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The Clinical Relevance of Circulating Tumour Cells and Tumour-Derived Extracellular Vesicles from Different Blood Compartments in Colorectal Cancer

Dissertation

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Dedicated to my family

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Summary

English

Colorectal cancer (CRC) represents the third most common cause of cancer-related mortality in Germany and the second most common in the USA. Following curative surgery, the selection of an optimal adjuvant treatment is of utmost importance. Liquid biopsies, as a tool to sample circulating tumour-derived elements, including circulating tumour cells (CTCs) and tumour-derived extracellular vesicles (tdEVs) in blood, may facilitate the adjuvant decision making. However, CTCs are currently limited in their informative value due to their low abundance, even in metastatic CRC patients. For my thesis, I investigated potential solutions to deal with the scarcity of CTCs. I evaluated the benefit of tumour-draining vein (DV) blood on detecting CTCs and tdEVs compared to central venous catheter (CVC) blood. Furthermore, my objectives were to gain insights into their correlation with the patient's clinicopathological information and to investigate the phenotype of DV biopsies. 510 blood samples from 364 gastrointestinal tumour patients and 93 from healthy participants were analysed. We employed the CellSearch system (CS), the current gold standard for CTC enrichment. The ACCEPT software and in-house developed R scripts were applied to objectify and automatize the enumeration process of CTCs. Following validation of gating settings for CTCs and tdEVs, the respective counts were correlated with the clinicopathological data of 227 blood samples from 142 CRC patients. Aside from the enumeration, ACCEPT provides a tabular output of different fluorescence signals, including size, morphology, and intensity. By implementing this output into analysis, CTC and tdEV phenotypes could be differentiated and diversity indices could be calculated. The first index was based on the Shannon index of clusters generated with the k-means algorithm after UMAP dimension reduction, and the second one was based on the standard deviation of 13 ACCEPT parameters. To make the optimized automated evaluation of CS and ACCEPT data accessible to other users in the future, I developed a Shiny app based on R. The results revealed that the detection rate of CTCs and the count of CTCs and tdEVs were highest in DV. As indicated in previous studies, tdEVs were more abundant than CTCs and exhibited a strong correlation with CTCs in our investigation. CVC-tdEVs reflected clinical indicators of tumour spread, while DV-tdEVs and DV-CTCs were associated with tumour size. In both compartments, the prognostic information of tdEVs surpassed that of CTCs in uni- and multivariate analyses and Kaplan-Meier estimates. CTCs and tdEVs in DV samples were larger, more eccentric, and displayed stronger CK intensities than CVC samples ($p < 0.05$). A higher CTC/tdEV diversity calculated by both indices was associated with shorter overall survival (OS) and improved patient stratification. The programmed Shiny app could be potentially used as a cost- and time-saving tool for organizing and interpreting CellSearch data when evaluating datasets from multiple studies. The present study demonstrates that DV biopsies have the potential to enhance biomarker detection. Moreover, tdEVs exhibited superior predictive accuracy compared to CTCs, and the analysis of phenotypes further improved the predictive accuracy. Taken together, implementing both biomarkers and their phenotypic characteristics can further personalize adjuvant decision-making strategies.

Summary

German

Das kolorektale Karzinom (KRK) ist die dritthäufigste krebsbedingte Todesursache in Deutschland und die zweithäufigste in den USA. Wenn eine kurative, das heißt operative Therapie möglich ist, stellt sich im adjuvanten Zeitraum die Frage, wie die Erkrankung weiter therapiert werden sollte. Die Flüssige Biopsie oder auch Liquid Biopsy genannt, bei der aus dem Blut zirkulierende Tumorzellen (CTCs) und tumor-assoziierte Vesikel (tdEVs) bestimmt werden, könnte die Entscheidungsfindung unterstützen. Ein wesentlicher Grund, wieso diese Biomarker noch kein etablierter Bestandteil in der Klinik sind, ist das seltene Vorkommen dieser, selbst in metastasierten Darmkrebspatienten. Für mein Projekt habe ich untersucht, welchen Einfluss das Blut aus der tumor-drainierende Vene (DV) auf die Detektion der CTCs und tdEVs im Vergleich zum zentralvenösen (CVC) Blut hat. Zudem erfolgte eine Korrelation dieser Biomarker mit klinikopathologischen Faktoren und eine phänotypische Charakterisierung. Ich analysierte insgesamt 510 Blutproben von 364 gastrointestinalen Krebspatienten und 93 Blutproben von gesunden Teilnehmern. Hierzu nutzten wir den Goldstandard unter den CTC-Anreicherungstechnologien, das CellSearch-System (CS). Um den Prozess der Enumeration zu objektivieren und zu automatisieren, nutzten wir die ACCEPT Software in Kombination mit R-Skripten. Nach der Validierung von Gate-Settings zur Enumeration von CTCs/tdEVs, setzte ich sie für 227 Blutproben von 142 KRK-Patienten ein. ACCEPT ermöglicht neben der Enumeration auch die Extraktion eines numerischen Outputs von verschiedenen Fluoreszenz-Signalen, z.B. der Größe, Morphologie und der Intensität. Zum einen ermöglichte die Bereitstellung der Fluoreszenz-Parameter den phänotypischen Vergleich und zum anderen die Berechnung zweier Diversitätsindizes. Diese Indizes basierten auf den Shannon-Index von k-means-generierten Clustern nach einer Dimensionsreduktion durch UMAP und auf der Standardabweichung selektierter Parameter. Für die zukünftige Evaluationen von CS-Daten und des ACCEPT-Outputs programmierte ich eine App innerhalb der R Umgebung. tdEVs waren im Vergleich zu CTCs in einer höheren Detektionsrate und Zahl im Blut zu finden. CTCs und tdEVs wiesen beide eine höhere Detektionsrate und Zahl im DV auf. CVC-tdEVs waren insbesondere mit der systemischen Ausbreitung des Tumors assoziiert, wohingegen sowohl DV-tdEVs als auch DV-CTCs vor allem mit der lokalen Tumorgröße und -invasion zusammenhingen. Unabhängig vom Blutentnahmeort wiesen tdEVs sowohl in der uni- als auch multivariaten Analyse einen höheren prognostischen Wert auf als CTCs. Die Evaluation der phänotypischen Eigenschaften ergab, dass beide Biomarker im DV verglichen zum CVC wesentlich größer und rundlicher waren und eine stärkere CK-Intensität aufwiesen. Eine erhöhte Diversität der CTCs/tdEVs war mit einem verkürzten Gesamtüberleben assoziiert. Die programmierte App erwies sich bei der Evaluation von weiteren Datensets als kosteneffektives und zeitsparendes Tool zur Organisation und Interpretation von CellSearch Daten. In meiner Dissertation zeige ich auf, dass die intraoperative Blutentnahme aus einer DV eine sichere Methode darstellt, die Detektion von Biomarkern effektiv zu steigern und zusätzliche Informationen zu gewinnen. tdEVs wiesen eine höhere prognostische Genauigkeit auf verglichen zu CTCs. Weiterhin erhöhte die Analyse der phänotypischen Eigenschaften beider Biomarker die prognostische Genauigkeit. Zukünftig könnte die Kombination beider Biomarker unter Einschluss derer phänotypischen Eigenschaften, die adjuvante Entscheidungsfindung wesentlich unterstützen.

List of Abbreviations

Abbreviation	Explanation
AACR	American Association for Cancer Research
ACCEPT	Automated CTC Classification, Enumeration and PhenoTyping
ACL	Anocutaneous line
aCT	Adjuvant chemotherapy
ADT	Androgen deprivation therapy
ARSI	Androgen receptor signalling inhibitors
ASCO	American Society of Clinical Oncology
At incl.	At inclusion
AUC	Area under the curve
Ben	Benign
BRAF	proto-oncogene B-Raf
CD	Cluster of differentiation
CEA	Carcinoembryonic Antigen
CI	Confidence Interval
CIN	Chromosomal instability
CK	Cytokeratin
CM	Contrast Maximization
CNA	Copy number alteration
CNPC	Castration-naïve prostate cancer
CRC	Colorectal cancer
CRC-LM	Colorectal cancer with liver metastases
CRPC	Castration-resistant prostate cancer
CS	CellSearch
CTC	Circulating tumour cell
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CTX	Chemotherapy
CVC	Central venous catheter
DAPI	4′,6-Diamidino-2-phenylindole
DFS	Disease free survival
DiOC	3,3′-Dihexyloxacarbocyanine iodide
DL	Deep learning
DLA	Diagnostic leukapheresis
DLRS	Derivative Log Ratio Spread
DNA	Deoxyribonucleic Acid
DOS	Day of surgery
DU	Düsseldorf cohort
DV	Tumour-draining vein
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EP	Expert panel
EPISPOT	EPithelial ImmunoSPOT
ESMO	European Society for Medical Oncology.
EU	European Union
EV	Extracellular vesicle
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Administration

FISH	Fluorescence in situ hybridization
FOBT	Faecal occult blood test
FOLFIRI	Folinic Acid + 5-Fluorouracil + Irinotecan
FOLFOX	Folinic Acid + 5-Fluorouracil + Oxaliplatin
FOLFOXIRI	Folinic Acid + 5-Fluorouracil + Oxaliplatin + Irinotecan
5-FU	5-fluorouracil
GAP	Genome alteration percentage
GI	Gastrointestinal
HE	Heidelberg cohort
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
HV	Hepatic vein
ICDO	International Classification of Diseases for Oncology
iFOBT	Immunological FOBT
IMV	Inferior mesenteric vein
IQR	Interquartile range
KRAS	Kirsten rat sarcoma virus
LA	Leukapheresis
LB	Liquid Biopsy
LCC	Left-sided colorectal cancer
LM	Liver metastases
L-TCF	Large tumour cell fragments
L-TMP	Large tumour microparticles
MASA	Mutant-allele-specific amplification
mBC	Metastatic breast cancer
MMR	Mismatch repair
MOA	Multi-obstacle architecture
mPC	Metastatic prostate cancer
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MsV	Mesenteric vein
NA	Not available
NGS	Next-generation sequencing
nmBC	Non-metastatic breast cancer
NPV	Negative predictive value
NS	Not significant
NSCLC	Non-small-cell lung cancer
OR	Odds ratio
OpRev	Operator review
OS	Overall survival
PA	Peripheral artery
PB	Peripheral blood
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
PFS	Progression-free survival
PNM	Paired non-metastatic

POV	Portal vein
PPV	Positive predictive value
PSA	Prostate-specific antigen
PSMA	Prostate-Specific Membrane Antigen
PV	Peripheral vein
PVB	Peripheral vein blood
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RC	Rectal cancer
RCC	Right-sided colorectal cancer
RD	Recurrence of disease
RNA	Ribonucleic acid
ROC	Receiver operating curve
RT	Radiotherapy
SD	Standard deviation
SI	Shannon diversity index
SMV	Superior mesenteric vein
SSA	Selective size amplification
S-TCF	Small tumour cell fragments
TNM	TNM Classification of Malignant Tumours
tdEV	Tumour-derived extracellular vesicle
UICC	Union Internationale Contre le Cancer
UMAP	Uniform Manifold Approximation and Projection
UPNM	Unpaired non-metastatic
USA	United States of America
USS	Uncertain statistical significance
UT	University Twente
VEGF	Vascular Endothelial Growth Factor
WB	Whole blood
WBC	White blood cell
WGA	Whole genome amplification
WGS	Whole genome sequencing
Wnt	Wingless/int1

Table of Contents

1. Introduction

1.1. The Clinical Problem

Colorectal cancer (CRC) refers to malignant tumours in the colon including the ascending, transverse, descending, and sigmoid colon, or the rectum. According to the international documentation system, tumours are considered rectal carcinomas if their distal border, as measured by a rigid rectoscope, is 16 cm or less from the anal verge.[2] CRC patients in the early stages are mostly curable by surgical resection of the tumour. However, approximately 30% of CRC patients in stage II-III experience a recurrence of the disease despite various therapeutic options in the adjuvant setting. Postoperative management can range from (intensive) adjuvant chemotherapy (aCT) to active surveillance, which includes regular disease monitoring.[3] The most beneficial treatment option must be identified to achieve the optimal survival outcome and minimize the risk of recurrent disease. Indeed, pathological staging, medical imaging, conventional tumour markers, and physical examination can help to determine the spread and aggressiveness of cancer disease, but their inability to detect minimal residual disease limits their clinical utility. The American Society of Clinical Oncology (ASCO) has reported a lack of clinically reliable features to identify high-risk patients who would benefit from intensive therapy, particularly in stages II and III.[4] aCT is currently recommended for all eligible stage III patients. However, approximately half of stage III patients do not benefit from adjuvant chemotherapy, and routine clinical tools fail to identify them.[3,5] In practice, this means that patients with excellent prognoses—evidenced by a 90% disease-free survival rate—are treated with the same adjuvant chemotherapy as high-risk patients, despite differing risk profiles. A relevant portion of these low-risk patients do not profit from it and some experience adverse effects like neuropathy. On the other hand, certain stage II patients are recommended only partial therapy using fluorouracil and folinic acid based on clinicopathological risk factors.[3] The administration of partial therapy in stage II patients can provide a marginal survival advantage compared to patients managed with observation alone.[6] Yet, several studies highlight the challenge of identifying this small group of patients with a favourable benefit-risk profile. A large retrospective study investigated 5-year overall mortality of more than 24,000 stage II patients and showed that neither patients with clinical risk factors (T4 disease, tumour perforation, resection of <12 lymph nodes) nor patients without risk factors would significantly profit from aCT.[7] In line with this finding, the European Society for Medical Oncology (ESMO) reported only a 3%–5% reduction in the risk of death for high-risk stage II patients receiving 5-fluorouracil (5-FU).[8] This highlights the demand for new biomarkers to identify patients that can truly benefit from adjuvant treatment. Despite considerable research on new predictive biomarkers, only a small fraction has successfully transitioned into clinical practice and there is a lack of biomarkers that can be used for patient prognostication in the stages II-III. In this context, carcinoembryonic antigen (CEA) is currently used as a tumour marker. CEA is a foetal glycoprotein that was first found in foetal and cancerous tissues of the digestive system and is detectable in malignant and non-malignant diseases.[9,10] Both the preoperative and postoperative measurement of CEA levels have prognostic relevance. CEA levels help in detecting recurrent disease for the follow-up period after CRC resection. Similarly, in metastatic CRC patients, higher CEA levels correlate with shorter overall survival (OS) and lower radiological response rate.[10–13] Yet, like other serological tumour markers, CEA's documented sensitivity and specificity are low, with 46% and 89% pooled values, respectively.[14] As another example, microsatellite instability (MSI) expression might influence treatment response because the generated mutations lead to neoepitopes and, therefore, to increased recognition by the immune system.[15]

Nevertheless, the pressing clinical problem remains that, to date, there is no biomarker that can adequately determine which patients should receive aCT and which should not due to statistical uncertainty. Therefore, research on novel biomarkers like circulating tumour cells (CTCs), tumour-derived extracellular vesicles (tdEVs), and circulating tumour DNA (ctDNA),

as tumour components detectable in the blood circulation and strong predictors of OS and disease-free survival (DFS), is urgently needed to offer accurate patient stratification.[16]

This dissertation specifically addresses the prognostic significance of tdEVs and CTCs taken from a tumour-draining vein (DV) and a central venous catheter (CVC), and their phenotypic diversity. Importantly, the role of tdEVs and phenotypic diversity in blood samples taken from DVs has not yet been addressed in the existing literature.

1.2. CRC Epidemiology

CRC is a global health issue as it is the second and third most common cancer worldwide in terms of mortality and incidence, respectively (**Figure 1**).[17]

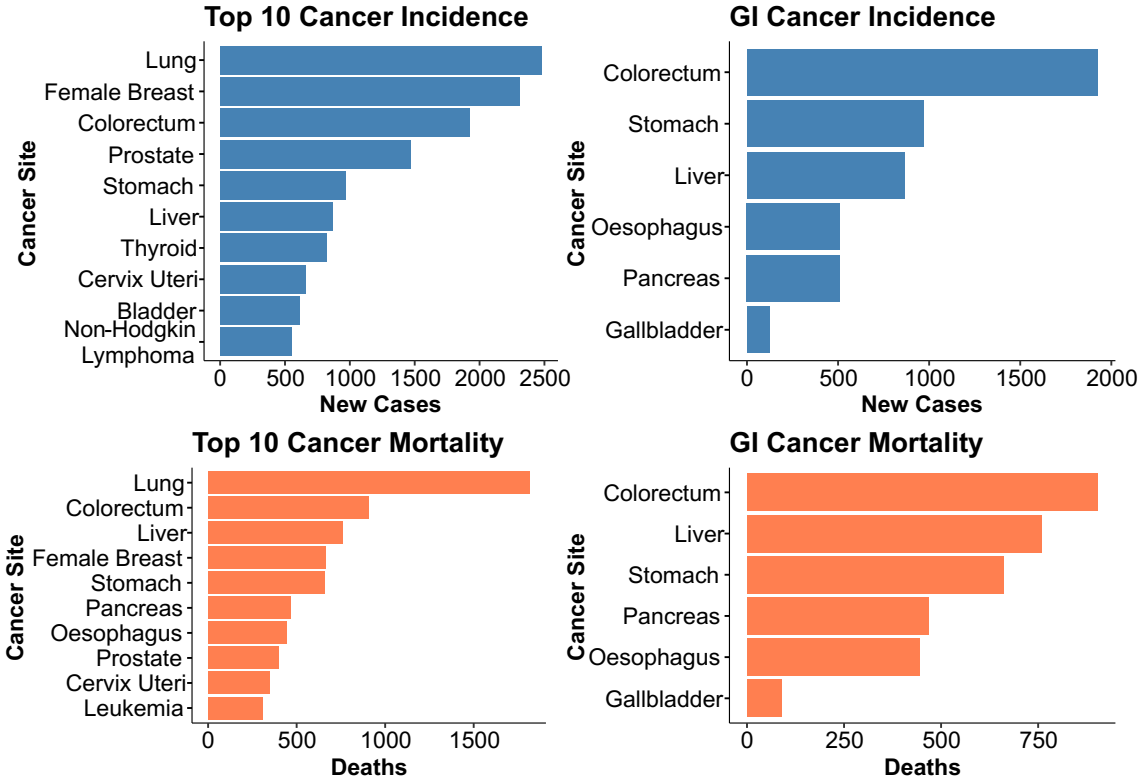

Figure 1**: Cancer Epidemiology**
The first row indicates the absolute number (multiplied by 1000) in incidence of cancer cases. The second row displays the absolute number of mortality (multiplied by 1000). On the left side, the ten most prevalent types of cancer are shown, while on the right side, the most pervasive GI cancer entities are demonstrated. Epidemiological data was obtained from the study by Bray et al. (2024).[18]

For the year 2040, it is estimated that 3.2 million cases of CRC will be diagnosed, resulting in 1.6 million deaths.[19] Because 60% of patients with CRC are diagnosed between 50 to 74 years of age and only less than 10% of patients under the age of 50, CRC can be regarded as a cancer primarily prevalent in the elderly population.[19] The 5-year survival rates are firmly stage-dependent. The 5-year survival rate is 91% for localized, 73% for regional, and only 14% for distant CRC patients.[20] The incidence rates are particularly elevated in industrialized countries of Europe and North America compared to developing countries. A combination of factors, including a lower level of physical activity, obesity, an unhealthy diet, and the consumption of alcohol and smoking, contribute to the higher incidence in industrialized countries.[21,22] Epidemiological trends in recent years indicate a decline in incidence rates across most age groups, which may result from the effective implementation of screening programs and improved access to healthy food.[20] Colonoscopy is an integral part of screening programs that enables the highly sensitive detection of suspicious lesions and their simultaneous removal (**Figure 2**).

This screening test is recognized as the gold standard, particularly in the opportunistic setting, although stool tests such as the "faecal immunochemical test" offer advantages in terms of cost-effectiveness and patient adherence (**Figure 2**).[23–25] In colorectal cancer screening, an opportunistic setting refers to colonoscopies performed on an ad-hoc basis during routine medical visits, rather than within a structured screening program.[26] In contrast to the decline in the total incidence rates, the recent epidemiological data suggests an increasing CRC incidence among young adults, which could be attributed to negative factors (lifestyle, environmental) outweighing the protective factors.[17]

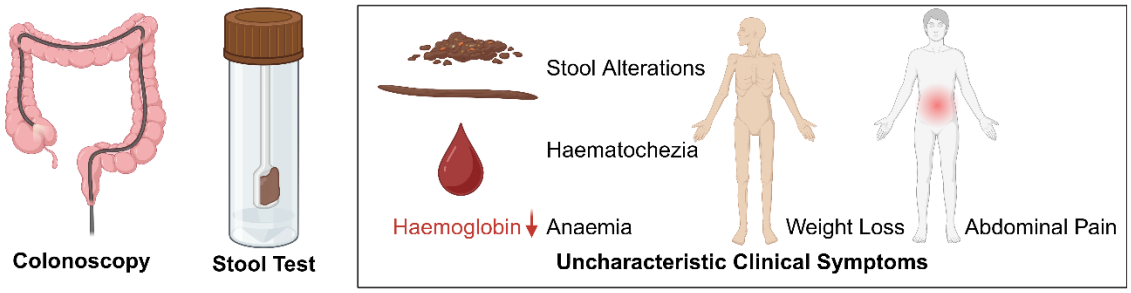

Figure 2: **CRC Screening and Diagnosis**
Colonoscopy is the gold-standard diagnostic test for detecting and removing suspicious lesions. Various stool tests such as the faecal occult blood test (FOBT), guaiac-based FOBT or the immunological FOBT (iFOBT) are also used for early detection (secondary prevention). There are no CRC-specific, characteristic early symptoms. On the right exemplary accompanying symptoms are portrayed. Created with BioRender.com

1.3. CRC Aetiology

CRC is not a homogenous group of cancer and is influenced by a multitude of factors. From an aetiological perspective, CRC arises predominantly (80-85%) sporadically due to environmental factors such as unhealthy or unbalanced diet, reduced physical activity, and overweight.[27] Most CRC cases arise from the classical pathway of the adenoma-carcinoma sequence (**Figure 3**).[28] The adenoma-carcinoma sequence implies that carcinomas develop gradually from benign to malignant adenomas after an initial inactivation of tumour suppressor genes, including the adenomatous polyposis coli (APC) gene, which negatively regulates proliferative signalling pathways like the Wingless/int1 (Wnt) pathway.[29] Further genetic alterations in tumour protein 53 (TP53) and Kirsten rat sarcoma virus (KRAS) genes, as well as changes in mismatch repair genes triggering microsatellite instability, lead to uncontrolled cell proliferation and the development of invasive cancer.[30–33] The speed of cancer development depends on the respective pathway: The pathway of CIN (chromosomal instability) might take 10 years, and the MSI pathway might take only a few years.[28] Also, tumour localization influences cancer development: Left-sided tumours are linked to tubular or villous adenocarcinomas, polypoid-like morphology and CIN-high tumours, while right-sided tumours tend to be associated with mucinous adenocarcinomas or sessile serrated adenomas, flat like morphology, MSI-high and mismatch repair deficient tumours.[34]

Aside from sporadic occurrence, CRC can be less frequently caused by inherited genetic changes and chronic gastrointestinal (GI) diseases, such as chronic colitis and Crohn's disease.[35] The most common genetic CRC disease is hereditary nonpolyposis colorectal cancer (HNPCC). HNPCC originates from mutations in DNA mismatch repair genes (e.g., MSH2 and MLH1), leading to microsatellite instability and, therefore, to a lower average age at onset (45 years) than the sporadic CRC type.[36] The genetic changes are associated with a 50-70% elevated risk of developing CRC and a higher risk for endometrial (20-60%), mammary, ovarian, and other cancers located in the urogenital and gastrointestinal tract. At-risk people for HNPCC are individuals fulfilling the Amsterdam or Bethesda criteria. If these criteria are fulfilled and molecular genetic testing of tumour tissue from an affected relative identifies a pathogenic mutation, genetic testing of the respective person via blood analysis

may be warranted. For these at-risk individuals, screening is recommended at an earlier age of 25 years or younger, and genetic consultation may be indicated.[33]

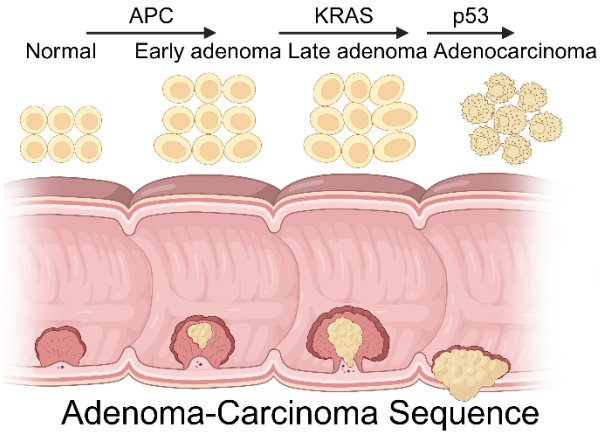

Figure 3: **CRC Cancer Development**
The transformation of a benign into a malignant lesion is an extended biological process that usually takes years.[28] *Created with BioRender.com.*
Abbreviations: APC – adenomatous polyposis coli; Kirsten rat sarcoma virus – KRAS; p53 – tumour protein 53

1.4. CRC Therapy (Germany)

The therapeutic management of CRC is tailored to the individual patient characteristics. All CRC patients are classified according to the *Union Internationale Contre le Cancer* (UICC) to assess the extent of cancer progression and support clinical decision-making (**Table 1**).[37] The therapy depends on the clinical stage, general health condition, and, at a later stage, on the molecular biology of the tumour tissue.[37] It is, therefore, essential that the presence of distant metastases, the extent of the primary tumour and lymph node involvement is assessed by preoperative imaging techniques such as CT or MR scan and abdominal ultrasound.[38] Treatment options include surgical removal, chemotherapy, targeted tumour therapy, and, exclusively for rectal carcinoma, (neo-) adjuvant radio (chemo-) therapy (**Table 2**).[39]

Table 1: **UICC Classification of CRC**

Stage	Primary Tumour	Lymphatic Metastases	Haematogenous Metastases
0	Tis	N-	M0
I	T1, T2	N-	M0
II	T3, T4	N-	M0
III	Any T	N+	M0
IV	Any T	Any N	M1

Lymphatic metastases are present from stage III onwards (local CRC), and haematogenous metastases from stage IV (metastatic CRC).
Abbreviations: M – presence of distant metastases (no or yes); N – spread of regional lymph nodes (no or yes); T – size/extent of the primary tumour (1-4)

1.4.1. Surgery

In early tumour stages, endoscopic resection can be offered with curative intention. In the case of locally advanced CRC or lymph node metastases, surgical resection of the primary tumour and affected lymph nodes is recommended. The extent of cancer resection depends on the tumour location and the vascular supply of the respective section.[40] Surgical treatment of colon cancer should include complete mesocolic excision (CME) and removal of affected adjacent structures. CME appears to be a deciding factor in the improvement of survival rates and in the decrease of locoregional disease over the last decades.[41] Minimal invasive laparoscopic and open resection are comparable surgical procedures regarding morbidity and postoperative

tumour recurrence, and both can be offered to the patient.[42] Intraoperative lymph node resection has a prognostic impact on the patient, even if all lymph nodes have been tested negative. Studies have shown that the more lymph nodes removed, the better the survival rate. Experts recommend removing 12 lymph nodes for a comprehensive histopathological examination. However, this number can vary and resection is not always possible.[43] The extent of residual tumour after primary resection is classified according to the R status: R0 (no residual tumour), R1 (microscopic residual tumour), or R2 (macroscopic residual tumour). An R1 tumour is defined as a tumour that extends to the resection margin and is associated with shorter DFS and OS.[44] Similarly, a circumferential safety margin of less than 1 mm is associated with an increased risk of local recurrence.[45] In selected cases after the administration of neoadjuvant radiochemotherapy, surgical resection may be omitted entirely if no tumour tissue is detectable clinically, endoscopically, and by other imaging methods as endosonography and magnetic resonance imaging (MRI). However, the patient must be fully medically informed and closely monitored (for at least five years) if resection is to be avoided in this case.[33]

Table 2: **Therapy for CRC Patients**

<table>
<tr><th>UICC</th><th>TNM</th><th>Therapy Colon Cancer</th><th>Therapy Rectal Cancer</th></tr>
<tr><td rowspan="2">0–I</td><td>Tis to T1</td><td>• Endoscopic resection
• No adjuvant chemotherapy</td><td>• Low-risk situation, tumour <3 cm:
Local (endoscopic) excision
• High-risk situation:
Rectal resection/rectal extirpation
• No neoadjuvant therapy</td></tr>
<tr><td>T2, N0, M0</td><td>• Radical surgical resection
• No adjuvant chemotherapy</td><td>• Rectal resection/rectal extirpation
• No neoadjuvant therapy</td></tr>
<tr><td>II</td><td colspan="2">• Radical surgical resection
• Optional: Adjuvant chemotherapy</td><td rowspan="2">• Middle/lower third
Rectal resection/rectal extirpation
Neoadjuvant radio-chemotherapy/ radiotherapy*
• Upper third
Rectal resection,
Adjuvant chemotherapy
<u>Optional:</u>
Neoadjuvant radio-chemotherapy/radiotherapy and rectal resection*</td></tr>
<tr><td>III</td><td colspan="2">• Radical surgical resection
• Adjuvant chemotherapy</td></tr>
<tr><td>IV</td><td colspan="3">• *Tumour board decision: Palliative vs. curative treatment
➔ Resection and/or systemic therapy (FOLFIRI/FOLFOX/FOLFOXIRI etc. and possibly combination with targeted substance)</td></tr>
</table>

