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Detection and characterization of circulating tumor cells in patients with testicular germ cell tumors and prostate cancer

Dissertation

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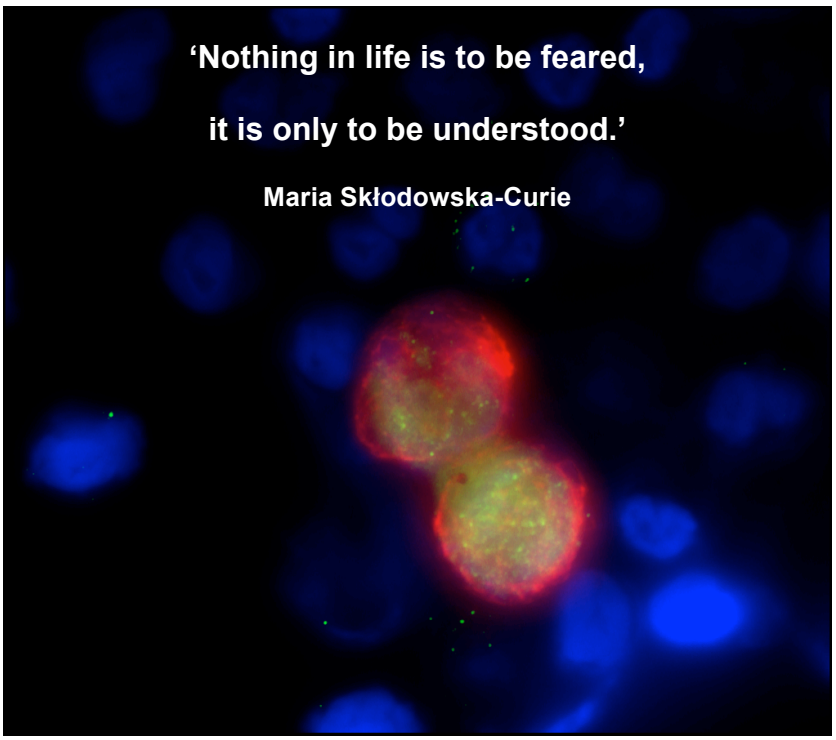
‘Nothing in life is to be feared,
it is only to be understood.’
Maria Skłodowska-Curie

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ABSTRACT

Circulating tumor cells (CTCs) are cells that have detached from primary tumor and are present in blood circulation. Detection of CTCs has been reported in urogenital malignancies including prostate and bladder cancer. In patients with castration-resistant prostate cancer, presence of CTCs has been associated with poor prognosis. This study aimed to investigate the prevalence, clinical relevance and characterize CTCs in testicular germ cell tumors and prostate cancer.

1. Germ cell tumors (GCTs) represent the most frequent malignancies among young men. Histologically, GCTs are divided into two classes: seminomatous and nonseminomatous germ cell tumors (NSGCTs). There is scant information about the presence of CTCs in blood of patients with GCTs. Considering heterogeneity of GCTs, CTCs were investigated using two independent assays targeting germ and epithelial cell-specific markers, and results were correlated with disease stage, histology, and serum tumor markers. CTCs were enriched from peripheral (n=143 patients) and testicular vein blood (n=19 patients) using Ficoll density gradient centrifugation. For CTC detection, a combination of germ (anti-SALL4, anti-OCT3/4) and epithelial cell-specific (anti-keratin, anti-EpCAM) antibodies was used. In parallel, 122 corresponding PB samples were analysed using the CellSearch® system. Additionally, in the cohort of 51 patients with NSGCTs, peripheral blood samples were examined for presence of potential therapeutical targets on level of CTCs: CD30 and Glypican-3. In total, CTCs were detected in 25/143 (17.5%) peripheral blood samples, whereas 11.5% of patients were CTC-positive when considering exclusively the CellSearch® assay. Twelve (23.5%) peripheral blood samples showed membranous expression of CD30, whereas Glypican-3 staining was found in CTCs from 6 (11.8%) patients. Five (9.8%) patients had CTCs positive for both CD30 and GPC3. The presence of CTCs in PB correlated to clinical stage (P<0.001) with 41% of CTC-positivity in patients with metastasized tumors, and 100% in patients with relapsed and chemotherapy-refractory disease. Histologically, CTC-positive patients suffered more frequently from nonseminomatous primary tumors (P<0.001)

than from seminomas, with higher percentage of yolk sac (P<0.001) and teratoma (P=0.004) components. Furthermore, CTC detection was associated with elevated AFP (P=0.025), βHCG (P=0.002) and LDH (P=0.002) serum levels. Incidence and numbers of CTCs in TVB were much higher comparing to PB. This is the first study reporting on CTCs in patients with GCTs. CTCs occur frequently in patients with more aggressive disease, and there is a gradient of CTCs with decreasing numbers from the tumor-draining vein to the peripheral blood vessels. Evaluation of CTCs in patients with GCTs might serve as potential diagnostic and/or prognostic biomarker as well as lead to therapy improvement.

2. Transrectal ultrasound guided prostate biopsy is a standard diagnostic procedure for prostate cancer diagnosis. However, the possibility of dissemination of cancer cells by biopsy is not negligible. The aim of this study was to investigate if prostate biopsy is associated with hematogenous dissemination of CTCs. Peripheral blood samples were obtained before and after performing prostate biopsy from 75 patients with serum prostate-specific antigen (PSA) levels >2 ng/mL. CTCs were detected with the usage of two methods - the established CellSearch® system and the EPISPOT assay that detects living PSA-secreting tumor cells. Using the CellSearch® system, in 8 (12.1%) patients CTCs were detected before, and 20 (29.7%) samples collected after biopsy were CTC-positive (P=0.017). With the usage of the EPISPOT assay, PSA-secreting CTCs were found in peripheral blood of 20 (28.5%) patients before biopsy, whereas 31 (44.3%) cases collected after performing biopsy were positive (P=0.027). When combining the results of both assays (27/75, 37.0%) patients were positive for CTCs before, and significantly more (42/75, 56.0%) of cases were detected CTC-positive after performing biopsy (P=0.009). This is the first report suggesting that prostate biopsy may cause dissemination of not only apoptotic but also viable prostate cells

Chapter 1: GENERAL INTRODUCTION

1. CANCER

Since decades cancer has become one of the leading clinical problems in the developed countries. Despite the efforts to fight this disorder, many patients do not benefit from common therapies and cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012 (WHO, 2012).

Cancer is a class of diseases caused by out-of-control cell growth. Its characterized by six biological capabilities ('hallmarks of cancer') acquired during the multistep development of human tumors that include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [Fig. 1.1 (Hanahan and Weinberg,2011)].

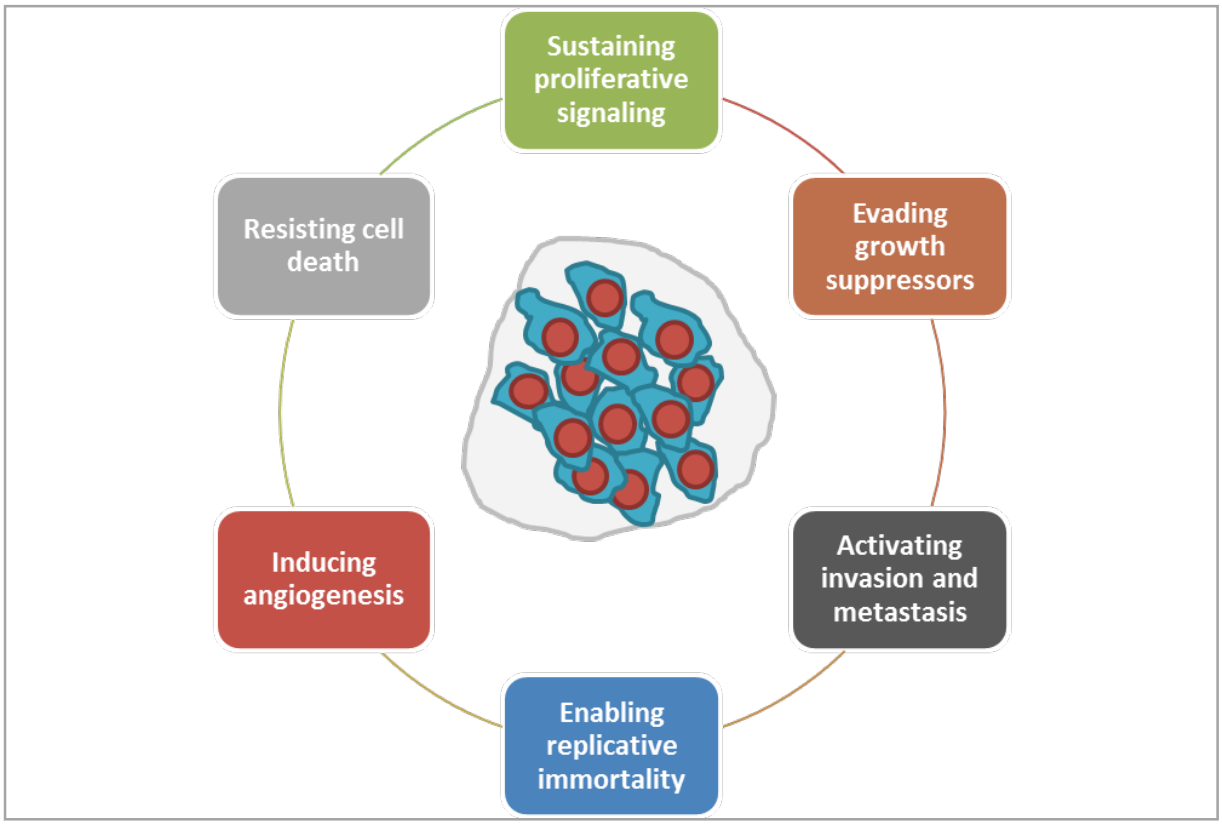

Figure 1.1 The six hallmarks of cancer (adapted from Hanahan and Weinberg, 2011).

Virtually all cell types in the body can give rise to cancer, but more than 80% of human tumors arise from epithelial tissues – the carcinomas. Tumors that arise from

epithelial cells forming protective cell layers are called squamous cell carcinomas and are prevalent in tissue types as skin, larynx, cervix. Many epithelia contain cells that are specialized in secretion of substances into ducts or cavities and this class of epithelia is said to be adenocarcionoma. The more common tissue sites of adenocarcinoma are colon, breast and prostate (**Böcker et al., 2001**).

However, tumors might also arise from various nonepithelial tissues throughout the body. Nonepithelial malignant tumors include sarcomas (derive from mesenchymal cell types), hematopoietic (arise from blood-forming tissues), neuroectodermal (arise from components of the central and peripheral nervous system), or germ cell tumors (derive from germ cells).

Tumors can be either benign that are localized and noninvasive or malignant with invasive features and forming metastases (**Weinberg et al., 2007**).

1.1 Metastatic cascade

Metastases are the cause of 90% of human cancer-associated deaths. Tumor progression towards metastasis is often described as a multistage process in which malignant cells spread from the tumor of origin to colonize distant organs. The following simplified order of metastatic process has been proposed — local invasion, intravasation (entry of tumor cells into the bloodstream), survival in the circulation, extravasation (exit of tumor cells from capillary beds into the parenchyma of an organ), and colonization leading to overt metastatic disease (**Pantel and Brakenhoff, 2004; Ignatiadis et al., 2012; Alix-Panabières and Pantel, 2013**).

2. MINIMAL RESIDUAL DISEASE

Minimal residual disease (MRD) is defined by the presence of malignant cells in distant organs that are undetectable by conventional imaging and laboratory tests used for tumor staging after curative surgery of the primary tumor. Circulating tumor cells (CTCs) have been defined as cancer cells of solid tumor origin found in the peripheral blood. It is generally thought that these cells detach from primary or secondary tumors of patients with advanced cancer prior to detection in the

circulation [(Pantel et al., 2009) Fig. 1.2]. Occult cancer cells can be also found in the bone marrow of carcinoma patients and are defined as disseminated tumor cells (DTCs). Both CTCs and DTCs are considered surrogate markers of MRD and potentially metastasis-initiating cells (Ignatiadis et al., 2012).

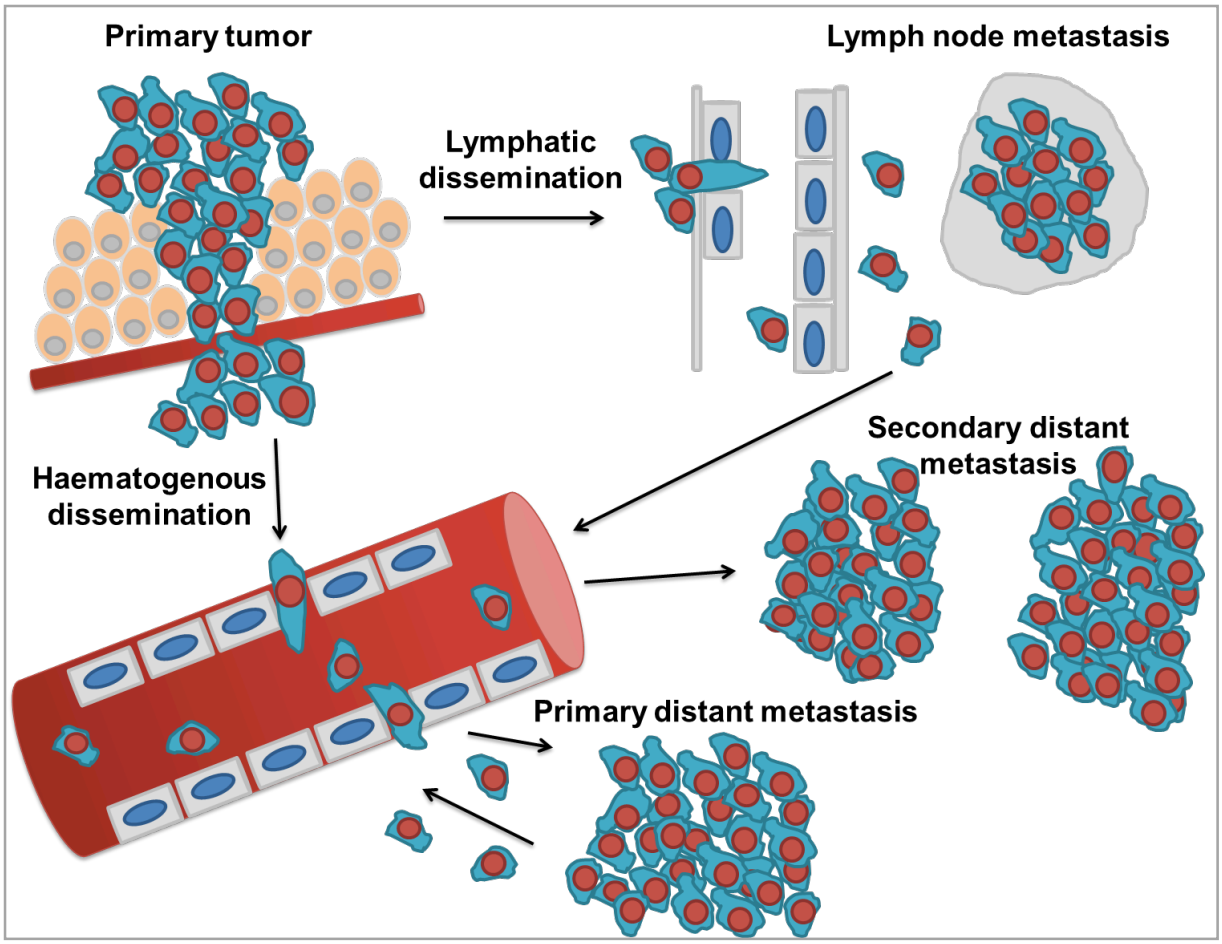

Figure 1.2. Dissemination of tumor cells. A portion of cells of primary tumor gain motility features and via lymph or/and blood circulation vessels migrate through a body until they find a proper niche to anchor and establish primary metastasis (adapted from Pantel and Brakenhoff, 2004).

2.1 Methods for enrichment and detection of circulating tumor cells

There are different methods for CTC detection and enrichment based on properties of CTCs that distinguish them from the surrounding normal hematopoietic cells, including physical properties (size, density, electric charges, deformability) as well as biological properties [cell surface protein expression, viability (Alix-Panabières and Pantel, 2013). The overview of different methods used in this study for enrichment and detection of circulating tumor cells is presented in the Fig. 1.3.

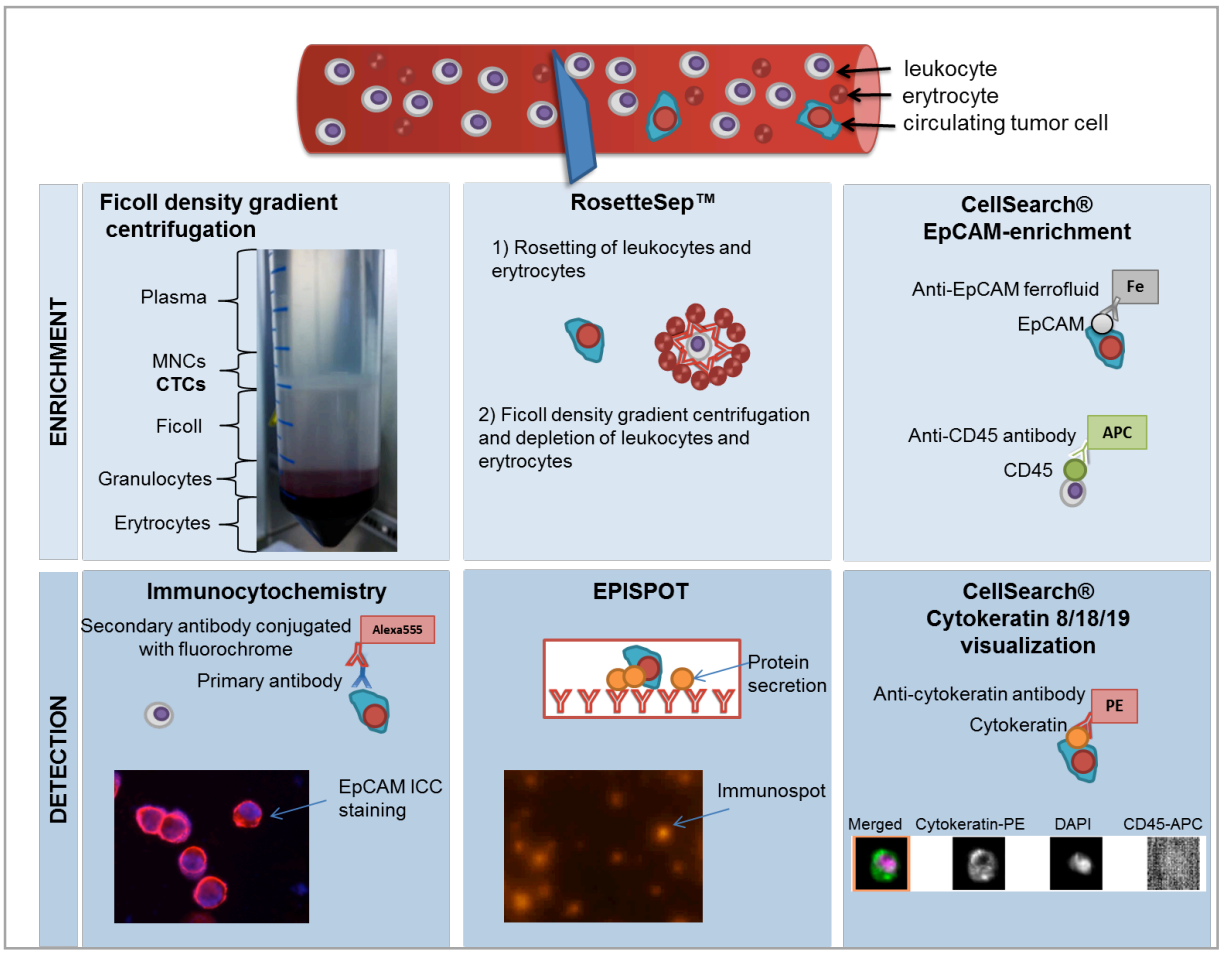

Figure 1.3 The overview of methods used in this study for enrichment and detection of circulating tumor cells.

2.1.1 Ficoll density gradient centrifugation followed by immunocytochemistry

Ficoll (d = 1.077 g/ml) density gradient centrifugation is performed to separate the mononuclear cells (MNC) and CTCs (d <1.077 g/ml) from the other cells (blood cells, granulocytes and tumor cells that have a d > 1.077 g/ml). This process generates a layered separation of cell types based on cellular density. In order to verify enriched CTCs from the undesired background of leukocytes, morphologic identification is combined with immunocytochemistry (ICC) analyses (**Pantel et al., 1994**). ICC refers to the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, or enzyme. The fluorescence staining of epithelial-specific antigens like cytokeratins (e.g., CK-8, -18, and -19), epithelial-specific adhesion molecules [e.g., epithelial cell adhesion molecule

(EpCAM)], or tumor-specific proteins [(e.g., estrogen receptor (ER), or the prostate-specific membrane antigen (PSMA)] can be used for the detection and characterization of CTCs from a patient-derived sample after the enrichment (**Pantel et al., 1994; Bednarz-Knoll et al., 2011; Gorges and Pantel 2011**).

2.1.2 The CellSearch® system

The semi-automated CellSearch® system is thus far the only assay cleared by the FDA for CTC detection, following the pivotal clinical studies in patients with metastatic breast, prostate, and colon cancer (**de Bono et al., 2008; Miller et al., 2010**) (**Fig. 1.4**). In this technology, CTCs are captured by EpCAM-coated magnetic beads and identified at the single cell level by immunostaining with antibodies against different keratins including 8, 18, and 19 (**Riethdorf et al., 2007**). An anti-CD45 antibody is used to exclude leukocytes and nuclei are counterstained with DAPI. After enrichment and immunocytochemical staining, immunomagnetically labeled cells are kept in a strong magnetic field and scanned using the CellSpotter Analyzer by Veridex. From the captured images, a gallery of images is presented and reviewed by a technologist/scientist who identifies tumor cells. In addition, the CellSearch® system allows evaluation of additional surface marker-of-interest on CTC-level using the extra fluorescent channel [e.g. HER2 (**Fehm et al., 2010; Rink et al., 2012**)].

