECKERT, B., MEINEL, T. R., ARNOLD, M., SCHAUMBERG, J., KRÜGER, S., DEB-CHATTERJI, M., MAGNUS, T., RÖTHER, J. & MINNERUP, J. 2021. Characterization of Extracranial Giant Cell Arteritis with Intracranial Involvement and its Rapidly Progressive Subtype. *Annals of Neurology,* 90, 118-129.

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9 Erklärung des Eigenanteils

Die in dieser Dissertation beschriebene Laborarbeit und Probensammlung wurde im Institut für Tumorbiologie am Zentrum für Experimentelle Medizin an dem Universitätsklinikum Hamburg-Eppendorf durchgeführt. Die Idee zum Projekt wurde gemeinsam mit PD Dr. Simon Joosse und PD Dr. med. Katharina Prieske entwickelt. Die Experimente wurden eigenständig mithilfe von Frau Sandra Lenz und weiteren medizinisch-technisch Angestellten im Institut für Tumorbiologie durchgeführt. Die Auswertung der Ergebnisse, die Schlussfolgerungen sowie die Verfassung dieser Dissertation wurden ohne weitere externe Mithilfe durchgeführt.

Datum Unterschrift

10 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Das gilt insbesondere auch für alle Informationen aus Internetquellen. Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet. Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe. Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum Unterschrift

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