Therapy modalities include surgery and chemotherapy for both colon and rectal cancer patients.
**Radio(chemo) therapy is only offered to rectal cancer patients. Primary resection is possible for cT1/T2 tumours (lower and middle third) with questionable lymph node involvement or cT3a/b tumours (middle third) with limited infiltration of perirectal fat and without lymph node metastases and extra mural vascular infiltration. For rectal cancers in the upper third, preoperative radio/radio-chemotherapy may be recommended for specific risk constellations such as T4 tumours or tumours with a positive magnetic resonance circumferential resection margin (mrCRM+). Treatment decisions for metastatic CRC patients are based on a multidisciplinary tumour board discussion. Metastatic CRC patients may benefit from surgical, chemotherapeutic, or palliative approaches.*
Abbreviations: FOLFIRI – Folinic Acid + 5-Fluorouracil + Irinotecan; FOLFOX – Folinic Acid + 5-Fluorouracil + Oxaliplatin; FOLFOXIRI – Folinic Acid + 5-Fluorouracil + Oxaliplatin + Irinotecan
Table 2 is based on the S3 guideline (2017).[33]

1.4.2. Adjuvant Therapy for Colon Cancer and Perioperative Therapy for Rectal Cancer

Commonly, adjuvant therapy is administered to patients with successfully resected (R0) and UICC stage II or higher stage of disease without present contraindications like poor general health conditions, liver cirrhosis, renal insufficiency, or severe infections. The potential toxicity of tumour therapy must always be considered with the associated benefits.[46] Colon cancer is usually sensitive to chemotherapy but does not respond to radiation, which culminated in the development of different chemotherapy regimens. Adjuvant oxaliplatin-based chemotherapy is indicated for patients with stage III colon carcinoma, e.g., by using the so-called FOLFOX scheme where oxaliplatin is combined with folinic acid and fluorouracil or, if contraindicated, by employing a monotherapy with fluoropyrimidines. In high-risk situations (like tumour perforation), stage II patients may benefit from adjuvant fluoropyrimidines-based therapy (**Table 2**).[33] It is important to emphasize that there is a relevant therapeutic uncertainty in UICC II patients. As of now, clinical markers are largely considered in the decision-making process including T4, tumour perforation, emergency surgery and an insufficient number of examined lymph nodes.[33] However, recent studies have indicated that the utilization of clinical criteria for patient inclusion can result in the over-treatment of a significant proportion (13%) of patients, without any corresponding increase in survival rates.[47] For rectal cancer patients, no pre- or postoperative therapy is indicated in stage I and radiochemotherapy and chemotherapy may be administered in the neoadjuvant and adjuvant settings of stages II to III (**Table 2**).[33]

1.4.3. Therapeutic Options in the Metastatic Situation

At the time of diagnosis, approximately 20% of the patients have metastases, and 50% will develop a metastatic disease as it progresses. Concerning UICC I-III patients, approximately 20% will develop metastases despite successful surgical resection.[48–50] Usually, metastases occur regionally in the lymph nodes and then spread to distant organs, including the liver and lungs, via the lymphogenic and haematogenous pathways.[51] Patients with rectal cancer experience relatively late recurrence of disease (RD) in the lungs, whereas colon cancer patients develop relatively early RD in the liver.[52] Despite the possibility of offering adjuvant therapy, most curatively resected patients develop RD within two years, and by that, an advanced cancer stage is associated with a poorer outcome. RD risk factors include certain pathological criteria, such as affected lymph nodes and vascular and perineural invasion. The formation of metastases is facilitated by multiple factors, including genetic abnormalities, epithelial-mesenchymal transition (EMT), and blood flow physiology. Notably, a relevant portion of mCRC patients can undergo curative surgical or systemic treatment despite its systemic spread, in contrast to often more aggressive cancer entities like pancreatic cancer. In this context, curative therapy options might encompass surgery of metastases (liver and other localizations), chemotherapy or targeted therapy (anti-epidermal growth factor receptor (anti-EGFR), anti-vascular endothelial growth factor (anti-VEGF)) tailored to the patient's overall condition and molecular profile.[15,33] The patient’s overall condition determines whether active therapy or a best supportive care (BSC) approach is preferred. In fit patients, the primary objective is maximum tumour reduction. If metastases are primarily resectable, surgical resection may be attempted. When surgery is not feasible in fit patients, systemic therapy is the alternative. The choice of chemotherapy or targeted therapy is guided by the RAS mutation status, tumour localization, and BRAF mutation status. In contrast, for unfit patients, chemotherapy is only feasible at a reduced intensity due to their limited treatment tolerance.[33,53]

1.4.4. Liquid Biopsy for Postoperative Monitoring

Notwithstanding the existence of laboratory and clinicopathological markers, the monitoring of patients in the adjuvant period remains inadequately validated. The so-called liquid biopsy offers the promise of precise patient stratification. Liquid biopsy is a non-invasive procedure using body fluids such as blood or urine instead of tissue samples to detect tumour material (**Figure 4**). Different tumour-associated components can be analysed, such as circulating tumour cells (CTCs), cell-free tumour nucleic acids, extracellular vesicles (EVs), and tumour-educated platelets.[54,55] The informative potential of LB lies not only in enumerating these tumour-associated elements but also in their molecular characterization, e.g. by genomic and proteomic analyses.[56] In contrast to the conventional tissue biopsy, LB is a minimally invasive procedure associated with barely any adverse events, and it can be easily repeatable in shorter time intervals. These practical aspects make LB a particularly suitable tool for monitoring the tumour's development, gaining longitudinal information, and assessing the heterogeneity of cancer diseases, especially in the metastatic stage when multiple sites are affected and where representative tissue biopsies are difficult.[57] To sum up, the implementation of various body fluids to extract circulating tumour-associated elements has the potential to enhance our understanding of drug response and facilitate the early detection of cancer.[56]

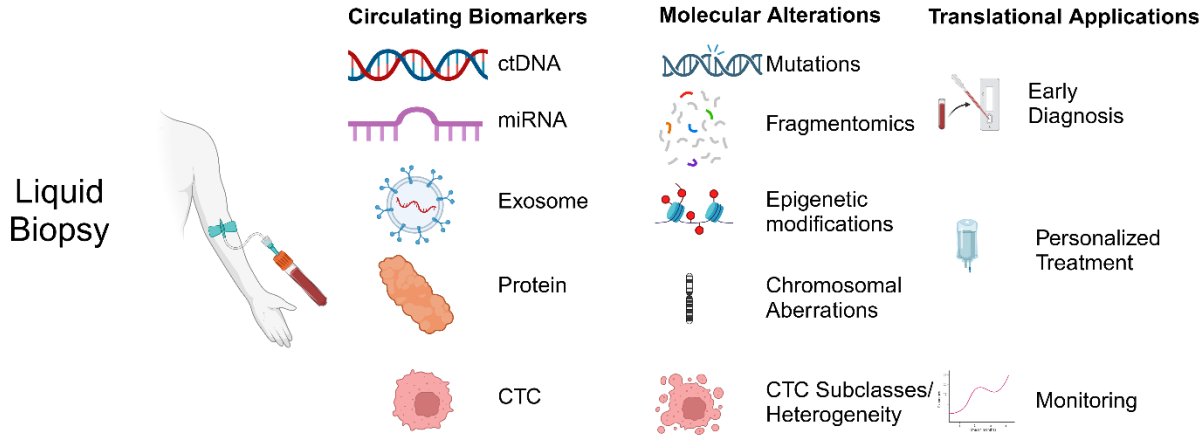

Figure 4: **Liquid Biopsy**
Tumour-associated biomaterials, including CTCs and ctDNA, are detectable in blood. Downstream analysis reveals mutations, modifications, and various subclasses. The obtained primary and downstream data advances personalized medicine as it enables patient monitoring and assessment of the patient's therapeutic response. Created with BioRender.com
Abbreviations: ctDNA – circulating tumour DNA; CTC – circulating tumour cell; miRNA – microRNA

1.5. Circulating Tumour Cells

In 1865, Thiersch explained that malignant cells might invade different vessels in their proximity and might have the ability to survive in human blood circulation.[58] Four years later, in 1869, Thomas Ashworth published the initial proof for the existence of CTCs: An autopsy revealed disseminated cancer cells in the blood circulation identical to the ones observed inside the tumour.[59] Today, CTCs are defined as cells from a malignant lesion that entered the systemic circulatory system by active invasion or passive detachment (**Figure 5**). Two models can explain cancer cell dissemination. The first assumes that the "fittest" tumour cell clones disseminate from the primary tumour and thus are highly similar to the cells of origin. The other model assumes that tumour cell dissemination occurs early in tumour evolution - possibly even in a pre-neoplastic state.[60] CTCs are commonly described as cells of epithelial origin, but epithelial-mesenchymal transition (EMT) can lead to loss of epithelial antigens.[56] Plus, depending on cancer type and stage, there are considerable differences in the morphology of CTCs. More generally speaking, CTCs can be characterized as large non-leukocytic nucleated cells that can appear as single cells or aggregates (e.g., attached to fibroblasts) in blood circulation. Aggregates could have a survival advantage over single cells as they might be

more protected against oxidative stress and immunological actions. The clinical potential of CTCs as biomarkers is envisioned to lie in (1) detecting cancer activity at the time of blood draw (real-time monitoring), (2) estimating the therapeutic response to anticancer agents, (3) early diagnosing cancer and predicting the development of non-malignant into malignant diseases.[56,61]

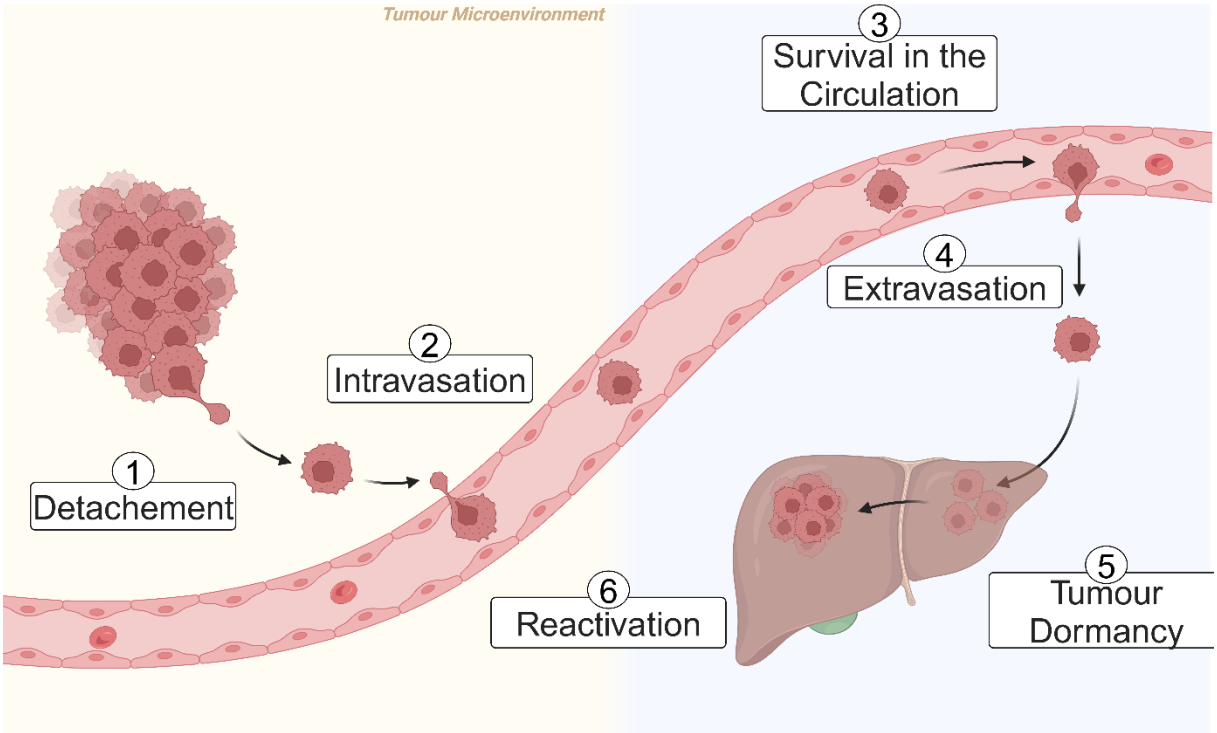

Figure 5: **Circulating Tumour Cells and Formation of Metastases**
Metastasis formation begins with the detachment of tumour cells from the primary tumour (1), which intravasate into the blood system by stimulating angiogenesis and active processes (2). Then, the invaded CTCs circulate in the blood system, where only a fraction of CTCs survive (3). Finally, after extravasation from the blood system and reaching the target organ (4), the CTCs can enter a dormant state where the regular cell cycle is exited (5). In this state, CTCs can be reactivated and contribute to the growth of metastases and the further cancer spread (6). The data was obtained from the study by Shin et al. (2023).[62] *Created with BioRender.com*

1.5.1. Enrichment Technologies

Unfortunately, the scarcity of CTCs in the bloodstream has remained an unresolved challenge for decades despite the growing number of sensitive and molecular CTC methodologies. It is estimated that there are only five to 250,000 CTCs in the total blood volume, making them extremely rare and difficult to detect because of the much larger cellular background of approximately one million leukocytes per CTC.[63–65] Technologies, based on either biological (antigen/protein expression) or physical/morphological properties (size, deformability, density), have been developed for the enrichment of CTCs.[66] The CellSearch system (CS) and technologies like Isoflux™ and the MagSweper utilize the epithelial cell adhesion molecule (EpCAM) as an enrichment marker. However, a significant number of CTCs that do not express EpCAM are missed due to the downregulation of surface markers on CTCs in the context of epithelial-mesenchymal transition (EMT).[67] Downregulation of surface markers results in a higher false negative rate of EpCAM-dependent technologies.[56] Therefore, EpCAM-independent technologies, like the epithelial immunospot (EPISPOT), were developed utilizing membrane-bound antibodies to detect the protein secretome of viable CTCs.[56] Technologies based on microfluidic- and nanotechnology have the potential to enhance sensitivity, specificity, yield and purity of CTC detection. The CTC-Chip considers a combination of EpCAM expression and morphological criteria by utilizing thousands of small antibody-labelled

microspots. By that, CTCs are targeted by their tumour-specific antigens, which are later imaged and analysed.[56,68]

Aside from biological targeting, physical CTC enrichment technologies incorporate size, elasticity, density, deformability, and electricity measures of blood cells.[69] The Parsortix Cell Separation System is an example of a physical liquid biopsy platform that uses size and deformability to enrich a high number of viable CTCs. The high quality of the enriched CTCs allows downstream analyses to be carried out afterwards.[70] Generally, CTCs are often considered to have a larger diameter than white blood cells (WBCs) and this can be used as a physical differentiation criterion. However, there are also CTCs that are smaller in size than WBCs, and there is evidence that smaller CTCs are of particular biological aggressiveness in certain entities. The SSA-MOA technology tries to solve this dilemma by combining selective size amplification (SSA) for target cells and a multi-obstacle architecture (MOA). This is made possible by polymer microbeads for selective size amplification of tumour cells and a precise multi-obstacle architecture filter that reduces cell deformation and ensures clear separation of WBCs.[71] Another unique technology is the DEPArray technology, which combines microelectronics and microfluidics and allows the isolation of pure single cells and their subsequent molecular analysis by next-generation sequencing (NGS).[72] Generally, physical technologies can be advantageous due to minimal costs and a good vitality of cells, but the overall low efficiency, specificity, and purity call for further improvements [56].

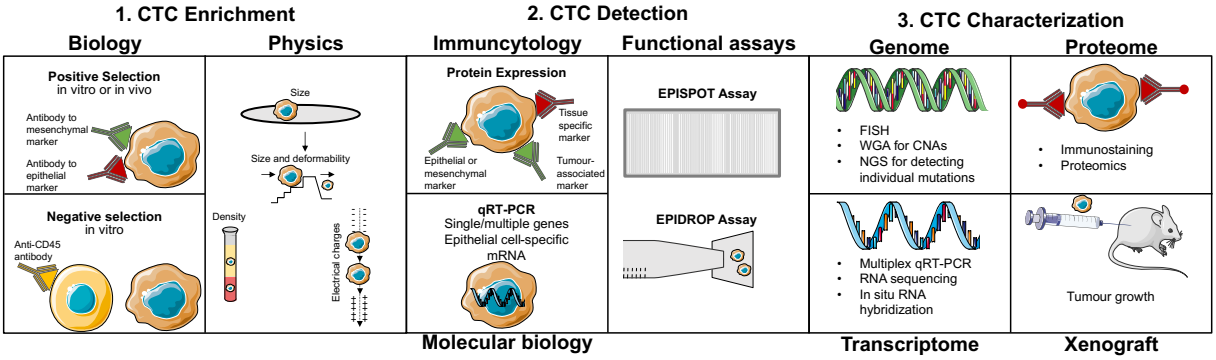

Figure 6: **CTC Detection Methods**
The image was modified after Alix-Panabières (2021).[55] Figures were partly generated using Servier Medical Art (https://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 unported license.
Abbreviations: CNA – copy number alteration; FISH – fluorescence in situ hybridization; mRNA – messenger ribonucleic acid; NGS – next-generation sequencing; RNA – ribonucleic Acid; WGA – whole genome amplification; qRT-PCR - quantitative reverse transcription polymerase chain reaction

1.5.2. CellSearch

Despite the variety of advantageous enrichment technologies, the CS System is the only FDA-approved technology to date, offering a highly standardized and reliable enumeration of epithelial CTCs.[73] Detected CTCs with the CellSearch system are associated with reduced survival and were validated in metastatic breast cancer (mBC), prostate cancer (PC), and CRC.[74–77] The preprocessing procedure includes the transfer of 7.5 mL blood to a 14 mL CS tube, two centrifugation steps for plasma separation, and the addition of dilution buffer. The CS system comprises the Autoprep and the CellTracks Analyser II components (**Figure 7**). Within the Autoprep, ferrofluids coupled to monoclonal antibodies target EpCAM to capture cells of epithelial origin. Then, three marker-conjugated monoclonal antibodies target specific cell structures: The antibodies conjugated with Phycoerthrin (PE) target cytokeratins 8, 18, and 19. 4´,6-diamidino-2-phenylindole, dihydrochloride (DAPI) dyes the nuclei of the enriched cells. Conversely, the antibodies conjugated with APC target the cluster of differentiation 45 (CD45) present on leukocytes and are used to differentiate between CTCs and non-CTCs.[78] So, within CS CTCs are defined as $EpCAM^+$, PE^+, $DAPI^+$, and APC^- cells, but two more markers can be added for the identification of therapeutic targets or CTC subgroups.[79] The Autoprep system

comprises nine stations, each of which performs a specific task within a predetermined time interval (**Figure 7**).[80] In the next step, the enriched and stained material is transferred to a cartridge that is scanned by the CellTracks Analyser II, a four-colour, semiautomatic fluorescence microscope for image evaluation. Finally, a selection of PE^+, $DAPI^+$, and APC^- events is presented to trained operators, who decide whether all CTC criteria are fulfilled (**Figure 7**).[80] Later, the enumerated CTCs can be isolated by downstream analyses such as the MoFlo XDP flow cytometry sorter. Once isolated, CTC can be extracted and amplified through methods such as whole genome amplification (WGA). Thereafter, comprehensive genomic analyses may be performed to detect copy number alterations (CNA) and mutations.[81,82]

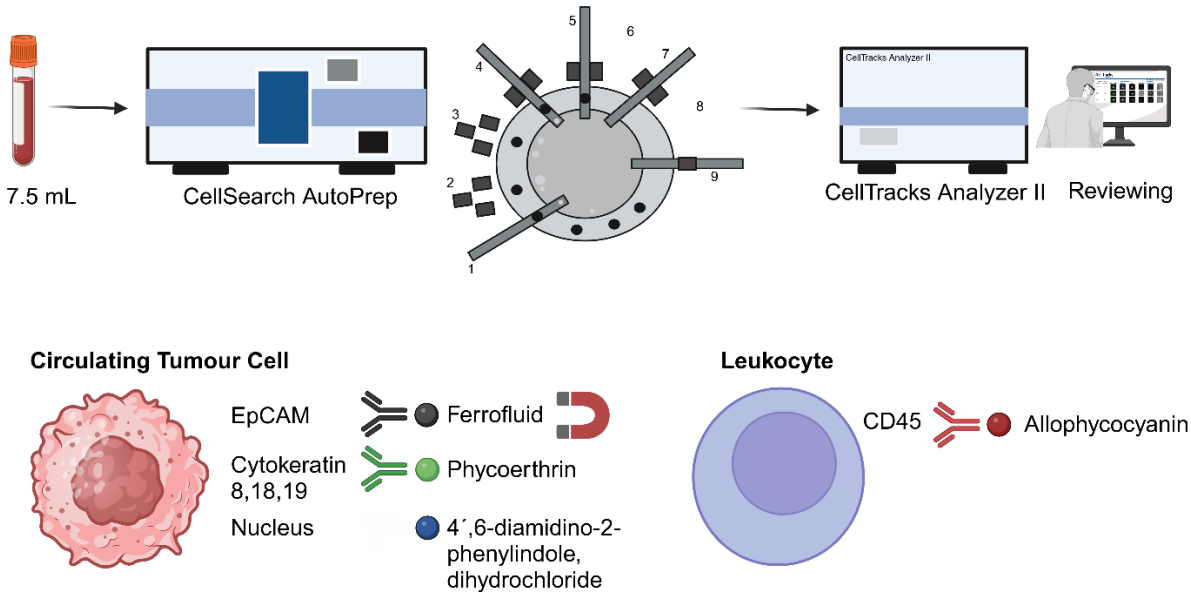

Figure 7: **CellSearch Method**
The image of the nine stations integrated in the AutoPrep system is a modified image from a publication by Coumans et al. (2015).[80]
Abbreviations: CD45 – cluster of differentiation 45; EpCAM – epithelial cell adhesion molecule
Created with BioRender.com

1.5.3. Automated CTC Detection

The fluorescence signals generated by CellSearch can be quantified and analysed using modern software, facilitating efficient evaluation and interpretation of blood samples.[83] CTCs exhibit significant morphological variability, and due to the growing number of emerging CTC technologies, it has become increasingly complex to define a CTC uniformly.[66] Even if considering exclusively CellSearch images of CTCs, there is still a large intra- and inter-reviewer variance. Approximately only 40% of all objects might be uniformly classifiable into a CTC or a non-CTC category by all reviewers.[84] Fluid transitions in morphology and staining between non-CTCs, such as leukocytes, and CTCs make unambiguous identification challenging. Because more generous morphologic inclusion criteria result in higher sensitivity but lower specificity and vice versa, the manual enumeration of CTCs faces a dilemma. To solve this dilemma, developments were made to automatize CTC identification and avoid selection bias. The development of the ACCEPT software (Automated CTC Classification, Enumeration, and PhenoTyping) and deep learning (DL) programs have paved the way for time- and cost-saving workflows, eliminating reviewer variability and achieving a high agreement compared to expert scoring.[84–86] The ACCEPT software is an open-source image analysis package (https://github.com/LeonieZ/ACCEPT) for automated CTC classification, enumeration, and phenotyping. It was developed within the EU Cancer-ID project. This software can process images from several CTC isolation platforms and utilizes a Bregman active contour technique to identify the contour of each event.[87] All events can be displayed and viewed in the ACCEPT sample visualizer (**Figure 8**).

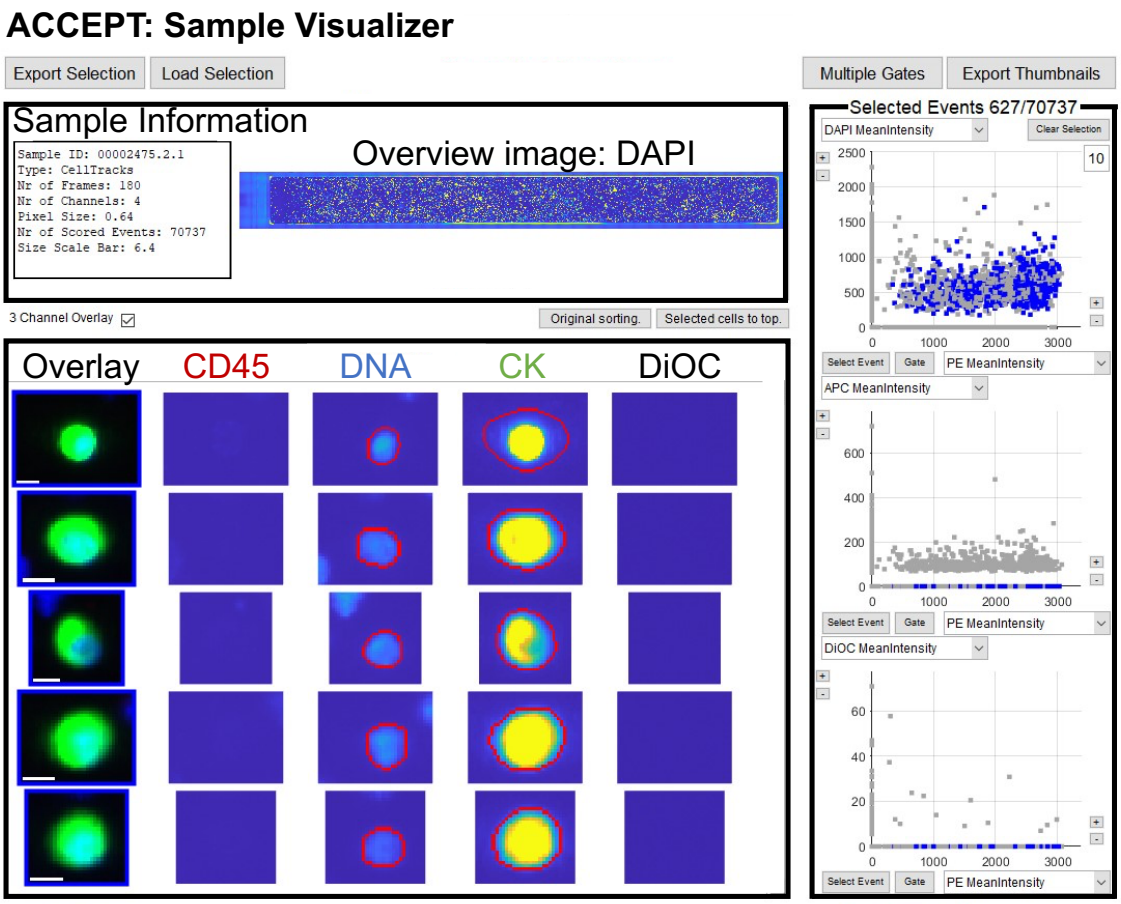

Figure 8: **ACCEPT Sample Visualizer**
Abbreviations: CD45 – cluster of differentiation 45; CK – cytokeratin; DAPI - 4´,6-Diamidino-2-phenylindole dihydrochloride; DiOC – 3,3'-Dihexyloxacarbocyanine Iodide

Four processors are implemented in ACCEPT to detect all objects within a cartridge (full detection) or to analyse the expression of extra markers (marker characterization). The full detection tool detects all events (**Figure 9**), including CTCs, tdEVs, leukocytes, and leukocyte-derived extracellular vesicles (ldEVs). The terms ldEVs and tdEVs were introduced by Nanou et al. to define $CD45^+$, $DAPI^-$, CK^- objects and $CD45^-$, $DAPI^-$, CK^+ objects.[88,89] It additionally offers an extensive numeric output of all objects reflecting several phenotypic attributes, including size (in µm²), eccentricity (value 0 corresponds to a perfect circle, 1 to a line; 0-1), perimeter (in pixels), mean, max and median intensity, standard deviation of the intensity, mass (sum of the intensity), perimeter to area ratio (P2A, 1 for a perfect circle), and relative overlay of each channel signal with the DAPI (DNA) signal. In the standard CTC assay fluorescence measurements are calculated for APC (CD45), DAPI (DNA) and PE (cytokeratin) channels, and optionally for one or two more markers that can be added to the assay. Using the full detection processor with a mask, the segmentation of an individually chosen channel can be transferred to another channel, which helps analyse low signal intensities and complex segmentations.[83]

ACCEPT users define linear gates by setting numeric cutoffs for these five channels which allow an automated selection or exclusion of objects. The platform's functionality and the positive correlation between ACCEPT-detected CTCs and patient outcomes have been demonstrated in several cancer entities (**Table 3**).[88,89]