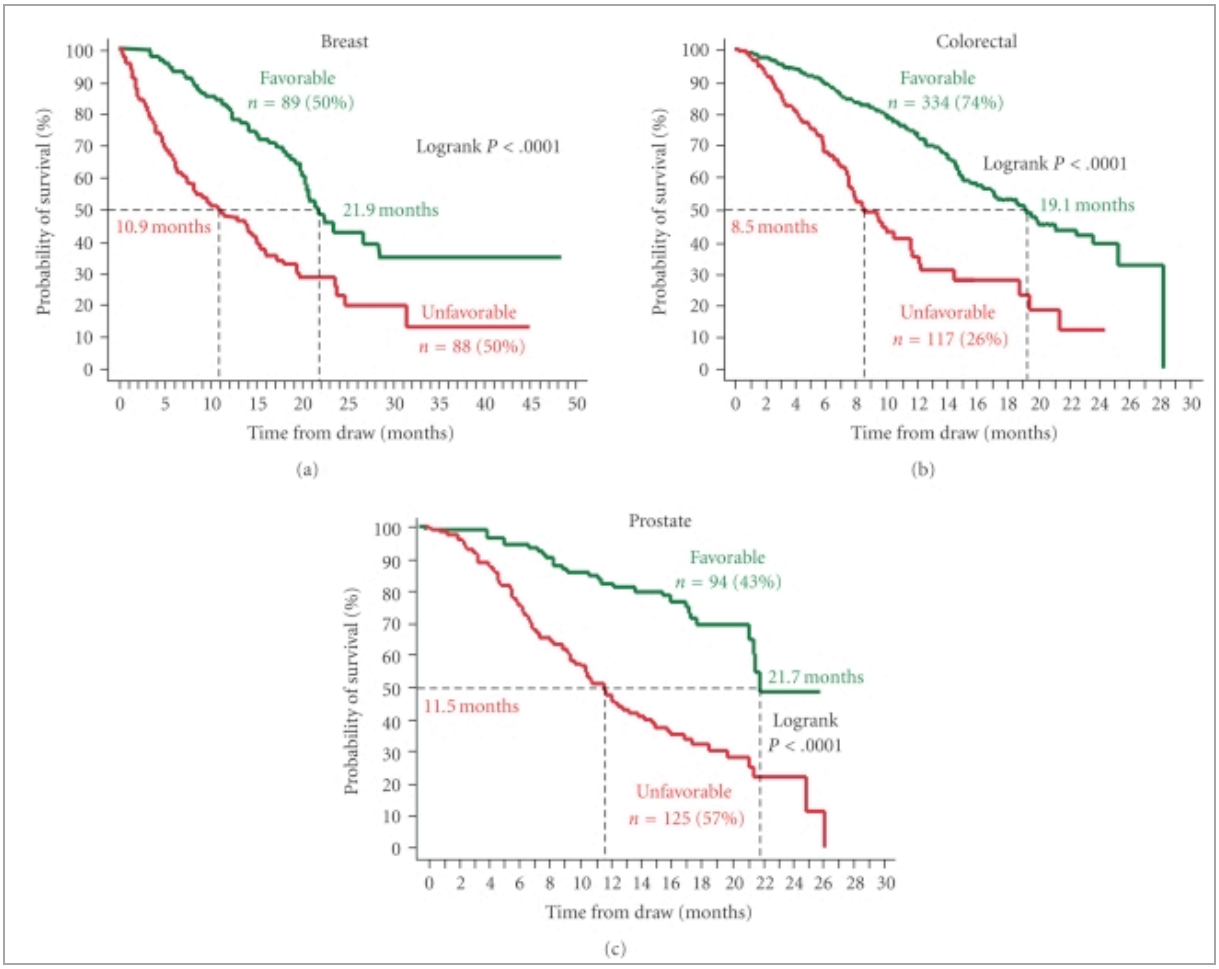

Figure 1.4 Kaplan Meier Analysis of overall survival before starting a new line of therapy for patients with metastatic breast cancer (a), metastatic colorectal cancer (b), and castration resistant prostate cancer (c). Patients were divided into those with Favorable and Unfavorable CTC. The cutoff value between favorable and unfavorable CTC was ≥5 CTC/7.5 mL blood for breast and prostate cancer and ≥3 CTC/7.5 mL blood for prostate cancer (from **Miller et al., 2010**).

2.1.3 The EPISPOT assay

The EPISPOT (EPithelial Immuno SPOT) assay is based on the detection of proteins secreted by functional CTCs combined with a negative enrichment (**Deneve et al., 2013; Ramirez et al., 2014**). In the first step, leukocytes are depleted. Then, cells are cultured for a short time on a membrane coated with antibodies that capture the released proteins which are subsequently detected by secondary antibodies labeled with fluorochromes. (**Alix-Panabieres et al., 2005**). Using the EPISPOT assay, CTCs releasing different proteins can be detected such as cytokeratin-19 (CK19), mucin-1 (MUC1), prostate-specific antigen (PSA) or fibroblast growth factor-2 (FGF2). As it has been demonstrated that the majority of circulating tumor cells

undergo apoptosis (**Larson et al., 2004**), an efficient metastasis process requires living CTCs. Therefore, in contrast to the other CTC technologies, the EPISPOT assay focuses on detection of viable CTCs.

2.2 Clinical utility of detection of circulating tumor cells

A 'liquid biopsy' may be defined as a blood test enabling single CTC detection among a billion normal hematopoietic cells (**Pantel and Alix-Panabieres, 2013**). The CTC technology could facilitate detection of early tumor cell dissemination, presence of CTCs might serve as a prognostic factor, and characterization of CTCs could lead to discovery of novel anti-cancer therapies (**Alix-Panabieres and Pantel, 2014**).

2.2.1 Detection of occult metastasis

Haematogenous dissemination of CTCs might be an indicator of recurrent disease in cancer patients that is a consequence of undetected metastasis (that occurred before initial diagnosis). Therefore, blood analysis for CTCs offers an appealing approach for the detection of occult metastasis in cancer patients (**Pantel et al., 2009; Ignatiadis et al., 2012**).

2.2.2 Prognostic marker

CTC detection is possible at both early and late stages of cancer development and might allow estimation of the risk of relapse and survival. Yields of these rare cells range from one identified cell up to several hundreds of cells per blood sample (**Bendarz-Knoll et al., 2011**). Detection of CTCs in early-stage breast cancer patients has been proposed as a prognostic factor (**Cristofanilli et al., 2010; Zhang et al., 2012; Bidard et al., 2014; Jaeger et al., 2014**). In patients with advanced non-metastatic bladder cancer, presence of CTC was proposed as predictive for early systemic disease (**Rink et al., 2011**). Additionally, presence of CTCs has been reported to have the prognostic effect on progression-free survival and overall survival metastatic breast cancer (**Cristofanilli et al., 2004; Bidard et al., 2014**) castration-resistant prostate cancer (**de Bono et al., 2008**), metastatic colorectal cancer (**Cohen et al., 2008; Miller et al., 2010**).

2.2.3 Predictor of response to therapy

Predicting patients' responses to therapeutic regimens is another application of CTC technology. These cells are obtained through a routine blood draw and could serve as an easily accessible marker for monitoring treatment effectiveness. Several studies have suggested that the presence of CTCs that have survived therapy might reflect a failure of systemic therapy (**Müller et al., 2005; Liu et al., 2009; Rack et al., 2010; Goldkorn et al., 2014**). In breast cancer patients, CTCs were found more frequently in patients with progressive disease than in patients with stable disease or remission (**Müller et al., 2005**). CTC enumeration has been proposed as an early metric to help redirect and optimize therapy in castration-resistant prostate cancer (**Goldkorn et al., 2014**).

2.2.4 Characterization of circulating tumor cells

CTCs can be not only a biomarker for cancer detection but also a source of molecular information. Characterization of CTCs on through immunocytochemistry, fluorescence *in situ* hybridization (FISH), and using the DNA and RNA assays might lead to better understanding the metastasis process and identification of potential targets for new anti-cancer therapies (**Swennenhuis et al., 2009; Riethdorf and Pantel, 2010; Lianidou and Markou, 2011; Ignatiadis et al., 2012**).

Immunocytochemistry

The usage of various ICC markers for CTCs could improve treatment of cancer patients in the era of target therapy. In metastatic breast cancer, CTCs expressiong human epidermal growth factor receptor (HER2) were detected in patients with HER2-negative primary tumors and HER2 status of CTCs should be considered in the clinical response on HER2-targeted therapies (**Fehm et al., 2010; Riethdorf et al., 2010**). CTCs isolated from metastatic breast cancer patients frequently lack estrogen receptor (ER) expression, comparing with primary tumors, which may reflect a mechanism to escape endocrine therapy (**Babayan et al., 2013**). In prostate cancer, measurement of pre- and posttreatment androgen receptor (AR)

signaling within CTCs may help target such treatments to patients most likely to respond to second-line therapies (**Miyamoto et al., 2012**).

Fluorescence in situ hybridization

Presence of genetic aberrations on single CTC level might be detected using FISH and may be used to support its malignant origin as well as could help to understand metastatic dissemination and therapy resistance. In prostate cancer, CTCs with heterogeneity of androgen receptor (*AR*) copy number gain, phosphatase and tensin homolog (*PTEN*) loss or breast cancer 1, early onset (*BRCA1*) loss have been reported (**Attard et al., 2009; Bednarz et al., 2010**). Additionally, in castration resistant prostate cancer, *ERG* oncogene after fusion with *TMPRSS2* present in CTCs, might predict resistance to abiraterone acetate therapy (**Attard et al., 2009**).

Molecular analysis

By quantitative genomic analysis of single circulating tumor cells, specific genomic aberrations and mutations can be detected that can be used as predictive or prognostic markers. It has been demonstrated that CTCs show a considerable intra- and inter-patient heterogeneity of genetic alterations for therapeutic targets like *EGFR*, *KRAS*, or *PIK3CA*, which might help to explain the variable response rates of different anti-cancer drugs (**Hannemann et al., 2011; Gasch et al., 2013**).

3. AIMS OF THE STUDY

The major aims of this study was to investigate the prevalence and clinical relevance of CTCs in urological tumors (testicular germ cell tumors and prostate cancer). The studies presented in this thesis belong to so called ‘translational research’ that aims to make findings from basic science useful for practical clinical applications. The detailed aims of each chapter of my thesis are listed below:

1) Evaluation of the incidence of CTCs in patients with testicular germ cell tumors using two independent assays (Ficoll-enrichment followed by ICC and the CellSearch® system) and correlation of the findings to clinical parameters such as tumor histology, stage of disease, and tumor marker levels in blood serum.
2) Determination whether transrectal ultrasound-guided prostate biopsy is associated with haematogenous dissemination of tumor cells with the usage of two methods in parallel - the CellSearch® system and the EPISPOT assay that detects PSA-secreting tumor cells.

Chapter 2: DETECTION AND CHARACTERIZATION OF CIRCULATING TUMOR CELLS IN PATIENTS WITH TESTICULAR GERM CELL TUMORS

1. INTRODUCTION

1.1 Epidemiology of testicular germ cell tumors

Testicular cancer represents between 1% and 1.5% of male neoplasms and 5% of urological tumours in general, with 3-10 new cases occurring per 100,000 males/per year in Western society. The predominant histological type, comprising of 90-95% of testicular cancers, are germ cell tumours [(GCTs) **EAU, 2012**]. Testicular GCTs generally affect young men in the third or fourth decade of life and are also the most frequent tumor type in young men between the age of 20 and 40.

In recent decades, rapid increases in incidence rates have been observed in white Caucasian populations (**Ruf et al., 2014; Znaor et al., 2014**).

1. 2 Classification of testicular germ cell tumors

Germ cell tumors are staged according to the International Union against Cancer (UICC) classification. The UICC staging system (including four stages I-III; **Tab. 2.2**) is based on the TNM status which describes a size, an extension of a tumor to other organs as well as level of serum tumor markers. The following TNM classification of testicular cancer has been proposed (**Tab. 2.1**).

Table 2.1 TNM classification for testicular cancer (EAU, 2012).

pT Primary tumor			
pTX Primary tumor cannot be assessed **pT0** No evidence of primary tumor (e.g. histological scar in testis) **pTis** Intratubular germ cell neoplasia (testicular intraepithelial neoplasia) **pT1** Tumor limited to testis and epididymis without vascular/lymphatic invasion: tumor may invade tunica albuginea but not tunica vaginalis **pT2** Tumor limited to testis and epididymis with vascular/lymphatic invasion, or tumor extending through tunica albuginea with involvement of tunica vaginalis **pT3** Tumor invades spermatic cord with or without vascular/lymphatic invasion **pT4** Tumor invades scrotum with or without vascular/lymphatic invasion			
N Regional lymph nodes clinical			
NX Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Metastasis with a lymph node mass 2 cm or less in greatest dimension or multiple lymph nodes, none more than 2 cm in greatest dimension **N2** Metastasis with a lymph node mass more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, any one mass more than 2 cm but not more than 5 cm in greatest dimension **N3** Metastasis with a lymph node mass more than 5 cm in greatest dimension			
pN Pathological			
pNX Regional lymph nodes cannot be assessed **pN0** No regional lymph node metastasis **pN1** Metastasis with a lymph node mass 2 cm or less in greatest dimension and 5 or fewer positive nodes, none more than 2 cm in greatest dimension **pN2** Metastasis with a lymph node mass more than 2 cm but not more than 5 cm in greatest dimension; or more than 5 nodes positive, none more than 5 cm; or evidence or extragonadal extension of tumor **pN3** Metastasis with a lymph node mass more than 5 cm in greatest dimension			
M Distant metastasis			
MX Distant metastasis cannot be assessed **M0** No distant metastasis **M1** Distant metastasis **M1a** Non-regional lymph node(s) or lung **M1b** Other sites			
S Serum tumor markers			
Sx Serum marker studies not available or not performed **S0** Serum marker study levels within normal limits			
	LDH (U/l)	**βHCG (mIU/ml)**	**AFP (ng/ml)**
S1	< 1.5 x N and	< 5,000 and	< 1,000
S2	1.5-10 x N or	5,000-50,000 or	1,000-10,000
S3	> 10 x N or	> 50,000 or	>10,000

N, upper limit of normal LDH assay; LDH, lactate dehydrogenase; βHCG, human chorionic gonadotrophin; AFP, alpha fetoprotein

Table 2.2 Stage grouping of testicular cancer (EAU. 2012).

Stage 0	pTis	N0	M0	S0, S1
Stage I	pT1-T4	N0	M0	SX
Stage IA	pT1	N0	M0	S0
Stage IB	pT2-pT4	N0	M0	S0
Stage IS	Any patient/TX	N0	M0	S1-3
Stage II	Any patient/TX	N1-N3	M0	SX
Stage IIA	Any patient/TX	N1	M0	S0
	Any patient/TX	N1	M0	S1
Stage IIB	Any patient/TX	N2	M0	S0
	Any patient/TX	N2	M0	S1
Stage IIC	Any patient/TX	N3	M0	S0
	Any patient/TX	N3	M0	S1
Stage III	Any patient/TX	Any N	M1a	SX
Stage IIIA	Any patient/TX	Any N	M1a	S0
	Any patient/TX	Any N	M1a	S1
Stage IIIB	Any patient/TX	N1-N3	M0	S2
	Any patient/TX	Any N	M1a	S2
Stage IIIC	Any patient/TX	N1-N3	M0	S3
	Any patient/TX	Any N	M1a	S3
	Any patient/TX	Any N	M1b	Any S

1.3 Histology of testicular germ cell tumors

Histologically, GCTs are divided into two classes: seminomatous and nonseminomatous germ cell tumors (NSGCTs). Seminomas resemble totipotent primordial germ cells, and nonseminomas are either undifferentiated (embryonal carcinoma) or differentiated, exhibiting different degrees of embryonic (teratoma) or extra-embryonic (yolk sac tumor and choriocarcinoma) patterning (**Ulbright et al., 2005**). The histological differentiation of GCTs in presented in **Fig. 2.1**. It has also clinical implications as NSGCTs tend to grow faster, occur at an earlier mean age, and show lower 5-year survival rate, compared to seminomas. Less than 10% of all germ cell tumors arise in extragonadal sites (e.g. mediastinum), and their management follows that of testicular GCTs (**Bosl and Motzer, 1997**).

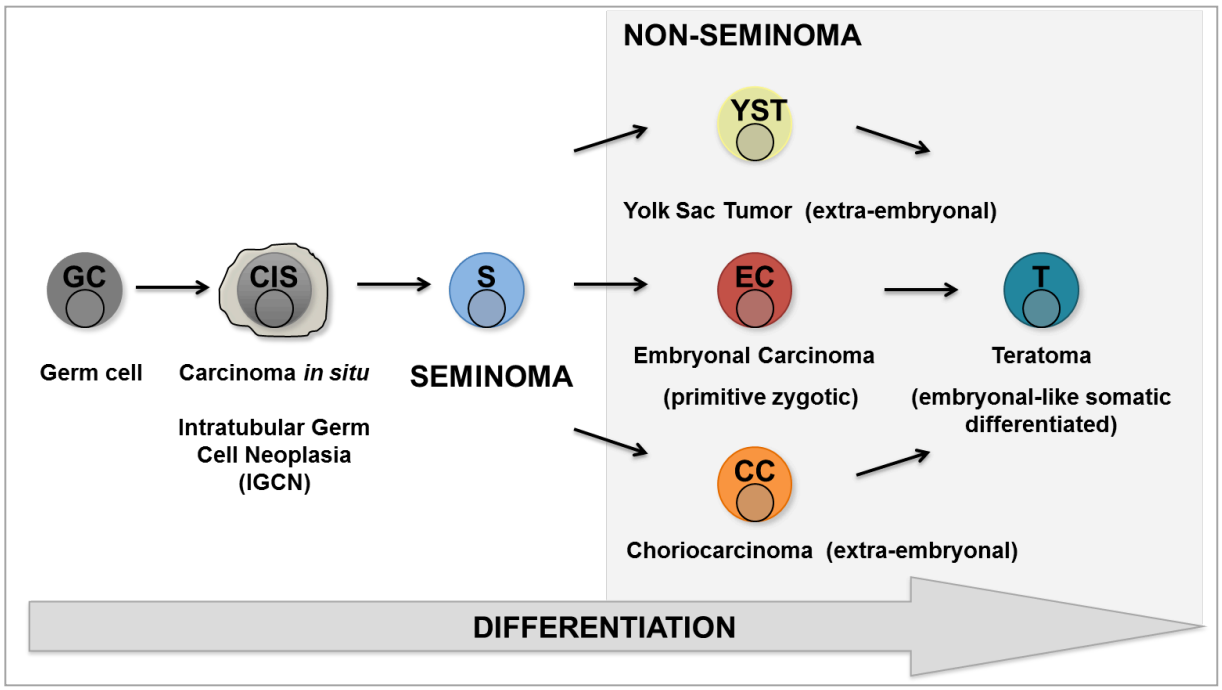

Figure 2.1 Histogenetic model for the development of germ cell tumors. CC, choriocarcinoma; EC, embryonal carcinoma; GC, germ cell; IGCNU, intratubular germ cell neoplasia, S, seminoma;T, teratoma; YST, yolk sac tumor, adapted from **Ulbright et al., 2005**.

1.4 Diagnosis and treatment of testicular germ cell tumors

The diagnosis of GCTs is based on physical and ultrasound examination. Additionally, serum tumour markers are prognostic factors and contribute to diagnosis and staging of GCTs. The following markers should be determined: alpha-fetoprotein (AFP), β-human chorionic gonadotropin (βHCG), and lactate dehydrogenase (LDH). In patients with nonseminomatous germ cell tumour, AFP is increased in 50-70% and βHCG is elevated in 40-60% of cases. Up to 30% of seminomas can present or develop an elevated βHCG level during the course of the disease. LDH is a less specific marker, and its concentration is proportional to tumor volume. (**Trigo et al., 2000; EAU, 2012**). However, the available serum markers are rarely elevated in pure teratoma.

The gold standard for detecting metastases in GCTs is computed tomography of the chest, abdomen, and pelvis. Patients with non-metastasized clinical stage I disease have an up to 50% risk of occult (invisible at the time of diagnosis) metastases, leading to relapse during surveillance (**EAU, 2012**).

The main treatment options for testicular GCTs are surgical removal of testis (orchidectomy), radiotherapy or chemotherapy (cisplatin-based). While treatment success depends on the stage and histology, the average survival rate after five years is around 95%, and stage I tumors cases (if monitored properly) have essentially a 100% 5-year survival rate (**EAU, 2012**).

1.5 Molecular markers of germ cell tumors

Germ cell tumors are characterized by very high heterogeneity. They express both germ cell-specific markers including placental-like alkaline phosphatase (PLAP), CD30, CD117 (C-KIT), a-fetoprotein (AFP), OCT3/4, SALL4, Glypican-3 (GPC3) as well as markers characteristic for epithelial cells like keratins and epithelial cell adhesion molecule epithelial (EpCAM) (**EAU, 2012**).

1.5.1 SALL4

Sal-like protein 4 (SALL4), a stem cell marker, is a zinc finger transcription factor and homologous to the Drosophila spalt (sal) gene. In Drosophila, sal acts as a region-specific homeotic gene involved in the specification of head and tail regions during embryonal development (**Jurgens et al., 1988; Reuter et al., 1989**). Similarly, in mice, Sall4 is essential to early embryogenesis, and homozygous mutant mice exhibit early embryonic lethality. In humans, *SALL4* is located on chromosome 20q13.13-13.2. As in other species, SALL4 is essential to human development, and mutations in SALL4 lead to Okihiro syndrome. In human embryonic stem cells, SALL4 is essential to maintain embryonal stem cell pluripotency and self-renewal by forming a regulatory network with OCT4, NANOG, and SOX2 (**Sakaki-Yumoto et al., 2006; Warren et al., 2007; Kohlhase et al., 2002; Cao et al., 2009**). SALL4 has been reported as a sensitive and specific diagnostic marker that is present in all GCT types. Strong expression of SALL4 in GCTs suggests that it might play some role in pathogenesis of germ cell tumors, however, the underlying mechanism is unknown (**Cao et el al., 2009**).

1.5.2 OCT3/4

The human OCT3/4 (POU5F1) transcription factor is encoded by the *POU5F1* gene that has been mapped to chromosome 6p21. The OCT3/4 and is currently regarded as one of the key regulators of pluripotency. The central role of OCT3/4 is keeping cells in an undifferentiated pluripotent state and preventing differentiation (**Niwa et al., 2000; de Jong and Looijenga, 2006**). In germ cell development prolonged expression of OCT3/4 is tightly linked to malignant transformation and the genesis of germ cell tumors of adolescents and young adults. OCT3/4 is a sensitive and specific marker especially for seminomas and embryonal carcinomas (**Looijenga et al., 2003; Jones et al., 2004**).

1.5.3 Keratins

Keratin is a family of fibrous structural proteins. The keratins that are most frequently expressed in germ cell tumors are keratins 8 and 18. These are keratins of simple epithelia the first two keratins expressed during mouse embryogenesis (**Jackson et al., 1980; Jackson et al., 1981**). Keratins 8 and 18 were shown to be expressed by seminomas and embryonal carcinomas (**Cheville et al., 2000; de Haas et al., 2008**). Additionally, keratin 19 as a marker for epithelial cells was found to be present in some seminomas, and the majority of nonseminomas (**Hildebrandt et al., 1998**).

1.5.4 Epithelial cell adhesion molecule

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein mediating calcium independent homotypic cell-cell adhesion in epithelia. EpCAM is also involved in cell signaling, migration, proliferation, and differentiation (**Münz et al., 2006; Maetzel et al., 2009**). It is also expressed in germline cells and spermatogonial stem cells (**Anderson et al., 1999; Dovey et al., 2013**). **Schoenberger et al.** (2013) showed that EpCAM is highly expressed in malignant GCTs such as yolk sac tumors and choriocarcinomas.