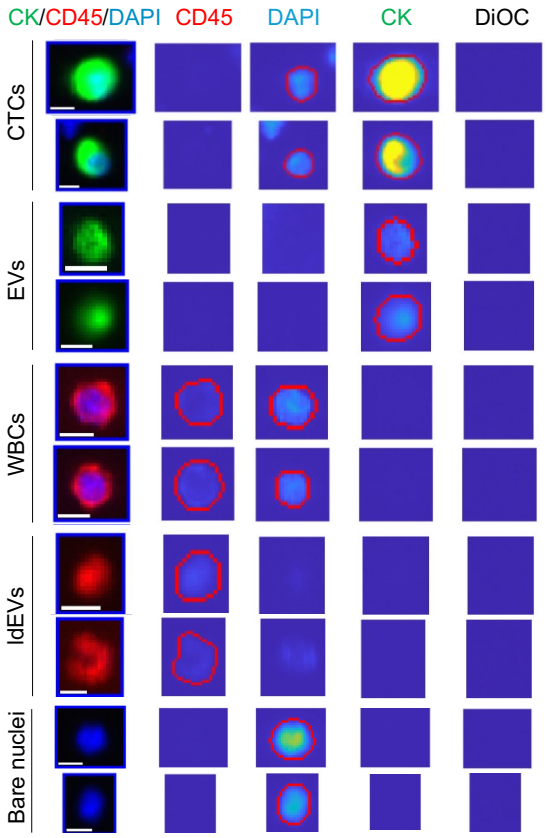

Figure 9: **ACCEPT Detection of Multiple Objects**
Abbreviations: CD45 – cluster of differentiation 45; CK – cytokeratin; DAPI - 4´,6-Diamidino-2-phenylindole dihydrochloride; DiOC – 3,3'-Dihexyloxacarbocyanine Iodide

Given that CTCs and tdEVs can express therapeutically targetable surface markers (e.g. human epidermal growth factor receptor 2 (HER2) or Prostate-Specific Membrane Antigen (PSMA)), the marker characterization processor helps in quantifying these signals of the pre-selected CTCs.[90] In a mBC study, ACCEPT enhanced the intra-reviewer concordance of HER2 evaluation and introduced a greater degree of standardization (**Table 3**).[91] The pre-selection

of CTCs is either directly performed via the CellTracks Analyser II system or a plugin. Marker expression can be analysed numerically by considering the numeric output or graphically by considering the respective CTC images and scatter plots within the sample visualizer.[83]

Table 3: **Studies on Automated CTC Detection**

Author	**Method & particles**	**Patient Cohort**
Nanou (2024, *PNAS nexus*)[85]	OpRev, ACCEPT, CM, DL CTC & tdEV	418 Ben, 6,293 nmBC, 2,408 mBC, 698 mPC
Nanou (2023, *JCO Precis Oncol*)[92]	ACCEPT CTC & tdEV	547 baseline & 284 follow-up mBC
Stevens (2022, *Cancers*)[87]	DL StarDist CTC & tdEV	533 & 601 various entities
Isebia (2022, *Cancers*)[93]	ACCEPT CTC & tdEV	104 CNPC & 66 CRPC
Oeyen (2021, *Prostate Cancer Prostatic Dis*)[94]	ACCEPT CTC & tdEV	170 CRPC
Zeune (2020, *Nat Mach Intell*)[95]	DL CTC, tdEV, WBC, bare nucleus	499 various metastatic entities & cell lines
Nanou (2020, *Br J Cancer*)	ACCEPT CTC & tdEV	190 CRPC, 450 mCRC, 179 mBC & 137 mNSCLC
Nanou (2020, *Breast Cancer Res*)[96]	ACCEPT CTC & tdEV	98 mBC
Nanou (2020, *Cells*)[97]	ACCEPT tdEVs, edEV, ldEV, leukocyte, nucleated cell	395 mCRC
Nanou(2019, *Cells*)[88]	ACCEPT ldEV & leukocyte	25 Healthy & 25 CRPC, 25 mCRC, 25 mNSCLC
De Wit (2019, *Int J Cancer*)	OpRev & ACCEPT CTC & tdEV	97 NSCLC
De Wit (2018, *Cancers*)[98]	ACCEPT DAPI+ nucleus	192 mNSCLC & 162 controls
Zeune (2018, *Cytometry A*)[84]	OpRev, ACCEPT, DL, EP CTC	100 (images) mPC & mNSCLC
Nanou (2018, *Oncotarget*)[99]	ACCEPT CTC & tdEV	129 CRPC
Zeune (2017, *PLOS ONE*)[91]	ACCEPT CTC	132 mBC
Zeune (2017, *SIAM J Imaging Sci*)[100]	ACCEPT Background/Development	Theoretical no samples
Scholtens (2011, *Cytometry A*)[101]	EP & Automated classifier Matlab Various CTCs & not related debris	31 primary BC/CRC, 37 metastatic entities, 9 healthy
Ligthart (2011, *PLOS ONE*)[101]	Matlab Algorithm CTC	185 CRPC & 68 healthy & 100 CRPC

Abbreviations methods: CM – contrast maximization; cancer; CTC – circulating tumour cell; DL – deep learning; DLA – diagnostic leukapheresis; edEV – endothelium-derived extracellular vesicle; EP – Expert panel; ldEVs – leukocyte-derived extracellular vesicles; OpRev – operator review; Pros – prospective; Retros – retrospective; tdEV – tumour-derived extracellular vesicle; WB – whole blood; WBC – white blood cell

Abbreviations patient cohort: Ben – benign; CNPC – metastatic castration-naive prostate; CRPC – metastatic castration-resistant prostate cancer; mBC – metastatic breast cancer; mCRC – metastatic colorectal cancer; mNSCLC – metastatic non-small cell lung cancer; mPC – metastatic prostate cancer; nmBC – non-metastatic breast cancer; NSCLC – non-small cell lung cancer

Despite offering a fast and reviewer-independent way of identifying CTCs, ACCEPT is still prone to errors due to insufficient segmentation and the dependence on technical differences, including camera properties and fluorescence filters. Therefore, the reliability or authenticity of the generated CTC count remains questionable. Deep learning (DL) platforms might hold the answer as they provide a more accurate segmentation and a stronger correlation to the patient outcome.[85]

1.5.4. Clinical Relevance of CellSearch-CTCs in CRC

In mCRC patients, a study from 2008, including 430 CellSearch samples, reported a detection rate of only 48% for ≥1 CTC.[85] Despite the lower yield of CTCs in CRC, CellSearch was validated and approved by the FDA for mCRC patients as CellSearch-CTCs - with the cutoff ≥3 CTCs - were strong and independent predictors for OS and PFS at baseline and at four time points after first-, second- or third-line therapy. Moreover, the conversion from an unfavourable to a favourable CTC count indicated an improved prognosis compared to consistently high CTC levels.[76] The CTC detection rates of mCRC patients are in most studies ≤48%[76,102–107] (**Table 4**) which is contrasted by considerably higher detection rates documented in metastatic castration-resistant prostate cancer (mCRPC) and mBC patients with ≥5 CTCs in 49% of the samples at the baseline.[74,77]

Table 4*:* **CellSearch Studies on PV-Sampling in CRC M1 Patients**

Author	Collective	Time Point	CTC Detection rate	Significance of OS/PFS
Arrazubi (2019, *Ann Surg Oncol*)[102]	44 mCRC	Pre- & postop	≥2 18.6% preop 29.5% postop	Yes OS & PFS (≥2)
Bidard (2019, *Cells*)[103]	132 mCRC*	Before LM surgery At inclusion 4 weeks	7% (≥1), 0% (≥3) preop; 42% (≥1), 19% (≥3) at incl.; 11% (≥1), 3% (≥3) 4w	Yes OS (≥3); 4w (≥1)
Dizdar (2019, *Mol Oncol*)[104]	32 mCRC	Preop	34.4% ≥3	Yes OS (≥3)
Nicolazzo(2019, *Cancers*)[105]	84 mCRC	Before any treatment*	≥1 46% RCC 39% LCC 38% RC	Yes PFS LCC; No RCC & RC
Bork (2015, *Br J Cancer*)[106]	48 mCRC*	Preop (1x) Postop day 3 & 7	≥1 18.8% preop. 17.6% 3d 21.4% 6d	NA for only mCRC
Seeberg (2015, *Ann Surg*)[107]	189 mCRC	At surgery	19.6% ≥1 13.8% ≥2	Yes OS & PFS (≥2)
Cohen (2008, *J Clin Oncol*)[76]	430 mCRC	Before therapy initiation	48% ≥1 33% ≥2 26% ≥3	Yes OS & PFS (≥3)

***Bidard** patients were treated with first-line triplet or doublet chemotherapy combined with targeted therapy; **Nicolazzo** sampled blood from patients before any first-line systemic treatment;*
***Bork** used peripheral and central venous blood.*
Abbreviations: At inclusion – at incl.; D – day; LCC – left-sided colorectal cancer; LM – liver metastases; NA – not available; NS – not significant; OS – overall survival; PFS – progression-free survival; Postop – postoperatively; Preop – preoperatively; RC – Rectal cancer; RCC – right-sided colorectal cancer; S – significant; W – week

In non-metastatic CRC patients, the scarcity of CTCs is an even more pronounced issue, with detection rates ranging from 2% to 35% (**Table 5**). 5-25%, 11-33%, and 8-39% of CRC patients tested positive for CTCs in stages I, II and III respectively.[106,108–113] Despite the low positivity rates in non-metastatic patients, CTCs are still considered strong predictors as they are associated with shorter OS and DFS. Owing to the challenging enrichment in locoregional CRC, the established cutoff of ≥3 CTCs in mCRC had to be lowered to mostly ≥1 CTC and often remained an independent predictor for OS and DFS. Yet, applying ≥1 CTC as a cutoff has to be viewed critically because of the subjectivity and technical hurdles associated with the enumeration process.[104,106,108,110,114] Utilizing CTCs from preoperative blood and also postoperative blood (2-3 years post-surgery) provided relevant prognostic information.[106,108,110] However, one study including 472 stage III patients 4-8 weeks after surgery showed no significant prognostic effect of CTCs (**Table 5**).[109] In regards to clinicopathological data, associations were found between elevated (preoperatively or postoperatively determined) CTC counts and positive lymph node status, advanced disease stage, tumour stage and neoadjuvant chemoradiotherapy.[106,108,110,112] Taken together, the available data present an inconsistent picture for non-metastatic CRC patients but simultaneously indicate promising associations with patient outcomes and clinicopathological parameters.

Table 5: **CellSearch Studies on PV-Sampling in CRC M0 or M0/M1 Patients**

Author	Collective	Time Point	CTC Detection rate	Significance of OS/PFS
Abdalla (2021, *PLOS ONE*)[108]	68 I-IV 34 I-III	Preop. for all; Postop. within 96h	29.4% preop. I-III; 19.1% postop. I-IV	Yes OS & PFS
Dizdar (2019, *Mol Oncol*)[104]	80 I-IV	Preop.	31.30% I-IV	Yes OS (≥1)
Sotelo (2015, *Ann Oncol*)[109]	472 III	Postop. 4-8 weeks	35%	No OS & PFS
Van Dalum (2015, *Int J Oncol*)[110]	183 I-III	Preop. Postop. 6 time points	24% preop.; 20% directly postop.	Yes OS & PFS* preop.
Bork (2015, *Br J Cancer*)[110]	287 I-IV 239 I-III	Preop. (1x); Postop. 3 & 7d	I-III (≥1): 7.9% preop; 10% postop 3d; 14.3% postop 7d	Yes OS & PFS (≥1) preop. I-III
Gazzaniga (2013, *Tumour Biol*)[112]	37 II-III (high risk)	Postop.*	22%	NA
Thorsteinsson (2011, *Anticancer res*)[111]	20 I-III	Preop. (3x: 1d, DOS); Postop. 30d	1.7%; preop.; 5.3% postop.	NA

Sotelo* postoperative blood was drawn just before starting adjuvant treatment; **Van Dalum the CTC count consists of the total number found in the four aliquots. The time points included before surgery, after surgery, after adjuvant therapy, one year after, two years after, three years after, and four years after. Significantly associated with DFS: CTCs before surgery, after adjuvant treatment, after two years, and after four years. Significantly associated with OS: CTCs before surgery, after adjuvant treatment, after two years, after three years, and after four years; **Bork** used peripheral and central venous blood; **Gazzaniga** has not specified the blood collection place. Peripheral blood is assumed because it is the standard blood sample. CTCs were counted after primary tumour resection and before the start of adjuvant therapy; **Thorsteinsson** two blood samples were taken the day before surgery. One blood sample was taken on the day of surgery and the fourth sample was taken 30 days after surgery.*
Abbreviations: D – day; DOS – day of surgery; NA – not available; OS – overall survival; PFS – progression free survival; Postop – postoperatively; Preop – preoperatively; W – week

CTC enumeration is just one of many possible clinical applications and can be complemented by analysis of its genetics, proteomics, marker expression and phenotypic diversity. Analysis of these properties provides additional insights into the molecular mechanisms driving cancer

progression, response to treatment, and disease relapse.[91,115–118] Heitzer et al. conducted a study that examined the mutation spectrum of the primary tumour, metastases and CTCs in mCRC patients. Their findings revealed the presence of relevant mutations in the driver genes within the CTCs. Intriguingly, the study also identified mutations that were exclusively present in the CTCs. Notably, some of these mutations were also observed at a subclonal level in the tissue of the primary tumour or metastases. This finding underscores the potential for identifying novel treatment targets, such as CDK8, that might otherwise have remained unconsidered. Consequently, these findings offer patients new treatment options through inclusion in clinical trials.[115] Moreover, the CellSearch system allows the inclusion of additional antibodies to detect treatment targets, such as PSMA in the prostate, HER2 in the breast or EGFR in CRC patients. Quantifying targetable markers and determining the CTC's phenotype can help in estimating the response to treatment and patient outcome.[91]

1.6. Potential Strategies to Improve Sensitivity of CellSearch Detection

1.6.1. Extracellular Vesicles

Extracellular vesicles (EVs) started gaining attention in cancer research around 2010, and they are thus relatively new biomarkers compared to CTCs.[119] According to the International Society for Extracellular Vesicles (ISEV), EV *is* a term for *"particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus"*.[120] EVs are present in multiple body fluids (blood, urine, cerebrospinal fluid) and are classifiable into different subclasses based on biological function and biogenesis. They originate from different cells like monocytes, endothelial cells, cardiomyocytes, or CTCs. There are different classes of EVs based on their cellular origin, cargo and biological significance: Exosomes originate from the endolysosomal pathway, and microvesicles and apoptotic bodies arise from the cell surface.[121,122] Similarly, the EV's cargo varies considerably as exosomes and microvesicles carry distinct proteins, lipids, and RNAs.[123] EVs are mediators of cell-to-cell interaction and their effect can be exerted directly by fusing with the recipient cell's membrane and releasing its cargo in the cytosol, or indirectly by activating receptors of the recipient cell. The importance of these particles stems from both their role in normal physiology and pathology of various diseases. Relevant for cancer progression, EVs can trigger the formation of a pre-metastatic tumour niche, immunosuppression, angiogenesis, metastasis, and EMT. Higher EV concentrations can be found in cancer patients compared to healthy individuals, and the EV concentration is correlated to disease progression.[56,123] Both the enumeration and characterization of EVs and their cargo, have the potential to be utilized in clinical applications as key player influencing tumour development and microenvironment.[123]

1.6.2. EV Enrichment by the CellSearch System

Due to their higher abundance and tumour specificity, tdEVs represent a promising complementary biomarker to CTCs. The CS system co-enriches large $EpCAM^+/CK^+/DAPI^-/CD45^-$ particles which are tdEVs.[89] tdEVs can be detected and enumerated using ACCEPT and more recently developed DL platforms. The Terstappen group showed that tdEVs are substantially more common in cancer patients than in healthy volunteers, and that the number of tdEVs is significantly associated with survival.[89,92,99] Detection of tdEVs with the CellSearch system is complicated due to the substantial cellular background and due to the lack of tdEV-specific targetable markers. Additionally, during the CellSearch procedure, the plasma is discarded, and only the large tdEVs, with a size range between 1 to 14 µm, constituting less than 1% of all tdEVs, are considered.[89,124,125] As for CTCs, the detection of EVs with the CellSearch system is further complicated by the absence or downregulation of EpCAM and insufficient expression of intracellular CK proteins. Although the molecular composition of the particles, and thus their exact cellular origin, remains to be deciphered, the particles can be phenotypically characterised by analysing the fluorescence signals of the markers and thus

conclusions can be drawn about the biological aggressiveness, allowing patients to be stratified into risk groups.[93,94,96]

The strong correlation between tdEV and CTC counts indicates an analogous biological function in cancer.[89] tdEV average and median counts in the blood are about 10-fold higher than CTCs, and tdEV quantities are in >99% of patient cases higher than CTCs.[89] Nevertheless, tdEVs as a biomarker lack cancer-specificity, because they can be detected in healthy participants as well.[89] Thus, to decrease the number of false-positive test results and increase the specificity in patient risk stratification a (physiological) reference range needs to be defined. Recently, Nanou et al. (2020) defined a reference range between 0 and 20 tdEVs, based on the observed tdEV distribution in a healthy cohort. The tdEV cutoff above the defined normal range was associated with shorter OS (HR ≈ 2) and higher HR in multiple metastatic cancer entities. In mCRC, tdEV counts enhanced risk stratification of patients compared to CTCs. It was found that a considerable proportion of mCRC patients (≈ 25%) exhibited a favourable CTC count (<3 CTCs) and could be further stratified, based on their tdEV counts leading to the identification of a subgroup with a worse prognosis (HR ≈ 2).[89]

1.6.3. Diversity of CTCs and tdEVs

Cancer is regarded as a dynamic process that often leads to increasing heterogeneity during disease progression and can manifest both within the same tumour lesion (over time: temporal heterogeneity) as well as between different tumour lesions (spatial heterogeneity).[126] This biological phenomenon significantly influences therapy resistance and helps in estimating the response to treatment (**Figure 10**).[127] This suggests that there is heterogeneity between patients with the same cancer entity. In addition, non-cancer related differences in lifestyle, environmental exposures, metabolism, genetics and other characteristics lead to differences in cancer development and response (**Figure 10**).[128] Similarly, CTCs constitute heterogeneous particles and promote therapy resistance by changing their molecular properties. Great genetic discrepancies between CTCs – of different individuals, within the same primary tumour and between different metastatic sites – were revealed by sequencing technologies.[116]

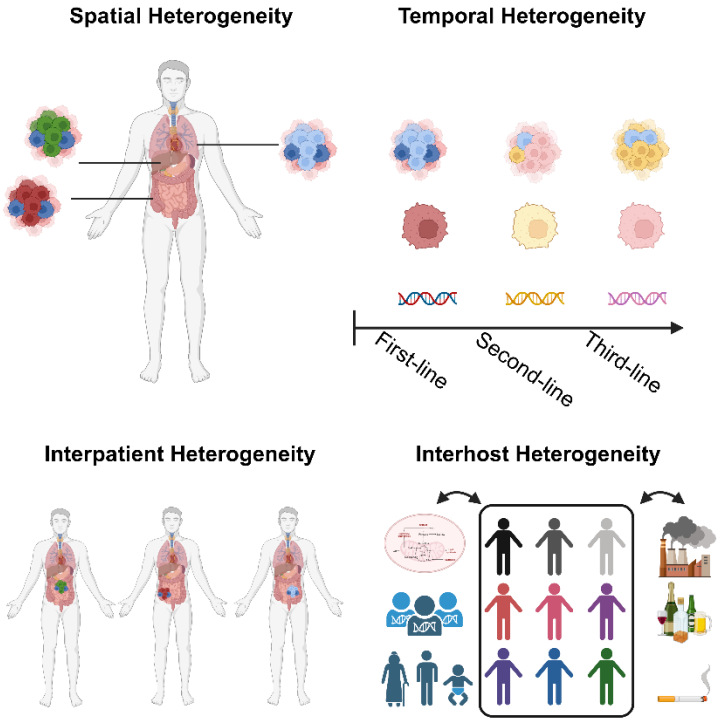

Figure 10: **Cancer Heterogeneity**
This figure was modified after Dagogo-Jack et al. (2018) and MacDonald et al. (2025).[127,128] Cancer Heterogeneity can be divided into spatial, temporal, interpatient and interhost heterogeneity, as explained in the main text. Created with BioRender.com

In this context, amplified heterogeneity and particular subclasses were often linked to adverse prognosis and aggressive tumour biology in prostate and breast cancer studies.[93,94,96,129] One way to measure diversity is through indices like the Shannon index, a measure of the entropy, first introduced by Claude Shannon.[130] The Shannon index quantifies the diversity of a system by considering the categories and the uniformity of their distribution. A higher uncertainty (entropy) to predict the upcoming groups means a higher heterogeneity. The proportion of the respective group in the total collective is multiplied by its logarithm. As a result, smaller proportions, which influence the overall distribution only marginally, have a larger effect on the diversity index compared to larger proportions. The products of all groups are finally added together meaning that a Shannon Index close to 0 represents a low diversity and a high index represents a high diversity. The Shannon index reaches its maximum when all categories are equally represented, combining high richness with perfect evenness (**Table 6**). In contrast, dominance of one or few categories results in a lower index, indicating reduced diversity (**Table 6**).[130] The Shannon index was originally developed in the context of information theory and was later adapted in the ecological research to quantify species diversity (**Equation 1**).

$$H' = -\sum_{i=1}^{S} p_i \cdot \ln(p_i) \quad (1)$$

- *H'*: Shannon diversity index, a measure of species diversity in a community
- *S*: Total number of species (species richness)
- *pi*: Proportion of individuals belonging to species
- *ln*: Natural logarithm

Table 6: **Calculation of the Shannon Index**

Diversity	Group	Proportion (p)	ln(p)	-p * ln(p)
High diversity	A	0.25	-1.3863	0.3466
	B	0.25	-1.3863	0.3466
	C	0.25	-1.3863	0.3466
	D	0.25	-1.3863	0.3466
	Total			1.3863
Low Diversity	A	0.85	-0.1625	0.1381
	B	0.05	-2.9957	0.1498
	C	0.05	-2.9957	0.1498
	D	0.05	-2.9957	0.1498
	Total			0.5875

Howard Scher et al. was the first to use the Shannon index to explore the diversity of CTCs (**Table 7**).[116] In this study, CTCs were classified into 15 phenotypic subtypes based on single-cell features to calculate the Shannon index. Higher Shannon diversity was associated with shorter survival when treated with androgen deprivation therapy and better survival when treated with taxane chemotherapy in prostate cancer patients. Oeyen et al. (2020) also investigated the Shannon index in 170 mCRPC patients starting a new line of androgen receptor signalling inhibitors (ARSi). Here the Shannon Index was based on five k-means generated clusters and the diversity for the respective sample was determined by the number of CTCs per CTC cluster per blood sample. They found that diversity was associated with adverse outcomes, typically reduced during therapy and increased in cases of progressive disease. Diversity enabled the early identification of patients at risk who were not identifiable by clinical or radiological signs alone. Moreover, specific phenotypic cell characteristics of 19,129 CTCs were correlated with survival. Patients who exhibited CTCs with low CK expression and smaller cell morphology had a shorter PFS. Large cells with oval nuclei or

oversized cells with high CK intensity were also associated with a poor prognosis in contrast to other cell classes.[94]

Table 7: **Overview of Current Studies on Diversity**

Author	Collective	Method	Particles	Diversity/Subclasses
Fehm (2024, *Clin Chem*)[131]	101 mBC	CS	CTCs	HER2 positive CTCs
Isebia (2022, *Cancers*)[93]	104 CNPC & 66 CRPC	CS & ACCEPT	CTCs & tdEVs	CTCs subdivided by OpRev into 6 subclasses
Oeyen (2021, *Prostate Cancer Prostatic Dis*)[94]	170 mCRPC	CS & ACCEPT	CTCs & tdEVs	SI based on k-means clusters
Nanou (2020, *Breast Cancer Res*)[96]	98 mBC	CS & ACCEPT	CTCs & tdEVs	Subclasses based on CK- & HER2 positivity
Nicolazzo (2019, *Cancers*)[105]	84 mCRC	CS	CTCs	Intact vs. apoptotic, epithelial-like vs. mesenchymal-like
De Wit (2018, *Oncotarget*)[129]	108 CRPC & 22 mBC	CS & MF of $EpCAM^{high}$ depleted blood	CTCs	$EpCAM^{high}$ vs. $EpCAM^{low}$
Scher (2017, *Cancer Res*)[116]	179 mCRPC	Epic Sciences platform	CTCs	SI of cell types
Deutsch (2016, *Breast Cancer Res Treat*)[132]	442 mBC	CS	CTCs	Intact vs. apoptotic
Kuboki (2013, *Anticancer Res*)[133]	63 mCRC*	CS	CTCs	EGFR expression
Coumans (2010, *Ann Oncol*)[134]	179 mCRPC	CS	EpCAM+ CK+	Intact CTC, granular CTC, L-TCF, L-TMP, S-TMP, CK+/CD45+

**Kuboki et al. included advanced CRC patients who were treated with cetuximab with or without Irinotecan as a third-line treatment.*
Abbreviations: CNPC – castration-naive prostate cancer; CRPC – castration-resistant prostate cancer; CS – CellSearch; CTC – circulating tumour cell; mBC – metastatic breast cancer; mCRC – metastatic colorectal cancer; L-TCF – large tumour cell fragments; L-TMP – large tumour microparticles; mCRPC – metastatic castration-resistant prostate cancer; MF – microfiltration; OpRev – operator review; SI – Shannon index; S-TMP – small tumour microparticles; tdEV – tumour-derived extracellular vesicle; WBC – white blood cell

Isebia demonstrated in her work that subclasses of CTC clusters and CTCs (**Table 7**, **Figure 11**) characterized by heterogenous CK expressions occurred more frequently in castration-resistant prostate cancer (CRPC) compared to castration-naive prostate cancer (CNPC) samples. In CRPC the higher abundance of these subclasses might point towards a particular aggressive tumour biology. Interestingly, the strong correlation between tdEVs and heterogeneously CK-expressing CTCs might be indicative of a tumorous or neoplastic origin from the primary tumour itself.[135] Apoptotic CTCs, on the other hand, influence patient survival as well. Impressively, Deutsch et al. could show with a large cohort of 442 mBC patients that CTC kinetics of apoptotic CTCs had a superior discriminatory power compared to intact CTCs.[132] In addition, not only the cellular origin but also the localization of the primary tumour influences the measurable phenotypic characteristics of CTCs. Nicolazzo et al. suggested that a higher number of apoptotic CTCs, but a lower number of mesenchymal CTCs originates from right-sided CRC compared to left-sided CRC. The differential distribution might be explained by the tumour microenvironment and the respective pattern of CTC dissemination.[105] To

emphasize the prognostic relevance of multiple subclasses rather than one, Coumans et al. demonstrated that subdividing EpCAM$^+$ and CK$^+$ particles into seven subclasses revealed a strong association between all EpCAM$^+$CK$^+$CD45$^-$ particles and OS. While the overall prognostic power of EpCAM$^+$CK$^+$ particles was evident, less stringent phenotypic criteria led to the inclusion of more events in healthy participants, thereby reducing specificity, as observed for large tumour microparticles (L-TMP) and small tumour microparticles (S-TMP). Moreover, similar to the findings of Oeyen et al., they demonstrated that large tumour cell fragments (L-TCF) exhibited a particularly strong association with short OS.[134,136]

Then, the incorporation of additional markers that label further therapeutic targets on CTCs and tdEVs can provide new insights into their phenotypic properties and can increase the proportion of informative samples (**Figure 11**).[137]

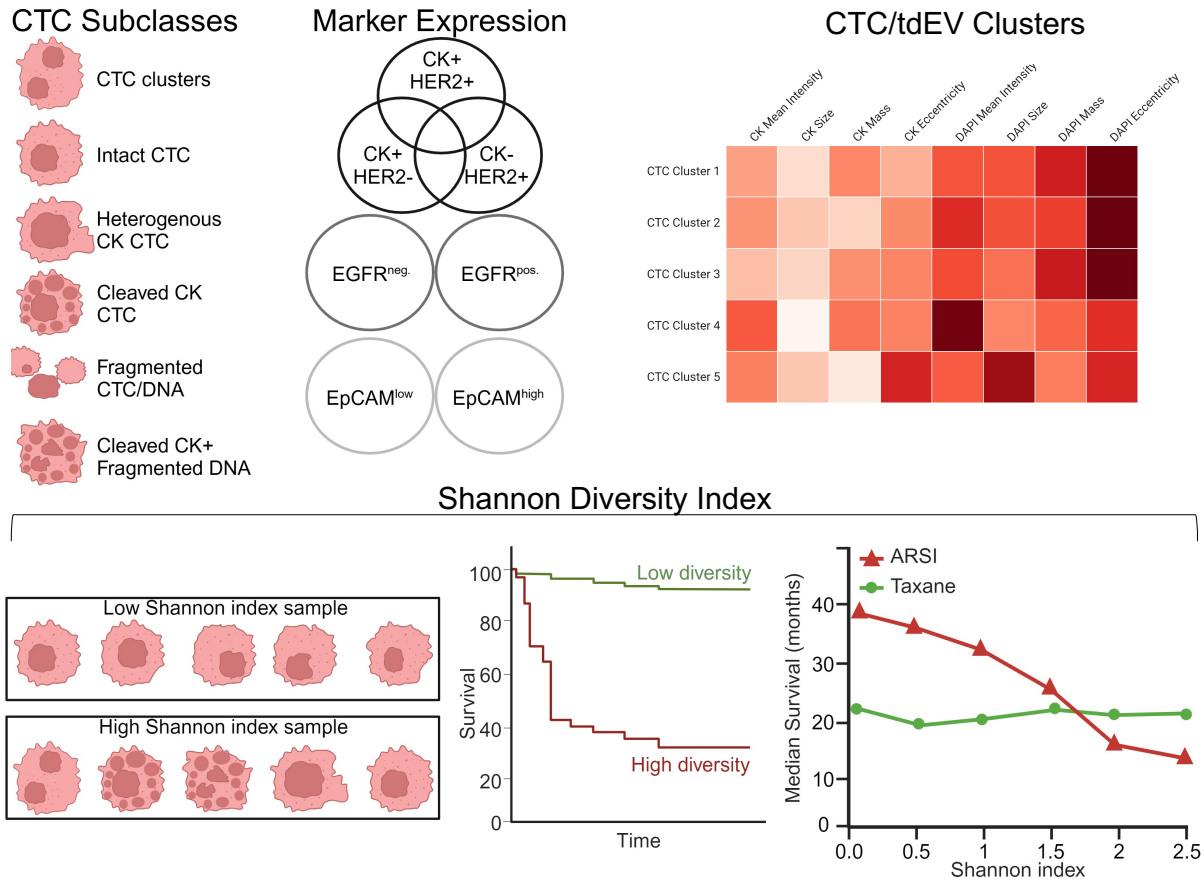

Figure 11: **Diversity of CTCs/tdEVs**
*CTCs can be defined in multiple subclasses based on their phenotypic attributes (**CTC subclasses**).*[93] *CTCs can show different levels of marker expression regarding tumour markers (**Marker expression**).*[96,129,131,133] *By incorporating a variety of phenotypic characteristics, CTCs can be grouped into clusters (**CTC/tdEV clusters**).* [94] *Here the Shannon index quantifies the diversity by considering the categories of CTCs and the uniformity of their distribution (**Shannon diversity index**). It was found that high Shannon diversity was associated with poor survival and particularly in mCRPC patients treated with androgen receptor signalling inhibitors (ARSI)* [94,116]*. Created with BioRender.com*
Abbreviations: ARSI – androgen receptor signalling inhibitors; CK – cytokeratin; CTCs – circulating tumour cell; EpCAM – epithelial cell adhesion molecule; EGFR – epidermal growth factor receptor; HER2 – human epidermal growth factor receptor 2

In the CellSearch system, additional staining markers are available for multiple cancer entities and enable a stratification into multiple phenotypes. In a study involving 98 mBC patients CTCs and tdEVs were additionally stained with the HER2 marker dividing CTCs/tdEVs into three distinct phenotypes – HER2$^+$CK$^-$, HER2$^-$CK$^+$, and HER2$^+$CK$^+$. Thereby, a remarkable 95% of

blood samples were tested positively for CTCs. Patients exhibiting multiple CTC and tdEV classes had a shorter OS compared to patients exhibiting only one class, reinforcing the premise that phenotypic heterogeneity is linked to poor prognosis.[96] An interventional randomized clinical trial conducted by Fehm et al. included HER2-negative mBC patients with HER2-positive CTCs who either received standard therapy with lapatinib or without it. This study demonstrated that patients with HER2 positive CTCs treated with lapatinib achieved a longer OS which supports the use of CTC-guided therapy.[131] In CRC patients, the use of additional markers is less established. In a study conducted by Kuboki et al. CRC patients receiving cetuximab therapy were divided into two groups based on the epidermal growth factor receptor (EGFR) expression - EGFR-positive vs. EGFR-negative patients. The results showed no significant difference in prognostic outcomes between the two groups.[133] EGFR expression varied among CTCs from the same patients, but their impact on prognosis remained unclear.[138] In summary, research on particle diversity provides a multitude of new information on tumour biology and enable a more accurate identification of patients with a poor prognosis (**Figure 11**). The inclusion of phenotypic properties increases the number of informative samples and represents a way to overcome the high false-negative rates.