1.5.5 CD30

CD30 is a transmembrane glycoprotein receptor that is normally found on the surface of activated T cells, but it was also detected on various cell types of hematopoietic and lymphatic cells of non T-cell origin (**Chiarle et al., 1999; Deutsch et al., 2011**) as well as on activated or neoplastic mesothelium (**Dürkop et al., 2000**). CD30 antigen has low expression on normal cells but is more highly expressed on Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma, and on T cells of other lymphoproliferative disorders (**Gattei et al., 1997**). Furthermore, expression of CD30 is an established marker of embryonal carcinoma cells (**Pallesen et al., 1988**), and shows differential expression between normal and transformed human pluripotent stem cells (**Herszfeld et al, 2006**). Expression of CD30 in human pluripotent stem cells enhances cell growth and inhibits apoptosis (**Chung et al., 2010**). Interestingly, there is a controversy regarding expression of CD30 in embryonal carcinoma after chemotherapy: while two reports described loss of CD30 after chemotherapy (**Berney et al., 2001; Sung et al., 2006**), a more recent report described persistent expression of CD30 post-chemotherapy, and even concluded that it constituted a negative prognostic factor for survival (**Giannatempo et al., 2013**). CD30 is also an interesting therapeutic target in the age of targeted therapy: Brentuximab vedotin is a clinically well-established antibody-drug conjugate (enabling the delivery of a cytotoxic drug to the target malignant cell) that combines the agent monomethylauristatin E with a CD30 specific monoclonal antibody (**Younes et al., 2010; Younes et al., 2012**).

1.5.6 Glypican-3

Glypican-3 (GPC3) belongs to the family of heparin sulfate proteoglycans and it is thought to regulate growth through interactions with morphogenic or growth factors such as Wnt5a, fibroblast growth factor 2, bone morphogenic protein 7, and tissue factor pathway inhibitor. GPC3 is normally expressed in trophoblasts and a wide spectrum of fetal tissues, but shows only limited expression in adult tissues. Expression has been found in tumor tissues such as hepatocellular carcinoma,

hepatoblastoma, and Wilms tumor. More recently, it has been reported as an immunohistochemical marker of GCTs, with particular expression in yolk sac tumor and choriocarcinoma components, but it is also been found in teratomas with immature elements and in embryonal carcinomas, although the latter has been controversially discussed (**Zynger et al., 2006; Zynger et al., 2010; Preda et al., 2011**). It has been discussed that it plays a role in tumor cell differentiation (**Zynger et al., 2006**). Currently, several therapeutic antibodies targeting GPC3, mainly for the treatment of hepatocellular carcinoma, are under development: GC33 (phase II clinical trial) and YP7 (preclinical development) are humanized mouse antibodies, whereas MDX-1414 and HN3 (both in preclinical development) are fully humanized antibodies (**Zhu et al, 2013; Feng et al., 2013; Feng and Ho, 2014**).

1.5.7 Gain of 12p chromosomal region

The most common chromosomal anomaly found in invasive GCTs is a relative gain of the short arm of chromosome 12 (12p), mediated by isochromosome 12p [i(12p)] formation in up to 80% of cases. The remaining 20% of i(12p)-negative GCTs also contain additional copies of the short arm of chromosome 12 (**Rodriguez et al., 1993; Suijkerbuijk et al., 1993**). It has been demonstrated that gain of 12p is related to invasive growth and that amplification of specific 12p sequences localized to 12p11.2-p12.1 (**Fig. 2.2**). This finding indicates that an increased copy number of one or more genes located on 12p plays a role in the development of GCTs (**Zafarana et al., 2002**).

Suppression of apoptosis/Sertoli cell-independence	*DAD-R* (12p11)	homolog of defender against cell death 1
Suppression of apoptosis	*EKI1* (12p12.1)	ethanolamine kinase 1
Malignant transformation and proliferation	*KRAS2* (12p12.1)	GTPase KRas; V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
	CCND2 (12p13.32)	cyclin D2
Stemness and maintenance of pluripotency	*STELLA* (12p13.31)	developmental pluripotency associated 3; putative DNA binding protein
	NANOG (12p13.31)	homeodomain, reported role in mES pluripotency
	EDR1 (12p13.31)	early development regulator 1
	GDF3 (12p13.31)	growth differentiation factor 3
	CD9 (12p13.31)	Tetraspanin surface antigen; ES/EC stem cell marker

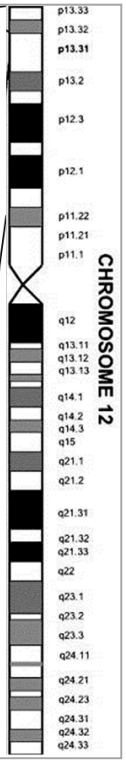

Figure 2.2 Candidate genes for involvement in the pathogenesis of germ cell tumors contained within amplified regions of 12p (Zafarana et al., 2002; Looijenga et al., 2003).

1.6 Circulating tumor cells in testicular germ cell tumors

There are few reports showing the presence of CTCs in blood of patients with GCTs. Few studies have previously assessed presence of tumor RNA or whole cells in apheresis products of patients undergoing peripheral stem cell transplants (Fan et al., 1998; Hildebrandt et al., 1998; Bokemeyer et al., 2001). Additionally, in a small number of studies, possible circulating tumor cells in peripheral blood of patients with testicular GCT were detected by reverse transcriptase chain reaction (RT-PCR) using alpha fetoprotein and human chorionic gonadotropin-specific mRNA as markers (Yuasa et al., 1999; Hautkappe et al., 2000).

1.7 Aim of the study

The aim of the present study was to determine the incidence of CTCs in patients with GCT using Ficoll density gradient centrifugation followed by ICC staining and the established CellSearch® system, and to correlate the findings to clinical parameters such as primary tumor histology, clinical stage of disease, and serum tumor marker levels.

2. MATERIAL AND METHODS

2.1 Clinical material

2.1.1 Peripheral blood

Nine to seventeen mL of peripheral blood was collected into EDTA-tubes preoperatively from 143 patients. One-hundred forty one patients suffering from histologically proven testicular GCTs and, 2 patients with mediastinal GCTs were included in this study. Detailed clinico-pathological parameters of all patients are summarized in **Tab. 2.3**. Pathologists experienced in pathology of GCT evaluated histologic diagnosis. All patients were treated between October 2011 and November 2013 in one of three participating centres: Department of Urology (Federal Armed Forces Hospital, Hamburg, Germany), Department of Urology or Department of Oncology, (University Medical Centre Hamburg-Eppendorf, Hamburg, Germany). Blood samples were taken with informed consent after ethical committee approval. One-hundred thirty seven blood samples were taken from patients at the time of initial therapy at the primary disease diagnosis. Additionally, 6 GCT patients who relapsed after conventional treatment were enrolled (**Tab. 2.4**). For patients with refractory disease, parameters concerning the primary tumor, except histology of the primary lesion, were not considered.

2.1.2 Testicular vein blood

Testicular vein blood (0.5 to 3.2 mL) was taken intra-operatively from 16 patients.

2.1.3 Primary testicular germ cell tumors

Twelve primary testicular germ cell tumors of different histological types were included.

2.3.4 Healthy and non-germ cell tumor controls

Seventeen individuals were included as control group, 7 suffering from non-germ cell tumor of the testis (e.g. Leydig Cell tumor), and 10 healthy male volunteers.

Table 2.3 Characterization of the study cohort.

Parameter		Entire cohort (n=143)	
		Number	% of valid cases
Age [years] mean: 37.7 (range: 16.9 -75; median:37.3)	<37.3	71	49.7
	≥37.3	72	50.3
Clinical stage	I	91	68.4
	II	27	20.3
	III	15	11.3
Tumor size [mm] mean: 34.3 (range: 1.4 -112; median: 30.0)	<34.3	69	60.0
	≥34.3	50	40.0
Seminoma mean: 32.7 (range: 2.2-105.0; median: 27.0)	Seminoma <40	42	64.6
	Seminoma ≥40	23	35.4
Nonseminoma mean: 29.6 (range: 1.4-112.0; median: 27.0)	Nonseminoma <29.6	25	49.0
	Nonseminoma ≥29.6	26	51.0
Primary tumor stage	pT1	73	61.3
	pT2	41	34.4
	pT3	4	3.3
Infiltration of *rete testis*	No	79	70.5
	Yes	33	29.5
Infiltration of *tunica albuginea*	No	47	69.1
	Yes	21	30.9
Lymphatic vessel invasion	No	69	69.0
	Yes	31	30.0
Vascular invasion	No	88	83.0
	Yes	18	17.0
Testicular Intraepithelial Neoplasia (TIN)	No	12	11.2
	Yes	95	88.8
Contralateral TIN	No	100	92.6
	Yes	8	7.4
Histology of primary lesion	Pure Seminoma	66	51.2
	Nonseminoma:	63	48.8
	≥1%Embryonal Carcinoma	47	36.4
	≥1%Yolk Sac Tumor	26	20.2
	≥1%Teratoma	36	27.9
	≥1%Choriocarcinoma	13	10.1
AFP [ng/mL] mean: 175.8 (range: 0.7-7600; median: 3.0)	Normal (< 7)	96	72.7
	Elevated (≥7)	36	27.3
βHCG [U/L] mean: 1182.9 (range: 0-121425; median:1.2)	Normal (<1)	62	47.0
	Elevated (≥1)	70	53.0
LDH [U/L] mean: 254.5 (range: 122-1972; median: 189.0)	Normal (<250)	99	78.0
	Elevated (≥250)	28	22.0

Note that due to the missing values not all numbers sum up to 143 cases.

Table 2.4 Characterization of relapsed, treatment-refractory germ cell tumor patients.

Case Nr	Site of primary GCT	Clinical stage (UICC) at primary diagnosis	Tumor markers at primary diagnosis	IGCCCG-defined risk	Site of metastases at blood sample collection	Chemotherapy regimen	Outcome
5	Testicular	I S	AFP=1760	Intermediate	Lungs, bone mediastinum	PEB, TIP, HD-CE, GOP, CP	Died
32	Testicular	III B	AFP=256.9 βHCG=36961 LDH=638	Intermediate	Lungs, skin, liver	PEB, PEI, HD-CE, GOP, oE, E	Died
33	Mediastinal	III C	AFP>38000	Poor	Bone	HD-PEI,GOP,HD-CE	Alive
35	Testicular	III C	βHCG=20000	Poor	Lungs, CNS	PEB, PEI, GOP, E	Died
36	Mediastinal	III C	AFP=3928.4 βHCG=25 LDH=419	Intermediate	-	PEI, HD-PEI, CEI	Alive
38	Testicular	III C	βHCG=225000	Poor	Lungs, CNS	PEB, PI	Alive

CE, carboplatin and etoposide; CEI, cisplatin, etoposide, ifosfamide; CP, carboplatin and paclitaxel; E, everolimus; GOP, gemcitabine, oxaliplatin, paclitaxel; HD-CE, high dose carboplatin and etoposide; HD-PEI, high dose cisplatin, etoposid, ifosfamide; oE, oral etoposide; PEB, cisplatin, etoposide, bleomycin; PEI, cisplatin, etoposid, ifosfamide; PI, paclitaxel and ifosfamide; TIP, paclitaxel, ifosfamide, cisplatin; CNS, central nervous system; UICC, International Union Against Cancer; AJCC, American Joint Committee on Cancer; IGCCC, International Germ Cell Consensus Classification

2.2 Cell lines and media

The list of germ cell tumor cell lines is presented in the Tab. 2.5.

Table 2.5 Germ cell tumor cell lines.

Cell line	Histology	Origin	Medium
TCam-2	seminoma	primary testicular tumor	A*
2102Ep	embryonal carcinoma/teratocarcinoma	primary testicular tumor	B*
NCCIT	malignant pluripotent embryonal carcinoma	mediastinal tumor	
NT2	embryonal carcinoma/teratoma	primary testicular tumor	

***Supp. material I**

2.3 Reagents

2.3.1 Antibodies

The list of antibodies is presented in the **Tab. 2.6**.

Table 2.6 Antibodies.

Type	Host	Antibody	Clone	Dilution	Company
Primary antibodies	mouse	anti-human SALL-4	6E3	1:750	Abnova, Taiwan
	goat	anti-human OCT3/4	sc-8629	1:750	Santa Cruz Biotechnology, US
	mouse	anti-human pan keratin A45-B/B3 directly labelled with Cy3	-	1:300	Micromet, Germany
	mouse	anti-human pan keratin A45/B3	-	1:300	Micromet, Germany
	mouse	anti-human EpCAM (NCL-ESA)	VU1D9	1:100	Novocastra, Germany
	mouse	anti-human CD30	Ber-H2	1:10	Dako, Denmark
	mouse	anti-human Glypican-3	1G12	1:500	Biomosiaics, , VT, US
	mouse	anti-human CD45 directly labelled with Alexa-488	HI30	1:150	Biolegend, CA, US
Secondary antibodies	mouse	anti-goat Alexa-488-conjugated	-	1:200	Life Technologies, CA, US
	rabbit	anti-mouse Alexa-488-conjugated	-	1:200	Life Technologies, CA, US
	rabbit	anti-mouse Alexa-546-conjugated	-	1:200	Life Technologies, CA, US

2.3.2 Immunohistochemical staining reagents

Immunohistochemical staining reagents are listed in the **Tab. 2.7**.

Table 2.7 Immunohistochemical staining reagents.

Name of the reagent	Working solution	Company
Fixation Solution B for Epithelial Cell Detection Kit	135µl diluted in 10 mL of 1xPBS	Micromet, Germany
AB blocking serum	diluted 1:10 in 1xPBS	Biotest, Germany
Dako REAL™ Antibody Diluent	-	Dako, Denmark
DakoCytomation Antibody Diluent with Background Reducing Components	-	Dako, Denmark
Vectashield® Mounting Medium with DAPI	-	Vector Laboratories, CA, US
Dako REAL™ Detection System, Peroxidase/DAB+,Rabbit/Mouse	-	Dako, Denmark
Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+,Rabbit/Mouse	-	Dako, Denmark
Cell and tissue staining goat kit (HRP-AEC system)	-	R&D systems, MN, USA
Trypsin	one pill diluted in 1mL of distilled water	Sigma, US
Citrate Buffer D	diluted 1:10	Dako, Denmark
Citrate Buffer B	diluted 1:10	Biogenex, US
Meyer's Hemalaum Solution	-	Merk, Germany
Eukitt® quick-hardening mounting medium	-	Sigma, US

2.3.3 FISH reagents

The list of FISH reagents is presented in the **Tab. 2.8**.

Table 2.8 Fluorescent in situ hybridization reagents.

Name of the regaent	Company
CEP12 Spectrum Green	Abbott Molecular, USA
Cot Human DNA	Roche, Germany
BioPrime® DNA Labeling System	Invitrogen, Germany
Human Placental DNA	Roche, Germany
dUTPs Spectrum Orange	Abbott Molecular, USA
Bio-Spin 30 Tris Columns	Bio-Rad Laboratories, USA
Spot-Light Tissue Heat Pretreatment Buffer	Zymed, USA
Pepsin Solution	Zytovision, Germany

2.3.4 FISH buffers and solutions

The list of FISH reagents is presented in the **Tab. 2.9**.

Table 2.9 Fluorescent in situ hybridization buffers and solutions.

Name of the buffer	Components	pH value
20 x SSC	175.3 g NaCl, 88.2 g Na_3-citrate * 2H20 in 1000 ml of H_20	7.0
2 x SSC-0.3 % NP-40	100 ml 20x SSC buffer, 3 ml NP-40 in 1000 ml of H20	7.0
Proteinase K Buffer	1 ml 1M Tris-HCl, 100 µl $CaCl_2$ * H_2O in 50 ml of H_20	-
Denaturation Buffer	35 ml formamide, 1.5 ml 20x SSC buffer in 400 ml of H_20	7.4
50%Formamide/2xSSC Buffer	200 ml formamide, 40 ml 20x SSC buffer in 400 ml of H_20	7.4
Hybridization Buffer	4 g dextran, 10 ml formamide, 4 ml 20x SSC buffer in 20 ml of H20	-
2% Formaldehyde	4 ml of 37% formaldehyde in 200 ml methanol	-

2.4 Cell line material preparation

2.4.1 Cell culture

Each cell line was cultured in an appropriate medium and conditions at 37°C (**Tab 2.5**). The passages were taken in every 2-3 days depending on a cell line and the confluence status. The cryo-preservation vials were prepared in a standard way with the usage of a proper medium and 10% DMSO (**Supp material II**).

2.4.2 Cytospin preparation

The microscopic slides were placed into the centrifugation pocket. Then, 250 – 300 µl of cell suspension containing 100 000 tumor cells, were put onto the slide surface. The slide was centrifuged for 3 min. at 300 x g in order to spin down the cells. The excess volume of 1xPBS was discarded. The slides were left overnight to air-dry at room temperature and used the following day for further analysis. If not, they were wrapped in an aluminum foil and stored at -80°C.

2.5 Detection of circulating tumor cells

2.5.1 Enrichment of CTCs

In order to assess the tumor cell recovery rate, blood samples from healthy donors were spiked with different numbers of tumor cells from each cell line and performed using Ficoll-Hypaque gradient centrifugation. Additionally, 500 cells of all four GCT cell lines were spiked into normal peripheral blood and processed in the CellSearch® system to assess detection thresholds of this system in GCT cells.

All blood samples were processed within a maximum of 24 h after collection. Mononuclear cells (MNCs) were enriched using the Ficoll-Hypaque (**Supp. material II**) gradient centrifugation. The blood was mixed 1:1 with 1xPBS, carefully layered on 20 mL Ficoll-Hypaque and centrifuged for 30 min. at 450 x g. The MNC fraction, preferably containing CTCs, was collected and re-suspended in 1 mL of 1xPBS and spun down for 3 min. at 300 x g to prepare the microscopic slides. The slides were

left overnight to air-dry at room temperature and were used within 24 hours for further analysis. For long-term storage, slides were wrapped in aluminium foil back to back and stored at -80°C.

For each double immunohistochemical staining, cytospins containing $3x10^6$ (for peripheral blood) or $1x10^6$ (for testicular vein blood) mononuclear cells were prepared as follows: The slides were fixed for 10 min. with the Fixation Solution B for Epithelial Cell Detection Kit (**Tab. 2.7**) and permeabilized for 5 min. in 0.1% Triton X in 1xPBS. Subsequently, the cells were incubated with 10% AB blocking serum (**Tab. 2.7**) for 20 min.

2.5.2 Double SALL4/keratin immunohistochemical staining

The slides were incubated for 45 min. with an anti-human SALL-4 mouse antibody (Tab. 3.4) in Dako REAL™ Antibody Diluent (**Tab. 2.7**). As secondary antibody, an anti-mouse Alexa-488-conjugated antibody (**Tab. 2.6**) in DakoCytomation Antibody Diluent with Background Reducing Components (**Tab. 2.7**) was used for 45 min. Subsequently, the cells were incubated with the anti-human pan keratin antibody A45-B/B3 directly labelled with Cy3 (**Tab. 2.6**) in Dako REAL™ Antibody Diluent (**Tab. 2.7**). A45-B/B3 is reactive with a common epitope of various keratins, including keratin 8, keratin 18 and keratin 19. Finally, the slides were counterstained with DAPI (**Tab. 2.7**) and covered with cover-slips.

2.5.3 Double OCT3/4/EpCAM immunohistochemical staining

Cells were incubated for 45 min. the primary goat antibody against human OCT3/4, (**Tab. 2.6**) in Dako REAL™ Antibody Diluent (**Tab.2.7**). As secondary antibody, an anti-goat Alexa-488-conjugated antibody (**Tab. 2.6**) in DakoCytomation Antibody Diluent with Background Reducing Components (**Tab. 2.7**) was used for 45 min. Subsequently, cells were incubated with mouse EpCAM antibody (**Tab. 2.6**) in Dako REAL™ Antibody Diluent (**Tab. 2.7**). Specimens were incubated with the secondary anti-mouse Alexa-546-conjugated antibody (**Tab. 2.6**) followed by counterstaining with DAPI (**Tab. 2.7**) and covered with cover-slips.

2.5.4 Immunocytochemical staining for CD30 and GPC3

For immunocytochemical staining, cytospins containing $1.4x10^6$ (for peripheral blood) or $0.5x10^6$ (for testicular vein blood) MNCs were prepared. Briefly, the slides were fixed for 10 min. with the Fixation Solution B for Epithelial Cell Detection Kit (**Tab. 2.7**) and permeabilized for 5 min. in 0.1% Triton X in 1xPBS. Subsequently, the cells were incubated with AB blocking serum (**Tab. 2.7**) for 20 min. Slides were incubated for 45 min. with an anti-human CD30 mouse antibody (**Tab. 2.6**) or anti-human Glypican-3 mouse antibody (**Tab. 2.6**) diluted in 10% AB blocking serum (**Tab. 2.7**). As secondary antibody, an anti-mouse Alexa-546-conjugated antibody (**Tab. 2.6**) in DakoCytomation Antibody Diluent with Background Reducing Components (**Tab. 2.7**) was used for 45 min.

2.5.5 Immunocytochemical staining for CD45

In order to visualize leukocytes, the slides were incubated with the anti-human antibody against CD45, directly labelled with Alexa-488 (**Tab. 2.6**) in 10% AB serum (**Tab. 2.7**). Finally, all the specimens were counterstained with DAPI (**Tab. 2.7**) to visualize cells' nuclei and covered with cover-slips.

2.5.6 CellSearch® analysis of circulating tumor cells

In parallel, 122 blood samples of 7.5 mL were collected in CellSave® tubes (**Supp. material III**) and performed with the usage of the CellSearch® system. All blood samples were measured within 96 h after collection. Blood samples were gently mixed with 6.5 mL of dilution buffer, centrifuged for 10 min. at 800 g at room temperature, and transferred into the CellTracks® AutoPrep system (**Supp. material III**). The CellTracks® AutoPrep system allows automation of the sample processing, including all reagent addition, mixing, incubation, and aspiration steps. The first step was aspiration of the plasma and dilution buffer layer. In order to capture epithelial cells, anti-EpCAM antibody–coated ferrofluids and capture enhancement reagent were added. After incubation and magnetic separation, unbound cells and remaining plasma were removed, and ferrofluid-labeled cells were re-suspended in buffer,

permeabilized, and fluorescently labelled using the phycoerythrin-conjugated anti-cytokeratin antibodies recognizing cytokeratins (predominantly cytokeratins 8, 18, and 19). Additionally, an antibody against CD45 conjugated with allophycocyanin to identify white blood cells was added. The cell nuclei were fluorescently labelled with a nuclear dye [4'6-diamidino-2-phenylindole (DAPI)]. After incubation and repeated magnetic separation, unbound staining reagents are aspirated, and a cell fixative is added. After enrichment and immunocytochemical staining, immunomagnetically labeled cells were kept for 20 min. in a strong magnetic field and scanned using the CellSpotter® Analyzer (**Supp. material III**). Results of the analyses were interpreted by researchers experienced with this system (**Riethdorf et al., 2007**).

2.5.7 Apoptotic cell evaluation

Apoptotic cells were assessed by characteristic morphology - presence of small pycnotic nuclei or apoptotic apoptotic bodies or speckled cytoplasmic staining.

2.6 Immunohistochemical staining on formalin-fixed paraffin sections

The scheme of immunohistochemical staining for SALL4, OCT3/4, Keratins 8/18/19 and EpCAM on formalin-fixed paraffin primary tumors is presented in the **Tab. 2.10**.

Table 2.10 Immunohistochemical staining scheme for formalin-fixed paraffin primary tumors.

IHC steps	Target protein(s)			
	SALL4	**OCT3/4**	**Keratins 8, 18, 19**	**EpCAM**
Deparaffinization	• Incubation for 2h at 60°C • Washing twice for 15 min. in xylene • Washing twice for 10 min. in 100% ethanol and • Shortly rinsing in series of ethanol (96%>80%>70%) • Incubation in distilled water for 5 min.			
Antigen retrieval	5 min. at 125°C in decloaking chamber in citrate buffer D (**Tab 2.7**)		5 min. at 125°C in decloaking chamber in citrate buffer B (**Tab 2.7**)	10 min. incubation with Trypsin (**Tab. 2.7**) for at 37°C
Washing	3 times for 3 min. in 1xTBST (**Supp. material II**)			3 times for 3 min. in 1xPBS
Antibody/dilution	anti-human SALL4 (**Tab. 2.6**) 1:300	anti-human OCT3/4 (**Tab. 2.6**) 1:1000	anti-human pan keratin A45/B3 (**Tab. 2.6**) 1:200	anti-human EpCAM (**Tab. 2.6**) 1:75
Antibody incubation	Overnight incubation at 4°C			Incubation for 60 min at room temperature
Visualization	Dako REAL™ Detection System, Peroxidase/DAB+ (**Tab 2.7**)	Cell and tissue staining goat kit HRP-AEC system (**Tab 2.7**)	Dako REAL™ Detection System, Peroxidase/DAB+ (**Tab 2.7**)	Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+ (**Tab 2.7**)
Washing	3 times for 3 min. in 1xTBST (**Supp. material II**)			3 times for 3 min. in 1xPBS
Staining of nuclei	Incubation for 2 sec. in Mayer's Hemalaum solution (**Tab 2.7**)			
Dehydration	• Incubation 2 times for 1 min.in 80% ethanol • Incubation 2 times for 1 min.in 96% ethanol • Incubation 2 times for 1 min.in 99% ethanol • Incubation 2 times for 2 min.in xylene			
Covering	• Sealing with a drop of Eukitt medium (**Tab 2.7**) • Covering with a cover-slip			

2.7 Fluorescent *in situ* hybridization

In order to confirm the germ-cell origin of CTCs, fluorescence *in situ* hybridization (FISH) was conducted using a probe derived from the Homo sapiens PAC clone 876C13 from region 12p11.23 (kindly provided by A. J. M. Gills and L. H. J. Looijenga). The probe overlaps the most frequently amplified region in GCTs identified as 12p11.2–p12.1 (**Looijenga et al., 2003**).

2.7.1 Probe preparation

1 µg of isolated PAC DNA was dissolved into 18 µl of H_20 and boiled for 5 min. Next, it was immediately cooled down on ice and the labelling reaction using BioPrime® DNA Labeling System (**Tab. 2.8**) was prepared according to the scheme presented in **Tab. 2.11**.

Table 2.11 FISH probe labelling reaction.

Reagent	Vol. (µl) per sample
1 µg PAC DNA in H2O	18
10x dNTPs2	5
0.5 mM labelled-dUTPs (spectrum orange)	5
2.5x random hexamers	20
Klenow fragment	1

The sample was mixed gently, centrifuged briefly and incubated for at least 3 hours at 37°C. The labelled probe was purified using the Bio-Spin 30 Tris Columns (**Tab. 2.8**) as it was described in the manufacturer's protocol. Next, the product was precipitated using 5 µl of 3M sodium acetate and 150 µl of 100% ethanol and left for a minimum 1 hour at 4°C. After that time, the probe was spun down for 30 min. at 20000 x g at 4°C. The supernatant was removed. The probe was air-dried, dissolved in 25 µl of Hybridization Buffer (**Tab. 2.9**) overnight at 37°C and stored at -20°C.

2.7.2 Fluorescent in situ hybridization on cytospins

Four samples from peripheral and one sample from testicular blood were analysed. Cytospins were incubated with denaturation solution (**Tab. 2.9**) for 5 min. at 75°C.

Then, the slides were dehydrated, and enzyme pre-treatment of cells was carried out with Proteinase-K (**Tab. 2.9**) solution for 7 min. at 37°C. Cytospins were washed, dehydrated, and air-dried before adding 3 µl of the probe mixed with 1 µl of Cot Human DNA (**Tab. 2.8**), 1 µl of Centromere 12 (**Tab. 2.8**) and 5 µl of hybridization buffer (**Tab. 2.9**). After denaturation at 75°C for 7 min., hybridization was carried out at 37°C overnight. Post-hybridization washes were carried out at 72°C and at room temperature in 2×SSC/0.3% NP-40 (**Tab. 2.9**) for 3 min. each. After dehydration in ascending concentrations of ethanol and air drying, slides were mounted with mounting medium containing DAPI (**Tab. 2.7**).

2.7.3 Fluorescent in situ hybridization on paraffin sections

The deparaffinized primary tissue was fixed 10 min. in 2% formaldehyde in methanol (**Tab. 2.9**) at -20°C and washed three times for 3 min. in 1xPBS at room temperature. The slides were pre-treated in Spot-Light Tissue Heat Pre-treatment Buffer (**Tab. 2.8**) for 10 min. at 97°C and left for 15 min. to cool down in the same chamber at room temperature. Afterwards, the specimens were washed twice for 3 min. in 1xPBS at room temperature and treated with 100 µl of Pepsin Solution (**Tab. 2.8**) for 10 min. at 37°C and then again washed three times for 2 min. in 1xPBS at room temperature. Next, slides were dehydrated as follows: 1 min. in 80% ethanol, 1 min. in 96% ethanol, 1 min. in 100% ethanol and left to air dry. Three microliters of the probe were mixed with 1µl of Cot Human DNA (**Tab. 2.8**), 1 µl of Centromere 12 (**Tab. 2.8**) and 5 µl of hybridization buffer (**Tab. 2.9**) and applied onto the slide surface. Then the slides were denatured for 3 min. at 95°C and hybridized overnight at 37°C. The next day samples were washed as follows: 2 min. in 2xSSC/NP-40 buffer (**Tab. 2.9**) at 72°C, 2 min. in 2xSSC/NP-40 buffer (**Tab. 2.9**) at room temperature, 2 min. in 1xPBS at room temperature, 1 min. in 70% ethanol, 1 min. in 80% ethanol, 1 min. in 96% ethanol, 1 min. in 100% ethanol and dried on air. A drop of DAPI mounting medium (**Tab. 2.7**) was put on the surface of the slide.

2.8 Microscopy

The slides were analysed under the microscope (**Supp. material V**). Micrographs were taken using the AxioVision software (**Supp. material IV**).

2.9 Statistical analysis

Statistical analysis was performed using SPSS software (**Supp. material IV**). Descriptive analyses were performed using Fisher's Exact test for categorical variables. Differences in variables with a continuous distribution across categories were assessed using Mann-Whitney U test. Results were considered statistically significant if $P<0.05$ and highly statistically significant if $P<0.001$.The survival curves were generated with the usage of Kaplan-Meier test verified by log-rank analysis.

3. RESULTS

3.1 Control material

3.1.1 Expression of selected markers in germ cell tumor cell lines

The expression of different germ cell tumor and epithelial-specific markers was examined in four germ cell tumor cell lines. Theses cell lines show diverse germ cell tumor histology (**Tab. 2.5**) and represent different stadiums of GCT differentiation. Strong expression of SALL4, OCT3/4, and keratins was found in >75% of the cells of all germ cell tumor cell lines (**Fig. 2.2 A**). In some cells of the TCam-2 and NT2 cell lines, keratins were detected in a dot-like pattern (**Fig. 2.2 C**). While EpCAM was strongly expressed in TCam-2 and 2102Ep cells, only weak or no expression was observed in NT2 and NCCIT cells (**Fig. 2.2 A**).

3.1.2 Expression of selected markers in primary germ cell tumors

All 12 primary tumors showed strong SALL4 and OCT3/4 expression, whereas only 8 (66.7%) of primary tumors exhibited strong or moderate keratin staining, of which 3 (25.0%) showed a dot-like pattern. EpCAM was expressed in 5 (41.6%) of the primary tumors (**Fig. 2.2 C**).

3.1.3 Spiking experiment with germ cell tumor cell lines

Using Ficoll-Hypaque gradient centrifugation followed by IHC staining with selected markers, tumor cells spiked into blood of healthy donors were recovered with 60-70% of sensitivity. Similarly, cells from all 4 cell lines were detected by the CellSearch® system (**Fig. 2.2 B**) with detection rate of 80-100%.

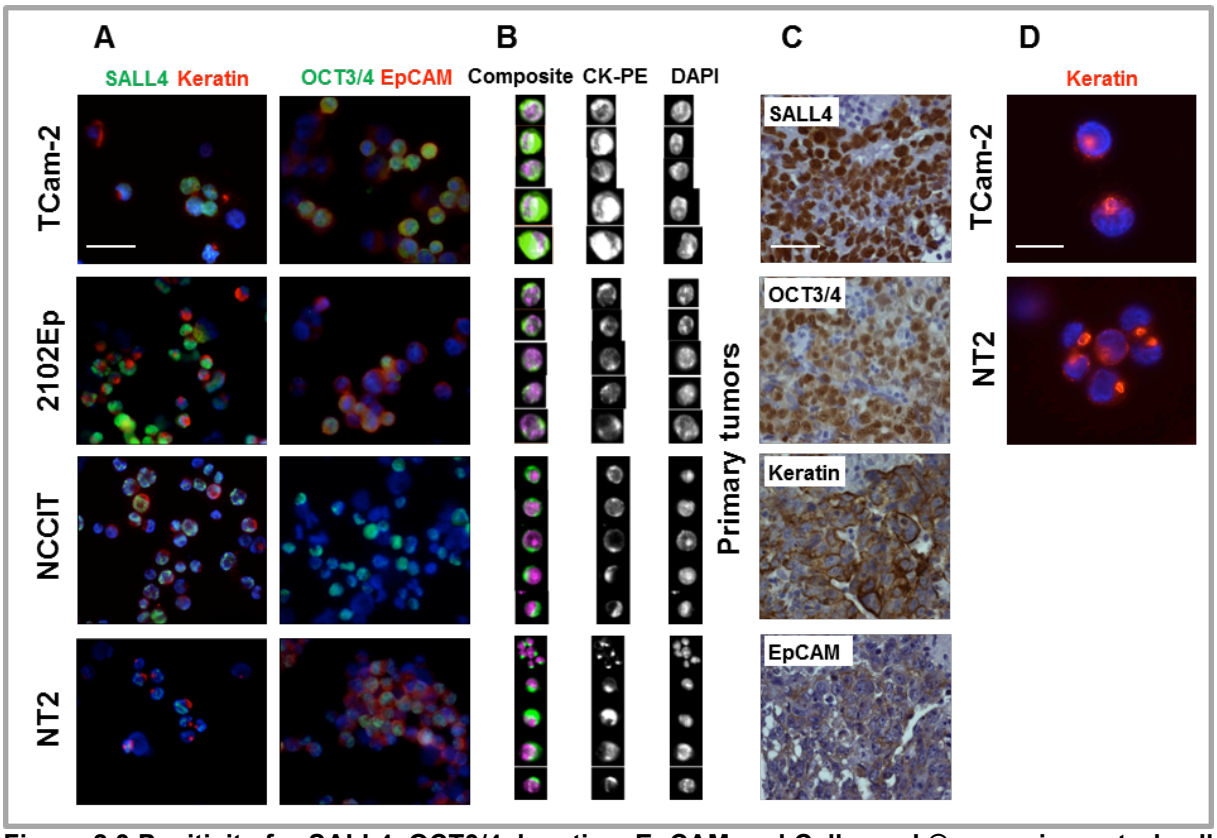

Figure 2.3 Positivity for SALL4, OCT3/4, keratins, EpCAM and Cellsearch® assay in control cell lines. A, Double fluorescence staining for SALL4 (green) / keratins (orange) and OCT3/4 (green) / EpCAM (orange) counterstained with DAPI (blue) to visualize cells' nuclei in 4 different germ cell tumor cell lines (magnification: 400x). Scale bar, 50 µm; B, Representation of the staining of tumor cells from 4 different germ cell tumor cell lines spiked into blood of healthy donors using the Cellsearch® assay; C, Representative images of primary testicular germ cell tumor samples positive for SALL4, OCT3/4, keratins and EpCAM immunohistochemical staining counterstained with Hematoxylin to visualize cells' nuclei (magnification: 400x, scale bar: 50 µm). (CK, cytokeratin; PE, phycoerythrin; APC, allophycocyanin; DAPI, 4′,6-diamidino-2-phenylindole); D, Dot-like pattern keratin (orange) staining in TCam-2 and NT2 cell lines, counterstained with DAPI (blue) to visualize cells' nuclei (magnification: 1000x). Scale bar, 20 µm.

3.1.4 Healthy and non-germ cell tumor controls

No positive cells for selected markers (SALL4, OCT3/4, Keratins and EpCAM) were found in the peripheral blood of 10 healthy volunteers or in non-germ cell tumor patients (acute monoblastic leukemia, Leydig cell tumour, Sertoli cell tumor).

3.2 Detection of circulating tumor cells in germ cell tumor patients

3.2.1 Circulating tumor cells in peripheral blood

Fourteen (9.8%) of 143 patients were positive for CTCs enriched by Ficoll-density gradient centrifugation and detected by subsequent staining with SALL4/keratins and/or OCT3/4/EpCAM (**Fig. 2.3**).

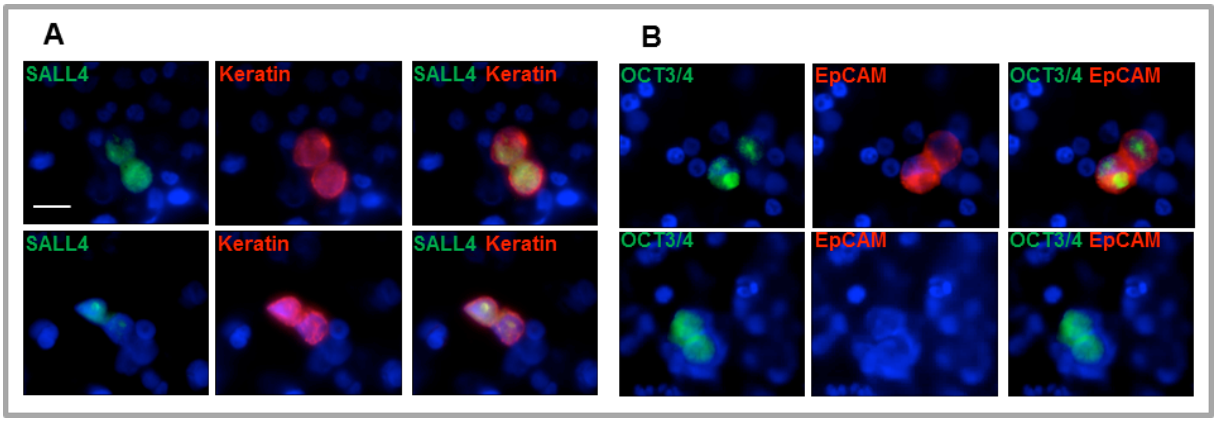

Figure 2.4 Representative images of tumor cells detected in peripheral blood of patients with germ cell tumors. Representative images of circulating tumor cells detected in peripheral blood, double-stained with SALL4 (green) / keratins (orange) (A) and OCT3/4 (green) / EpCAM (orange) (B) counterstained with DAPI (blue) to visualize cells' nuclei (magnification: 1000x, scale bar: 25 µm; DAPI, 4′,6-diamidino-2-phenylindole).

Fourteen (11.5%) of 122 patients were classified as positive for CTCs after processing with the CellSearch® system (**Fig. 2.3 B**). In total, CTCs were found in 25 (17.5%) of 143 patients with GCTs, irrespective of the method. Interestingly, only in 3 patients, CTCs were found in parallel with both detection methods.

The mean number of CTCs enriched by Ficoll density gradient centrifugation was 13 in $3x10^6$ of MNCs (range: 2-60; median: 8.5). Using the CellSearch® system, the mean number of CTCs was 2.6 per 7.5 ml of blood (range: 1-14; median: 1). CTCs showed heterogeneous staining of SALL4, OCT3/4, keratins and EpCAM within individual cells from the same patient (**Tab. 2.12**) and in 5 (20.0%) of CTC-positive patients we observed clusters of 3-5 cells.

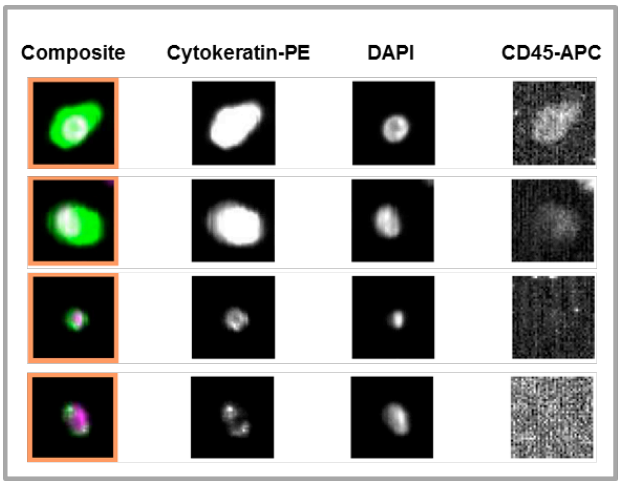

Figure 2.5 Representation of the staining of circulating cells using the Cellsearch® assay. CTCs are defined as CK-PE(+), DAPI(+), and CD45(−) cells. (CK, cytokeratin; PE, phycoerythrin; APC, allophycocyanin.

3.2.2 Tumor cells in testicular vein blood

In total, 12 (63.2%) of 19 testicular vein blood samples were positive for tumor cells. The mean tumor cell number was 45 (range: 4-120; median: 16) per $1x10^6$ of MNCs. In 7 (58.3%) patients, CTCs were forming clusters of 3 to 7 cells (Fig. 2.4). In one patient, CTCs were found both in testicular vein and peripheral blood and no patient had CTCs in peripheral vein only. Due to the small number of samples, no further statistical analysis was done for tumor cells detected in testicular vein blood samples.

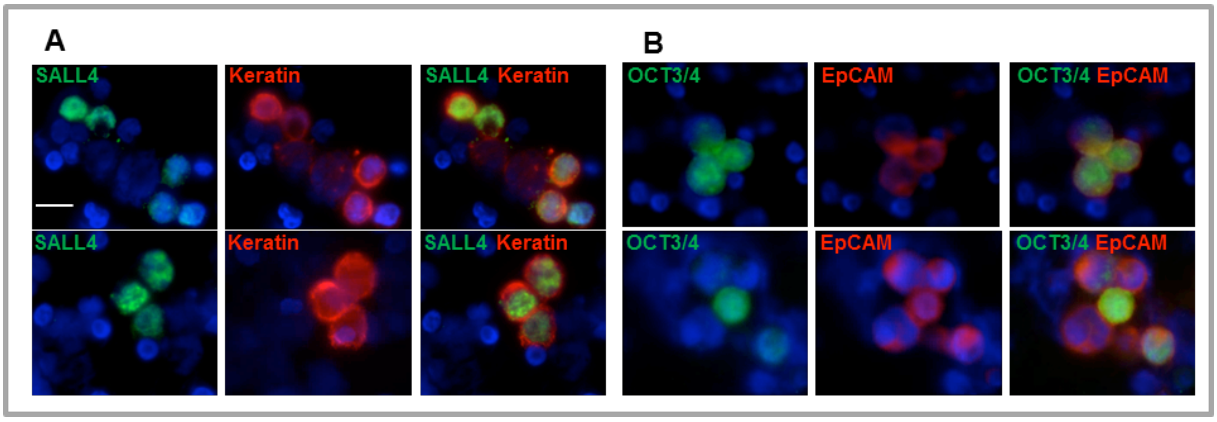

Figure 2.6 Representative images of tumor cells detected in testicular veins of patients with germ cell tumors. Images of tumor cells detected in testicular vein blood, double-stained with SALL4 (green) / keratins (orange) and OCT3/4 (green) / EpCAM (orange) counterstained with DAPI (blue) to visualize cells' nuclei (magnification: 1000x, scale bar: 25 µm).

Table 2.12 Characterization of patients positive for circulating tumor cells.

	Case Nr	Clinical stage (UICC)	Histology	Tumor Markers	No. CTCs/3 x 10^6 MNC				No. CTCs in CellSearch®	Apoptotic
					SALL4	Keratin	OCT3/4	EpCAM		
Treatment-naive patients	131	I	PS	Not elevated					1	
	3	II A	PS	βHCG = 3 LDH = 298					3	
	20	I S	PS	βHCG = 5					1	
	28	I S	PS	LDH=253					2	
	210	I	90% EC, 5% YST, 5% CC	AFP=385 βHCG=206					3	
	23	I	60% EC, 40%YST	AFP=4351 βHCG = 2026					1	
	153	II C	30% S, 70% T	AFP=1210 βHCG = 1920 LDH = 261			10	10		
	10	I	20% S,80% T	AFP=12		5		5	1	
	142	II B	70% EC, 30% T	LDH=400					1	
	112	III	80% EC, 2% YST, 18% T	AFP=115 βHCG = 330 LDH = 547					1	
	158	II B	78% EC,2% YST, 20% T	AFP=181 βHCG = 13					1	
	161	I S	45% EC,50% YST,5% CC	βHCG = 3373	10	10				
	168	nd	83% EC, 17% T	AFP=92 βHCG = 525 LDH = 252					1	
	2	II	55% EC, 5% YST, 40%T	AFP=7600 βHCG = 2029 LDH = 470		15		15	4	1
	16	III	33% EC,33% YST, 33% T	AFP=14 βHCG = 7 LDH = 253	2		2			
	151	II	100% T	Not elevated	10	10				
	44	II C	25% EC, 25% YST,25% T, 25% CC	Not elevated	7	7				
	139	III	nd	nd	1	15				
	89	III	nd	nd		60		10		5
Treatment-refractory patients	5	III	50% EC, 50% YST	Not elevated	15	15	35			
	32	III	50% EC, 25% YST, 25% CC	βHCG = 121425					14	3
	33	III	100% YST mediastinal	AFP=1589		4		1		2
	35	III	20% EC,80% T	βHCG = 792		5		2	2	2
	36	III	100% T mediastinal	AFP=3928		2	2			1
	38	III	50% EC, 50% CC	βHCG =10000	2	2				

PS, pure seminoma; S, seminoma; EC, embryonal carcinoma; YST, yolk sac tumor; T, teratoma; CC, choriocarcinoma; MNC, mononuclear cell; AFP [ng/mL], alpha fetoprotein; βHCG [U/L], beta human gonadotropin; LDH [U/L], lactate dehydrogenase; UICC - Union for International Cancer Control; nd, no data available

3.2.3 Detection of gains in 12p chromosomal region of circulating tumor cells

In 4 patients, CTCs with gains of the 12p chromosomal region were found in peripheral blood (**Fig. 2.5 B; Tab. 2.13**). In one tested sample from testicular vein, four tumor cells with 5-12 signals from the 12p region of interest and 5-7 centromere 12 signals were found which was similar to aberrations observed in the primary tumor tissue (**Fig. 2.5 A; Tab. 2.13**). Leukocytes present on slides showed 2 signals for 12p and centromere 12 each.