1.7. Tumour-Proximal Biopsy

1.7.1. Anatomical Blood Routes

Metastasis, the spread of cancer from its original site to other parts of the body, can be influenced by two mechanisms. First, the location of the primary tumour and the potential of its cancer cells to reach the vicinity of the target lesion. Second, the target organ's role in supporting or suppressing the growth of CTCs.[139] Blood drainage of CRC follows clearly defined anatomical routes (**Table 8**, **Figure 12**).[140,141] Blood from all colon sections and the upper rectum drains through the superior (SMV) or inferior mesenteric vein (IMV). The IMV drains into the splenic vein, whereas the SMV and the splenic vein join to form the so-called portal vein confluence. Blood first passes through the capillary bed (small sinusoids) when it reaches the liver. Here, the nutrient-rich portal blood from the digestive organs and the spleen mix with oxygen-rich blood from the hepatic propria artery. After that, blood flows from the hepatic veins (HV) into the inferior vena cava to the heart and lung capillaries. Once these capillaries have been passed, blood circulates back to the heart and into the arterial circulation, from where it can flow to all possible anatomical sites. Beyond the most commonly occurring hepatic drainage, venous blood from the lower rectum is drained via the inferior rectal vein into the internal iliac vein, then the common iliac vein, and finally into the inferior vena cava, from where it is transported to the right heart and into the pulmonary circulation.[140,141] In summary, metastatic spread of colorectal cancer occurs via the portal and caval venous systems (**Table 8**) whereas most blood drains into the liver, potentially explaining why the liver represents the most common site of metastases. Other sites of metastases include the lungs, skeleton, brain and the surrounding tissue through direct metastasis.[142–145]

Table 8: **Metastatic Routes**

Location	Distance from ACL	Lymphatic path	Haematogenous path
Colon cancer	≥16 cm	Mesenteric	Portal vein → liver hepatic veins → inferior vena cava → lung → further spread in the skeleton and brain
Rectal Cancer	Upper third (12-16 cm)	Paraaortic	Superior rectal vein → inferior mesenteric vein → splenic vein → portal vein → liver → further spread in the lung, skeleton and brain
	Middle third (6-<12 cm)	Paraaortic + pelvic wall	Middle and inferior rectal veins→ internal iliac vein → inferior vena cava → lung → further spread in the skeleton and brain
	Lower third (<6 cm)	Paraaortic + pelvic wall + inguinal	

The information on metastatic routes was obtained from the studies by Zurcher et al.[141]*, Harkins et al.*[140] *and Riihimäki et al.*[145]

Abbreviations: ACL - anocutaneous line

1.7.2. Clinical Potential of Tumour-Proximal Biopsy

The median cubital vein, i.e., the peripheral vein, is considered the standard blood collection site for routine clinical use because of its easy accessibility, high reproducibility for the clinician, low risk of complications and adverse side effects for the patient, resulting in high patient compliance.[146] However, in contrast to breast cancer and pancreatic cancer patients, the informative value of peripheral CTCs in CRC patients is limited due to drastically lower quantities which can be explained by the liver that could filter and withhold a relevant proportion of CTCs.[147] Due to the larger size of CTCs compared to WBCs, liver metastases might develop through the mechanical arrest of CTCs in the capillary bed (diameter between 3-8 µm). The

mechanical arrest of CTCs depends on deformability, blood pressure, and cell size to capillary diameter ratio. For the CTCs to survive and grow in the initial capillary bed, the level of compatibility between the respective CTC subpopulation and the tumour environment could play a significant role.[142,148] By aiming to increase tumour cell yield in these patients, tumour-draining veins (DVs) have been explored to capture these cells prior to their potential hepatic arrest (**Table 9**, **Figure 12**).[147] This approach could be an alternative to apheresis, which processes a larger volume of blood and detects CTCs from the mononuclear cell fraction, significantly increasing CTC detection.[82,149]

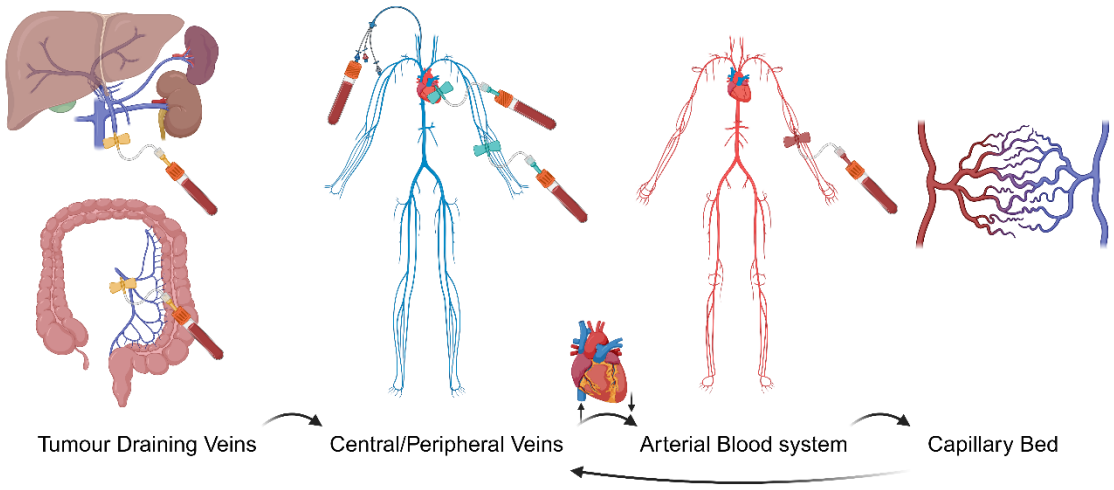

Figure 12: **Tumour-Proximal Biopsy**
Tumour-proximal veins encompass the mesenteric and portal veins. After the liver passage, blood enters the central venous compartment and flows into the heart, which pumps blood to the lungs and arterial system. The microcirculatory system comprises arterioles, capillaries, and venules, which serve as a place for the oxygen exchange of red blood cells, the exchange of hormones and nutrients, and the regulation of microvascular blood flow. After passage through the microcirculatory system, blood enters the venous system again. Created with BioRender.com

1.7.3. Sampling Techniques

Reaching clear conclusions from the existing research (**Table 9**) is challenging due to the variation in CTC detection technologies[150–157], and enrichment technologies for tumour mRNA, CK20 and other transcripts.[158–170] Previous studies utilizing DV blood applied CellSearch, Epispot, other cytology-based methods for CTC detection, and PCR-based methods for the detection of mRNA, CK20, and other transcripts (**Table 9**). Blood from draining veins can be obtained before, during, and after surgery, with each time point delivering unique informative value. In patients undergoing resection of the primary CRC tumour, the mesenteric vein (MsV), the portal vein (POV), or a vein directly draining the tumour can be sampled (**Figure 12**). The existing literature indicates that blood is usually taken during surgery prior to tumour resection. Although less commonly used, blood collection after tumour resection can be considered a valid alternative as it avoids additional tumour tissue manipulation and delay.[150,153] In mCRC patients undergoing liver metastasectomy, mostly the HV, as the primary outflow vein, and POV, as the primary inflow vein, are sampled before or after mobilization of the liver.[152,154] In fact, mobilization of the liver increases CTC numbers in the HV.[154] The knowledge about surgery-triggered tumour cell dissemination led to changes in the use of surgical techniques.[160] Nowadays, the intraoperative release of CTCs into the systemic circulation can theoretically be reduced by implementing the no-touch isolation technique, in which the lymphovascular structures are ligated before tumour manipulation. However, this approach has not proven to be superior to conventional surgical techniques in terms of long-term outcomes.[171]

All studies uniformly found higher CTC quantities in DVs compared to PVs or CVCs. The detection rate for DVs ranged between 35% and 70% for CellSearch, 66% for Epispot, between 8% and 76% for PCR-based methods, and between 52% and 96% for other CTC detection technologies (**Table 9**).

Table 9: **Tumour-Draining Vein Studies**

Author	Method	Samples	Time	DV	Rate (%)	Prog.
Yang (2024, *Eur J Surg Oncol*)[157]	CS	343 M0 & M1	+	First d.	35.9-37.1	NA
Joosse (2018, *Clin Chem*)[172]	CS & CK19-ES	69 M0 & M1	-	MsV	CS + ES = 39.1*	NA
Zhao (2017, *Oncotarget*)[151]	CS	59 M0 & M1	-	POV	46-70	NA
Rahbari (2016, *Ann Surg*)[152]	CS	105 M1 (liver)	-	HV POV	HV/POV 38.2-54/11.4-19.6	NA
Connor (2016, *Ann Surg Oncol*)[154]	CS	50 M1 (liver)	-	HV	54	Yes OS & DFS
Deneve (2013, *Clin Chem*)[155]	CS & CK19-ES	34(CS) 41(ES) M0 & M1	-	MsV	CS/ES 56/66	NS
Rahbari (2012, *Ann Surg Oncol*)[156]	CS	80 M0 & M1	-	MsV	35	NA
Iinuma (2006, *Int J Oncol*)[173]	RT-PCR*	167 M0 & M1	-	MsV	34	Yes OS & DFS
Sadahiro (2005, *Ann Surg Oncol*)[159]	RT-PCR*	100 M0	-	POV*	49	No OS & DFS
Zhang (2003, *World J Gastroenterol*)[164]	RT-PCR*	58 M0	-	POV*	74	NA
Sunouchi (2003, *Dis Colon Rectum*)[165]	RT-PCR*	37 M0 & M1	-	MsV*	43	Yes OS
Uchikura (2002, *Hepatogastroenterology*)[166]	RT-PCR*	16 M1	NA	POV	NA	NA
Guller (2002, *Ann Surg*)[174]	qPCR*	39 M0	-	MsV	8	NA
Koch (2001, *Arch Surg*)[167]	RT-PCR*	40 M0 and M1	-	MsV	50	NA
Yamaguchi (2000, *Ann Surg*)[168]	RT-PCR*	52 M0 & M1	-	MsV	37-38	Yes OS
Luo (1999, *Zhonghua Wai Ke Za Zhi*)[170]	RT-PCR*	54 M0 & M1	-	POV	76	USS LM
Hayashi (1999, *Surgery*)[160]	MASA	27 M0 & M1	-/=/+	POV*	33*	NA
Tsutsuyama (2019, *PLOS ONE*)[150]	Cyto-based	26 M0 & M1	+	MsV	96	NA
Märkl (2016, *World J Clin Oncol*)[153]	CK18 ICC	56 M0 & M1	+*	MsV	52	NS

**__Joosse__ Patients with CTCs detected in the CellSearch or EPISPOT assay, or both, were counted as CTC positive; Iinuma: RT-PCR for CEA & CK20 detection; __Sadahiro__ RT-PCR was used for CEA detection. Catheter inserted into the umbilical vein or the inferior mesenteric vein for portal vein sampling; __Zhang__ RT-PCR detected CK20. Cannulae were utilized to aspirate blood (5-10 mL) from the portal vein through the right gastroepiploic vein; __Sunouchi__ RT-PCR detected CEA. Mesenteric vein and*

artery. 8% of artery samples were positive; ***Uchikura*** *RT-PCR detected CEA;* ***Guller*** *Real-Time qPCR detected CEA & CK20. Mesenteric qPCR positive patients had local or metastatic recurrence (3/3, no statistic);* ***Koch*** *RT-PCR to detect CK20;* ***Yamaguchi*** *RT-PCR to detect CEA and CK20. 38.% detection of CEA and 36.5% detection CK20;* ***Luo*** *RT-PCR detected CK20;* ***Hayashi*** *Venous catheter was placed into the portal vein from the right gastroepiploic vein;* ***Märkl*** *blood was taken postoperatively.*
Abbreviations: CS – CellSearch; D – drainage; DV – draining vein; HV – hepatic vein; ICC – immunocytochemistry; LM – liver metastases; MASA – mutant-allele-specific amplification; MsV – mesenteric vein; NS – not significant; PNM – paired non-metastatic; POV – portal vein; Prog. – prognostic; S – significant; UPNM – unpaired non-metastatic; USS – uncertain statistical significance
Symbols: **-** *before resection;* **=** *During resection;* **+** *after resection*

Considering only mCRC patients, the detection rate for draining veins ranged between 38.2% and 85.7% for CellSearch. Thus, there is a significant variability in the detection rates (range: 7.7%-96%) that may have resulted from differences in the distribution of clinicopathological data. However, a study incorporating training and validation groups suggests that the numeric differences could be attributed to the small cohort sizes.[152] Studies included mostly less than a hundred patients - eight studies included ≤50 patients, another eight studies between >50 and ≤100 patients and three studies >100 patients. The largest patient cohort involved 343 CRC patients.[157]

Despite higher CTC quantities and the associated higher potential informative value, a single intraoperative blood sample cannot reflect dynamics in CTC levels and the biological meaning of intraoperative CTCs/tdEVs remains to be depicted. In addition to intraoperative blood sampling, postoperative blood sampling might reflect the preoperative baseline, the intraoperative CTC shedding, and the apoptotic loss of newly released tumour cells.[175]

1.8. Aims of My Thesis

In the present doctoral thesis, I have examined four aspects in detail (**Figure 13**). First, the comparison of CTC and tdEV detection rates and numbers between the DV and CVC. Second, the correlation of CTCs and tdEVs with the patient's clinicopathologic parameters and survival to test whether an elevated biomarker count would be associated with shorter OS and more advanced disease indicators. Third, the isolation and sequencing of DV-derived CTCs to prove their malignant origin. Fourth, the phenotypic differences of CTCs and tdEVs detected in DV and CVC and their clinical informative potential to enhance patient stratification.

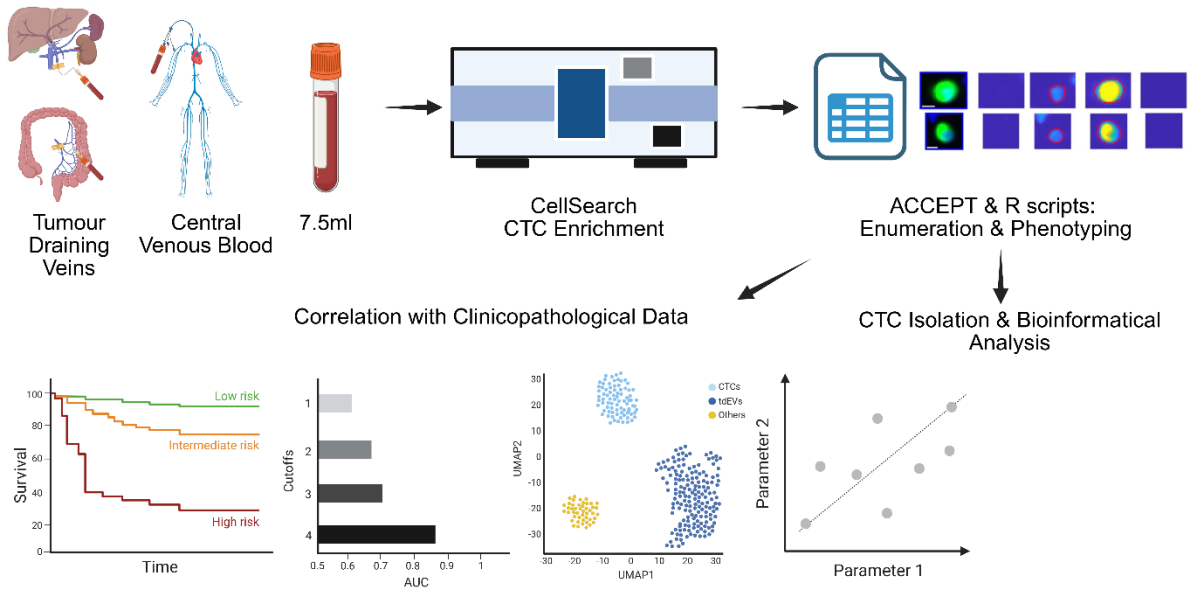

Figure 13: **Workflow of my Dissertation**
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2. Publication

Phenotypic diversity of CTCs and tdEVs in liquid biopsies of tumour-draining veins is linked to poor prognosis in colorectal cancer.

Journal: Journal of Experimental & Clinical Cancer Research

Year: 2025

Relevant publication:

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Journal of Experimental & Clinical Cancer Research

RESEARCH Open Access

Phenotypic diversity of CTCs and tdEVs in liquid biopsies of tumour-draining veins is linked to poor prognosis in colorectal cancer

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Abstract

Background Circulating tumour cells (CTCs) and tumour-derived extracellular vesicles (tdEVs) have great potential for monitoring therapy response and early detection of tumour relapse, facilitating personalized adjuvant therapeutic strategies. However, their low abundance in peripheral blood limits their informative value. In this study, we explored the presence of CTCs and tdEVs collected intraoperatively from a tumour-draining vein (DV) and via a central venous catheter (CVC) prior to tumour resection.

Methods CellSearch analyses of 395 blood samples from 306 patients with gastrointestinal tumours and 93 blood samples from healthy donors were used to establish and validate gates for the automated detection of CTCs and tdEVs with ACCEPT software and R scripts. The selected gate settings were applied to 227 samples of 142 patients with colorectal cancer (CRC) from two independent collectives. Phenotypic features were obtained via numeric analysis of their fluorescence signals (e.g. size, shape, and intensity) and were used for calculating diversity using Shannon index (SI) of clusters generated via the k-means algorithm after Uniform Manifold Approximation and Projection (UMAP) pre-processing, and standard deviation (SD).

Results CTCs and tdEVs were more abundant in the DV samples compared to CVC samples ($p < 0.05$). tdEVs were detected in higher numbers than CTCs in both compartments. Importantly, tdEVs in CVCs were associated with tumor spread, whereas CTCs in DVs were linked to tumor size. In both compartments, the prognostic value of tdEVs for overall survival (OS) surpassed that of CTCs, as demonstrated by univariate, multivariate, and Kaplan-Meier analyses. CTCs and tdEVs in DVs were phenotypically distinct, being larger, more eccentric, and displaying stronger cytokeratin intensities ($p < 0.05$) compared to those in CVC samples. Furthermore, increased diversity in CTC and tdEV phenotypes was significantly associated with shorter survival, validating the prognostic relevance of the SD-diversity metric.

[†]Nikolas H. Stoecklein and Rui P. L. Neves shared senior authorship.

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Conclusion Our study demonstrates that DV sampling significantly enhances the detection of prognostically relevant CTCs and tdEVs in CRC patients, underscoring the superior prognostic significance of tdEVs compared to CTCs. Importantly, the combined phenotypic diversity of both markers emerges as a more powerful biomarker than their enumeration alone. These findings suggest that comprehensive, automated analysis of CTCs and tdEVs in DVs may open new avenues for tailoring individualized therapies in CRC patients.

Keywords Circulating tumour cells, CTCs, Tumour-derived extracellular vesicles, tdEVs, Colorectal cancer, CRC, Tumour-draining vein, Intraoperative blood sampling, CellSearch, Diversity, Single cell analysis

Introduction

Approximately 70% of patients diagnosed with colorectal cancer (CRC) have a local or regional disease (AJCC Stage I-III). For this group of patients, surgical resection is the primary treatment with curative intent and the 5-year survival rate ranges from 91% (local disease) to 73% (regional disease) [1]. However, even after successful tumour resection, approximately 20% of patients experience metastatic relapse within five years [2–4] and in such cases, the 5-year survival rate drastically decreases to only 14% [1]. Identifying the patients at a higher risk of relapse after surgery could improve clinical management and aid in tailoring adjuvant therapeutic strategies.

The molecular mechanisms driving disease progression and relapse are not yet fully understood. Nonetheless, it is recognized that circulating tumour cells (CTCs), as cells that left the primary tumour and retain the potential to seed metastases, play a crucial role in tumour spread [5, 6]. As a result, CTCs have emerged as a promising prognostic tumour biomarker for a variety of malignancies including CRC [7]. CTCs can be efficiently detected via the FDA-cleared CellSearch system, which defines them as EpCAM+/CK+/DAPI+/CD45- cells [8, 9], for which accuracy, precision, linearity, reproducibility and specificity, have been previously described [8–15]. In advanced CRC, approximately 30% of the patients have more than three CellSearch CTCs (CS-CTCs) (in 7.5 mL of blood) and this is associated with poorer clinical outcomes (10, 16–17). In patients with operable CRC, the lower counts and detection rates limit the value of CS-CTCs as biomarker and contribute to conflicting data regarding their prognostic utility [18–22]. In this group of patients, sampling of the tumour or metastasis outflow can increase CS-CTC detection [23–30], and the presence of CS-CTCs in the tumour-proximal mesenteric vein correlates with the serological biomarker CA19-9 [26], but the prognostic value of such cells has not yet been clearly demonstrated [23, 25].

CTC identification with the CellSearch system has some inherent subjectivity. To further enhance standardization of CTC identification in the CellSearch assay, the ACCEPT tool was developed [31, 32]. This tool analyses the raw CellSearch images, segments all objects, quantifies multiple fluorescent parameters, and enables users to objectively discriminate the cells or particles of interest via the quantified parameters. In addition to CTCs, the ACCEPT tool can be used to identify large (>1 µm) tumour-derived extracellular vesicles (tdEVs), which are co-enriched in CellSearch and defined as EpCAM+/CK+/DAPI-/CD45- particles [33, 34]. tdEVs are important mediators of intercellular communication in both physiological and pathological processes, including cancer [35, 36]. In the peripheral circulation of patients with metastatic CRC, CellSearch tdEVs (CS-tdEVs) are significantly more abundant than CTCs, and their abundance is an independent risk factor for shorter overall survival (OS), suggesting a greater potential as biomarker [37, 38]. However, the presence and clinical relevance of CS-tdEVs in tumour-proximal veins in operable CRC has not yet been explored.

Sampling blood from tumour-proximal veins intraoperatively presents a unique opportunity to improve CTC recovery, and we hypothesize that the same applies to tdEVs. Using the CellSearch and the ACCEPT tool, we analysed CS-CTCs and CS-tdEVs in tumour-proximal blood collected prior to CRC tumour resection to explore the prognostic potential of these two biomarkers for the adjuvant period. Previous studies have shown that the phenotypic heterogeneity of peripheral vein CTCs can be pictured by the CellSearch system and quantified via the ACCEPT tool, and suggest that diversity during patient follow-up could inform on disease progression [39, 40]. Considering that tdEVs are also a diverse class of particles [41], in the present work we investigated the phenotypic heterogeneity of CS-tdEVs and CS-CTCs and its clinical relevance.

Methods

Patients and processing of blood samples

A total of 510 samples from 364 patients with gastrointestinal tumours were used in this study (Suppl. Table 1). These included patients admitted at the Department of General, Visceral and Paediatric Surgery, University Hospital of the Heinrich-Heine University Duesseldorf (DU cohort), and a cohort of patients admitted at the Department of General, Visceral and Transplantation Surgery, Heidelberg University Hospital, which has been previously described (HE-cohort) [23]. All patients were admitted for surgery with tumour curative intention. The study was carried out in accordance with Good Clinical

Practice guidelines and the Declaration of Helsinki and was independently approved by the Ethics Committees of the Medical Faculty of the Heinrich-Heine University Duesseldorf and the Ruprecht-Karls University Heidelberg. All patients provided written informed consent prior to sample collection. For prognostic analyses in CRC, we used a sub-collective of patients where successful surgical resection of the tumour margins was achieved (R0 CRC) (Suppl. Table 3). From these patients, blood samples from the tumour draining vein (DV, DU-cohort $N=76$, HE-cohort $N=58$) were collected intra-operatively prior to tumour resection by puncturing the colic and mesenterico-portal vessels. The blood was collected initially into a syringe and then immediately transferred into CellSave tubes (Menarini, Bologna) for cell preservation. Samples from the central vein catheter (CVC, DU- cohort $N=36$, HE-cohort $N=57$) were collected via a central line (either in the internal jugular or subclavian vein) directly into CellSave tubes. All blood samples were processed using the CellSearch Circulating Tumour Cell Kit (Menarini) in the CellSearch AutoPrep system (Menarini) according to the manufacturer's protocol. Additionally, we used ACCEPT data from 93 healthy volunteers from the IMMC06 clinical trial (NCT00133913) [8, 37].

Identification of CTCs and tdEVs

The enumeration of CTCs was initially performed manually according to the CellSearch protocol by trained operators. The digitally stored CellSearch image files were subsequently re-analysed with the ACCEPT tool for the automated identification of CTCs (CK+/DAPI+/CD45-), and tdEVs (CK+/DAPI-/CD45-) [42], using the set of gates previously described [34] (Suppl. Table 5). For the present work, we defined three additional selection criteria: one directly in the ACCEPT tool (Eccentricity < 0.9); and two upon downstream analysis of the ACCEPT-output tabular data with an R-based in-house developed script (CK size > DAPI size; CK mean intensity > DAPI mean intensity, or CK mean intensity > 150) (Suppl. Table 5). We validated these criteria in the GI and healthy collectives (Suppl. Methods). In addition ACCEPT was also used to identify white blood cells (WBCs, CK-/DAPI+/CD45+), lymphocyte-derived extracellular vesicles (ldEVs, (CK-/DAPI-/CD45+), and bare nuclei (CK-/DAPI+/CD45-). As part of the standard CellSearch Circulating Tumour Cell assay, samples were scanned for fluorescence in DiOC channel (the 4th channel of the system to detect FiTC) despite the fact that no marker was used for that channel. Fluorescence in the DiOC channel was used to exclude events with high autofluorescence (Suppl. Table 5).