Table 2.13 Fluorescent in situ hybridization analysis of circulating tumor cells.

Case nr	Material	Cell	12p11.23	Centromere 12
2	Peripheral blood	#1	3	2
5	Peripheral blood	#1	4	2
		#2	3	2
44	Peripheral blood	#1	5	3
38	Peripheral blood	#1	3	2
6	Testicular vein blood	#1	5	5
		#2	3	2
		#3	12	7
		#4	11	5
	Primary tumor tissue	100 cells	mean: 6.1 (range: 3-12, median: 5)	mean: 5.1 (range 2-11, median: 4.5)

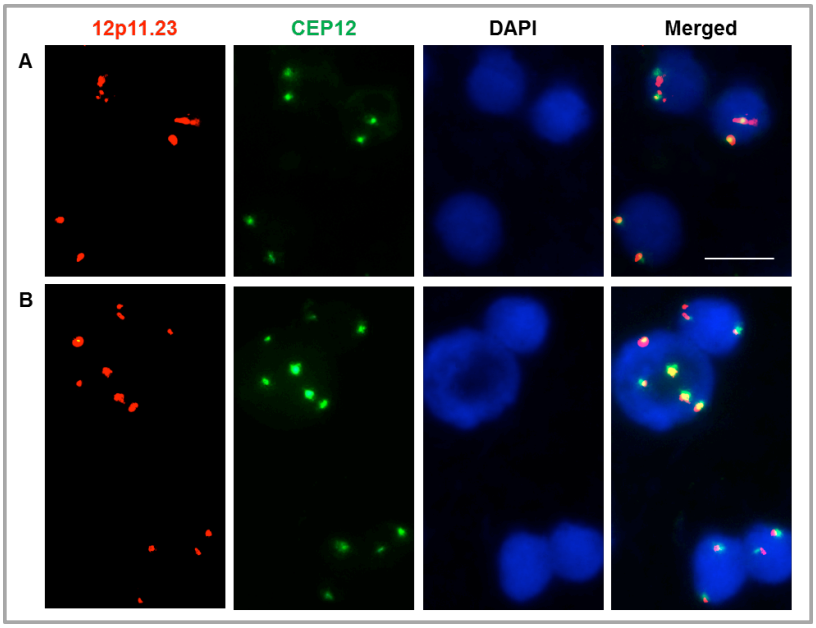

Figure 2.7 Representative images of fluorescent in situ hybridization for 12p11.23 on CTCs isolated from patients with germ cell tumors. A, Two CTCs isolated from peripheral blood show 4 (upper cell on the left), and 3 (upper cell on the right) 12p11.23 signals (red) and 2 signals for centromere 12 (green). A leukocyte (cell below) shows non-aberrant pattern. DAPI (blue) staining was used to visualize cells' nuclei (magnification: 630x, scale bar: 10 µm). B, Two tumor cells isolated from testicular vein blood show 3 (upper cell on the right), and 5 (upper cell on the left) 12p11.23 signals (red) as well as 2 and 5 signals for centromere 12, respectively. Two leukocytes (cells below) show non-aberrant pattern. DAPI (blue) staining was used to visualize cells' nuclei (magnification: 630x, scale bar: 10 µm).

3.3 The incidence of CD30 and Glypican-3 expression on circulating tumor cells in of nonseminomatous patients

3.3.1 Expression of CD30 and GPC3 in leukocytes from healthy volunteers

In the MNC fraction isolated from healthy blood donors, only cells positive for both markers, CD30 and CD45, were found. The mean number of CD30(+)CD45(+) cells was 2 per $1.4x10^6$ of MNC (range: 0-8; median: 0.5). These cells were not considered to be of GCT origin. GPC3-positiviy was never observed in the MNC fraction isolated from peripheral blood of healthy volunteers.

3.3.2 Expression of CD30 and GPC3 in germ cell tumor cell lines

Strong membranous expression of CD30 was found in >50% of 2102Ep cells and <25% of NCCIT cells showed weak to moderate CD30 staining (**Fig. 2.8 A**). No CD30 staining was found in NT2 cell line. Strong GCP3 staining was present in >75% of cells from NT2 and >50% of NCCIT cell line with a cytoplasmic/membranous localization and a characteristic granular pattern (**Fig. 2.8 B**). 2102Ep cell line showed no GPC3 expression. None of the cell lines showed staining positive for CD45.

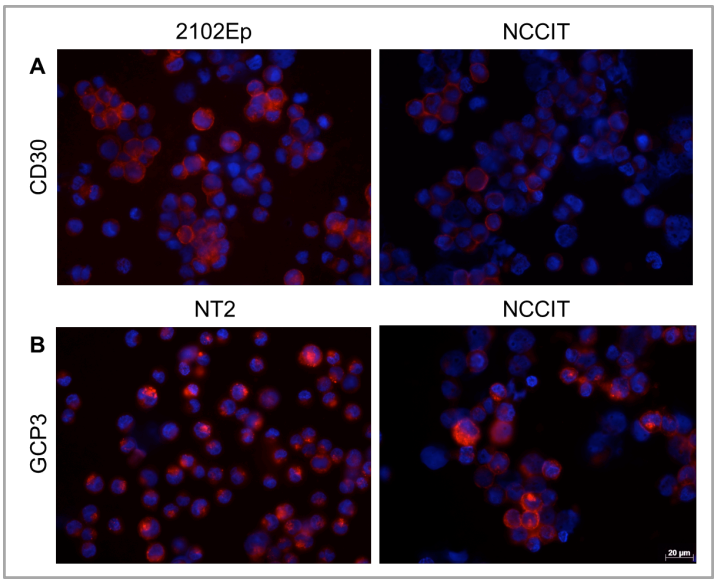

Figure 2.8 Representative photos of CD30 (A) and GPC3 (B) staining in germ cell tumor cell lines.

3.3.3 Expression of CD30 and GPC3 in circulating tumor cells of nonseminomatous germ cell tumor patients

Twelve (23.5%) peripheral blood samples showed membranous expression of CD30 (**Fig. 2.9 A**) in candidates for CTCs with a mean number of 4 cells in $1.4x10^6$ MNCs (range: 1-10; median: 3).

Granular cytoplasmic expression of GCP3 was found in CTCs from 6 (11.8%) patients (**Fig. 2.9 B**) with a mean number of 3.2 cells in $1.4x10^6$ MNCs (range: 1-8; median: 1.5).

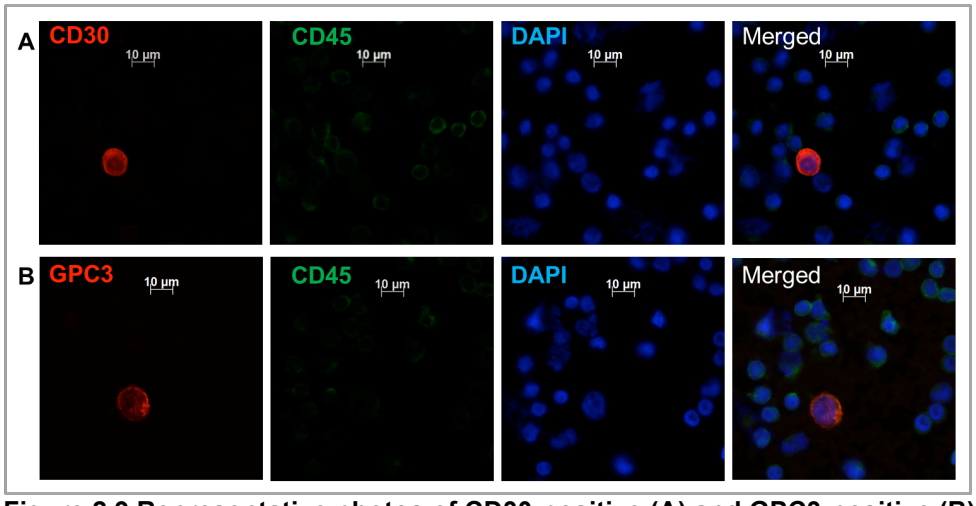

Figure 2.9 Representative photos of CD30-positive (A) and GPC3-positive (B) circulating tumor cells.

Table 2.14 Characterization of patients positive for CD30(+) and GPC3(+) circulating tumor cells.

Case number		Clinical stage (UICC)	Histology					Tumor markers			No. CTCs/1.4 x 10^6 MNCs		Positive by CellSearch®
			%S	%EC	%YST	%CC	%T	AFP	βHCG	LDH	CD30	GPC3	
Treatment-naïve	107	I	12	85	1	1	1	34	451		1	2	
	128	I		5			95	54	15		4		
	10	I	20				80				10		1
	166	I					100	12			5		
	168	na		83			17	93	525	252	3		1
	173	II C		100				13	4	446	7		
	44	II C		25	25	25	25				3	8	
	143	II C	50	3	7		40	704	1099	264	3	1	
	6	III		80			20			334	2		
Treatment-refractory	5	III		50	50							6	
	33	III (mediastinal)			100			1589			3	1	
	35	III		20			80		792		2		2
	38	III		50		50			10000		5	1	

PS, pure seminoma; S, seminoma; EC, embryonal carcinoma; YST, yolk sac tumor; T, teratoma; CC, choriocarcinoma; MNC, mononuclear cell; AFP [ng/mL], alpha fetoprotein; βHCG [U/L], beta human gonadotropin; LDH [U/L], lactate dehydrogenase; UICC - International Union Against Cancer; nd, no data available

Five (9.8%) of 51 patients had CTCs positive for both CD30 and GPC3 markers, and only one patient showed cells positive for GPC3 but negative for CD30. Additionally, 3 (5.2%) samples were positive for CTCs using the CellSearch® system in parallel (**Tab. 2.14**). All of these samples were positive for CD30, but none showed expression of GPC3 on CTCs. Additionally, 6 patients were positive for at least 1 CTC using the CellSearch® system, and negative for CD30 and GPC3.

3.4 Associations with clinico-pathological parameters

In order to assess clinical characteristics associated with the detection of CTCs, correlations between the presence of CTCs and different clinico-pathological parameters of patients were analysed (**Tab. 2.15**).

3.4.1 Circulating tumor cell detection and clinical stage

CTCs were found more frequently in patients with more advanced clinical stages (II or III) as compared to stage I (Fisher's Exact Test, 2-sided P<0.001). Patients with metastatic disease were 5 times more often positive for CTCs than patients with

stage I tumors [17/42 (41%) *versus* 7/91 (7.7%); Fisher's Exact Test, 2-sided P<0.001]. Ten (66.7%) of 15 patients with distant metastases were positive for CTCs.

3.4.2 Circulating tumor cell detection and primary tumor histology

Regarding histology of the primary tumor, CTCs were more frequently found in patients with nonseminomas compared to pure seminomas (Fisher's Exact Test, 2-sided P<0.001). Patients positive for CTCs had significantly higher percentages of yolk sac tumor (Mann-Whitney U test, P<0.001) and teratoma (Mann-Whitney U test, P=0.004) histological components within the primary tumor. Not unexpectedly, CTC-positivity after CellSearch® processing was associated with ≥50% of embryonal carcinoma component (Fisher's Exact Test, 2-sided P=0.002), and CTCs detected by Ficoll-enrichment followed by ICC staining showed an association with an increased content of choriocarcionoma component in primary tumors (Mann-Whitney U test, P=0.037).

Table 2.15 Descriptive stratification of the study cohort by circulating tumor cell status.

Parameter		CTC positive (n=25)		CTC negative (n=118)		P value (Fisher's Exact Test, 2-sided)
		Number	% of valid cases	Number	% of valid cases	
Clinical stage	I	7	29.2	84	77.1	**<0.001**
	II	7	29.2	20	18.3	
	III	10	41.6	5	4.6	
Tumor size [mm]	<34.3	5	31.3	64	62.1	**0.028**
	≥34.3	11	68.7	39	37.9	
	Seminoma <40	2	50.0	40	65.6	0.610
	Seminoma ≥40	2	50.0	21	34.4	
	Nonseminoma <29.6	3	25.0	22	56.4	0.097
	Nonseminoma ≥29.6	9	75.0	17	43.6	
Primary tumor stage	pT1	9	56.3	64	62.1	0.124
	pT2	5	31.2	36	35.0	
	pT3	2	12.5	2	1.9	
Infiltration of *rete testis*	No	10	71.4	69	70.4	1.000
	Yes	4	28.6	29	29.6	
Infiltration of *tunica albuginea*	No	3	50.0	44	71.0	0.363
	Yes	3	50.0	18	29.0	
Lymphatic vessel invasion	No	7	53.8	62	71.3	0.215
	Yes	6	46.2	25	28.7	
Vascular invasion	No	9	75.0	79	84.0	0.424
	Yes	3	25.0	15	16.0	
Testicular Intraepithelial Neoplasia (TIN)	No	2	14.3	10	10.8	0.656
	Yes	12	85.7	83	89.2	
Contralateral TIN	No	12	92.3	88	92.6	1.000
	Yes	1	7.7	7	7.4	
Histology of primary lesion	Pure Seminoma	4	17.4	62	58.5	**<0.001**
	Nonseminoma	19	82.6	44	41.5	

Note that due to the missing values not all numbers sum up to 25 (CTC positive) and 118 (CTC negative) cases.

3.4.3 Circulating tumor cell detection and serum tumor markers

The presence of CTCs also correlated with elevated levels of serum tumor markers. Detection of CTCs was significantly associated with elevated levels of AFP (Mann-Whitney U test, P=0.025), βHCG (Mann-Whitney U test, P=0.002), and LDH (Mann-Whitney U test, P=0.002). However, it is noteworthy that 4 patients with normal levels of serum markers were positive for CTCs.

3.4.4 Preliminary follow-up evaluation

The mean follow-up time was 13.7 months (range: 0.66-24.1; median: 14.9) for patient cohort in this study. Although the number of events (recurrences=5, deaths=3) was small, the preliminary follow-up analysis resulted in a significantly reduced disease-free (log rank test, $p<0.001$) and overall survival (log rank test, $p<0.001$) in CTC-positive compared to CTC-negative patients (**Fig. 2.10**). It is noteworthy that all six patients with treatment-refractory disease that were included in this analysis were positive for CTCs. Of these patients, three died within less than 3 months after blood collection. In PB of one patient, CTCs were detected using Ficoll-enrichment followed by ICC staining, the second patient showed CellSearch®-positivity, and the third patient was positive by both detection methods.

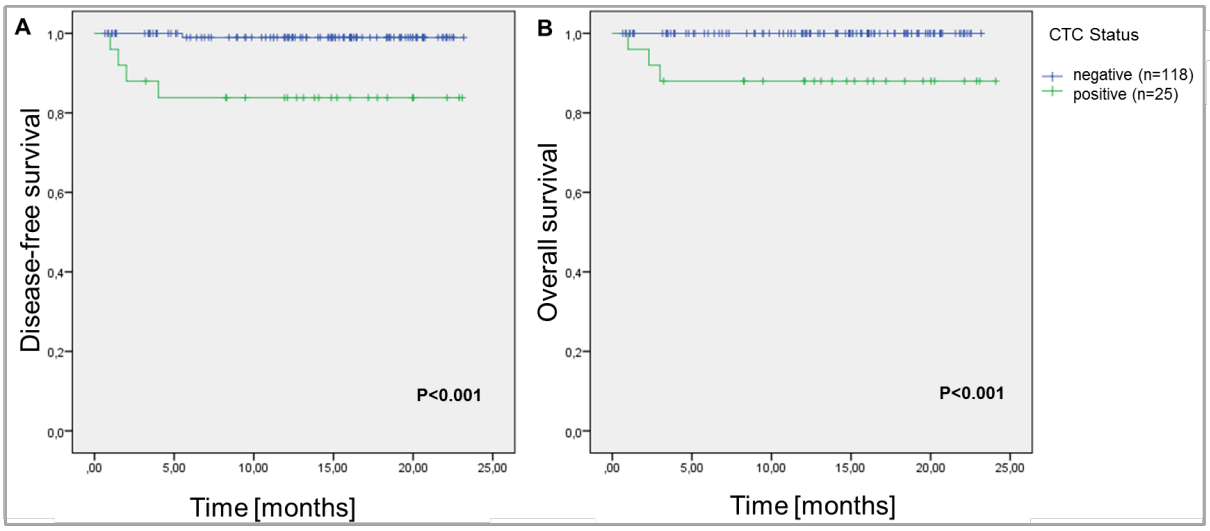

Figure 2.10 Kaplan Meier Analysis of disease-free (A) and overall survival (B) for patients with germ cell tumors. Patients were divided into those with negative (no CTCs, in blue) and positive CTC status (≥1 CTC, in green).

4. DISCUSSION

The main aim of this study was to investigate the incidence and possible clinical utility of CTCs in patients with GCTs. This is the first study demonstrating the presence of CTCs in a large number of GCT patients including all clinical stages and all histological subtypes.

4.1 Clinical material and methods

The choice of the right study cohort and a reproducible method is the basic but very crucial and potentially error-prone step in design of studies screening for putative diagnostic and prognostic markers.

4.1.1 Patients and blood samples

In this study, peripheral blood samples from 143 patients with GCTs were analysed. The study cohort was large especially that GCTs are rather rare and only 10 new cases diagnosed per 100,000 males/per year in Germany (**Ferlay et al., 2012**). To extensively investigate presence of CTCs in GCTs, patients included in this study were of all clinical stages (I-IIIC) and had tumors of different histological subtypes and approximately the equal number of patients had seminomatous and nonseminomatous GCTs. As additional material, blood samples from tumor-draining testicular vein were obtained from 19 patients during orchidectomy. Previously, CTC detection in tumor-draining mesenteric blood of colorectal cancer patients (**Deneve et al., 2013**) and in the central venous compartment in patients with metastatic breast cancer has been reported (**Peeters et al., 2011**). This is the first study analysing the presence of tumor cells in this very unique material. Furthermore, primary testicular germ cell tumors of different histological types were included in this study.

4.1.2 Enrichment and detection of circulating tumor cells

Finding reliable method to separate and detect CTCs from peripheral blood of cancer-affected patients as well as their further characterization is one of the main goals of researchers working in the field of CTCs. In the previous studies reporting on CTCs in patients with GCTs, mainly reverse transcriptase PCR method detecting RNA from tumor cells was used (**Tab. 2.16**). In this study, CTCs were analysed in parallel with the use of two methods allowing the visualization of CTCs. One method was enrichment by Ficoll density gradient centrifugation followed by ICC with germ cell and epithelial cell-specific markers and the other was the standard CellSearch® system.

Ficoll density gradient centrifugation is performed to separate the mononuclear cells and CTCs from the other blood cells based on cellular density. Afterwards, immunocytochemistry is performed in order to detect CTCs. The biggest advantage of this method is its marker-independence. However, this procedure might result in high cell loss. In order to prevent the cell loss, several attempts has been made as carefulness during blood layering and MNC fraction aspiration, optimization of centrifugation parameters (temperature, brake off) and cytospin preparation.

The CellSearch® system is a semi-automated CTC-detection method. It has been well-established in patients with metastatic breast, prostate, and colon cancer (**Miller et al., 2010**). This is the first study reporting its usage in patients with GCTs. The limitation of this system is its dependence on EpCAM- and keratin expression.

There was little overlap between two assays. In only 3 of 25 (12%) patients CTCs were found using both methods, demonstrating that by the combination of both assays, detection of CTCs in patients with GCTs can be improved. The discrepancy between the two methods can be explained by the fact that Ficoll-based assay captures CTCs based on physical properties and also adds germ cell markers to the epithelial cell markers used by the CellSearch® system.

Table 2.16 The summary of literature review on circulating tumor cells in germ cell tumors.

No of patients	Disease clinical stage	Method	Targets	Material	% of positive results	Other results/comments	Reference
28	advanced	RT-PCR	βHCG	PBSC harvests	12.7% of apheresis products	CTCs can be present in a significant number of germ cell patients undergoing autologous transplantation.	Fan et al., 1998
20	advanced	RT-PCR, IHC	EGFR, GCAP	peripheral blood, PBSC harvests	15% (IHC) 35% (RT-PCR)	RT-PCR for GCAP is suitable for the sensitive detection of residual germ cell tumor cells in peripheral blood and progenitor cell harvests	Hildebrandt et al., 1998
5	variable	Nested RT-PCR	AFP	peripheral blood, PBSC harvests	100%	Single cancer cell could be detected in 10^6 peripheral blood mononuclear cells.	Yuasa et al., 1999
119	variable	Nested RT-PCR	AFP, βHCG	peripheral blood	26.5% (combination of markers)	Association to higher stage of patient was found. Positive results were found mostly in embryonal carcinoma.	Hautkappe et al., 2000
50	mainly advanced	RT-PCR	fibronectin, XIST, βHCG, CD44, EGFR, GCAP, human endogenous retrovirus type K (ENV and GAG)	PBSC harvests	58% (combination of markers)	3-year survival rates were 68% for RT-PCR-positive and 58% for RT-PCR-negative patients.	Bokemeyer et al., 2001
34	variable	ELISA	Caspase-Cleaved CK18 (M30), caspase-cleaved and intact CK18 (M65)	peripheral blood	-	M65 and M30 levels appear to reflect chemotherapy-induced changes that correlate with changes of available serum tumor markers.	De Haas et al., 2008
74	variable	Real-time PCR	Cell-free DNA fragments of actin-106/193/384	Peripheral blood	Increased in all patients with GCTs	This method distinguished patients with cancer from healthy individuals with 87% sensitivity and 97% specificity. Cell-free DNA levels were more frequently increased in patients with advanced clinical stage.	Ellinger et al., 2009

AFP, alpha fetoprotein; CK, cytokeratin; EGFR, epithelial growth factor receptor; ELISA, enzyme-linked immunosorbent assay; GCAP, germ cell alkaline phosphatase; IHC, immunohistochemistry; PBSC, peripheral blood stem cell; RT-PCR, reverse transcriptase polymerase chain reaction

4.1.3 Markers of germ cell tumors

Finding reliable markers for the identification of CTCs in patients with GCTs is challenging because of the high histological diversity of these tumors comprising both pure seminomas but also tumors with different nonseminomatous tumor components. Therefore, these tumors present with very heterogeneous expression patterns of both germ cell and epithelial cell-specific proteins. In our study, we performed an immunocytochemical analysis applying a combination of novel germ cell-specific (anti-SALL4, anti-OCT3/4) and epithelial cell-specific (anti-keratin, anti-EpCAM) antibodies. In order to select suitable markers for CTCs detection, different germ cell tumor cell lines and primary tumors were stained with these markers, showing high expression of SALL4, OCT3/4, keratins, and EpCAM.

The only previously published immunocytochemical CTC analysis in GCT patients using the pan-anti-keratin antibody A45-B/B3 identified only 3/20 (15%) of patients with advanced and/or relapsed GCTs as positive for CTCs (**Hildebrandt et al., 1998**). Fourteen patients (9.8%) had CTCs, positive for at least two of the selected markers, and the selected marker combination consisting of SALL4, OCT3/4, keratins 8, 18, 19, and EpCAM seemed to be sensitive and specific to detect different histological types of GCTs.

4.1.4 FISH for 12p

Using FISH technique, the detected CTCs showed gain of the 12p11.23 chromosomal region which is a cytogenetic hallmark of GCTs, present in about 80% of invasive tumors (**Zafarana et al., 2002; Looijenga et al., 2003**). This finding additionally confirms the germ-cell origin of CTCs and thus specificity of our assays and supports the invasive potential of isolated CTCs.

4.2 Clinical relevance of circulating tumor cells in patients with germ cell tumors

4.2.1 Circulating tumor cells and patients' survival

Although the mean follow-up time was short (13.7 months) and the number of events (deaths=3, recurrences=5) was small, a significant correlation between CTC-positivity and shorter disease-free and overall survival was found. This might support the prognostic potential of CTC detection in patients with GCTs. Nevertheless, to investigate whether the detection of CTCs in GCT patients is an independent prognostic factor, as shown for other tumor entities (**Pantel et al., 2009; Kang and Pantel, 2013**); future long term follow-up evaluations are required.

4.2.2 Circulating tumor cells and patients' clinical stage

A strong correlation was found between the presence of CTCs and more advanced clinical stages of disease. Additionally, all patients with treatment refractory disease included in this analysis were positive for CTCs. These findings might additionally, corroborate the prognostic potential of CTCs in patients with GCTs. In a much smaller cohort study, **Hautkappe et al., (2000)** previously reported that AFP- and/or βHCG-mRNA detection in PB of patients with testicular germ cell tumors was associated with the higher tumor stage (**Hautkappe et al., 2000**). Thus, the significance of CTC detection as complementary biomarker for the identification of patients with high risk of recurrence deserves further attention.

4.2.3 Circulating tumor cells and histology of primary tumor

Nonseminomatous tumors tend to be more aggressive and are more prone to metastasize than seminomatous tumors. In this study, CTCs were more frequently found in patients with nonseminomatous than in seminomatous tumors, especially in those with higher percentages of yolk sac and teratoma components. Teratomas and yolk sac tumors have been reported as the most common types of tumors observed in patients with late relapses (**Michael et al., 2000; Atsü et al., 2003; Mayer et al.,**

2011). Teratomas are not producing common tumor markers such as AFP, βHCG or LDH. Therefore, evaluation of CTCs might be of special value to detect metastasis or relapse in patients suffering from teratomas. In several studies, the presence of predominantly embryonal carcinoma components has been reported as a factor for poor prognosis (Dunphy et al., 1988; Nicolai et al., 1995; Atsü et al., 2003; Albers et al., 2003). Hautkappe et al. (2000) found AFP- and/or βHCG- mRNAs mostly in patients with embryonal carcinomas. In the current study, a strong correlation between the presence of CTCs and a higher percentage (≥50%) of embryonal carcinoma component within primary tumors was only found in blood samples analysed with the CellSearch® system, suggesting that this approach might be particularly useful to detect CTCs derived from embryonal carcinomas expressing both EpCAM as well as one of the detected keratins.

4.2.4 Circulating tumor cells and serum tumor markers

Concentrations of AFP, βHCG and LDH serum concentration represent standard tumor markers in GCTs, but only 10-60% (nonseminomatous tumors), 10-40% and 40-60% of patients, respectively, have elevated concentrations at primary diagnosis (Gilligan et al., 2010). In the present study, CTCs were significantly associated with elevated serum concentrations of AFP, βHCG, and LDH. Higher levels of serum tumor markers after orchiectomy are associated with worse outcome in metastasized nonseminoma (IGCCC, 1997). An association between elevated serum tumor markers and the presence of CTCs might indicate a diagnostic and prognostic significance of CTCs. However, CTCs were found also in 4 marker-negative patients, suggesting that determination of CTCs might help to minimize the diagnostic gap of conventional tumor markers.

4.3 Incidence of tumor cells in blood from testicular vein

To the best of our knowledge, this is the first study investigating intra-operatively collected blood from the testicular vein of patients with GCTs. The testicular vein carries deoxygenated blood from testis to the inferior vena cava or one of its tributaries and might be the first path of haematogenous tumor-cell spread in testicular GCTs (**Kara et al., 2012**). Our results seem to support this hypothesis. Of note, we observed very high numbers of tumor cells of up to 120 per $1x10^6$ MNCs in TVB. In the testicular vein, the CTC yield was higher in comparison to PB, which is similar to the gradients observed in breast and colorectal cancers, where significantly more CTCs could be detected in the central venous blood or the mesenteric vein, respectively (**Peeters et al., 2011; Deneve et al., 2013**). In more than half of GCT patients, clusters of 3-7 tumor cells were observed, which was much more frequent than in PB (20 %). These findings suggest that a high number of isolated and clustered CTCs is shed from the primary tumor into the local blood stream, and during the circulation CTCs may undergo anoikis or they might be filtered in the lungs (or other organs), which may cause the observed gradient between TVB and PB (**Fig. 2.11**).

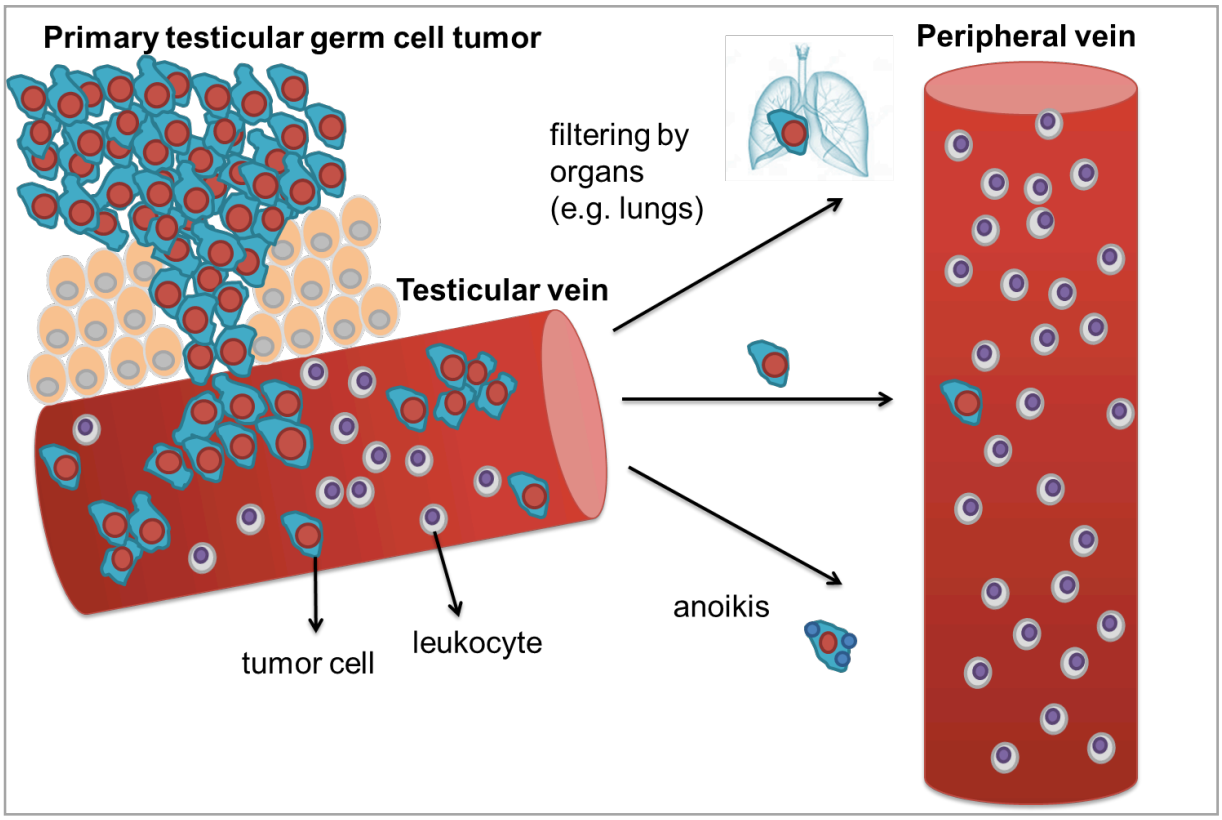

Figure 2.11 A possible mechanism of dissemination of tumor cells from testicular germ cell tumors. A high number of isolated and clustered tumor cells is shed from the primary tumor into the tumor-draining testicular vein, and during the circulation tumor cells may undergo anoikis or might be filtered in the lungs (or other organs).

4.4 CD30 and Glypican-3 expression in circulating tumor cells of nonseminomatous patients

In our cohort of 51 NSGCT patients, 23.5% showed CTCs positive for CD30(+) and 11.8% of cases were GPC3(+). To best of our knowledge, the current study is the first report on the presence of CD30(+) and GPC3(+) circulating tumor cells in GCTs. CD30 and GPC3 might be promising targets for personalizing the treatment approach, especially in patients with advanced NSGCTs.

4.4.1 CD30 as potential therapeutical target

CD30 is a marker of malignant Hodgkin/Reed-Sternberg cells present in Hodgkin lymphoma (**Irsch et al., 1998**). In germ cell tumors, its expression has been reported as an independent prognostic factor of poor prognosis in relapse/chemoresistant

patients. Brentuximab vedotin has been recently approved by the United States Food and Drug Administration, and the European Medicines Agency to treat Hodgkin lymphoma that relapses after salvage autologous stem cell transplantation or the failure of 2 multi-agent regimens (**De Claro et al., 2012; Younes et al., 2012; Pro et al., 2012**). Additionally, promising early results were reported in 3 highly pretreated patients with GCT enrolled in an ongoing, multicenter phase 2 trial of brentuximab vedotin for CD30 positive nonhematological malignancies (**Albany et al., 2013**). Our study suggests that brentuximab vedotin could target not only CD30-positive primary tumors, but in addition eliminate CD30(+) circulating tumor cells in patients with GCT.

4.4.2 GPC3 as potential therapeutical target

GPC3 has been proposed as a factor of poor prognosis in hepatocellular carcinoma (**Shirakawa et al., 2009; Fu et al., 2013**). Detection of GPC3 mRNA was found in 76%-100% in peripheral blood of hepatocellular carcinoma patients and has been suggested to indicate metastatic disease (**Yan et al., 2011; Wang et al., 2011**). GPC3 expression by GCTs showing yolk sac tumor and choriocarcinoma histology has been previously reported (**Zynger et al., 2006**). It is also noteworthy that a high proportion of patients with relapsed or refractory GCTs were positive for GPC3 on CTCs, indicating a drugable target in this population with limited therapeutic options. Therefore, this study supports clinical trials using an antibody against GPC3 in resistant/refractory GCT patients.

5. CONCLUSIONS

This study showed that CTCs can be detected in the peripheral blood of about 18% of 143 patients diagnosed with GCTs, both using the established CellSearch® system, as well as using the Ficoll-based enrichment and a combination of immunocytochemical markers. The presence of CTCs was associated with histologically more aggressive nonseminomatous tumors, advanced clinical stages, increased serum concentrations of tumor markers (AFP, βHCG and LDH), and chemotherapy refractory relapse. Additionally, in the cohort of 51 patients with nonseminomatous GCTs, expression of potential therapeutical targets was evaluated showing that 23.5% of NSGCTs patients had tumor cells positive for CD30(+) and in 11.8% of cases CTCs were expressing GPC3(+). The current study indicates the diagnostic potential of CTCs as prognostic biomarker. Further studies with longer follow-up periods are needed to evaluate the association of CTCs with outcome and in particular survival of patients with GCTs. Molecular characterization of CTCs might reveal gene mutations and genetic aberrations responsible for cell invasive potential (**Bednarz-Knoll et al., 2011; Alix-Panabieres et al., 2013**) and may serve as a "liquid biopsy" assessing potential targets for therapy that might be promising for personalizing the treatment approach (**Nagata et al., 2005; Feng et al., 2013**) or gene mutations relevant to targeted therapy [e.g., c-KIT or BRAF (**Honecker et al., 2009**)].This opens new perspectives, not only with regards to the assessed markers, but also for other drug targets in the ever growing era of targeted therapy. Sequential blood analyses during therapy may also hold the promise to gain insights into drug resistance in individual patients. Thus, the present work opens a new avenue to personalized medicine in patients with GCTs.

Chapter 3: HAEMATOGENOUS DISSEMINATION OF TUMOR CELLS DURING PROSTATE BIOPSY

1. INTRODUCTION

1.1 Epidemiology of prostate cancer

Prostate cancer (PCa) is the most common cancer in men (**Fig 3.1**), with an incidence rates varying by more than 7-fold (25–193 per 100,000) among European Union countries. The highest rates were estimated in Northern and Western European countries, and the lowest in Central and Eastern European countries (**Ferlay et al., 2013**).

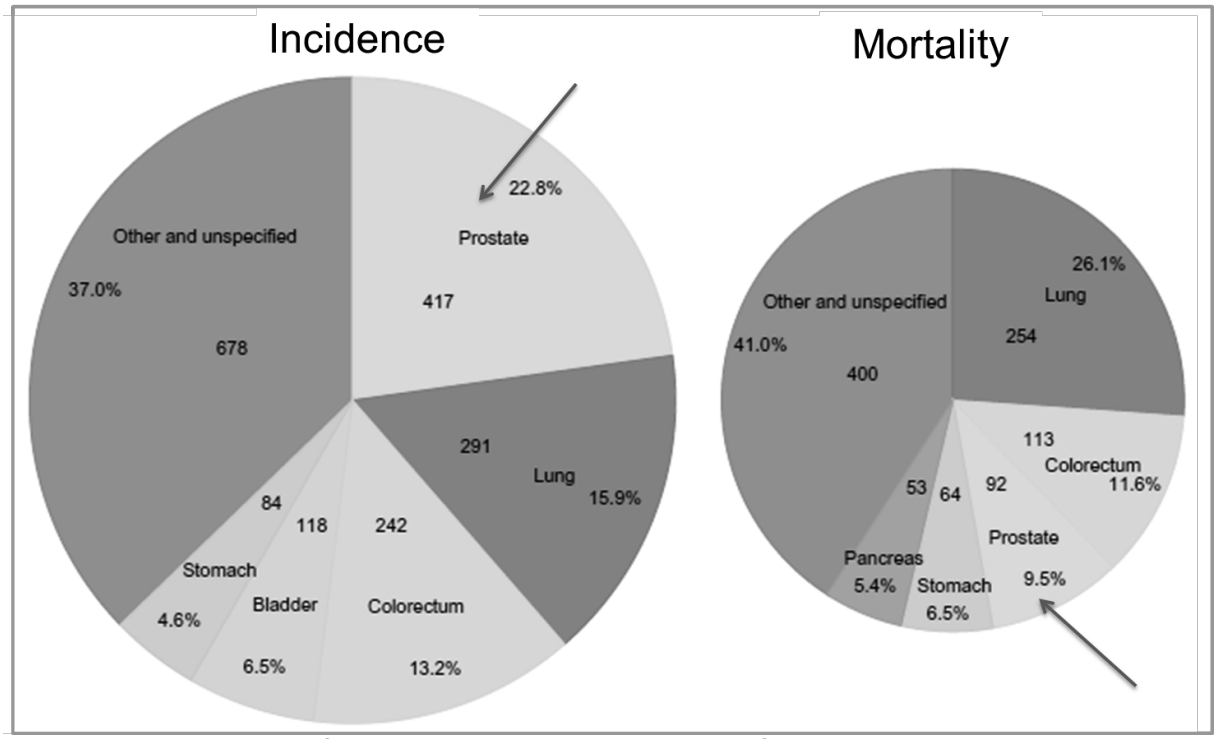

Figure 3.1 Distribution of the expected cases and deaths for the 5 most common cancers in Europe 2012 in males (from Ferlay et al., 2013).

1.2 Classification of prostate cancer

PCa stage is classified according to different scales among which the most common and informative is the one recommended by International Union against Cancer (UICC). The UICC staging system (including four stages I-IV; **Tab. 3.2**) is based on the TNM status (**Tab. 3.1**) which describes a size, an extension of a tumor to other organs as well as histopathological grading (Gleason score). The Gleason score (1-5) measures the dedifferentiation degree of PCa cells. It is evaluated in two biggest tumor foci separately and defined as two added numbers – first from a bigger focus, second from a smaller one (e.g. 4 + 3). The higher the number is, the more dedifferentiated tissue structure is, the more space is observed between the glands and the more cells infiltrate the surrounding normal tissue (**IARC, 2004**).

Table 3.1 TNM classification of carcinomas of the prostate (IARC, 2004).

pT Primary tumour
pTX Primary tumour cannot be assessed **pT0** No evidence of primary tumour **pT1** Clinically inapparent tumour not palpable or visible by imaging **pT1a** Tumour incidental histological finding in 5% or less of tissue resected **pT1b** Tumour incidental histological finding in more than 5% of tissue resected **pT1c** Tumour identified by needle biopsy (e.g., because of elevated PSA) **pT2** Tumour confined within prostate **pT2a** Tumour involves one half of one lobe or less **pT2b** Tumour involves more than half of one lobe, but not both lobes **pT2c** Tumour involves both lobes **pT3** Tumour extends beyond the prostate **pT3a** Extracapsular extension (unilateral or bilateral) **pT3b** Tumour invades seminal vesicle(s) **pT4** Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, or pelvic wall
N Regional lymph nodes
NX Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Regional lymph node metastasis
M Distant metastasis
MX Distant metastasis cannot be assessed **M0** No distant metastasis **M1** Distant metastasis **M1a** Non-regional lymph node(s) **M1b** Bone(s) **M1c** Other site(s)
G Histopathological grading
GX Grade cannot be assessed **G1** Well differentiated (Gleason 2-4) **G2** Moderately differentiated (Gleason 5-6) **G3–4** Poorly differentiated/undifferentiated (Gleason 7-10)

Table 3.2 Stage grouping of prostate carcinoma (IARC, 2004).

Stage I	pT1a	N0	M0	G1
Stage II	T1b	N0	M0	G2, 3-4
	T1b, c	N0	M0	Any G
	T1, T2	N0	M0	Any G
Stage III	T3	N0	M0	Any G
Stage IV	T4	N0	M0	Any G
	Any T	N1	M0	Any G
	Any T	Any T	M1	Any G

1.3 Diagnostics of prostate cancer

PCa diagnosis has been based mostly on a palpation or ultrasound investigation, digital rectal exam as well as biochemical measurements of increased level of serum concentration of prostate specific antigen (PSA) and transrectal ultrasound-guided (TRUS) core biopsy.

1.3.1 PSA screening

PSA belongs to the family of glandular kallikrein-related peptidases secreted by the epithelial cells of the prostate gland. A supposed physiological role of PSA is liquefying the seminal fluid. Prostate cancer causes PSA release into the circulatory system, increasing the level in blood up to 10^5-fold (**Lilja et al., 2008**). Although it is the best tumor marker of prostate cancer currently available in clinical practice, PSA is also often elevated in men with benign prostatic hyperplasia, prostatitis and other non-malignant disorders (**Gao et al., 2003**).

A majority of PSA in blood occurs in stable covalent complexes with protease inhibitors. The non-complexed forms, known as free PSA (fPSA), are unreactive with plasma protease inhibitors. Epitopes exposed by fPSA enabled the development of assays specifically measuring fPSA or complexed PSA as complements to the conventional PSA assay, which measures total PSA (tPSA) (**Lilja et al., 2008**).

1.3.2 Transrectal ultrasound-guided prostate biopsy

TRUS biopsy has become the standard way to obtain material for histopathologic examination in order to diagnose or exclude PCa (**Graefen and Schlomm, 2013**). This extensive sampling is required because prostate cancer is a multifocal disease in many patients and the current imaging technologies do not allow identifying the suspicious lesions with sufficient precision. A series of core needle biopsies (18 G, and 1.25 mm diameter) are taken according to a routine scheme, 5–8 biopsies from each side under ultrasound guidance. Histologic diagnosis is performed according to the Gleason score defining the two most common cell growth patterns in the specimen (**Heidenreich et al., 2014**). However, there is limited number of reports showing that TRUS biopsy may cause hematogenous dissemination of PSA-mRNA-bearing cells (**Moreno et al., 1997; Hara et al., 2001**) and cellular epithelial material (**Ladjevardi et al., 2014**).

1.4 Circulating tumor cells in prostate cancer

The detection of circulating tumor cells in patients with PCa has been reported in multiple studies. The presence of CTCs was shown to correlate with the higher grade of PCa and reduced patients' overall survival (**de Bono et al., 2008; Olmos et al., 2009; Scher et al., 2009**). CTCs were reported to predict overall survival even better than standard serum PSA-concentration measurements (**de Bono et al., 2008; Scher et al., 2009**). The significance of these phenomena emphasises the fact that in February 2008 CellSearch® System was accepted by FDA as an aid in the monitoring of metastatic prostate cancer patients (**Shaffer et al., 2007; Danila et al., 2007; de Bono et al., 2008**). Cytokeratin-positive CTCs are reported as good pre- but not post-operative predictors of PCa progression (**Weckermann et al., 2009**). They aggregated more often than in other types of solid tumours into clusters (**Brandt et al , 1996; Wang et al., 2000; Schmidt et al., 2004; Stott et al., 2010**) and were reported to express both apoptotic and proliferation markers (**Schmidt et al., 2004**). CTCs were be also detected in blood of non-metastatic (localized) PCa patients (**Stott et al., 2010;Thalgott et al., 2013; Shao et al., 2014**)

1.5 Aim of the study

During the transrectal ultrasound-guided prostate biopsy procedure, a needle penetrates the prostate tissue causing significant risk for local trauma releasing prostate cells into the blood vessels. This study aimed to investigate whether prostate biopsy is associated with haematogenous dissemination of tumor cells using two different methods: the standard CellSearch® system and the EPISPOT assay that focuses on detection of living PSA-secreting cells.