Calculation of diversity

Diversity was calculated considering all tdEV and CTC events within each cartridge via two different approaches: a cohort-dependent (referred to as the Shannon diversity index) and cohort-independent (referred to as the SD-diversity index).

For Shannon diversity, all nine CK-PE and nine DAPI fluorescence-based parameters quantified by ACCEPT plus the DAPI overlay with CK parameter were considered. ACCEPT data were normalized using min-max normalization and further processed through Uniform Manifold Approximation and Projection (UMAP). UMAP was executed in Python 3.11.5 with the packages "umap-learn" 0.5.3 and "plotly" 5.15.0 and the following settings: "random_state" of 42, "n_neighbors" of 45, "n_components" of 18, "min_dist" of 0.0, and the Euclidean metric. Subsequently, k-means was applied considering different numbers of expected clusters, and Shannon diversity index was calculated for each cartridge on the basis of the distribution of particles through each cluster definition. Following multiple tests on, the distribution across 18 clusters was chosen. For SD-diversity, six CK-PE and six DAPI fluorescence-based parameters quantified by ACCEPT plus the DAPI overlay with CK were considered. All values of the 13 parameters were standardized via z-score normalization, and these normalized values were transformed into their absolute values. For each cartridge individually, we calculated the standard deviation (SD) for each of the 13 parameters, and finally we calculated the mean value of the 13 SD values. The resulting value constituted the SD-diversity index value for the respective cartridge.

Results

CTC detection rates increase in DV blood samples

Our initial aim was to test the hypothesis that the detection frequency of both CTCs and tdEVs can be increased by DV blood sampling. For a more systematic and unbiased assessment of the particles enriched with CS, we used the ACCEPT tool. Using previously defined gates and a set of parameters that we re-defined and validated for different cancer entities (Suppl. Methods, Suppl. Figure 1), we analysed 93 CVC and 134 DV samples from R0 CRC patients (Suppl. Table 3). The set of gates resulted in five groups of particles clearly distinguishable visually, and upon dimensional reduction of data in a UMAP plot: CTCs and tdEVs, as well as co-enriched white blood cells (WBCs), lymphocyte-derived extracellular vesicles (ldEVs) and bare nuclei (Fig. 1A and B). In CVC samples, the CTC positivity rate of 22.6% was perfectly in line with previous studies in M0 CRC patients analysed with CS (average 23%). In DV samples, we indeed observed significantly higher CTC detection rates and counts compared to CVC samples (detection rate: 37.3% vs. 22.6%,

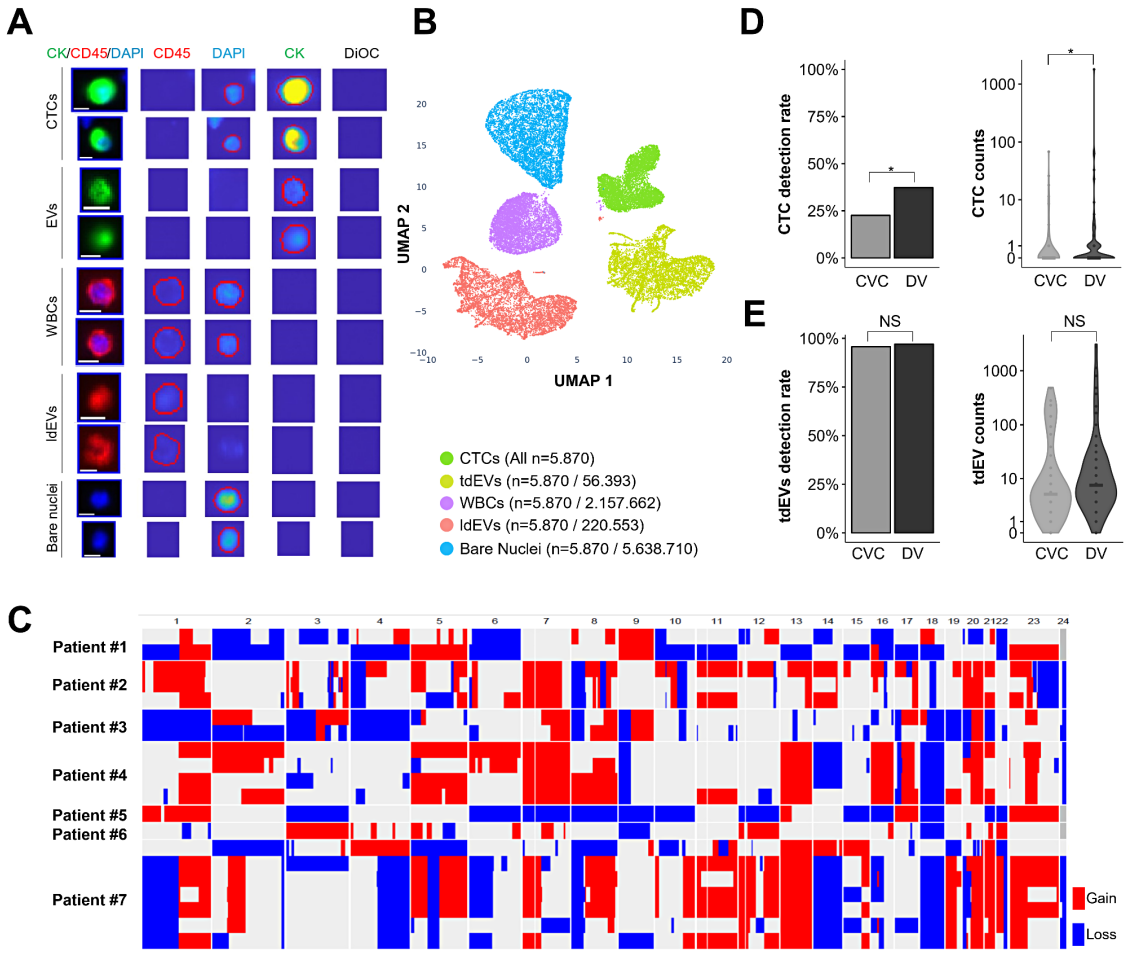


Fig. 1 Identification of CTCs and tdEVs with the CellSearch system. **(A)** Representative circulating tumour cells (CTCs), tumour-derived extracellular vesicles (tdEVs), white blood cells (WBCs), lymphocyte-derived extracellular vesicles (ldEVs) and bare nuclei identified using the ACCEPT tool. For every object, a thumbnail image overlay of the three fluorescent channels (CD45, DAPI, PE) and each channel separately is shown. The red contour indicates the detected boundary of each object. The scale bar represents 10 pixels equivalent to 6.4 µm. **(B)** UMAP visualization of the different types of events identified with ACCEPT in samples from CRC R0 patients from the combined DU + HE cohort ($N = 93$ CVCs; $N = 134$ DVs). All detected CTCs ($N = 5870$) are represented, while for purposes of better visualization, for all other populations, we randomly selected $N = 5870$ objects. **(C)** Heatmap representation of chromosomal copy number alterations (CNAs) of 20 single CTCs obtained by low-pass NGS after single-cell whole-genome amplification. **(D)** Detection rates (positivity rates) and counts of CTCs and **(E)** tdEVs in samples from CRC R0 patients from the combined DU + HE cohort ($N = 93$ CVCs; $N = 134$ DVs). The horizontal lines represent the median. *$p < 0.05$

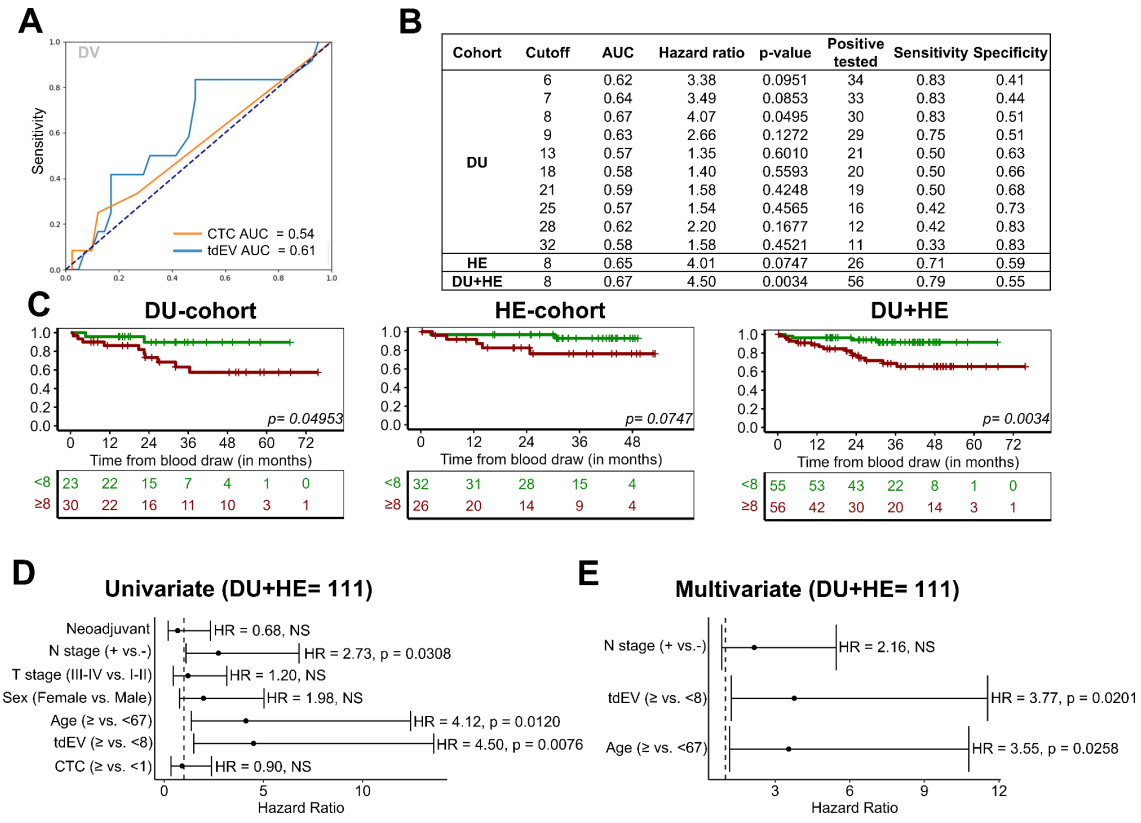
$p = 0.0271$; mean count: 16 vs. 2, $p = 0.0423$; range: 0-1790 vs. 0–68) (Fig. 1D). When we investigated the subgroup of M0 patients ($n = 118$) we found a similar distribution, but the differences did not reach statistical significance (detection rate: 35.1% vs. 23.5%, $p = 0.1100$; mean count: 19 vs. 2, $p = 0.1287$; range: 0-1790 vs. 0–68) (Suppl. Figure 2 A). To confirm the malignant nature of the CVC-derived CTCs and the more frequent DV-derived CTCs, we successfully performed low-pass WGS in 20 single CS-CTCs (from six DV samples and one CVC sample) and detected copy number aberrations characteristic of CRC (Fig. 1C). In line with previous reports, we detected tdEVs in greater numbers compared to CTCs (Fig. 1E). Strikingly, as for CTCs, higher tdEV counts were detected in DV samples (median: 8; mean: 89; range = 0-3108) when compared to CVC samples (median: 5; mean: 43; range: 0-492), but these differences did not reach statistical significance ($p = 0.065$) (Fig. 1E). For the M0-subgroup similar results were obtained (Suppl. Figure 2B). Notably, the numbers of CTCs and tdEVs were positively correlated (Suppl. Figure 3).

Differential association of CTCs and tdEVs from DV and CVC with clinicopathological parameters

Next, we investigated the association of CTCs and tdEVs with clinicopathological parameters (Suppl. Figure 4). Independent of the sampling site, tdEV counts were stronger associated with clinical parameters than CTC counts. In the DV, tdEV counts were strongly associated with higher pT stages ($p=0.0008$ by Mann-Whitney U test; $\rho=0.332$ and $p<0.0001$ by Spearman correlation analyses) and, to a lesser extent, also with pN stage ($p=0.0232$ by Mann-Whitney U test; $\rho=0.231$ and $p=0.0071$ by Spearman correlation) (Suppl. Figure 4). Notably, the CTC counts in the DV were also significantly associated with the pT stage ($\rho=0.226$ and $p=0.0087$ by Spearman correlation) (Suppl. Figure 4), suggesting that the DV sampling site more accurately reflects the status of the primary lesion than the CVC site. This positive correlation between pT stage and DV-tdEVs was likewise observed in the M0-subroup (Suppl. Figure 5).

tdEVs in DV have high prognostic value

In order to evaluate the clinical relevance of CTCs and tdEVs we investigated their prognostic impact. For this we focused only on the R0 M0 patients (UICC I-III) and analysed initially a test cohort (DU-cohort) with 60 patients (DV=53 samples, CVC=28 samples). First, we determined the best cut-off considering both hazard ratios and AUC values collectively, and identified ≥8 tdEVs per sample as the optimal cut-off (HR=4.07, AUC=0.67) (Fig. 2B-C). Notably, we generally observed a superior prognostic accuracy of tdEVs for OS compared with that of CTCs, as reflected by their higher area under the curve (AUC) upon receiver operating characteristic (ROC) analysis (Fig. 2A). As expected, elevating the tdEV cut-off value increased the specificity



Cohort	Cutoff	AUC	Hazard ratio	p-value	Positive tested	Sensitivity	Specificity
DU	6	0.62	3.38	0.0951	34	0.83	0.41
	7	0.64	3.49	0.0853	33	0.83	0.44
	8	0.67	4.07	0.0495	30	0.83	0.51
	9	0.63	2.66	0.1272	29	0.75	0.51
	13	0.57	1.35	0.6010	21	0.50	0.63
	18	0.58	1.40	0.5593	20	0.50	0.66
	21	0.59	1.58	0.4248	19	0.50	0.68
	25	0.57	1.54	0.4565	16	0.42	0.73
	28	0.62	2.20	0.1677	12	0.42	0.83
	32	0.58	1.58	0.4521	11	0.33	0.83
HE	8	0.65	4.01	0.0747	26	0.71	0.59
DU+HE	8	0.67	4.50	0.0034	56	0.79	0.55

Fig. 2 Prognostic value of CS-tdEVs detected in DV samples of CRC R0 M0 patients. **(A)** Receiver operating characteristic (ROC) curves for CTCs and tdEVs and the respective area under the curve (AUC) values calculated for CTCs and tdEVs detected in DV samples of patients from the DU-cohort ($N=53$). **(B)** Ten most relevant tdEV cut-offs identified in the DV samples from CRC R0 M0 patients of the DU-cohort ($N=53$), and validation of the 8 tdEV cut-off in the HE ($N=58$) and DU + HE ($N=111$) cohorts. **(C)** Kaplan-Meier estimates of overall survival for patients dichotomized on the basis of the 8 tdEV cut-off in the DU ($N=53$), HE ($N=58$), and DU + HE ($N=111$) cohorts of patients. **(D)** Univariate analysis of clinicopathological factors (including the ≥ 8 tdEV cut-off) in the DU + HE cohort of patients ($N=111$). **(E)** Multivariate analysis of clinicopathological factors, including the ≥ 8 tdEV cut-off, in the DU + HE cohort of patients ($N=111$)

while decreasing the sensitivity. Further validation of the prognostic value of the tdEV cut-off was sought through analysis of DV samples from an independent cohort (HE-cohort, $n=58$) and the combination of both DU- and HE-cohorts. Strikingly, we could validate this cut-off in the combined DU+HE cohort of 111 DV samples (HR=4.50, AUC=0.67, log rank $p=0.0034$, NPV=93%), and the cut-off demonstrated a result close to statistical significance in the HE-cohort (HR=4.01, AUC=0.65, $p=0.0747$) (Fig. 2B and D). Furthermore, in the DU+HE cohort, the negative impact of ≥8 DV-tdEVs on survival was independent from other parameters according to uni- and multivariate analyses (HR=3.77, $p=0.0201$) (Fig. 2E). In CVC samples, ≥8 tdEVs lost its discriminatory power. In these samples, the cut-off of ≥4 tdEVs had significant prognostic value in the DU-cohort, but it could not be validated in the HE-cohort (Suppl. Figure 6 A). In the case of CTCs, ≥1 CTC in DV and CVC was not associated with worse prognosis (Suppl. Figure 6B-D). The prognostic performance of CTCs was inferior to that of tdEVs in the same sampling site (Suppl. Figure 6 A and 6 C). Finally, we investigated the complete DU+HE cohort (M0/M1) and observed similar results using adapted thresholds with stronger significance (Suppl. Figures 7 and 8).

Phenotypic diversity of tdEVs and CTCs associates with poor survival

Considering the observed phenotypic diversity of CTCs/tdEVs (Fig. 1B), we were interested whether higher diversities in blood samples are associated with a poor prognosis, as previously observed for CTCs in peripheral blood [39, 40, 43–45]. Intriguingly, all phenotypic parameters quantified with ACCEPT were elevated in DVs compared with CVCs, indicating that CTCs and tdEVs from DVs were larger, more eccentric, had stronger CK intensities and, in the case of CTCs, had stronger DAPI signal intensities compared to their CVC counterparts (Suppl. Figures 9–10). In the next step, we assessed the diversity to test its prognostic impact. As an initial strategy, we explored diversity of CTCs and tdEVs by pre-processing the data with UMAP, performing k-means clustering, and calculating the Shannon index (Suppl. Figure 11). As hypothesized, higher diversity strongly correlated with worse prognosis, and we could identify≥0.4214 as a cut-off that significantly dichotomized patients according to OS in the DU-cohort (HR: 3.36, AUC: 0.65), in the HE-cohort (HR: 7.34, AUC: 0.61) and in the DU+HE cohort (HR: 5, AUC: 0.64, log rank $p=0.0002$, NPV=88%) (Suppl. Figure 11). Subsequently, for each individual CS sample (cartridge), we calculated a diversity index by averaging the standard deviations of 13 selected parameters across combined tdEVs and CTCs, referred to as the SD-diversity index.

Using this approach, higher diversity was strongly associated with worse prognosis. In DV samples from the DU cohort, the optimal patient dichotomization was achieved with an SD-diversity index threshold of ≥0.6389 (HR: 3.83, AUC: 0.70). Notably, this cut-off was validated in the HE cohort (HR: 6.20, AUC=0.71) and the combined DU+HE cohorts (HR=4.88, AUC=0.70, log-rank $p=0.0004$, NPV=92%) (Fig. 3A and C, Suppl. Figure 12). In addition, the SD-diversity index remained significant in the multivariate model for the combined DU+HE cohort of patients with DV samples (HR: 4.88, $p=0.0014$) (Fig. 3B), even when the ≥8 tdEVs cut-off was included in the model (HR: 3.92, $p=0.0457$) (Suppl. Figure 12C). Across all three cohorts, the OS predictive value of SD-diversity≥0.6389 was superior to the previously identified tdEV enumeration cut-off (≥8 tdEVs) (Suppl. Figure 12A). Notably, applying the same diversity index cut-offs demonstrated a similar prognostic impact in the combined M0/M1 cohort (Suppl. Figures 13–14). Furthermore, higher particle diversity in CVC samples also identified patients with worse prognosis, with the DU cohort cut-offs successfully validated in the HE and combined DU+HE cohorts (data not shown). Finally, to evaluate the robustness of the newly established SD-diversity metric, we aimed to validate the general observation that higher phenotypic SD-diversity correlates with worse survival outcomes, rather than focusing on a specific cut-off. Given the limited availability of DV data from CS, we re-analyzed previously published CS data from peripheral blood samples of a CRC M0 cohort (IMMC-26) with OS and progression-free survival (PFS) data [20]. Although we had to adjust the cut-off for SD-diversity due to differences between peripheral blood and DV samples—likely reflecting the higher diversity in DV samples—SD-diversity remained a significant prognostic biomarker for both OS and PFS, independent of T- and N-stage (Suppl. Figure 15).

Discussion

Classic clinicopathological parameters have limitations when deciding on adjuvant therapy in primary CRCs. In this context, circulating biomarkers in liquid biopsy samples hold promise for improving clinical decision-making. This study evaluated whether the phenotypic diversity of EpCAM-enriched, cytokeratin-positive objects (i.e., CTCs and tdEVs) in CS images of blood samples can identify operable CRC patients with poor prognosis. Our approach identifies patients with a high diversity of CK-positive objects who are at risk for poor survival outcomes. To conduct this study, we performed two CellSearch assays per patient on two intra-operatively collected blood samples: one from a central venous catheter (CVC) and the other from a tumour-draining

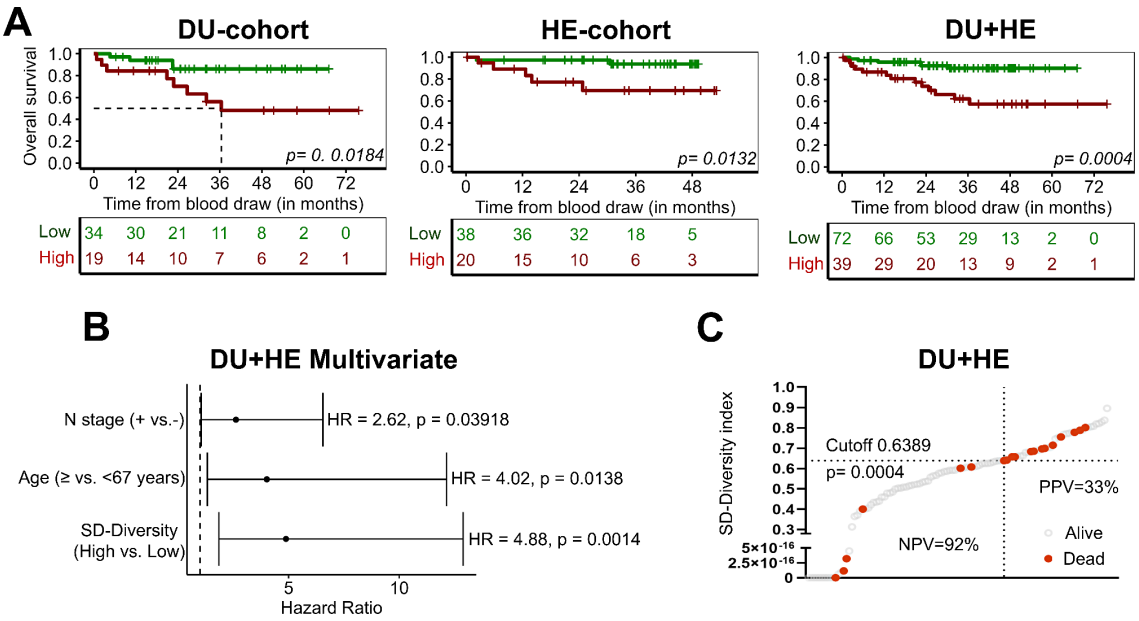


Fig. 3 Prognostic value of diversity in DV samples of CRC R0 M0 patients. **(A)** Kaplan–Meier estimates of overall survival for patients dichotomized on the basis of the SD-diversity cut-off of 0.6389 in the DU ($N=53$), HE ($N=58$), and DU + HE ($N=111$) cohorts of patients. **(B)** Multivariate analysis was performed including variables that were found to be significant in the univariate model. **(C)** SD-diversity index calculated for each of the $N=111$ patients of the DU + HE cohort showing the positive predictive value (PPV) and negative predictive value (NPV) of the cutoff 0.6389 as a biomarker. In red are indicated the patients that died

vein (DV), located closer to and downstream of the tumour, before the blood passed through the liver.

Our data confirmed previous investigations demonstrating that both CTCs and tdEVs are significantly more prevalent in DV samples [25, 26, 46–53], presumably due to their higher concentration proximal to the tumour, where tumour-derived material invades and leaks directly into the bloodstream. This was further validated by our ACCEPT analysis of a second, independent, and previously published DV collective [23]. The rarity of CTCs in CVC blood together with the observed markedly higher prevalence of biomarkers in DV blood led us to discontinue analysing the CVC samples in the DU cohort.

Importantly, we can make three new vital observations. First, CTCs and tdEVs in DVs have different morphologic features than their CVC sample counterparts. The automated CS image analysis via ACCEPT enabled us to record up to 19 parameters per identified CK-positive object (19 for CTCs and 9 for tdEVs), clearly showing that DV samples were dominated by larger and more intensely CK-stained CTCs and tdEVs. Together with the observed reduction in the frequency of CTCs and tdEVs in CVC blood, these data suggest their arrest during the liver passage [25]. Therefore, it is tempting to speculate that CS analysis captures information on two critical components for hepatic metastasis: CTCs, which are potential precursors of hepatic metastasis [54–56], and tdEVs, which might facilitate metastasis by releasing their cargo to support pre-metastatic niche formation in the liver [57–61].

The second intriguing observation of our study concerns the differential association of tdEV/CTC frequency with tumour stage. While the correlations with pN- and M-stages were similar in DV and CVC samples, the frequencies of CTCs and tdEVs correlated significantly with the pT-stage only in the DV sample. This observation suggests that DV blood can effectively enable the detection of CK-positive tumour material directly released from the primary tumour. This is further supported by the finding that there was a greater likelihood of obtaining genomically aberrant profiles typical for CRC in CTCs isolated from DVs than in CVC samples. Although we did not perform a systematic analysis of the genomic profiles here, it was interesting to observe the CNAs in CS-detected CTCs of non-metastatic CRC, which, to the best of our knowledge, have not been described before, likely owing to their shallow concentration in peripheral blood at this disease stage.

Third, we observed that the prognostic information of DV samples clearly outperformed that of CVC samples. Nevertheless, the prognostic value of CS-CTCs could not be validated in the HE and pooled DU+HE cohorts. We posit that this inconsistency arises most likely from the combination of relatively small patient cohorts and the comparatively low frequency of CS-CTC detection,

which, even with tumour-proximal sampling, remains notably lower than in patients with metastatic CRC. Remarkably, none of the previous studies could confirm a prognostic role for CS-CTCs in DV blood of non-metastatic CRC patients. Further research involving larger patient cohorts might be necessary to clarify the prognostic value of CS-CTCs in DV blood. However, our data show that the more abundant tdEVs can be complementary and that there is no need for the exclusive analysis of CS-CTCs. The tdEVs alone were significantly associated with poor survival over a broader range of thresholds, for which the best threshold could be confirmed in the independent HE cohort and in the pooled analysis. Similar to our findings, a previous study described tdEVs in the peripheral blood of patients with metastatic CRC and reported higher OS hazard ratios than CTCs did [38]. The observed prognostic impact, particularly of tdEVs, and the acknowledgment of the morphologic diversity of both CK-positive CS-CTCs and CS-tdEVs prompted our investigation into the prognostic significance of this diversity. Our underlying hypothesis posited that highly invasive and thus more aggressive cancers would exhibit a greater prevalence of CK-positive cells and tdEVs, yielding greater morphologic diversity in the DV sample before hepatocellular transit. As a starting point to test this hypothesis, we used k-means clustering and Shannon diversity index to analyse diversity on the basis of the morphological features of the CK-positive biomarkers, as previously described [39, 44]. In contrast to these previous studies, we pre-processed the initial dataset with UMAP since it was reported to improve the accuracy of subsequent clustering tools, including k-means [62, 63]. Our comprehensive analysis of the phenotypic attributes of both CTCs and tdEVs unveiled substantial diversity within and between DV samples. Interestingly, our data revealed that increased phenotypic diversity of CTCs and tdEVs correlated with adverse prognostic outcomes. Furthermore, we validated the prognostic impact of SD-diversity in a relatively large, independent CS dataset from M0 CRC patients. Given the limited availability of DV data in CRC, we utilized peripheral blood sample data for this purpose. However, the findings confirmed that SD-diversity is not confined to a specific sample type but represents a broader phenomenon, strongly reinforcing our hypothesis with this dataset. In prostate cancer, the diversity in the phenotypic attributes of CS-CTCs, as determined with ACCEPT, has been found to have a significant inverse correlation with progression-free and overall survival. CTC diversity has been associated with the development of therapy resistance [39], and specific morphologic subclasses of CS-CTCs have been linked to more advanced disease stages [43]. In this context, it is worth emphasizing that Scher and colleagues were the first, using the Epic Sciences CTC platform, to investigate the role of the Shannon index as a diversity measure of CTC morphology for predictive purposes. These results indicate a clear association between low CTC phenotypic heterogeneity and improved survival in patients treated with androgen receptor signalling inhibitors. In contrast, high heterogeneity was associated with better OS in patients treated with taxane chemotherapy [44].