2. MATERIAL AND METHODS

2.1 Clinical material

A total of 75 patients with serum PSA levels >2 ng/mL were selected for this study. Informed consent approved by an institutional review board was obtained from all patients. Transrectal ultrasound-guided core biopsies were performed between August, 2012 and January, 2014. Histologic diagnosis of 8-12 tissue cores was carried out according to the Gleason score by an experienced pathologist. Detailed patient characteristics are presented in **Tab. 3.3**.

Table 3.3 Characteristics of patients.

Biopsy result	Number of patients (%)	Age at diagnosis [years] (mean)	Total PSA [ng/mL] (mean)	Free PSA [ng/mL] (mean)	Free PSA/ Total PSA ratio [%] (mean)	Gleason score (mean)
No PCa	26 (34.7)	39 – 82 (62.1)	2.2 - 18.0 (8.2)	0.2 - 2.8 (1.3)	8.0 - 23.0 (15.3)	-
PCa	49 (65.3)	50 – 79 (67.3)	4.4 - 54.0 (10.5)	0.4 - 3.2 (1.0)	2.0 - 36.0 (11.5)	6 - 9 (6.8)
Total	75 (100)	39 – 82 (65.5)	2.2 - 54.0 (9.7)	0.2 - 3.2 (1.1)	2.0 - 36.0 (12.8)	-

PCa, prostate cancer; PSA, prostate-specific antigen

2.2 Blood sample collection

Peripheral blood samples were obtained before and within 30 min. after prostate biopsy. Researchers analyzing the samples were blinded to all patient-related data including blood sampling.

2.3 Cell line culture

An LNCaP cell line is a PSA-positive adenocarcinoma prostate cancer cell line that originates from PCa lymph node metastasis. It was cultured in a proper medium and conditions at 37°C (**Supplementary Material I.A**). The passages were taken in every 2-3 days depending on a cell line and the confluence status. The cryo-preservation

vials were prepared in a standard way with the usage of a proper medium and 10% DMSO (**Supp material II**).

2.4 Kits and assays

The list of kits and assays is presented in the **Tab. 3.4**.

Table 3.4 Kits and assays.

Name of the kit	Company
RosetteSep® human circulating epithelial tumor cell enrichment Cocktail	Stem Cell Technologies, Canada
Alexa Fluor® 555 Mononoclonal Antibody Labeling Kit	Molecular Probes, USA

2.5 Antibodies

Two anti-PSA antibodies of concentration 500ng/well were used: a coating antibody (clone H50) and secondary antibody (clone H117). Both antibodies were provided by Dr. Mari Peltola, (University of Turku, Finland).

2.5.1 Antibody labelling

To visualize immunspots, the secondary anti-PSA antibody (clone H117) was labelled with Alexa-555 dye using the Alexa Fluor® 555 antibody labelling kit (**Tab. 3.4**). 100 µg of the antibody (1 mg/mL) was transferred to the vial of reactive dye, inverted few times and incubated for 1 h at room temperature. The antibody was purified by centrifugation in spin columns for 5 min. at 1100 g. Finally, the solution of purified antibody was collected and stored at -20°C.

2.6 Statistical analysis

Statistical analyses were performed using the SPSS software (**Supp. material IV**). Comparisons between related samples were made using the McNemar's test for matched pairs. Independent variables were correlated using the Mann-Whitney U test. Results were considered statistically significant if $P<0.05$ and highly statistically significant if $P<0.001$.

2.7 Detection of circulating tumor cells

The detection of CTCs was performed using both methods: the CellSearch® system (Veridex, Raritan, NJ, USA) and the EPISPOT assay (**Fig**).

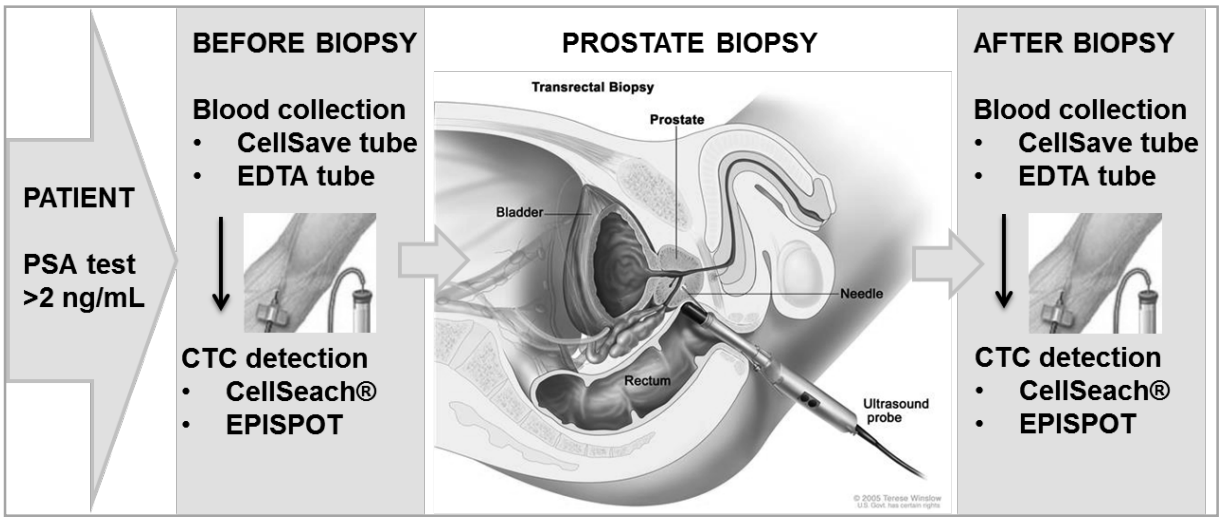

Figure 3.2 The scheme of the peripheral blood samples collection in this study. Illustrations adapted from **NCI, 2013**.

2.7.1 CellSearch® system

In total, 66 peripheral blood samples (7.5 ml) were collected in CellSave® tubes (**Supp. material III**) and measured within 96 h. The CellSearch® system was used as described in Chapter 2 (**2.5.6 CellSearch® analysis of circulating tumor cells**).

2.7.2 EPISPOT assay

The EPISPOT assay was performed from 70 peripheral blood samples of 6-7 mL collected in EDTA tubes.

Enrichment of CTC was performed using the RosetteSep® Human Circulating Epithelial Tumor Cell Enrichment Cocktail (**Tab. 3.4**). Briefly, blood was transferred into a 50 ml Falcon tube and RosetteSep® Human Circulating Epithelial Tumor Cell Enrichment Cocktail was added (20 µl per 1 mL of the whole blood). Tube was gently mixed and incubated for 20 min. at room temperature. Then, the sample was diluted with an equal amount of 2%FCS/1xPBS and carefully transferred on the top of Ficoll-Hypaque gradient (**Supp. material II**). The sample was centrifuged for 21 min. at

1200 x g with a brake off and the plasma phase including the enriched cells containing Ficoll-plasma interphase was carefully transferred into a new 50 mL Falcon tube. Afterwards, the enriched cells were 2 times washed with 2% FCS/1xPBS and centrifuged for 10 min. at 1200 x g. Finally, the supernatant was discarded and the cell pellet was dissolved in 800 µl µmetastatic medium (**Supp. material I.C**) or diluted in 1 mL of freezing solution (90% FCS with 10% DMSO) and stored in liquid nitrogen.

The nitrocellulose membranes (**Supp. material VI**) of the EPISPOT plate were coated with coating anti–PSA antibody (**Material and methods 2.5**) and incubated at 4 °C for 12 h. After membrane blocking with 100 µL of 5% BSA/1xPBS at 37 °C for 2 h, enriched CTCs were loaded and incubated at 37 °C with 5% CO_2 atmosphere for 18 h in µmetastatic medium (**Supp. material I.C**). Membranes were then washed 6 times using 0.1% Tween/1xPBS and 3 times with 1xPBS. The PSA protein released during the cell culture was detected by a second Alexa555-conjugated anti-PSA antibody (**Material and methods 2.5**). After incubation at 4°C for 12 h, membranes were washed 3 times in 0.1% Tween/1xPBS and 3 times with PBS and air-dried. As a positive control, cells from PSA-positive prostate cancer cell line LNCaP were used. For a negative control leukocyte-depleted peripheral blood from 20 healthy donors was utilized. Immunospots were visualized and enumerated (**Supplementary Material V**).

3. RESULTS

3.1 Control material

For PSA-secreting prostate cancer cell line (LNCaP), multiple immunospots were observed (Fig. 3.3 B) as well as spiked tumor cells were detectable using the CellSearch® system (Fig. 3.3 A). No immunospots were detected in leukocyte-depleted peripheral blood from 20 healthy donors.

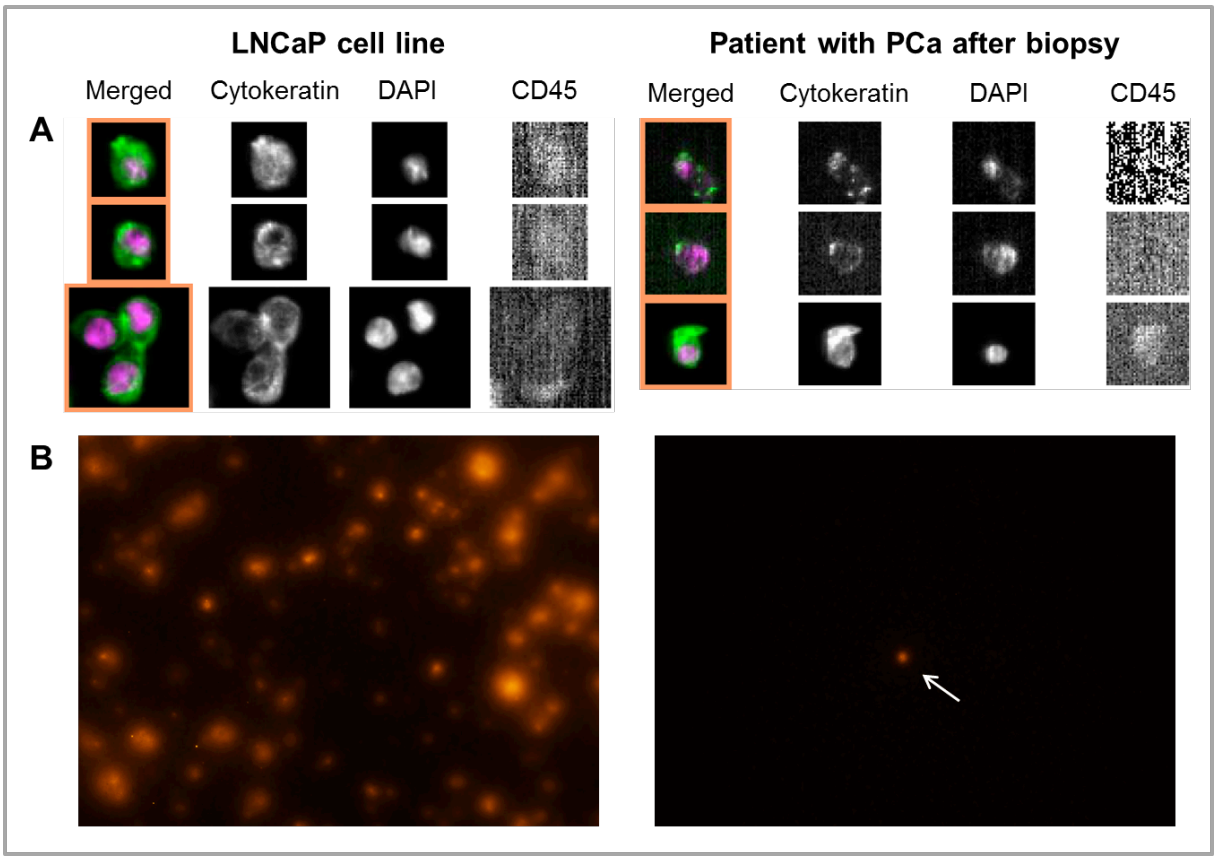

Figure 3.3 Representative example of positive cells. A, Image galleries after CellSearch® processing. The LNCaP cell line was used as positive control (left). Positive cells from patient with prostate cancer after performing biopsy – two upper ones were classified as apoptotic and the lower one as intact tumor cell (right). B, Analysis of viable PSA-releasing cells using the EPISPOT assay. The LNCaP cell line was used as positive control (left). Representative photo of single PSA immunospot corresponding to viable PSA-releasing cell from a prostate cancer patient after biopsy (right).

3.2 Detection of circulating tumor cells in blood samples collected before and after performing biopsy

3.2.1 CellSearch® results

Using the CellSearch® system, in 8 (12.1%) patients cells fulfilling CTC criteria were detected before, and in 20 (29.7%) samples collected after biopsy (McNemar's test, P=0.017) (**Fig. 3.4 A**). In 6 (9.1%) peripheral blood samples obtained before biopsy and in 11 (16.7%) of patients at least one cell with apoptotic morphology cells was observed.

3.2.2 EPISPOT results

With the usage of the EPISPOT assay, PSA-secreting circulating tumor cells were found in peripheral blood of 20 (28.5%) patients before biopsy, whereas 31 (44.3%) cases collected after performing biopsy were positive (McNemar's test, P=0.027) (**Fig.3.3 B**).

3.2.3 Combination of both methods

When combining the results of both assays, 27 (37.0%) patients were positive for CTCs before, and significantly more 42 (56.0%) of cases showed the presence of CTCs after performing biopsy (McNemar's test, P=0.009). Only 1 (1.6%) patient sample before biopsy was positive using both methods of CTC-detection, and in 9 (15.3%) blood samples collected after performing biopsy CTCs were detected with the usage of two methods in parallel.

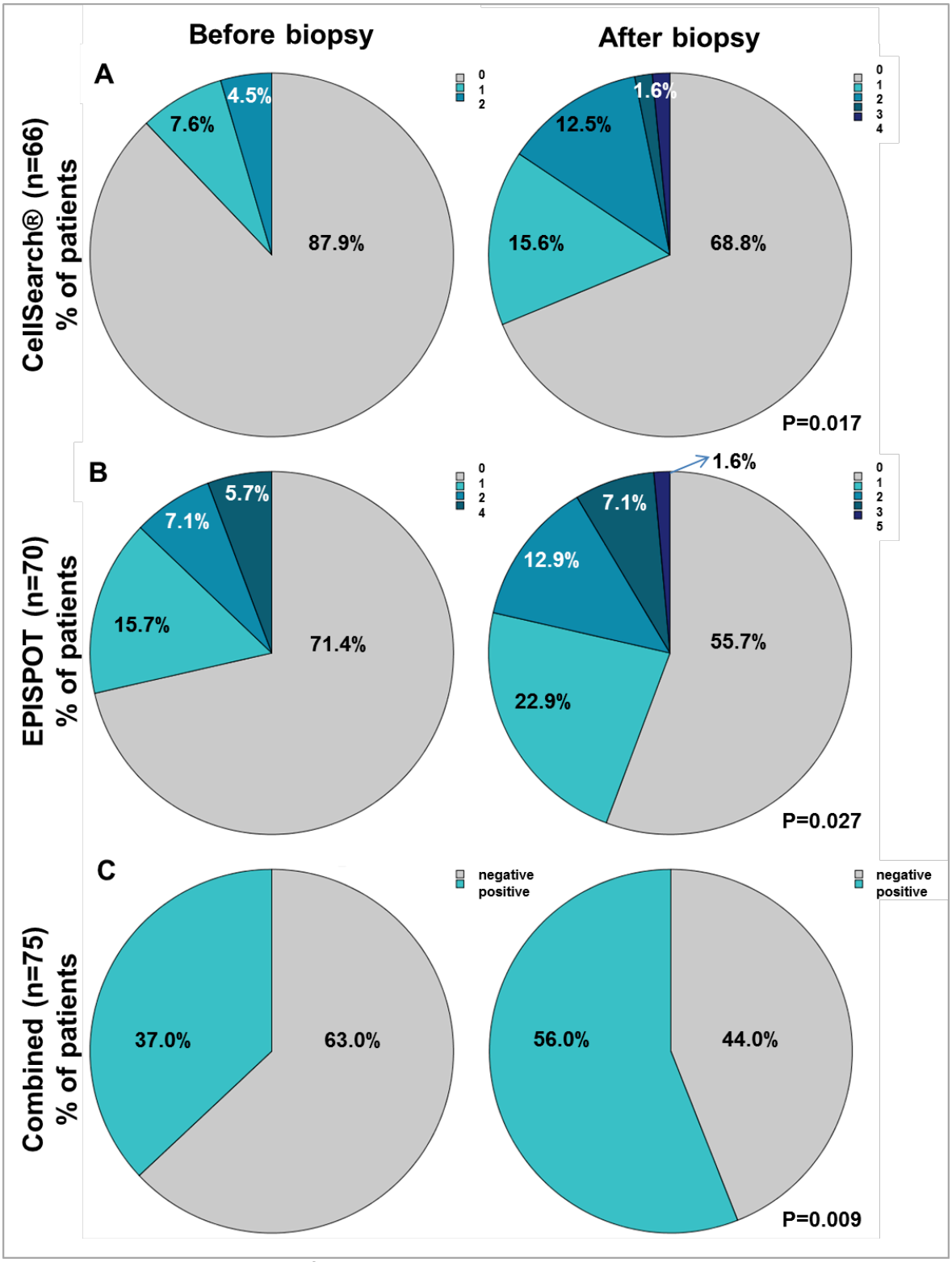

Figure 3.4 The distribution of circulating tumor cells among total peripheral blood samples collected before and after performing biopsy using the CellSearch® system (A), EPISPOT assay (B) and the combination of both methods (C).

3.3 Comparison of clinico-pathological parameters and detection of circulating tumor cells

Presence of CTCs was correlated to clinical and pathological parameters of patients undergoing prostate biopsy. For further analysis, patients were divided in two groups – the ones diagnosed with PCa and a group that had a negative biopsy result (no prostate cancer has been diagnosed).

3.3.1 Detection of circulating tumor cells in prostate cancer patients

Of 75 patients undergoing prostate biopsy, 49 (65.3%) were diagnosed with prostate cancer. Among these patients, there was a significant increase in number of CTCs after performing biopsy using the CellSearch® method [(McNemar's test, P=0.003 (**Fig. 3.5 A**)] and combing both assays [(McNemar's test, P=0.021 (**Fig. 3.5 C**)]. With the usage of EPISPOT assay no significant increase in number of PSA-secreting cells was observed in the PCa group (**Fig. 3.5 C**).

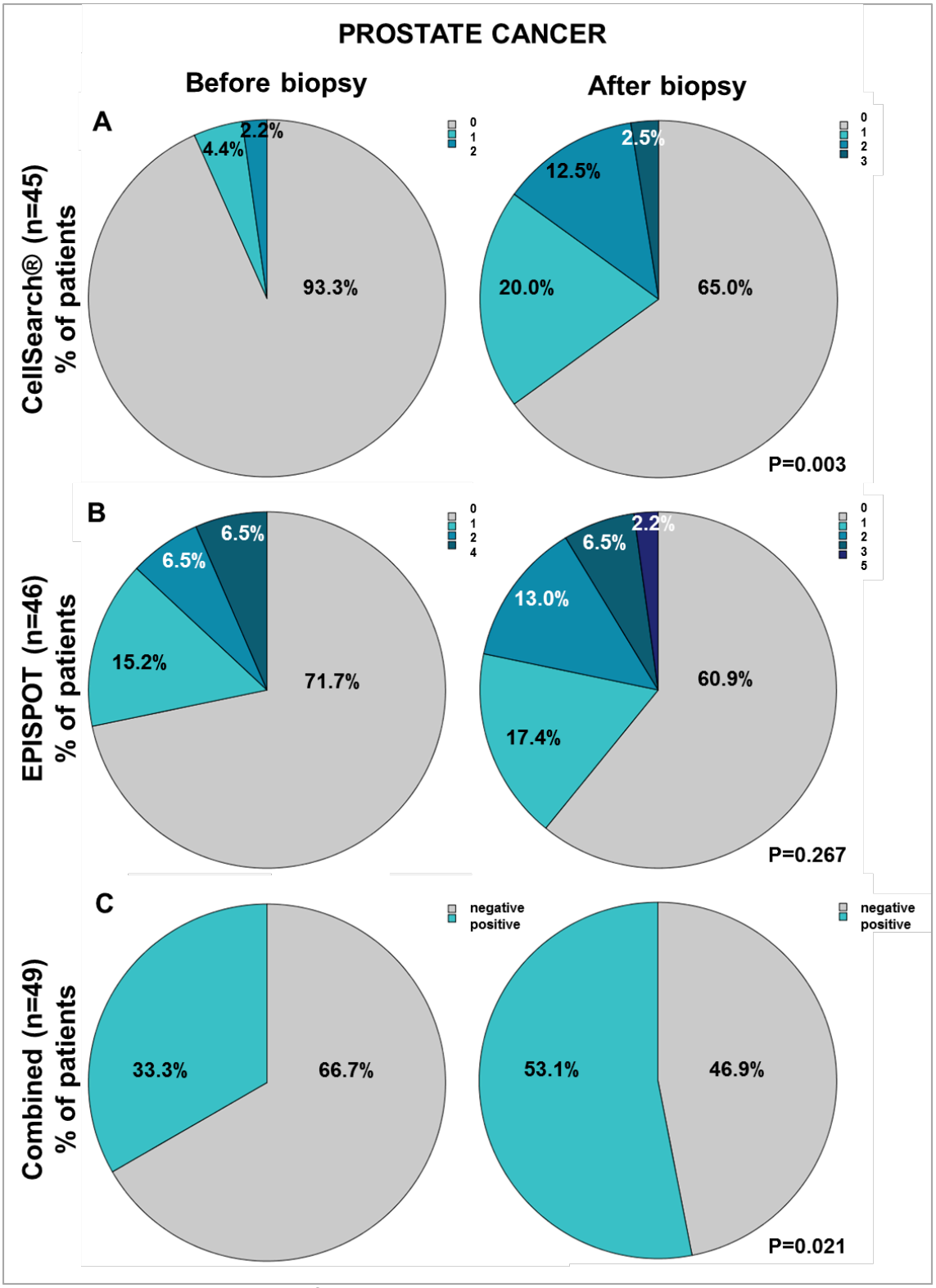

Figure 3.5 The distribution of circulating tumor cells among peripheral blood samples from patients diagnosed with prostate cancer collected before and after performing biopsy using the CellSearch® system (A), EPISPOT assay (B) and the combination of both methods (C).

3.3.2 Detection of circulating tumor cells in non-prostate cancer patients

For 26 (34.7%) patients that were classified as non-PCa after biopsy evaluation there were no statistically significant elevation of CTC number in blood samples collected after performing biopsy (**Fig. 3.6**). However, using the EPISPOT assay, there was tendency to increased number of PSA-secreting cells in peripheral blood after performing prostate biopsy in the non-prostate cancer group [(McNemar's test, P=0.070 (**Fig. 3.6 B**)].