Notably, previous works [39, 44] and our approach to describe diversity on the basis of the Shannon index require retrospective data from the complete sample set for cluster definition. This implies that a prospective analysis of individual samples cannot be performed, posing a limitation of this method. Because the number of particles varies greatly between samples, clustering tools and the resulting number of clusters are highly influenced by this factor. Consequently, if such clustering would be performed separately for each individual sample, the basis for calculating diversity would be sample specific, and the calculated value of diversity could not be compared between samples. This motivated us to explore a simpler alternative to measure diversity. As an easily applicable approach, we calculated a diversity index as a mean of the standard deviation of the morphological attributes (SD-diversity index). In contrast to the Shannon index, this index can be applied prospectively to individual blood samples. Importantly, our simplified method confirmed the worse prognosis resulting from high diversity, supporting the use of this method in prospective studies. In metastatic breast cancer patients, the presence of particles belonging to different classes of CTCs and tdEVs, as defined by their expression of CK and HER2 measured with CellSearch and ACCEPT, was linked to poorer clinical outcomes than in patients where only one class of particles was detected [40]. In conclusion, these and other similar observations highlight the added clinical value that can be obtained from CTCs and tdEVs by conducting a more comprehensive characterization beyond simple enumeration.

Conclusions

In conclusion, our study demonstrates that DV sampling enhances the detection of prognostically relevant CTCs and tdEVs in non-metastatic CRC. Importantly, this work highlights the potential of CTC and EV phenotypic diversity to enhance patient prognostication to potentially improve clinical decision-making for CRC patients in the adjuvant setting. Future confirmatory studies could evaluate our approach for assessing CS-CTC/CS-tdEV diversity in comparison to other minimal residual disease markers, such as ctDNA, in terms of prognostic accuracy and cost-effectiveness. Additionally, investigating whether a combination of these markers could further improve prognostic performance may offer new insights into optimizing patient management strategies.

Abbreviations

CTCs	Circulating tumour cells
tdEVs	Tumour-derived extracellular vesicles
DV	Tumour-draining vein
CVC	Central venous catheter
CRC	Colorectal cancer
SI	Shannon index
SD	Standard deviation
OS	Overall survival
AJCC	American Joint Committee on Cancer
CS-CTCs	CellSearch circulating tumour cells
EpCAM	Epithelial cell adhesion molecule
CK	Cytokeratin
DAPI	−4′,6-diamidino-2-phenylindole
WGS	Whole-genome sequencing
UMAP	Uniform Manifold Approximation and Projection
AUC	Area under the curve
HR	Hazard ratio
NPV	Negative predictive value
GI	Gastrointestinal
HE	Heidelberg (referring to a specific cohort)
DU	Düsseldorf (referring to a specific cohort)
NPV	Negative predictive value
WBC	White blood cell
ldEV	Lymphocyte-derived extracellular vesicle

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13046-024-03259-6.

Supplementary Material 1

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Author contributions

S.A.C. performed the ACCEPT analysis of all patient samples, analysed the clinical data, implemented bioinformatics tools, developed a pipeline for the analysis of ACCEPT data, contributed to in the single-cell profiling, and contributed to the writing of the manuscript. A.G.Z. implemented the bioinformatics tools, developed a pipeline for the analysis of ACCEPT data, analysed the data, and contributed to the writing of the manuscript. C.D. performed the CellSearch analyses of patients with GI tumours from the DU-cohort, and performed the single-cell profiling. M.S. implemented the pipeline and supported the analysis of single-cell NGS data. J.P.C. supported the development and implementation of the bioinformatics pipeline for the analysis of ACCEPT data. G.F., L.D., A.K., S.V., H.A., S.F. and M.W. collected the intraoperative samples from the DU-cohort, and collected, analysed and curated the clinical data. L.W.M.M.T. and A.N. analysed the samples from healthy donors, provided ACCEPT data on these samples, and assisted in data interpretation and analysis. H.N. helped in data interpretation, provided funding support, and contributed to the writing of the manuscript. N.N.R. provided raw CellSearch data from the HE-cohort, provided respective clinical information and assisted in the analysis. W.T.K. collected the intraoperative samples from the DU-cohort, analysed the clinical data and provided funding. N.H.S. and R.P.L.N. conceptualized the project, designed the study, analysed the data, provided funding support and were major contributors to the writing of the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Collection of samples from the DU-cohort was approved by the Ethics Committees of the Medical Faculty of the Heinrich Heine University Duesseldorf (Ref-No: 4664/2016). The samples from the HE-cohort are part of a study published by NNR [23], and at that time approved by the Ruprecht Karls University Heidelberg. The healthy control samples were collected as part of the IMMC06 clinical trial (NCT00133913) [8, 37] and the independent M0 R0 cohort from the IMMC-26 study [20], both at that time approved by the institutional review boards of the participating centres. All individuals provided written informed consent prior to participation.

Consent for publication

Not applicable.

Competing interests

N.H.S. declares financial ties to Menarini Silicon Biosystems (CellSearch Assay) in the form of third-party funding for research support, as well as to Illumina (NGS products) in the form of lecture fees. The other authors declare that they have no competing interests.

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Publisher's note

Supplementary Information

Phenotypic diversity of CTCs and tdEVs in liquid biopsies of tumour-draining veins is linked to poor prognosis in colorectal cancer

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Table of Contents

Supplementary Methods

Validation of ACCEPT criteria for enumeration of CellSearch-CTCs from CRC samples

For more automated and user-unbiased enumeration of CellSearch-CTCs (CS-CTCs), we employed the ACCEPT tool (Zeune2017). In order to validate the ACCEPT settings previously used for identification of CTCs (ACCEPT-UT settings) (**Suppl. Table 5**) (Nanou2018), we analysed CellSearch results of a total of 395 samples from GI/lung tumour patients and 93 control individuals with ACCEPT and compared the ACCEPT-CTC counts to counts obtained upon manual enumeration, according to the standard CS protocol (**Suppl. Fig. 1**). The described ACCEPT settings (**Suppl. Table 5**) resulted in a substantial increase in the CTC counts compared to those obtained with manual enumeration (**Suppl. Fig. 1**). We noted that this was due to the inclusion of several artefacts which triggered us to adapt the ACCEPT settings to our collective of samples. To the criteria reported previously we added a lower limit for eccentricity in order to exclude events with less round morphologies, and we considered the relation between CK and DAPI signals to exclude events with nucleus area larger than cytoplasm (ACCEPT-DU settings) (**Suppl. Table 5**). Taking the results obtained upon manual CTC enumeration as reference, in the collective of all malignant 362 GI/lung samples the new ACCEPT-DU settings were more specific (92%) and less sensitive (65%) compared to the previous settings (64% and 86%, respectively) (**Suppl. Fig. 1A**). Moreover, the agreement between CTC counts assessed manually and automatically with ACCEPT improved when using the ACCEPT-DU settings compared to the UT settings, as indicated by a higher Cohen's kappa coefficient (0.59 compared to 0.43, $p<0.0001$) and Spearman rank-order correlation (0.67 compared to 0.59, $p<0.0001$) (**Suppl. Fig. 1 A-C**). Furthermore, in samples obtained from healthy donors (N=93), or patients with benign tumours (N=33), the DU settings decreased the positivity rate from 32% to 13%, and from 27% to 12%, respectively, values more in line with the ones obtained manually (3% and 15%, respectively) (**Suppl. Fig. 1 A**). Taken together, the results collected indicate that the ACCEPT-DU settings are more stringent and have a performance more similar to the manual enumeration. This suggests that the new settings can be confidentially used for CTC enumeration in CRC samples.

Visualization of particle diversity in CellSearch-enriched samples

Initial visualization of particle diversity in CellSearch cartridges (**Figure 1B**), was based on all features extracted by ACCEPT (total of nine features per fluorescence channel). Uniform Manifold Approximation and Projection (UMAP) was used to reduce data dimensions to two and to plot the data, using the following settings: "random_state" of 42, "n_neighbors" of 30, "n_components" of 2, "min_dist" of 0.1 (0.4 in some cases) and a Euclidean metric. The total number of CTCs detected was plotted (n=5870), but the number of particles belonging to the other groups was limited to that same number of 5870, for purposes of computational efficiency and better visualization. The class of the particles was determined by the gates in ACCEPT (**Suppl. Table 5**), and this was used as a particle attribute to colour the particles in the final UMAP plot.

Isolation and copy number aberration profiling of single CTCs

To confirm the malignant nature of the identified CTCs we performed genome-wide profiling of chromosomal copy number aberrations (CNAs). For that, we isolated single DAPIpos/CKpos/CD45neg cells from the CellSearch cartridges by flow cytometry using a

MoFlo XDP sorter (Beckman Coulter, Germany) as we previously described (Neves2014). Single cells were sorted into individual empty PCR tubes and stored at -20 °C until analysis.

Whole genome amplification (WGA) was performed using MseI-based adapter-linker PCR as previously described (Klein1999, Stoecklein2002), commercialized as Ampli1TM WGA Kit by Menarini Silicon Biosystems (Bologna, Italy). The quality of the WGA product was evaluated by a control multiplex PCR as previously described (Knijnenburg2007) and good quality was defined as ≥3 signals in the control PCR (Mohlendick2013). Illumina-compatible libraries were prepared from good quality WGA products using Ampli1^{TM} LowPass kit for Illumina (Menarini Silicon Biosystems, Bologna, Italy) according to the manufacturer instructions. For high-throughput and more standardized processing, the manufacturer procedure was implemented in a fully automated workflow on a STARlet Liquid Handling Robot (Hamilton, Reno, NV, USA). Resulting libraries were sequenced on MiSeq or HiSeq instruments (Illumina, Hayward, CA, USA). The WGS-generated sequences were aligned to the human reference genome (hg19) using Burrows-Wheeler Alignment Tool (BWA 0.7.15). CNAs were predicted by using QDNAseq 11.0 with a window size of 500 kb. “Gain” and “loss” calls were filtered out by residual (> 4 standard deviations, SD i.e a default setting) and segmented copy number data of each sample were extracted in log2Ratio values. To assess the quality of the generated profiles, sequence-aligned reads were randomly sub-sampled closer to 200,000 reads and copy number analysis was performed with QDNAseq using a window size of 500 kb. Samples with high Derivative Log Ratio Spread (DLRS >= 0.35) in sub-sampled profiles or samples with high interquartile range (IQR >= 0.35) were excluded from further analyses due to high noise. Samples were classified as aberrant if the whole genome alteration percentage (GAP) was above 2.5%, a threshold defined based on a dataset of WBCs (data not shown).

Basic data visualization

We utilized the ggplot2 package (version “3.4.2”) in R to create violin-, bar-, correlation-, ROC- and forest- plots. GraphPad Prism (Version 7.03, GraphPad Software, San Diego, CA, USA) was also used for data visualization.

Statistical analyses

All the data was organized and analysed using in-house developed R and Python scripts. The Kolmogorov-Smirnov test was used to confirm non-normal distribution of CTC and tdEV counts across the different subsets of patient samples. Non-parametric two-tailed test (Mann – Whitney U-test), Wilcoxon Signed Rank test, and Chi-Square test were used for computing statistical significance for independent, matched, and collectives with nominal values respectively. In Cox regression analyses, the significant variables from the univariate analysis ($p<0.05$) were fit in multivariate. Receiver operating characteristic (ROC) curves (considering the variable “dead/alive”), the respective area under the curve (AUC), as well as the sensitivity, specificity and Youden Index values for different cutoffs were determined using R.

Survival analyses

A vector file containing all metric values of the respective biomarker was analysed by an in-house R script in a loop to automate the analysis of all possible cutoffs. The packages used were: (i) The 'survival' package (version 3.5-5) to perform survival analysis by calculating relevant prognostic values, including hazard ratio and log-rank p-value. (ii) The 'pROC' package (version 1.18.0) to compute area under the curve (AUC) values. The best cutoff chosen was the one that provided the best combination of the lowest p-value in the log-rank test and the highest AUC value in the ROC analysis. The “survminer” package (version “0.4.9”) was chosen to create Kaplan-Meier curves together with risk tables.

Supplementary References

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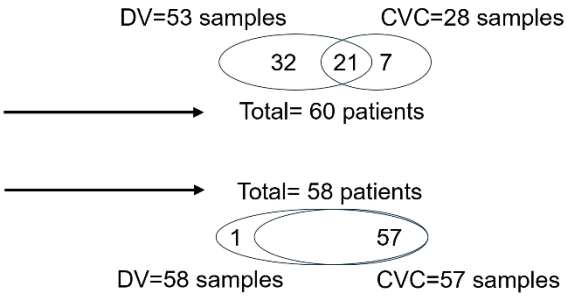
Supplementary Table 1 – Overview of all samples used in this study.

Cohort	Tumor entity	Samples	Patients/Donors
DU-cohort	Colorectal R0 (DV/CVC)	112	84
	Colorectal R0 (DV/CVC) M0	81	60
	Others GI malignant tumors	250	200
	Benign	33	22
HE-cohort	Colorectal R0 (DV/CVC) M0	115	58
	total:	510	364
IMMC06	Healthy	93	93
IMMC026	Colorectal M0 Preoperative	150	150



R0 - cases where successful surgical resection of the tumour margins was achieved; DV - draining vein; CVC - central venous catheter; GI - gastrointestinal

Supplementary Table 2 – ICD-O information on the samples used for the gate validation.

ICDO Classification	ICDO Description	Sample Number
C18; C19; C20	Colorectal (R1)	86
C18; C19; C20	Colorectal (Not Primary; Metastases)	23
C15	Malignant neoplasm of the esophagus	9
C16	Malignant neoplasm of the stomach	25
C17	Malignant neoplasm of the small intestine	2
C22	Malignant neoplasm of the liver and intrahepatic bile ducts	8
C24	Malignant neoplasm of other and unspecified parts of the biliary tract	5
C25	Malignant neoplasm of the pancreas	81
C34	Malignant neoplasm of the bronchus and lung	11
	Total Other Cancer Entities:	**250**
D12.2	Benign neoplasm of the ascending colon	2
D12.5	Benign neoplasm of the sigmoid colon	1
D12.7	Benign neoplasm of the rectum	1
D13.5	Benign neoplasm of the pancreas	2
D13.6	Benign neoplasm of the extrahepatic bile ducts	7
D13.7	Benign neoplasm of the liver and intrahepatic bile ducts	2
D37.7	Neoplasm of uncertain or unknown behavior of the endocrine glands	7
K85.9	Acute pancreatitis, unspecified	2
K86.0	Alcohol-induced chronic pancreatitis	5
K86.1	Other chronic pancreatitis	3
K86.2	Cyst of pancreas	1
	Total Benign:	**33**

Supplementary Table 3 – Clinicopathological information on the patients with colorectal cancer from the DU- and HE-cohorts where successful surgical resection of the tumour margins was achieved (R0) and from whom samples were collected intraoperatively from the draining vein (DV) and/or the central venous catheter (CVC).

		DV=134 samples		CVC = 93 samples	
		DU-cohort DV N=76	**HE-cohort DV N=58**	**DU-cohort CVC N=36**	**HE-cohort CVC N=57**
Time of observation Median in months (range)		24.4 (0.5-75.7)	32.8 (0.3-53.1)	47.5 (0.5-75.7)	33.4 (0.3-53.1)
Age Median in years (range)		68 (35-94)	65.5 (35-86)	70.5 (35-94)	66 (38-86)
Sex	**Male**	35	37	17	36
	Female	41	21	19	21
UICC	**I**	14	17	9	17
	II	17	24	7	23
	III	22	17	12	17
	IV	23	/	8	/
T stage	**1**	11	4	4	4
	2	12	15	7	15
	3	42	37	17	36
	4	11	2	8	2
N stage	**0**	39	41	18	40
	N1-N3	37	17	18	17

Supplementary Table 4 – Clinicopathological information on the M0 patients with colorectal cancer from the DU- and HE-cohorts where successful surgical resection of the tumour margins was achieved (R0) and from whom samples were collected intraoperatively from the draining vein (DV) and/or the central venous catheter (CVC).

		DV=111 samples		**CVC = 85 samples**	
		DU-cohort DV N=53	**HE-cohort DV N=58**	**DU-cohort CVC N=28**	**HE-cohort CVC N=57**
Time of observation Median in months (range)		27.2 (0.8-75.7)	32.8 (0.3-53.1)	48.6 (0.8-75.7)	33.4 (0.3-53.1)
Age Median in years (range)		68 (35-94)	65.5 (35-86)	74.5 (35-94)	66 (38-86)
Sex	**Male**	22	37	13	36
	Female	31	21	15	21
UICC	**I**	14	17	9	17
	II	17	24	7	23
	III	22	17	12	17
T stage	**1**	7	4	4	4
	2	11	15	7	15
	3	31	37	14	36
	4	4	2	3	2
N stage	**0**	31	41	16	40
	N1-N3	22	17	12	17
Neo-adjuvant therapy	**No**	45	40	24	39
	Yes	8	18	4	18
Adjuvant therapy	**No**	NA	44	NA	43
	Yes	NA (~22)	14	NA (~12)	14

DV - draining vein; CVC - central venous catheter; UICC - Union for International Cancer Control

Supplementary Table 5 - Parameters used for enumeration of circulating tumour cells (CTCs), tumour-derived extracellular vesicle (tdEVs), white blood cells (WBCs), lymphocyte-derived extracellular (ldEVs) and bare nuclei using ACCEPT tool and R script.

	Channel (Marker)	Parameter	CTC	tdEV	WBC	ldEV	Bare Nucleus
ACCEPT-UT*	DAPI (DNA)	Mean Intensity	>45	≤5	>30	n.a.	>30
	DAPI (DNA)	Max Intensity	n.a.	n.a.	>50	n.a.	n.a.
	DAPI (DNA)	Size	n.a.	n.a.	>16	n.a.	n.a.
	DAPI (DNA)	Standard Deviation	n.a.	n.a.	n.a.	≤5	n.a.
	PE (CK)	Mean Intensity	>60	>60	n.a.	n.a.	≤5
	PE (CK)	Max Intensity	n.a.	>90	n.a.	n.a.	n.a.
	PE (CK)	Size	>16 and ≤400	≤150	n.a.	n.a.	n.a.
	PE (CK)	Overlay with DNA	>0.2	n.a.	n.a.	n.a.	n.a.
	PE (CK)	Perimeter	n.a.	>5	n.a.	n.a.	n.a.
	PE (CK)	P2A	n.a.	≤1	n.a.	n.a.	n.a.
	PE (CK)	Eccentricity	n.a.	≤0.8	n.a.	n.a.	n.a.
	PE (CK)	Standard Deviation	n.a.	n.a.	≤5	≤5	n.a.
	APC (CD45)	Mean Intensity	≤5	≤5	>30	>30	≤5
	APC (CD45)	Max Intensity	n.a.	n.a.	>50	>50	n.a.
	APC (CD45)	Perimeter	n.a.	n.a.	n.a.	>5	n.a.
	APC (CD45)	Size	n.a.	n.a.	n.a.	≤150	n.a.
	APC (CD45)	Eccentricity	n.a.	n.a.	n.a.	≤0.85	n.a.
	DiOC (Empty) 1	Mean Intensity	≤5	≤5	≤5	≤5	≤5
	PerCP (Empty) 2	Mean Intensity	≤5	≤5	≤5	≤5	≤5
ACCEPT-DU	PE (CK) (in ACCEPT)	Eccentricity	<0.9	n.a.	n.a.	n.a.	n.a.
Additional criteria	CK / DNA (R script)	Size	CK > DNA	n.a.	n.a.	n.a.	n.a.
	CK / DNA (R script)	Mean Intensity	CK > DNA or CK >150	n.a.	n.a.	n.a.	n.a.

Notes: *Values for CTCs and tdEVs were from Nanou2018 and values for WBCs and ldEVs were from Nanou2019; CTC - circulating tumour cells; tdEV - tumour-derived extracellular vesicle; WBC - white blood cell; ldEV - lymphocyte-derived extracellular vesicle

Supplementary Figure 1

A

Group	Samples	Settings	Detection	Median (mean;range)	Sens.	Spec.	C.K.	S. C.	S.C. p-value
CRC+Othermalignant tumours	362	ACCEPT-DU	26.24%	0 (8.7348; 0-1790)	0.65	0.92	0.59	0.68	<0.0001
		ACCEPT-UT	52.21%	1 (18.7818; 0-3334)	0.86	0.64	0.43	0.59	<0.0001
		Manual	32.04%	0 (32.0635; 0-8284)	/	/	/	/	/
Benign	33	ACCEPT-DU	12.12%	0 (0.1212; 0-1)	0.20	0.89	0.10	0.09	1
		ACCEPT-UT	27.27%	0 (0.7879; 0-11)	0.60	0.79	0.29	0.38	0
		Manual	15.15%	0 (0.1818; 0-2)	/	/	/	/	/
Healthy	93	ACCEPT-DU	12.9%	0 (0.1505; 0-2)	0.67	0.89	0.23	0.31	0.0027
		ACCEPT-UT	32.26%	0 (0.6667; 0-20)	1.00	0.70	0.13	0.32	0.0018
		Manual	3.23%	0 (0.0323; 0-1)	/	/	/	/	/

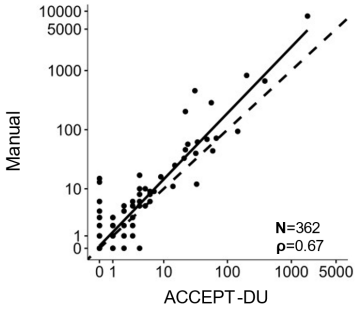
Notes: Detection - Detection rate; Sens. - Sensitivity; Spec. - Specificity; C.K. - Cohens Kappa; S. C. - Spearman rank order correlation coefficient; S. C. p-value - Spearman rank order correlation p-value

B

		ACCEPT-DU		Chi^2	Chi^2 (Yates corr)
		Neg	Pos		
Manual counting	Neg	226	20	130.12	127.22
	Pos	41	75		

		ACCEPT-UT		Chi^2	Chi^2 (Yates corr)
		Neg	Pos		
Manual counting	Neg	157	89	79.07	77.07
	Pos	16	100		

C



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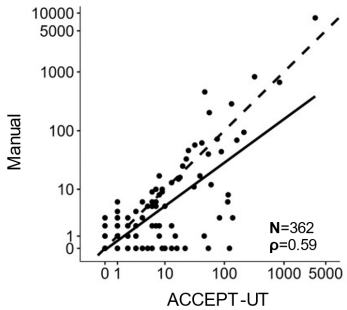
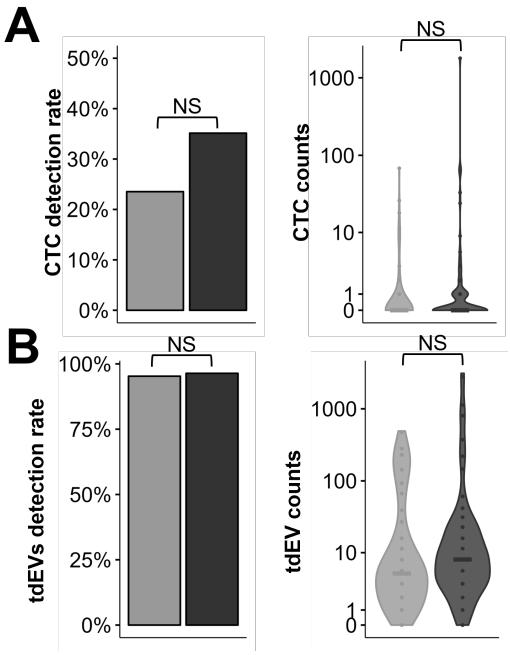


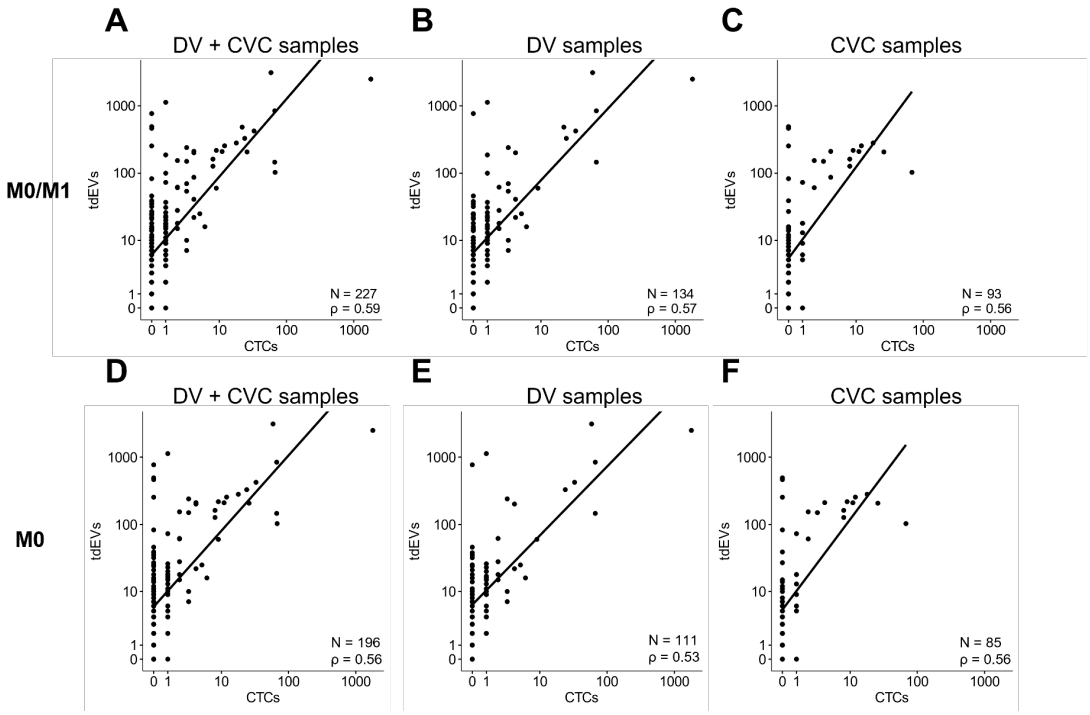
Suppl. Figure 1 – Validation of the ACCEPT-DU gates. (A) CellSearch-CTC counts obtained with ACCEPT software using the settings previously described (ACCEPT-UT), the new settings (ACCEPT-DU) and upon manual enumeration (Manual). Considered were 362 R0 CRC (DV/CVC) and other malignant tumours from the DU-cohort, the 33 benign from the same cohort, and the 93 samples from healthy donors of the IMMC06 cohort. Statistics for ACCEPT-DU and ACCEPT-UT were done using the manual counts as reference. **(B)** Sample positivity and negativity determined with the manual settings and with the ACCEPT-DU and ACCEPT-UT settings in the 362 samples from patients with malignant tumours as in (A). **(C)** Counts obtained manually and with the ACCEPT-DU settings for the complete collective of 362 samples as in (A). **(D)** Counts obtained manually and with the ACCEPT-UT settings for the complete collective of 362 samples as in (A).

Supplementary Figure 2



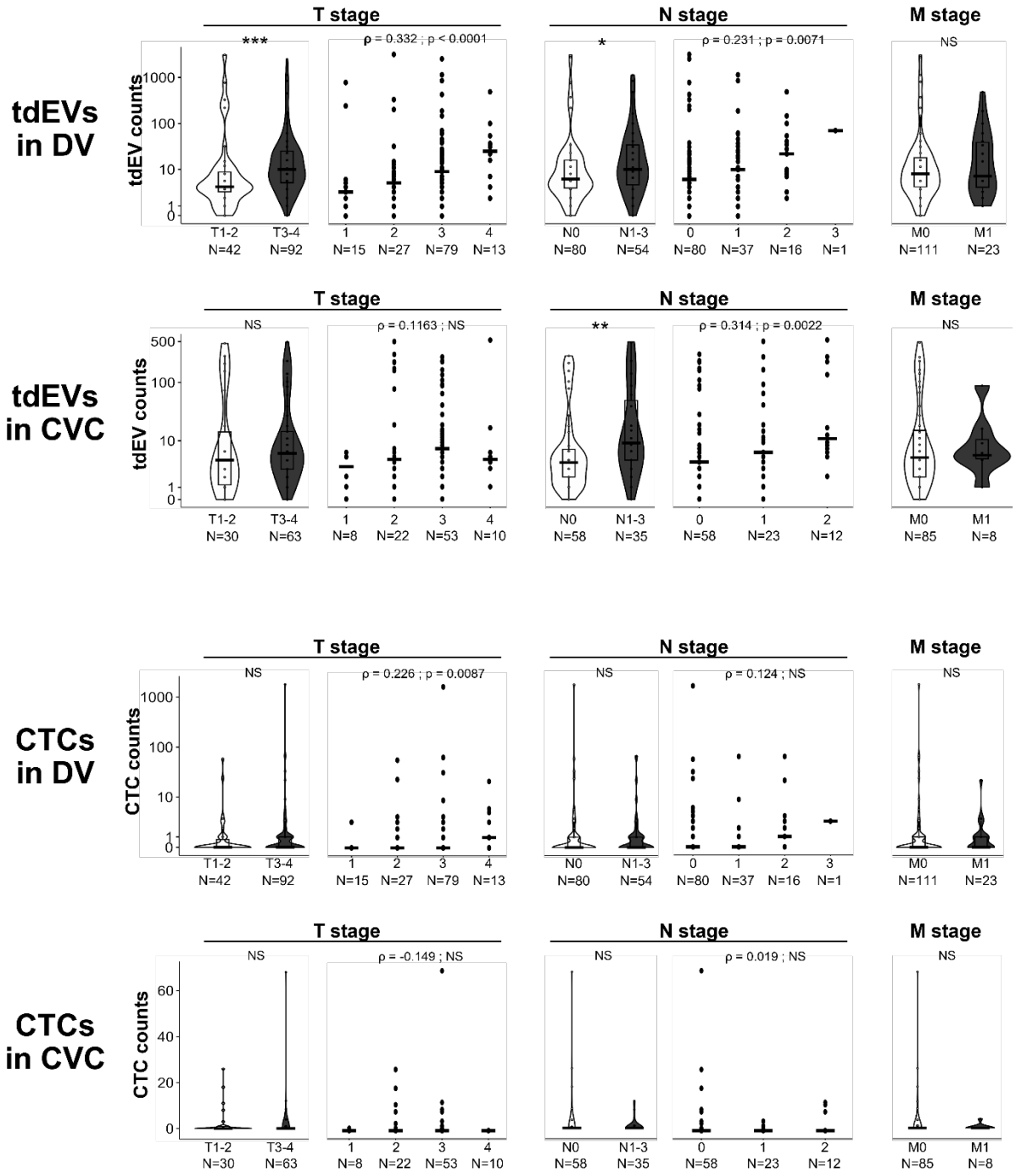
Suppl. Figure 2 – Counts and detection rate of CTCs and tdEVs (A) Detection rates (positivity rates) and counts of CTCs and **(B)** tdEVs in samples from CRC R0 M0 patients from the combined DU+HE cohort (N=85 CVCs; N=111 DVs). The horizontal lines represent the median.

Supplementary Figure 3



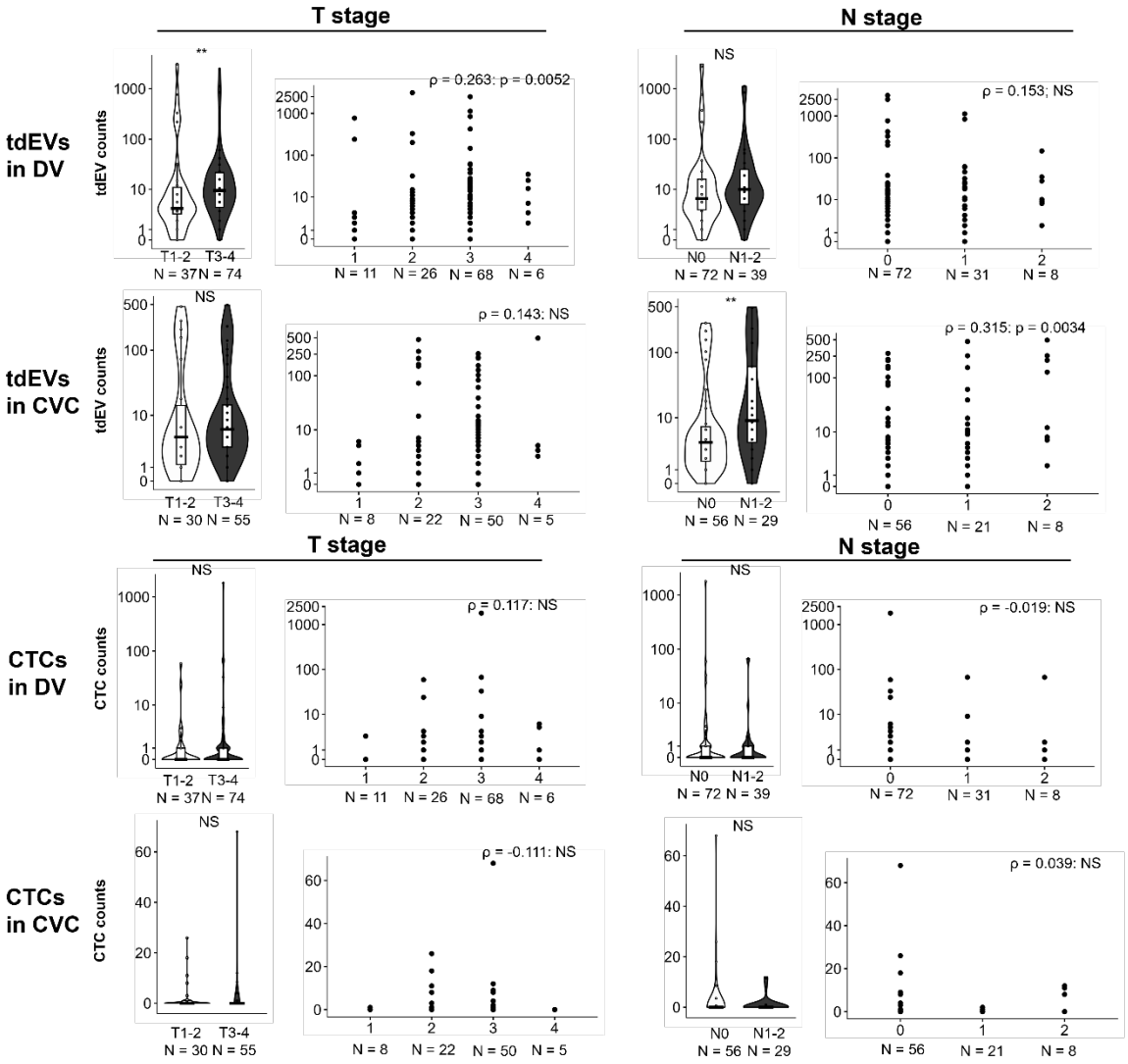
Suppl. Figure 3 – Correlation between CTCs and tdEVs in samples from colorectal R0 patients from the DU+HE cohort. (A+D) Draining vein (DV) and central venous catheter (CVC) samples (M0/M1 N = 227, p <0.0001; M0 N = 196, p <0.0001). **(B+E)** DV samples (M0/M1 N = 134, p <0.0001; M0 N = 111, p <0.0001). **(C+F)** CVC samples (N = 93, p <0.0001; M0 N = 85, p <0.0001).

Supplementary Figure 4



Suppl. Figure 4 – Association between tdEVs and CTCs detected in the draining vein (DV) and central venous catheter (CVC), and clinicopathological features. Particle counts in the collective of patients with colorectal cancer where successful surgical resection of the tumour margins was achieved (R0) and their association with pT-, M- and pN-staging of the respective patients. The statistics for grouped stages were performed with Mann-Whitney U testing while the statistics for individual stages were performed with Spearman correlation analyses.

Supplementary Figure 5



Suppl. Figure 5 – Association between tdEVs and CTCs detected in the draining vein (DV) and central venous catheter (CVC), and clinicopathological features of M0 patients. Particle counts in the collective of patients with M0 colorectal cancer where successful surgical resection of the tumour margins was achieved (R0) and their association with pT- and pN-, stages of the respective patients. The statistics for grouped stages were performed with Mann-Whitney U testing while the statistics for individual stages were performed with Spearman correlation analyses.

Supplementary Figure 6

A tdEVs in CVC (N = 85)

Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	4	0.70	7.36	0.0270	51.8	34.3	18 (64%)	0.90	0.50
HE	4	0.65	4.58	0.1227	37.1	27.8	34 (60%)	0.86	0.44
DU+HE	4	0.67	5.95	0.0073	41.6	30.0	52 (61%)	0.88	0.46

B CTCs in DV (N = 111)

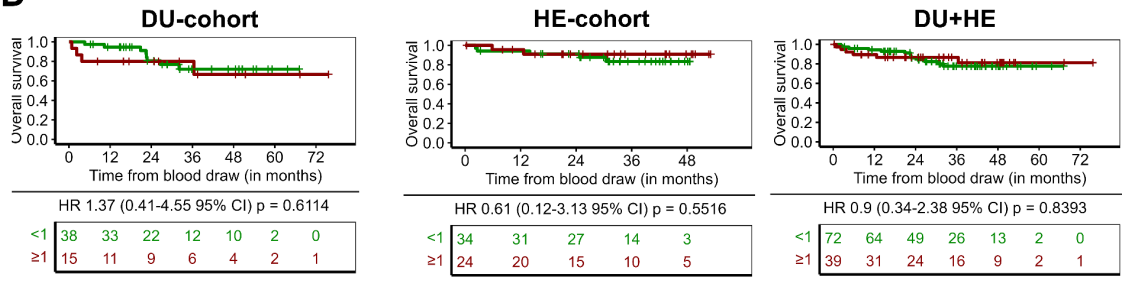
Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	1	0.53	1.37	0.6100	31.7	29.9	15	0.33	0.73
HE	1	0.43	0.61	0.5475	31.6	31.0	24	0.29	0.57
DU+HE	1	0.48	0.90	0.8392	31.6	30.6	39	0.32	0.64

C CTCs in CVC (N = 85)

Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	1	0.52	1.19	0.8687	40.5	41.8	2 (7%)	0.10	0.94
HE	1	0.73	7.47	0.0060	35.2	23.6	18 (32%)	0.71	0.74
DU+HE	1	0.57	2.37	0.0823	37.3	25.4	20 (24%)	0.35	0.79

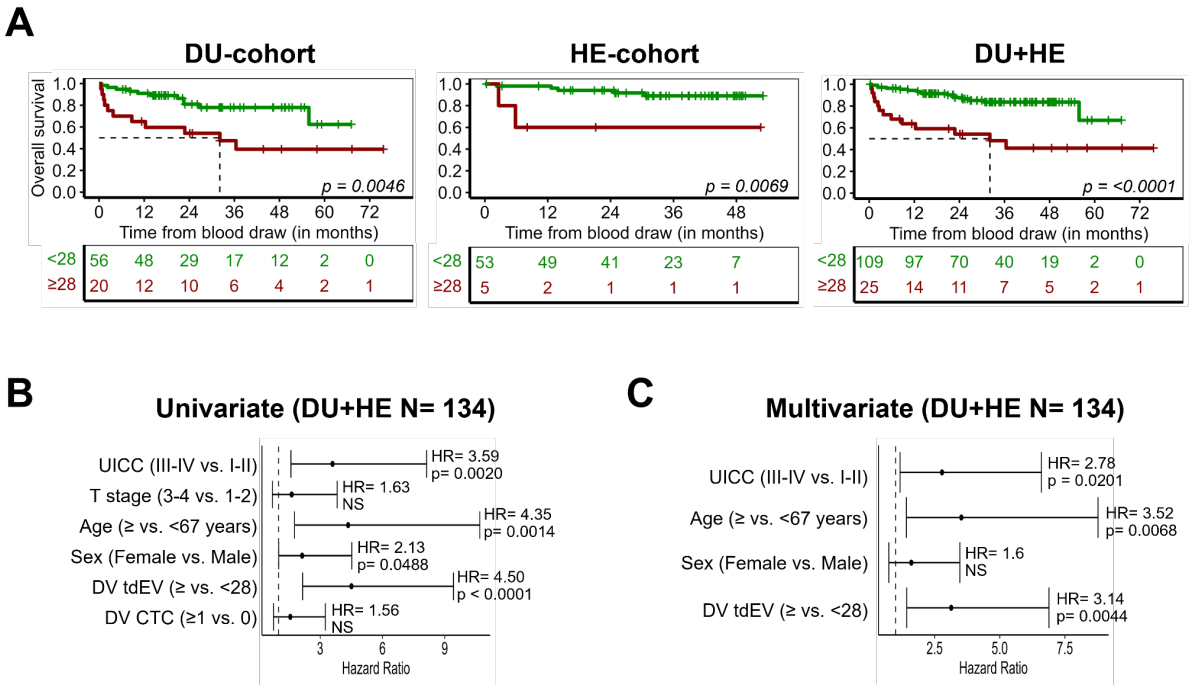
AUC - area under the curve; OS - overall survival

D



Suppl. Figure 6 – CTC and tdEV cutoffs of M0 patients. (A) Most relevant cutoffs for tdEV counts in CVC samples of M0 patients from the DU-cohort (N=28) and validation on the HE (N=57) and DU+HE cohorts (N=85). **(B)** Most relevant cutoffs for CTC counts detected in DV samples of M0 patients from the DU-cohort (N=53) and validation on the HE (N=58) and DU+HE cohorts (N=111). **(C)** Most relevant cutoffs defined for CTC counts detected in CVC samples of M0 patients from the DU-cohort (N=28) and validation on the HE (N=57) and DU+HE cohorts (N=85). **(D)** Kaplan-Meier estimates of overall survival for patients dichotomized based on the absence or presence (≥1 CTC cutoff) in DV samples of the DU- (N=53), HE- (N=58), and DU+HE (N=111) cohorts of M0 patients.

Supplementary Figure 7



Suppl. Figure 7 – Prognostic value of CS-tdEVs detected in DV samples of CRC R0 patients. (**A**) Kaplan-Meier estimates of overall survival for patients dichotomized on the basis of the 28 tdEV cut-off in the DU (N=76), HE (N=58), and DU+HE (N=134) cohorts of patients. (**B**) Univariate analysis of clinicopathological factors (including the ≥28 tdEV cut-off) in the DU+HE cohort of patients (N=134). (**C**) Multivariate analysis of clinicopathological factors, including the ≥28 tdEV cut-off, in the DU+HE cohort of patients (N=134).

Supplementary Figure 8

A tdEVs in CVC

Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	4	0.69	7.22	0.0265	51.6	34.6	25 (69%)	0.93	0.45
HE	4	0.65	4.58	NS	37.1	27.8	34 (60%)	0.86	0.44
DU+HE	4	0.67	6.29	0.0048	41.8	30.7	59 (63%)	0.90	0.44

AUC - area under the curve; OS - overall survival

B CTCs in DV

Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	1	0.61	2.33	0.0414	30.1	23.9	26 (34%)	0.5	0.72
HE	1	0.43	0.61	NS	31.6	31.0	24 (41%)	0.29	0.57
DU+HE	1	0.55	1.56	NS	30.7	27.3	50 (37%)	0.45	0.65

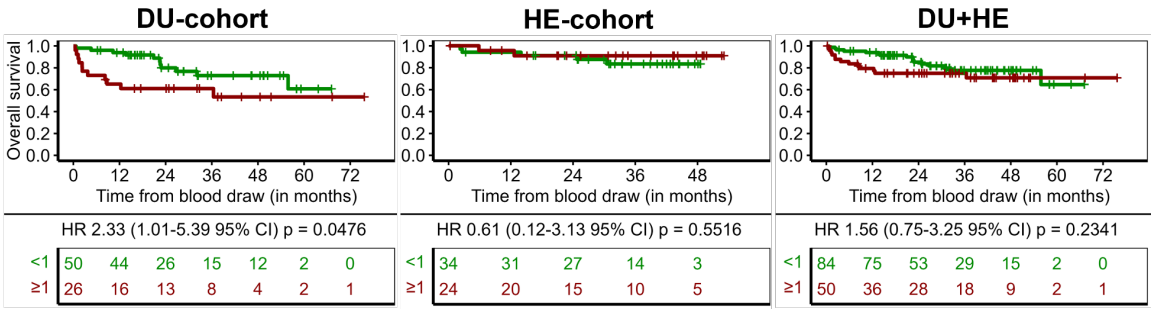
AUC - area under the curve; OS - overall survival

C CTCs in CVC

Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	4	0.54	8.24	0.0241	40.6	12.3	1 (3%)	0.0714	1
HE	4	0.65	5.29	0.0156	33.2	22.8	9 (16%)	0.43	0.88
DU+HE	4	0.55	3.26	0.0280	36.3	21.8	10 (11%)	0.19	0.92

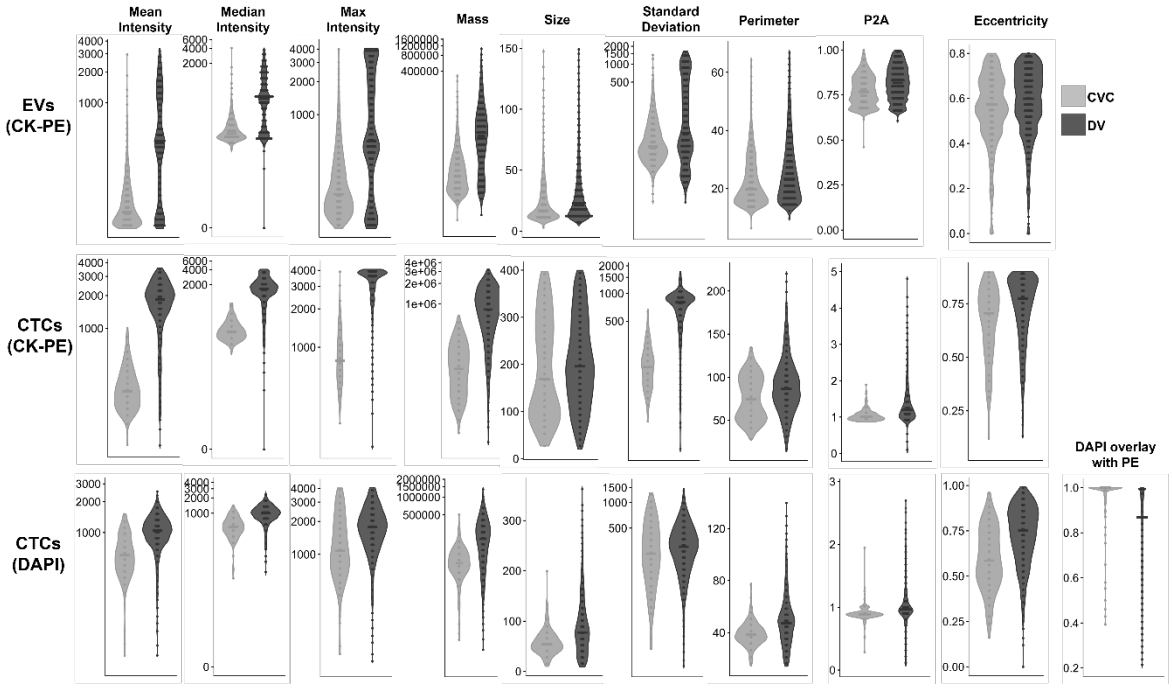
AUC - area under the curve; OS - overall survival

D



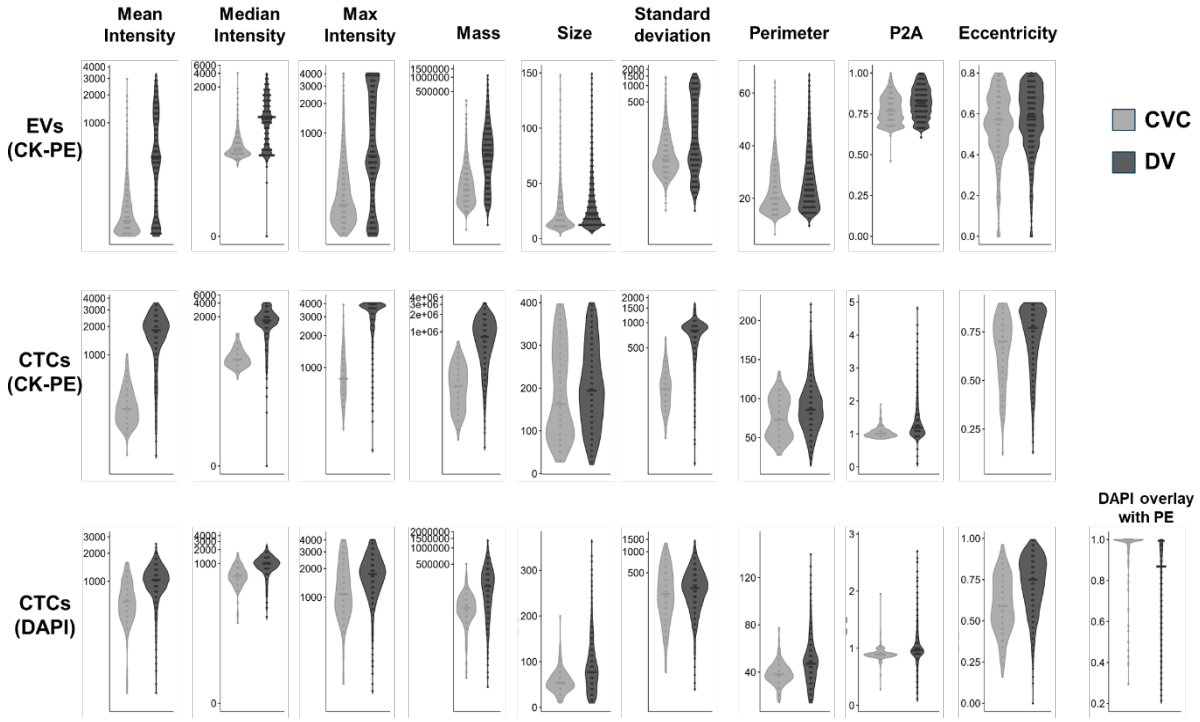
Suppl. Figure 8 – CTC and tdEV cutoffs. (A) Most relevant cutoffs for tdEV counts in CVC samples of patients from the DU-cohort (N=36) and validation on the HE (N=57) and DU+HE cohorts (N=93). **(B)** Most relevant cutoffs for CTC counts detected in DV samples of patients from the DU-cohort (N=76) and validation on the HE (N=58) and DU+HE cohorts (N=134). **(C)** Most relevant cutoffs defined for CTC counts detected in CVC samples of patients from the DU-cohort (N=36) and validation on the HE (N=57) and DU+HE cohorts (N=93). (**D**) Kaplan-Meier estimates of overall survival for patients dichotomized based on the absence or presence (≥1 CTC cutoff) in DV samples of the DU- (N=76), HE- (N=58), and DU+HE (N=134) cohorts of patients.

Supplementary Figure 9



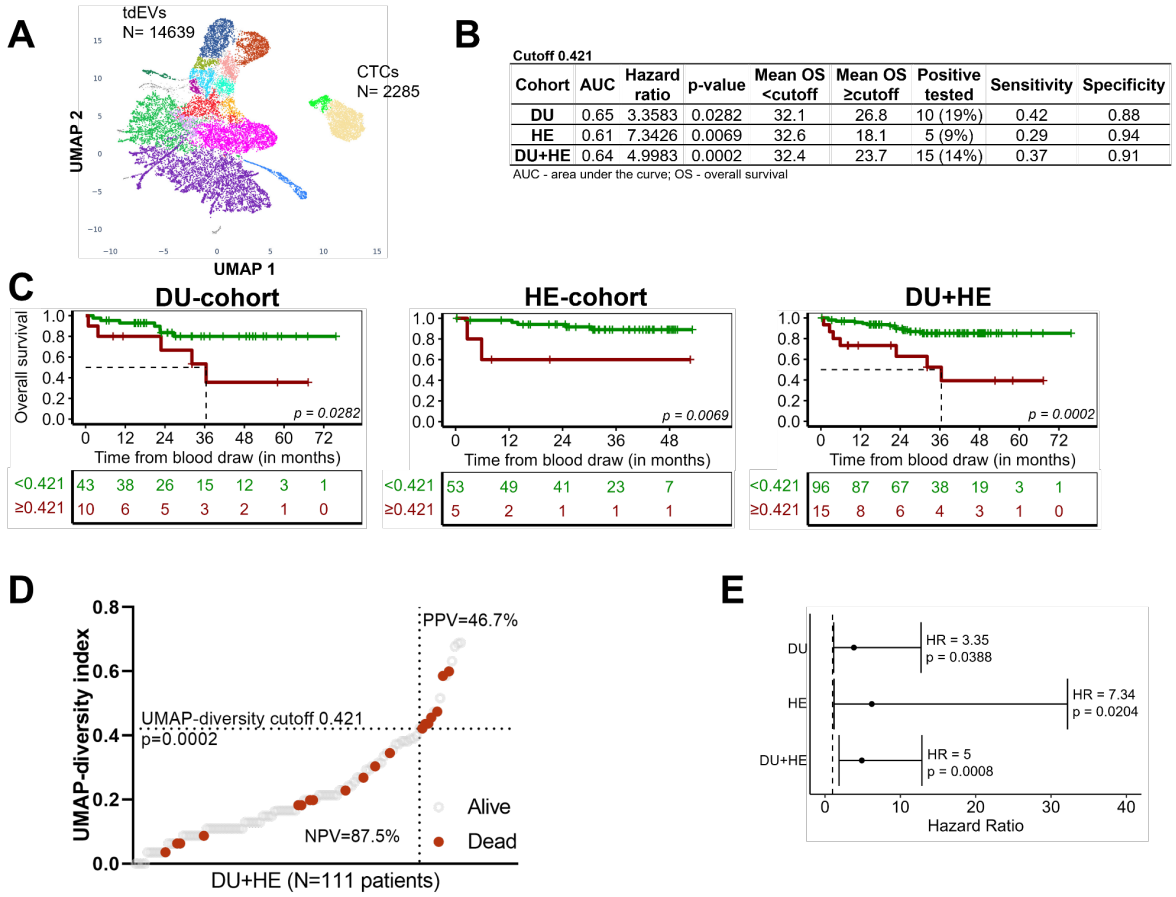
Suppl. Figure 9 – Phenotypic characterization of tdEVs and CTCs in draining vein (DV) and central venous catheter (CVC) samples of M0 R0 patients. Quantification by ACCEPT of nine parameters extracted from the Phycoerythrin (PE) signal detected in tdEVs and CTCs, as well as nine parameters extracted from the DAPI signal in CTCs. In addition, there is also the overlay of DAPI and PE signals determined with R-script based on the ACCEPT data. For analysis were considered R0 M0 CRC patients of the DU+HE cohort (DV= 111 samples, CVC= 85 samples) containing N=10756 DV-tdEVs; N=3883 CVC-tdEVs; N=2106 DV-CTCs; N=179 CVC-CTCs. Statistical tests to compare the quantified parameters between the CVC and DV were highly significant ($p < 0.0001$) aside from the comparison of DAPI standard deviation for CTCs ($p < 0.05$).

Supplementary Figure 10



Suppl. Figure 10 – Phenotypic characterization of tdEVs and CTCs in draining vein (DV) and central venous catheter (CVC) samples. Quantification by ACCEPT of nine parameters extracted from the Phycoerythrin (PE) signal detected in tdEVs and CTCs, as well as nine parameters extracted from the DAPI signal in CTCs. In addition, there is also the overlay of DAPI and PE signals determined with R-script based on the ACCEPT data. For analysis were considered R0 CRC patients of the DU+HE cohort (DV= 134 samples, CVC= 93 samples) containing N=11866 DV-tdEVs; N=4016 CVC-tdEVs; N=2145 DV-CTCs; N=183 CVC-CTCs. Statistical tests to compare the quantified parameters between the CVC and DV were highly significant ($p < 0.0001$) aside from two comparisons of PE size and DAPI standard deviation for CTCs ($p < 0.05$).

Supplementary Figure 11



Cohort	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	0.65	3.3583	0.0282	32.1	26.8	10 (19%)	0.42	0.88
HE	0.61	7.3426	0.0069	32.6	18.1	5 (9%)	0.29	0.94
DU+HE	0.64	4.9983	0.0002	32.4	23.7	15 (14%)	0.37	0.91

AUC - area under the curve; OS - overall survival

Suppl. Figure 11 – Shannon diversity calculated for CTCs and tdEVs found in the draining vein samples of M0 patients. (A) UMAP distribution of tdEVs and CTCs found in the DU+HE cohort, and the 18 clusters defined according to the UMAP clustering tool. **(B)** Most relevant Shannon diversity index cutoff. **(C)** Kaplan-Meier estimates of overall survival (OS) for patients dichotomized based on the Shannon diversity cutoff of 0.4214 in the DU (N=53), HE (N=58), and DU+HE (N=111) cohorts of patients. **(D)** Shannon diversity index calculated for each of the patients of the DU+HE cohort (N=111) showing the positive predictive value (PPV) and negative predictive value (NPV) of the cutoff 0.4214 as a biomarker. In red are indicated the patients that died. **(E)** Hazard ratio (HR) of patients with Shannon diversity index ≥0.4214.