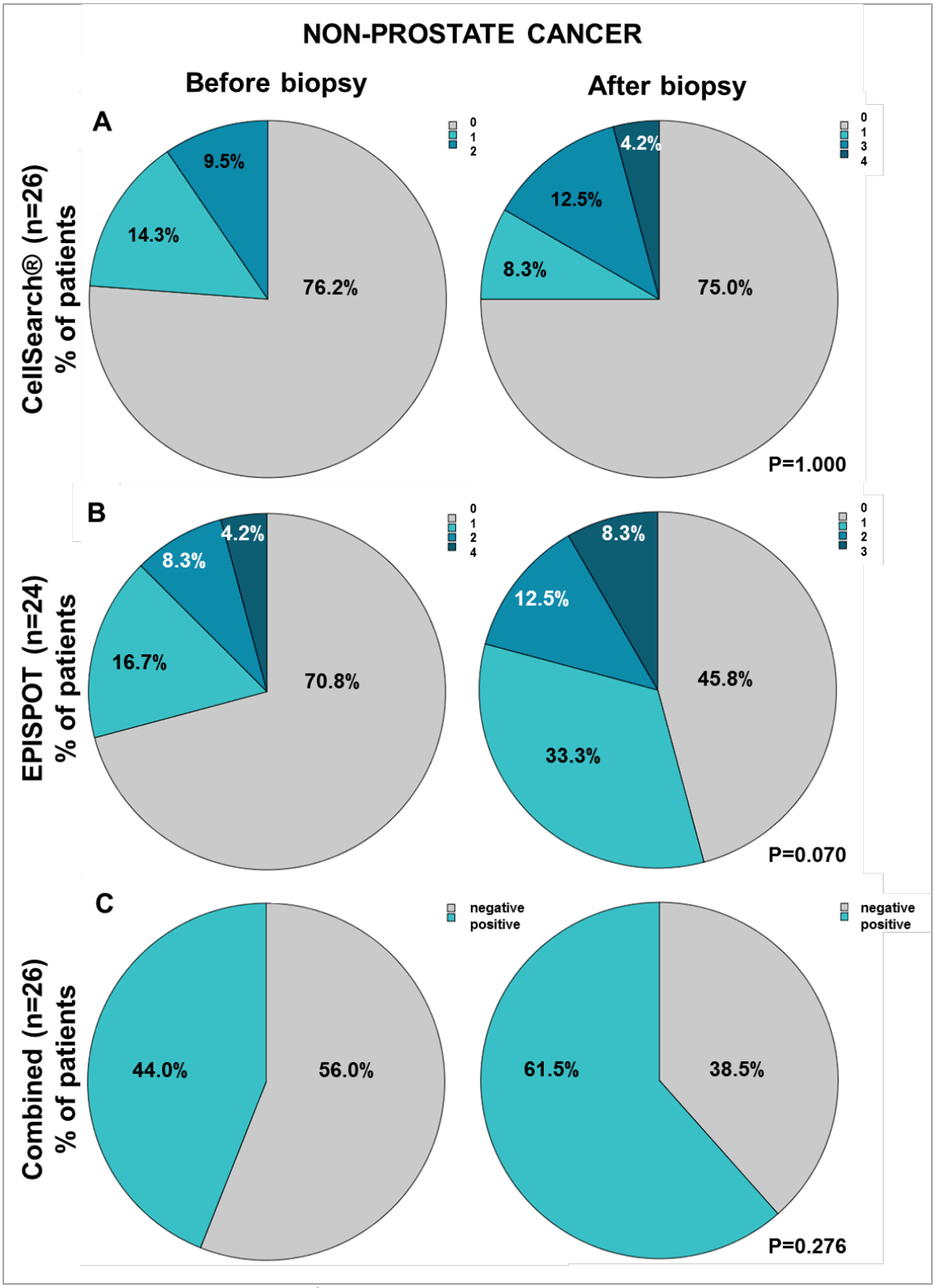

Figure 3.6 The distribution of circulating tumor cells among peripheral blood samples from non-prostate cancer cases collected before and after performing biopsy using the CellSearch® system (A), EPISPOT assay (B) and the combination of both methods (C).

3.3.3 Circulating tumor cells and PSA-level

Detection of PSA-secreting cells using the EPISPOT assay in blood samples taken after prostate biopsy was associated with higher percentage free-to-total PSA ratio (Mann-Whitney U test, P=0.039). Additionally, when restricted only to the patients with a serum PSA value between 2 and 10 ng/mL, the correlation was even more prominent (Mann-Whitney U test, P=0.015). No other significant correlations with presence of CTCs and PSA serum levels were found.

Table 3.5 Characteristics of patients positive for circulating tumor cells.

	CTC-positive patients before biopsy			**CTC-positive patients after biopsy**		
	CellSearch® n=8 (%)	**EPISPOT n=20 (%)**	**Combined n= 27 (%)**	**CellSearch® n=20 (%)**	**EPISPOT n=31(%)**	**Combined n= 42 (%)**
Biopsy result						
No PCa (n=26)	5 (23.8)	7 (29.20)	11 (44.4)	6 (25.0)	13 (54.2)	16 (61.5)
PCa (n= 49)	3 (6.7)	13 (28.3)	16 (33.3)	14 (35.0)	18 (39.1)	26 (53.1)
PIN						
No (n=31)	3 (10.7)	5 (17.2)	7 (23.3)	9 (33.3)	11 (37.9)	16 (51.6)
Yes (n=44)	5 (13.2)	15 (36.6)	20 (46.5)	11 (29.7)	20 (48.9)	26 (59.1)
Gleason score						
6 (n=17)	0 (0.0)	5 (31.1)	5 (31.3)	3 (20.0)	7 (43.8)	9 (52.9)
≥7 (n= 32)	3 (10.3)	8 (26.7)	11 (34.4)	11 (44.0)	11 (34.4)	17 (53.1)
Total PSA level [ng/mL]						
≤10 (n= 57)	6 (12.5)	17 (29.8)	22 (40.0)	13 (28.3)	28 (49.1)	34 (59.6)
>10 (n=17)	2 (11.2)	3 (17.2)	5 (27.8)	7 (38.9)	3 (17.6)	8 (44.4)
Free PSA level [ng/mL]						
≤1 (n=37)	4 (12.5)	14 (37.8)	17 (44.7)	8 (27.6)	17(45.9)	22 (57.9)
>1 (n=38)	4 (11.8)	6 (18.2)	10 (28.6)	12 (34.3)	14 (42.4)	20 (54.1)
Free PSA/Total PSA ratio [%]						
≤10 (n=30)	3 (11.1)	11 (36.7)	13 (46.4)	8 (32.0)	11 (36.7)	17 (60.7)
>10 (n=45)	5 (12.8)	9 (20.0)	14 (31.1)	12 (30.8)	20 (44.4)*	25 (53.2)

*percent of valid cases. CTC, circulating tumor cell; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen

4. DISCUSSION

In this study, the standard CellSearch® system and the EPISPOT assay, which specifically detects living PSA-secreting CTCs, were used, to assess circulating tumor cells in peripheral blood samples (before and after biopsy) of patients undergoing prostate biopsy.

4.1 Clinical material and methodology

The crucial step in every clinical study is choosing the proper cohort of patients as well as usage of a reproducible methodology.

4.1.1 Patients

In this study, a cohort of 75 patients undergoing prostate biopsy was included. In total, 150 peripheral blood samples of the same volumes were obtained from each patient both before and after performing biopsy. The majority (65%) was diagnosed with prostate cancer and 35% of patients had negative biopsy result, which means that no tumor cells were found. This was particularly important to compare frequencies of CTCs in PCa and non-PCa patients.

4.1.2 Methodology

Two different methods allowing assessment of CTCs were used in parallel. The CellSearch® system is the FDA-cleared semi-automated system for CTC detection in PCa patients (**Shaffer et al., 2007; Danila et al., 2007; de Bono et al., 2008**; **Wang et al., 2000; Schmidt et al., 2004; Swennenhuis et al., 2009**). This system allows visualizing CTCs of epithelial origin (positive for cytokeratin and EpCAM) and enables assessment of its morphology, what is particularly important in evaluation of apoptotic cells. While the EPISPOT assay specifically focuses on functional characterization of living prostate tumor cells by its secretion of PSA protein.

However, in only one sample collected before biopsy and 9 cases taken after performing biopsy, CTCs were detected in parallel with the usage of both methods.

These discrepancies might be explained by different bases of CTC-detection of both methods. The CellSearch® system allows capturing the cells of epithelial origin while the EPISPOT detects live PSA-releasing cells. Additionally, using the CellSearch® system, apoptotic cells were observed in approximately 9% and 17% of samples collected before and after biopsy, respectively.

Nevertheless, a possible limitation of both methods is detection of cells of epithelial origin or PSA-secreting as these features do not have to be specific only for malignant but also might characterize normal prostate cells. Thus, it cannot be excluded that a fraction of the 'CTCs' detected in our present study are normal epithelial cells released from the prostate during biopsy.

The CTC numbers detected in patients within this study were very low of maximum 4 cells (for CellSearch®) and 5 (for EPISPOT assay). The majority of patients showed exclusively one CTC. The presence of single CTC could represent background 'noise' from inflammatory cells rather than a true CTC. However, the specificity of the CellSearch® was previously tested. **Allard et al. (2004)** found only 8/145 (5.5%) samples from healthy women with 1 CTC/7.5 mL blood and no sample with ≥2 CTCs. Additionally, **Riethdorf et al. (2007)** found no positive results in seven healthy volunteers.

4.2 Haematogenous dissemination of circulating tumor cells caused by prostate biopsy

There are few reports showing that TRUS prostate biopsy can be associated with haematogenous dissemination of either PSA-mRNA-bearing cells (**Moreno et al., 1997; Hara et al., 2001**) or cellular epithelial material (**Ladjevardi et al., 2014**). In the study by **Moreno et al. (1997)** and **Hara et al. (2001)**, a reverse transcriptase-polymerase chain reaction (RT-PCR) targeted against prostate-specific antigen (PSA) mRNA was used to detect tumor material in the circulation. **Moreno et al. (1997)** reported that 4/43 (9.5%) patients converted to PSA-mRNA-positive after performing biopsy and none of the samples collected before biopsy were positive. **Hara et al. (2001)** showed that 3/46 (6.5%) of patients were positive before and significantly more - 21/46 (45.7%) of patients diagnosed with PCa were PSA-mRNA-

positive after performing biopsy. **Ladjevardi et al., 2014** showed that dissemination of epithelial cellular material occurred in venous blood samples after diagnostic core needle biopsy in 19/23 (82.6%), comparing to 9/23 (39.1%) samples positive before performing biopsy, in patients diagnosed with PCa.

Our study corroborates these previous reports showing that blood samples collected after performing biopsy contain significantly higher number of tumor cells and suggesting that prostate biopsy might be associated with dissemination of tumor material. Additionally, this is the first study that allowed visualization and counting of such cells showing that prostate biopsy may cause spread of not only apoptotic but also living prostate cells with the potential to colonize distant organs.

Interestingly, in patients diagnosed with PCa, the CellSearch® system showed the highest increase in frequency of CTCs in samples collected after performing prostate biopsy. The possible solution could be that this method allows the evaluation of cell morphology and therefore, could be more specific in detecting CTCs.

4.3 Clinical implications

TRUS biopsy is a standard method to obtain histological material in order to make a clinical diagnosis in PCa. However, in other urological tumors like renal cell cancer, biopsy is not a common modality for diagnosis, since it might cause cancer dissemination into blood stream or surrounding tissues and contribute to disease progression (**Smith et al., 1984; Kiser et al., 1986**). Our results suggest that prostate biopsy is associated with spread of viable prostate cells that. Thus, there exists a need for improved, imaging-guided, biopsy procedures to limit the tumor dissemination and to focus the biopsies to the most aggressive area of possible PCa. Another solution could be the usage of non-invasive methods as diffusion-weighted magnetic resonance imaging (MRI) that seems to have similar diagnostic accuracy as biopsy results (**Thoermer et al., 2014**).

5. CONCLUSIONS

This study showed that approximately 20% of patients undergoing prostate biopsy had significantly increased frequency of tumor cells in peripheral blood after, comparing to the samples collected before performing biopsy. Our findings might suggest that prostate biopsy procedure causes haematogenous dissemination of tumor material including both apoptotic and living prostate cells. Therefore, there is a need for improvement of biopsy procedures to possibly limit the tumor dissemination. Future follow up analysis of patients will provide information whether the observed increase in CTCs might lead to an increase in metastasis. Molecular characterization of detected CTCs might confirm the aggressive features of disseminated tumor material and help to improve the knowledge about metastasis formation. The present findings might stimulate these future diagnostic developments towards an improved prostate cancer diagnosis.

Chapter 4: SUMMARY

This study showed potential different clinical applications of detection and characterization of circulating tumor cells as potential diagnostic, prognostic and therapy-related marker in urological tumors – testicular germ cell tumors and prostate cancer.

Different methods for enrichment and detection of CTCs were used and compared within this study including Ficoll gradient centrifugation followed by multi-marker immunohistochemical staining, the EPISPOT assay that detects living, protein-secreting cells as well as the standard, semi-automated CellSeach® method. Each of these techniques has both advantages and limitations that were accurately considered. However, all these methods seemed to be specific and sensitive to detect CTCs for a particular biological and clinical purpose.

The findings presented in this thesis showed various clinical aspects of detection of CTCs. In patients with testicular germ cell tumors, presence of CTCs in peripheral blood might serve as a marker of occult metastasis and could be of prognostic relevance to predict patients' outcome. Additionally, detection of tumor cells in testicular vein might help to better understand dissemination of cancer cells and metastasis formation. In patients undergoing diagnostic prostate biopsy, detection of CTCs might indicate haematogenous dissemination of tumor material.

In addition, it was shown that CTCs can be used not only as a biomarker for cancer detection and prognosis but also as a source of molecular information. To characterize CTCs both fluorescent *in situ* hybridization and immunohistochemistry were applied. Characterization of CTCs in patients with germ cell tumors using fluorescent *in situ* hybridization might support its malignant properties. Immunohistochemical characterization of CTCs can identify potential targets for anti-cancer therapy for testicular germ cell tumors such as CD30 and GPC3.

Taken together, data collected within this study showed that detection of CTCs might be a powerful method of diagnostic and prognostic potential. Molecular characterization of CTCs may serve as a 'liquid biopsy' leading to improvement of anti-cancer therapy for patients with germ cell tumors and prostate cancer. Presence

of tumor cells in peripheral blood of patients undergoing diagnostic prostate biopsy might indicate dissemination of tumor material and could lead to improvement in this invasive procedure. The studies presented in this thesis indicated that results from basic science might be translated into clinical applications and will stimulate further diagnostic and prognostic developments in germ cell tumors and prostate cancer.

ABBREVIATIONS

AFP, alpha-fetoprotein

AJCC, American Joint Committee on Cancer

AR, androgen receptor

AUA, American Urological Association

BiTE®, bispecific T cell engagers

BRCA1, breast cancer 1, early onset

CK19, cytokeratin-19

CTC, circulating tumor cell

d, density

DAPI, 4',6-diamidino-2-phenylindole

DNA, deoxyribonucleic acid

DTC, disseminated tumor cell

EAU, European Association of Urology

EGFR, epithelial growth factor receptor

EpCAM, epithelial cell adhesion molecule

EPISPOT, EPithelial Immuno SPOT

ER, estrogen receptor

FCS, fetal calf serum

FGF2, fibroblast growth factor-2

FISH, fluorescent *in situ* hybridization

fPSA, free PSA

GCT, germ cell tumor

GPC3, glypican-3

HER2, human epidermal growth factor receptor 2

ICC, immunocytochemistry

IGCCC, International Germ Cell Consensus Classification

KRAS, kirsten rat sarcoma viral oncogene homolog

LDH, lactate dehydrogenase

mL, milliliter

MNC, mononuclear cell

MRD, minimal residual disease

mRNA, messenger ribonucleic acid

MUC1, mucin-1

NSGCT, nonseminomatous germ cell tumor

PB, peripheral blood

PCa, prostate cancer

PCR, polymerase chain reaction

PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase

PSA, prostate specific antigen

PTEN, phosphatase and tensin homolog

RNA, ribonucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

SALL4, sal-like protein 4

TRUS, transrectal ultrasound-guided

TV, testicular vein

UICC, International Union Against Cancer

WHO, World Health Organization

βHCG, β human chorionic gonadotropin

µl - microliter

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SUPPLEMENTARY MATERIAL

I. Cell culture media

A) RPMI-Medium (37 °C; 5 % CO_2)

90 % RPMI 1640 Medium (Gibco, Eggenstein, Germany)

10 % FCS (PAA Laboratories GmbH, Pasching, AU)

0.5 % Penicillin-Streptomycin Mix (50 U/mL, Gibco, Eggenstein, Germany)

2mM L-Glutamine (Gibco, Eggenstein, Germany)

B) DMEM-Medium (37 °C; 10 % CO_2)

90 % DMEM Medium (Gibco, Eggenstein, Germany)

10 % FCS (PAA Laboratories GmbH, Pasching, AU)

0.5 % Penicillin-Streptomycin Mix (50 U/mL, Gibco, Eggenstein, Germany)

2mM L-Glutamine (Gibco, Eggenstein, Germany)

C) Micro-metastatic medium (37 °C; 5 % CO_2)

90 % RPMI 1640 Medium (Gibco, Eggenstein, Germany)

10 % FCS (PAA Laboratories GmbH, Pasching, AU)

0.5 % Penicillin-Streptomycin Mix (50 U/mL, Gibco, Eggenstein, Germany)

2mM L-Glutamine (Gibco, Eggenstein, Germany)

1 % Insulin-Transferrin-Selenium (Gibco, Eggenstein, Germany)

50 ng/mL Epithelial Growth Factor

10 ng/mL β-Fibroblast Growth Factor

D) DMEM/RPMI (37 °C; 5 % CO_2)

40 % RPMI 1640 Medium (Gibco, Eggenstein, Germany)

40 % DMEM Medium (Gibco, Eggenstein, Germany)

20 % FCS (PAA Laboratories GmbH, Pasching, AU)

0.5 % Penicillin-Streptomycin Mix (50 U/mL, Gibco, Eggenstein, Germany)

2mM L-Glutamine (Gibco, Eggenstein, Germany)

II. Buffers and solutions

Name of reagent	Company
BSA (Bovines Serum Albumin)	Invitrogen, Germany
DMSO (Dimethylsulfoxide)	Serva Heidelberg, Germany
Ethanol absolute for analysis	Sigma-Aldrich, US
Ethanol 99%	Walter CMP, Germany
Ethanol 96%	Walter CMP, Germany
Ethanol 80%	Walter CMP, Germany
Ficoll	Amersham Biosciences, Germany
Formaldehyde 37 %	Merck, Germany
Triton X 100	Sigma-Aldrich, US
Trypan blue solution (0.4%)	Sigma-Aldrich, US
Tween-20	Merck, Germany
1xPBS	Gibco, Germany
Xylene	Sigma-Aldrich, US
Isopropanol	Sigma-Aldrich, US
Trypsin-EDTA 10 x	Gibco BRL, Life Technologies, Germany
HCl	Merck, Germany
Methanol	J.T. Baker, Netherlands
NaCl	Carl Roth GmbH, Germany
Name of buffer	**Components**
10xTBST	181.65 g Tris 262.98 g NaCl 15 g Tween-20 170 ml 35%HCl pill up to 3 l with distilled water and pH=7.6

III. The CellSearch® system (Veridex) components

Name	Components	Application
CellSave®Tube		Blood collection and preservation
CellSearch® CTC Test	Anti-EpCAM ferrofluid	CTC-enrichment and detection components
	Capture enhancement reagent	
	Permeabilization reagent	
	Cell Fixative	
	Nucleic acid dye	
	Staining reagent	
	Dilution solution	
Apparatus	Automated CELLTRACKS® AUTOPREP® System	Automated sample preparation
	CELLTRACKS® ANALYZER II	Sample analysis

IV. Software

Name	Application	Company/Source
SPSS software ver. 21.0 licensed for the University Medical Centre Hamburg-Eppendorf, Germany	Statistical analysis	IBM Corporation, US
Microsoft Office 2010	Writing, database and figure preparation	Microsoft, US
NCBI	Literature	www.ncbi.nlm.nih.gov
AxioVision	Micrograph preparation	Zeiss, Germany
Adobe Photoshop CS5	Figure preparation	Adobe Systems, US

V. Apparatus

Name	Company
Centrifuge	
Rotofix 32	Hettich, Germany
Centrifuge 5417R	Eppendorf, Germany
Centrifuge 5418	Eppendorf, Germany
Haraeus Multifuge 3S-R	Thermo Scientific, Germany
Sorvall RC5C plus	Thermo Scientific, Germany
Microscope	
Axiostar plus	Carl Zeiss, Germany
Axioplan 2 imaging	Carl Zeiss, Germany
Ebq 100 Isolated Fluorescence Lamp	Leica, Germany
Brightfield microscope	Helmut Hund GmbH, Germany
Cell culture	
Incubator Hera 150	Heraeus Kendro, Germany
Herasafe HS12	Heraeus Kendro, Germany
Neubauer cell counting chamber	Menzel Gläser, Germany
General laboratory apparatus	
Spectrophotometer ND 1000	NanoDrop, USA
Decloaking Chamber	Biocare Medical, USA
Thermomixer comfort	Eppendorf, Germany
Roller Mixer SRT1, StuartR	Bibby Sterilin, UK
Tissue Drying Oven 66	Medite, Germany
Waterbath GFL-1003	Hettich, Germany
Vortex-Genie 2	Scientfic Industries, USA
Severin Microwave 800	Severin, Germany
pH-meter	InoLab WTW, Germany
Certomat R H	B. Braun Biotech International, Germany
Certimat R IS	B. Braun Biotech International, Germany

VI. General Consumables

Material	Company
Fixogum Rubber Cement	Promega GmbH, Madison, US
Microscopic slides Super Frost / Plus	Assistent Glasfabrik Karl Hecht, Sondheim, Germany
Nitrocellulose membrane	Milipore, Germany
Cover-slides	Marienfeld, Germany
Cell culture flasks	Biochrom AG, Germany
Sterile Syringe Filter 0,2 µm PES	Carl Roth GmbH, Germany
Eppendorf tubes	Eppendorf, Germany
Falcon tubes 15 mL	Sarstedt, Germany
Falcon tubes 50 mL	Sarstedt, Germany

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CURRICULUM VITAE

The author of this thesis, Paulina Nastały was born on the 29th of August 1987 in Wejherowo, a small city located in the north of Poland. After obtaining her high-school certificate in 2006, she commenced her academic career studying biotechnology at the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk. In 2008, she went for semester abroad to the University of Bremen. In 2009 she obtained Bachelor title and two years later she obtained her Master of Science diploma in cancer research at the Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk. In October 2011, she started her PhD research project on the circulating tumor cells in patients with testicular germ cell tumors and prostate cancer under the supervision of Prof. Dr. med. Klaus Pantel and Dr. Sabine Riethdorf which has culminated in this thesis.

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