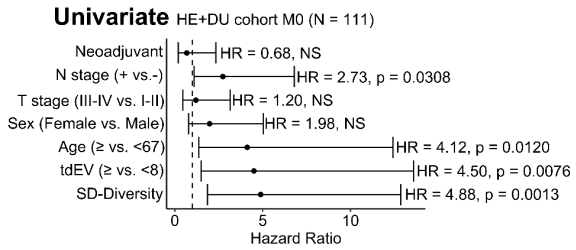
Supplementary Figure 12

A

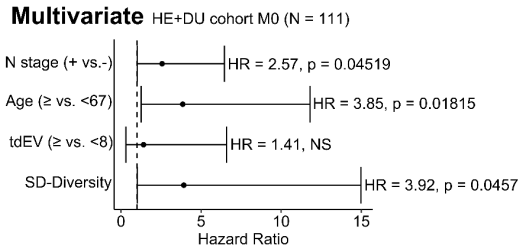
Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	0.6389	0.70	3.83	0.01839	31.6	30.4	19 (36%)	0.67	0.73
HE	0.6389	0.71	6.20	0.01320	34.4	25.6	20 (34%)	0.71	0.71
DU+HE	0.6389	0.70	4.88	0.00038	33.1	27.9	39 (35%)	0.68	0.72

AUC - area under the curve; OS - overall survival

B

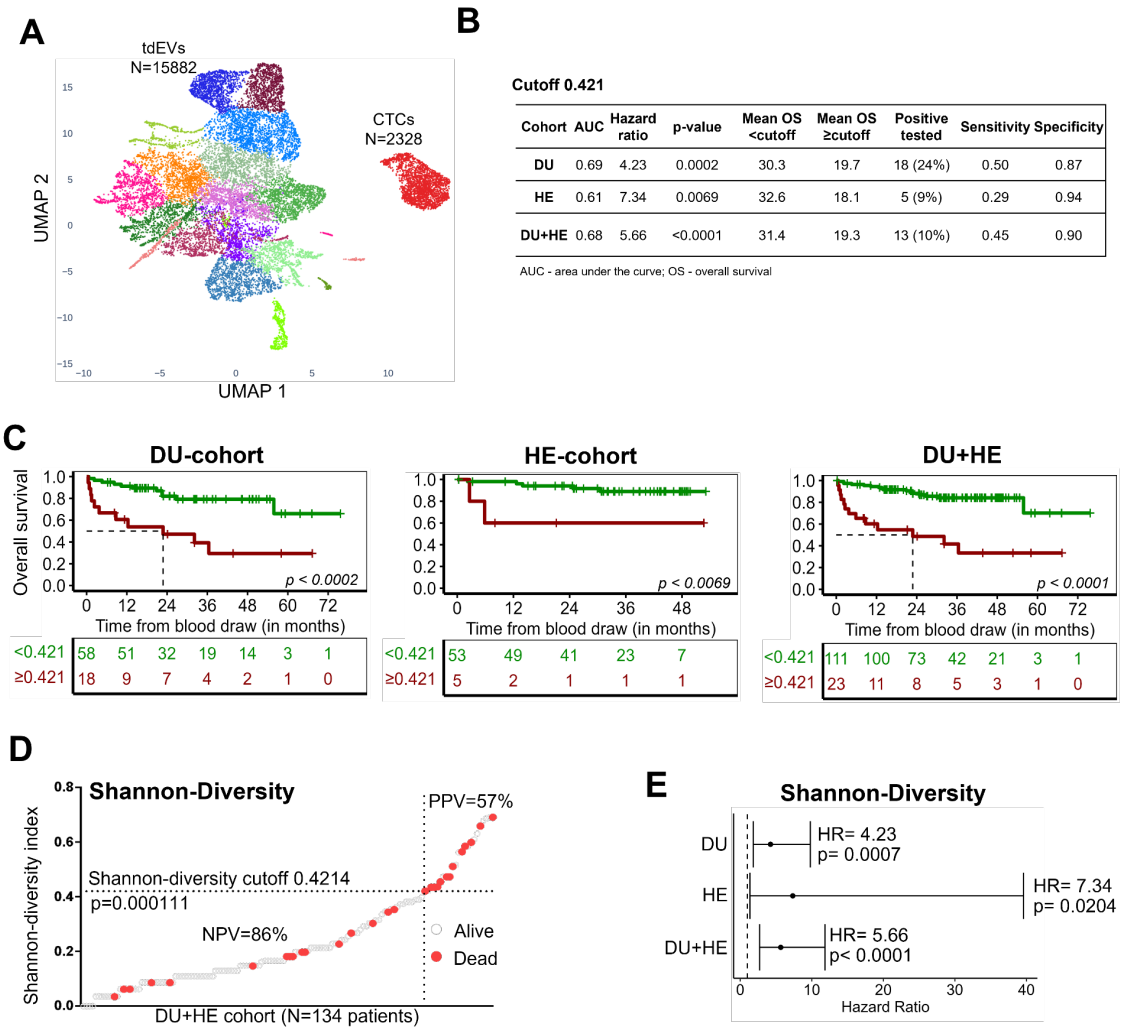


C



Suppl. Figure 12 – SD-diversity calculated for CTCs and tdEVs found in the DV samples of R0 M0 patients. (A) Most relevant SD-diversity index cutoff tested in the DU, HE and DU+HE cohorts. **(B)** Association of clinicopathological features, tdEVs and SD-diversity (≥0.6389) with the survival of patients from the DU+HE-cohort (N=111) by univariate analysis. **(C)** Multivariate analysis including DV tdEV count and SD-diversity in the model of patients from the DU+HE-cohort (N=111).

Supplementary Figure 13



Cohort	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
DU	0.69	4.23	0.0002	30.3	19.7	18 (24%)	0.50	0.87
HE	0.61	7.34	0.0069	32.6	18.1	5 (9%)	0.29	0.94
DU+HE	0.68	5.66	<0.0001	31.4	19.3	13 (10%)	0.45	0.90

AUC - area under the curve; OS - overall survival

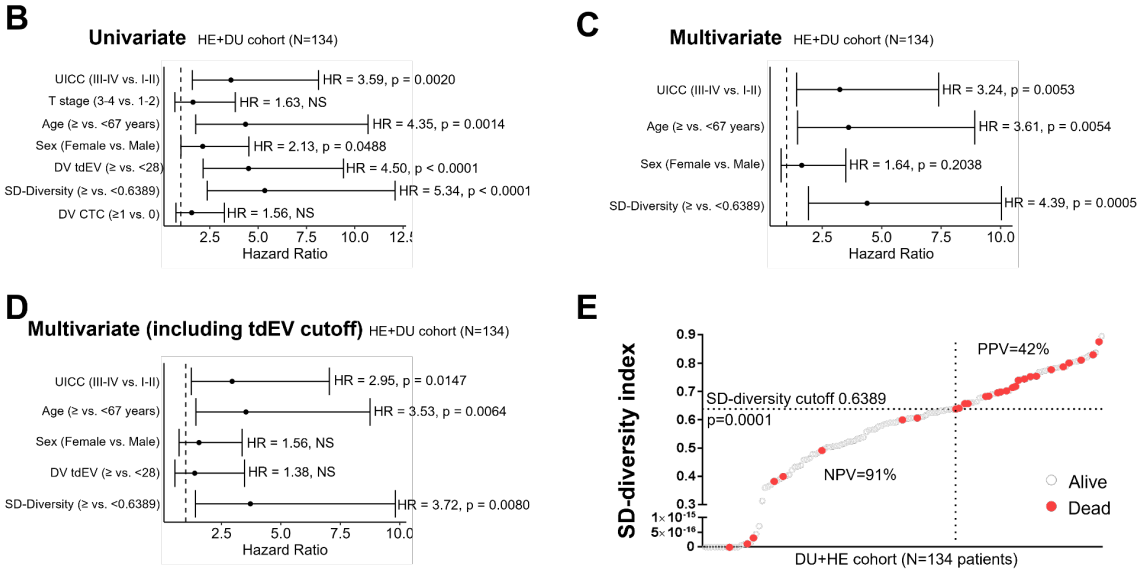
Suppl. Figure 13 – Shannon diversity calculated for CTCs and tdEVs found in the draining vein (DV) samples (UICC I-IV). (**A**) UMAP distribution of tdEVs and CTCs found in the DU+HE cohort, and the 18 clusters defined according to the UMAP clustering tool. (**B**) Most relevant Shannon diversity index cutoff. (**C**) Kaplan-Meier estimates of OS for patients dichotomized based on the Shannon diversity cutoff of 0.4214 in the DU (N=76), HE (N=58), and DU+HE (N=134) cohorts of patients. (**D**) Shannon diversity index calculated for each of the patients of the DU+HE cohort (N=134) showing the positive predictive value (PPV) and negative predictive value (NPV) of the cutoff 0.4214 as a biomarker. In red are indicated the patients that died. (**E**) Hazard ratio (HR) of patients with Shannon diversity index ≥0.4214.

Supplementary Figure 14

A

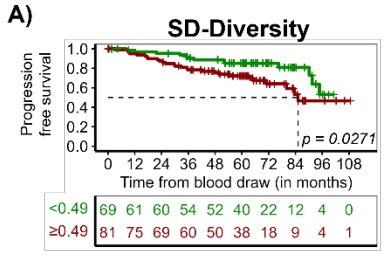
	Cohort	Cutoff	AUC	Hazard ratio	p-value	Mean OS <cutoff	Mean OS ≥cutoff	Positive tested	Sensitivity	Specificity
SD-diversity	DU	0.6389	0.73	4.66	0.0004	29.5	25.5	30 (39%)	0.73	0.74
	HE	0.6389	0.71	6.20	0.0132	34.4	25.6	20 (34%)	0.71	0.71
	DU+HE	0.6389	0.72	5.34	<0.001	31.7	25.5	50 (37%)	0.72	0.72

AUC - area under the curve; OS - overall survival



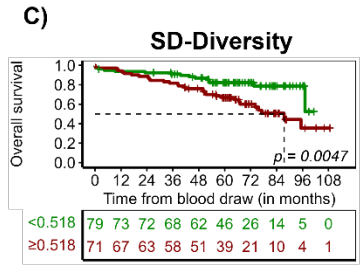
Suppl. Figure 14 – SD-diversity calculated for CTCs and tdEVs found in the DV samples (UICC I-IV). (A) Most relevant SD-diversity index cutoff tested in the DU, HE and DU+HE cohorts. **(B)** Association of clinicopathological features, tdEVs, CTCs and SD-diversity (≥0.6389) with the survival of patients from the DU+HE-cohort (N=134) by Univariate analysis. **(C)** Multivariate analysis excluding the DV tdEV count from the model. **(D)** Multivariate analysis including DV tdEV count and SD-diversity in the model. **(E)** SD-diversity index calculated for each of the N=134 patients of the DU+HE cohort showing the positive predictive value (PPV) and negative predictive value (NPV) of the cutoff 0.6389 as a biomarker. In red are indicated the patients that died.

Supplementary Figure 15



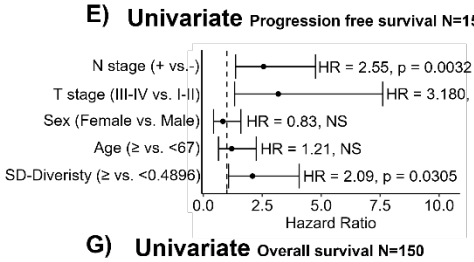
B) SD-Diversity – Progression free survival

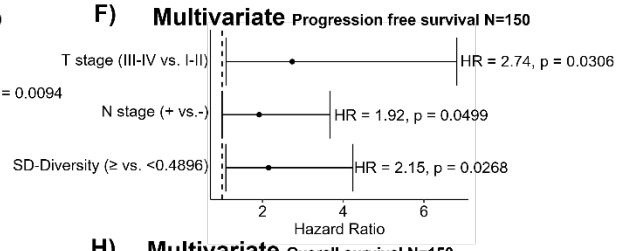
Cohort	Cutoff	AUC	Hazard ratio	p-value	Positive tested	Sensitivity	Specificity
IMMC026	0.4437	0.58	2.05	0.0419	91	0.73	0.44
	0.4537	0.59	2.10	0.0349	90	0.73	0.45
	0.4815	0.58	2.01	0.0417	87	0.70	0.46
	0.4841	0.58	1.99	0.0395	83	0.68	0.49
	0.4851	0.59	2.05	0.0318	82	0.68	0.50
	0.4896	0.59	2.09	0.0271	81	0.68	0.51
	0.4905	0.58	1.97	0.0385	79	0.65	0.52
	0.4938	0.59	2.01	0.0331	78	0.65	0.53
	0.5116	0.59	1.93	0.0413	75	0.63	0.55
	0.5175	0.59	1.90	0.0433	71	0.60	0.57

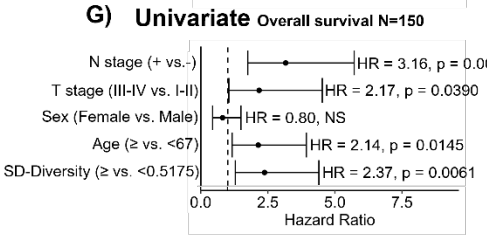


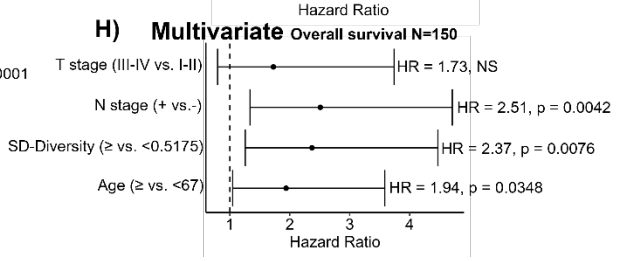
D) SD-Diversity – Overall survival

Cohort	Cutoff	AUC	Hazard ratio	p-value	Positive tested	Sensitivity	Specificity
IMMC026	0.4896	0.63	2.34	0.0074	81 (54%)	0.72	0.54
	0.4938	0.63	2.25	0.0095	78 (52%)	0.70	0.56
	0.5116	0.63	2.19	0.0108	75 (50%)	0.67	0.58
	0.5123	0.63	2.25	0.0080	74 (49%)	0.67	0.59
	0.5164	0.64	2.30	0.0064	73 (49%)	0.67	0.60
	0.5174	0.64	2.33	0.0056	72 (48%)	0.67	0.61
	0.5175	0.64	2.37	0.0047	71 (47%)	0.67	0.62
	0.5237	0.63	2.17	0.0106	70 (47%)	0.65	0.62
	0.5321	0.64	2.25	0.0071	69 (46%)	0.65	0.63
	0.5383	0.63	2.09	0.0136	68 (45%)	0.63	0.63









Suppl. Figure 15 – SD-diversity calculated for CTCs and tdEVs found in preoperative samples of the IMMC-26 study. (A) Kaplan-Meier estimate of progression-free survival (PFS) for patients dichotomized based on the SD-diversity diversity cutoff of 0.49 in the IMMC-26 cohort (N=150). **(B)** Most relevant SD-diversity cutoffs based on PFS. **(C)** Kaplan-Meier estimate of overall survival for patients dichotomized based on the SD-diversity cutoff of 0.52 in the IMMC-26 cohort (N=150). **(D)** Most relevant SD-diversity cutoffs based on OS. **(E)** Association of clinicopathological features including the SD-diversity cutoff of 0.49 with PFS of patients from the IMMC-26 study. **(F)** Multivariate analysis including SD-diversity based on PFS. **(G)** Association of clinicopathological features including the SD-diversity cutoff of 0.49 with the OS of patients from the IMMC-26 study. **(H)** Multivariate analysis including SD-diversity based on OS

3. Additional research

3.1. Real-World Problems Associated with CellSearch Analysis

Extracting the informative value of CS cartridges for enumeration and statistical purposes is operator-dependent and time-consuming. Long lists of samples are frequently reviewed, and each data frame is manually copied, which is time-consuming. In addition, the human-led documentation of the respective patient ID, cartridge number, and other information is problematic, mainly due to the thousands of samples being processed yearly. Consequently, incorrect documentation and, thus, incorrect evaluation are virtually unavoidable. A human operator-dependent procedure will become increasingly time-consuming and error-prone in light of a steadily growing number of CS samples from various studies. Furthermore, automatic enumeration platforms for CS samples like ACCEPT are limited in their informative potential due to rigid gate settings and the impossibility of calculating the diversity of the total tumour load.

3.2. Developing a User-friendly App

A solution to the error-prone and time-consuming manual process by human operators might be a tool that automates these processes. I created a Shiny app using R-scripts to automatically extract the CS information stored in .xml files and the cartridge files. The application also includes the option to automatically enumerate ACCEPT objects and calculate a SI diversity index of the combined tumour. On top of that, the user can visually depict the distribution and correlation of ACCEPT parameters for a specifically chosen data set. Aside from preparing CS data for further analysis, the app also allows the user to assess the association of enumerated events with clinicopathological data by providing various statistical metrics and selecting the best-performing ones.

3.2.1. App Capabilities

The app includes four major options for evaluating CS data (**Figure 14**, **Figure 15**). To obtain a tabular overview of the CS information of specific .iso files, the user must enter the path to the .iso files and the path of the desired storage location. The app automatically generates a tabular output after extracting the relevant CS information stored in the respective .xml files. This output includes the sample type, cartridge, patient ID, scan date, disk number, and implemented extra marker. The second option allows the user to extract all or user-defined cartridges. As an outcome, the cartridges are automatically transferred into a folder for evaluation by platforms like ACCEPT. The third option enables the enumeration of multiple ACCEPT objects and produces a tabular output. Complementing the ACCEPT platform, the application allows the user to set more complex gate settings and calculate a diversity index of the combined tumour load. Beyond that, the user can optionally specify an extra marker (e.g., PSMA or HER2), which automatically applies gates that positively select the respective extra marker. Fourth, the user can visualize the ACCEPT parameters of an individually defined number of cartridges with dot plots, histograms, and a UMAP. Aside from the four major options included for the CS analysis, the app can provide structured information on the association between the enumerated events and clinicopathological data by presenting various statistical metrics and selecting the best-performing ones.

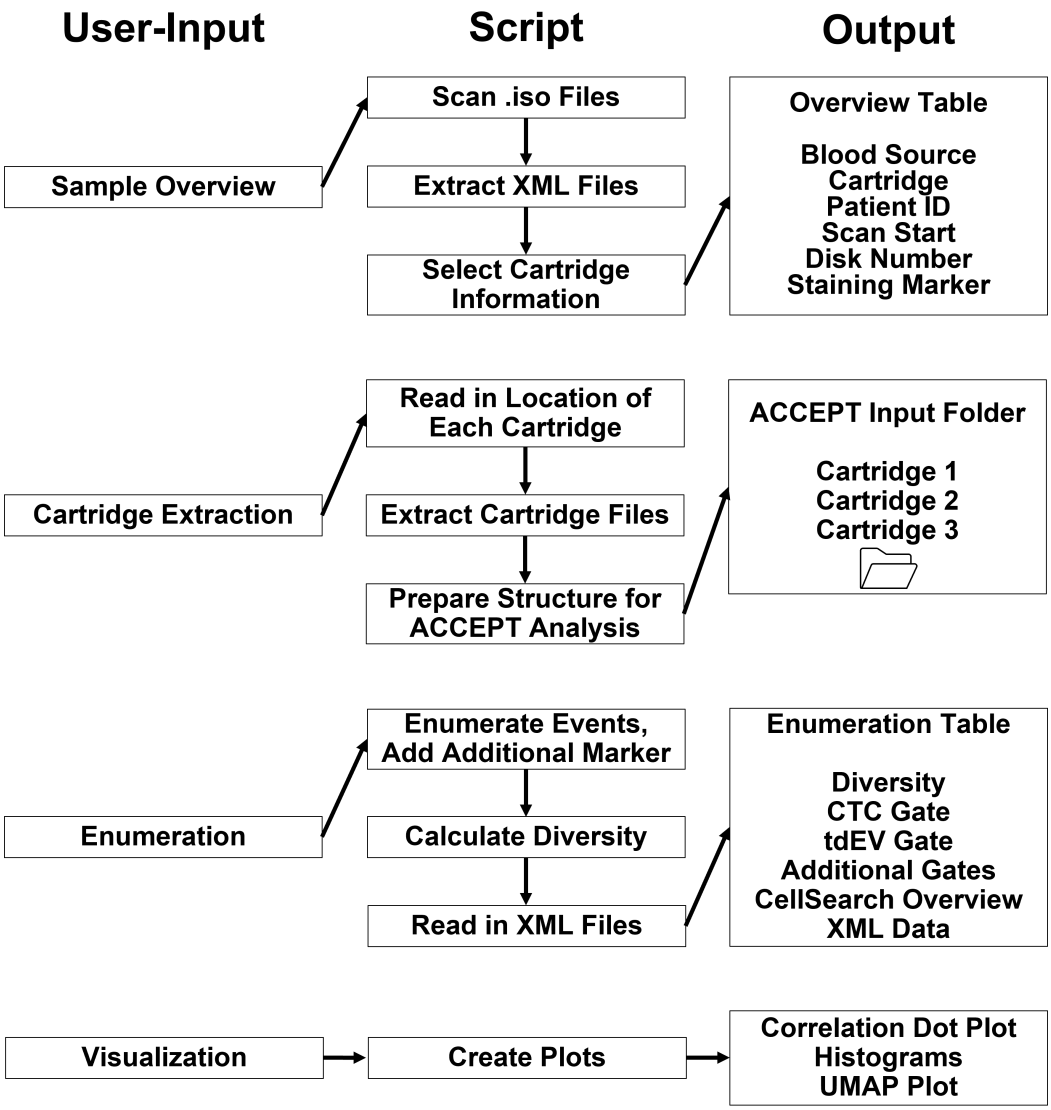


Figure 14: **Options Available in the Shiny App for CS Evaluation**

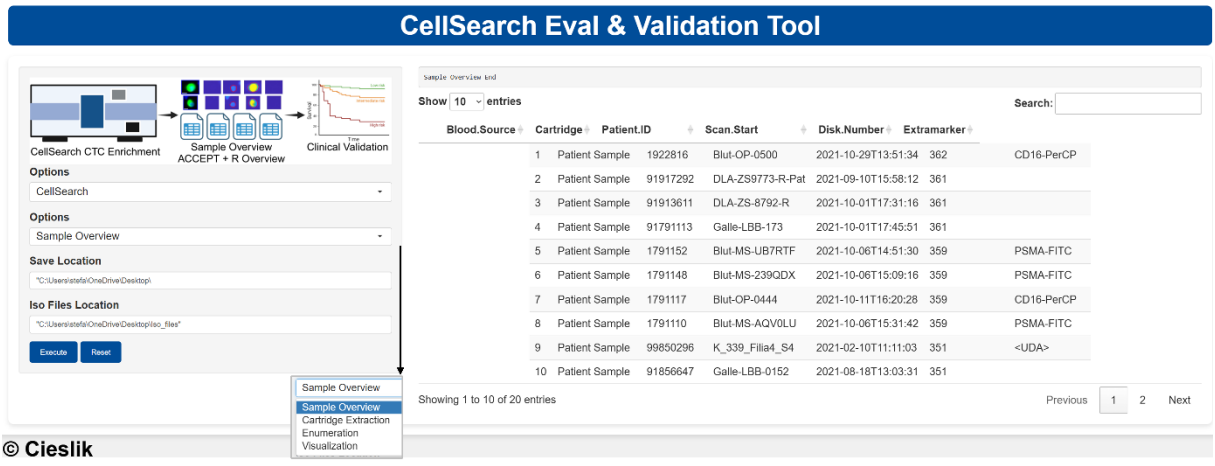


Figure 15: **User Interface of the Shiny App**
The Sample Overview option was executed to illustrate the interactive table within the application.

3.2.2. Future Aims

The application is currently being used internally in our research group to improve CellSearch-related processes and to identify and address any weaknesses in the tool. Once the internal evaluation is complete, we plan to make the application and its code available under an open-source license. By making the tool more widely accessible, we aim to establish a standardized and time-efficient approach for CellSearch analyses, potentially leading to more meaningful and efficient study results.

4. Discussion

4.1. Clinical Relevance of Tumour-Proximal Biopsy

The challenge in early-stage colorectal cancer is that established clinicopathological markers lack sufficient accuracy to reliably distinguish between high- and low-risk patients. This results in incorrect treatment recommendations, which in turn lead to overtreatment with toxic side effects or undertreatment with a worsened outcome. A relevant gain in information is expected from CTCs as circulating biomarkers. Due to the scarcity of CTCs, especially in stage I-III CRC patients, and technical limitations, resulting in low sensitivity and specificity values, the application of CTCs in tumour boards, clinical routine, and general decision-making is still hindered. Although significant progress was made with many new technologies, there is still no standardized and clinically approved CTC enrichment method for curative CRC patients.[176] It is assumed that by sampling blood proximal to the tumour that has not passed through any filtrating organ (liver or lung), higher quantities of CTCs might be detected, and additional biological insights can be gained.[147]

4.1.1. Impact on Detection Rates and Technical Limitations

In the present study, we observed a higher abundance of CTCs in the DV compared to the CVC, which could be explained by a multitude of factors: First, the blood has not yet passed through the liver, which could retain or eliminate CTCs via the numerous tiny capillaries alongside the immune system. Second, it can be assumed that there is an increased transfer of CTCs from the primary tumour into the circulation. Third, due to the proximity to the primary tumour, the influx from other veins of healthy tissues and, consequently, the dilution effect is less pronounced. Fourth, anatomical proximity also results in a reduced circulation time of CTCs in the bloodstream, meaning that DV-CTCs are less exposed to mechanical and immunological stress.[157] Most studies that evaluated the presence of CTCs in comparable (heterogenous) collectives with the CellSearch method show highly similar detection rates. Two other studies described slightly increased detection rates (ranging between 35% and 55.9%).[151,155–157,172] According to these numbers, our described CTC detection rate of 37.4% aligns with previous CS studies. Since Epispot and PCR-based technology enhanced the CTC yield compared to CS, methodical adaptations like including extra markers or analysing a larger blood volume need to be considered. In our study, we complemented CTCs and increased the informative value for CTC-negative patients by enumerating large tdEVs co-enriched by the CellSearch system and found in virtually every sample.[89] To date, no other study has quantified CellSearch-tdEVs in the DV of curative CRC patients. Although we successfully increased the tdEV yield, the difference did not reach statistical significance, most probably because of the limited number of study participants. A higher bioavailability of DV-tdEVs, particularly smaller exosomes, was also found in studies with lung cancer patients.[177–180] DV-tdEVs are still relatively understudied and require further investigation. A major limitation of our study is that it retrospectively assessed blood sampling at a single time point. A prospective randomized study is required to assess the longitudinal impact of CTCs and tdEVs on decision-making, incorporating repetitive postoperative blood sampling alongside intraoperative collection.

4.1.2. Impact on Assessing Tumour Biology and Prognostics

Subsequently, our study deals with the association between the described biomarker counts and clinicopathological factors. Initially, our focus was the correlation with TNM and UICC stages. It turned out that DV-tdEVs were mainly linked to the primary tumour size (pT stage). CVC-tdEVs, on the other hand, were associated predominantly with pathological markers of disease dissemination, including pN. Reinforcing our premise, the available literature suggests that DV-CTCs reflect tumour biology and prognosis in metastatic and non-metastatic CRC patients. Numerous clinical factors - such as tumour invasion, stage, and CEA levels - were associated with DV-CTCs.

Heterogenous and Non-Metastatic Patient Collectives

Due to the known anatomical blood pathways, which include portal-venous drainage from the colon and rectum, it is hypothesized that CTCs tend to flow into the liver. The significantly higher abundance of CTCs in MsV compared to CVC blood, observed by Rahbari et al., reinforces that the liver might act as a filter. The invasive potential of some of these trapped CTCs may contribute to the frequent metastatic spread into the liver.[156] A large-scale study with 343 participants using DV blood at a linear distance of less than 10 cm from the primary tumour pointed to the potential of CTCs to predict the development of liver metastases: CTC-positive patients had a 6.3-fold increased risk of developing liver metastases, which outperformed other clinical markers. By Combining CTCs with established clinicopathological information - T stage, vascular invasion, and CEA – they could create a nomogram that performed remarkably well with AUC values near 0.9 on predicting liver metastases. Thus, CTCs or nomograms, including CTCs, could be helpful in the early detection and intervention of liver metastases.[157] In our study, there was no link between the presence of metastases and DV-CTCs or DV-tdEVs. Given the absence of information on liver metastases, we could not investigate the impact of CTC-positive or high tdEV loads on the development of liver metastases in the follow-up period. Similar to Rahbari et al., we observed that CTC quantities from the CVC and DV were correlated (data not shown). Therefore, the liver's filtering capacity appears to be limited, which could be explained by particular CTC subtypes overcoming the liver's barrier. Rahbari found that higher numbers of DV-CTCs were associated with higher CVC-CTC counts, which points to a volume-dependent efficiency of the liver's filtering process.[156]

In contrast to the hepatic drainage, blood from the lower rectum drains via the internal iliac vein into the inferior vena cava.[140,145] Consequently, CTCs moving along this anatomical blood route might be detected more frequently by CVC sampling compared to CTCs passing through the portal-venous system. Indeed, in Rahbari's study, CRC patients with their primary tumour located in the lower rectum had significantly more CVC-CTCs than patients with their tumour localized in the upper rectum. In addition, MsV-CTCs were found almost twice as often in colon cancer patients than in rectal cancer patients, underlining that CTCs follow predefined anatomical blood routes. Just like in our study, CVC-CTCs were particularly correlated to advanced (UICC) disease stages. In contrast to our study, Rahbari found no association between MsV-CTCs and primary tumour size (T3-4 vs T1-2).[156] Yet, recent studies highlighted the potential of MsV-CTCs to reflect both local and advanced disease indicators, including local tumour invasion and tumour markers such as CA19-9 and CEA.[150,151] Iinuma et al. positively associated CEA/CK20 detected in the DV with depth of invasion, vascular invasion, lymph node metastasis, liver metastasis, and disease stage.[173]

In contrast to PB-CTCs, the impact of CTCs sourced from the DV and CVC on patient survival remains significantly less elucidated. Deneve found that a threshold of >27 CTCs indicated worse survival.[155] Similarly, Iinuma (2006), Sunouchi (2003), and Yamaguchi (2000) reported that CTC positivity in the DV correlated with poorer outcomes.[165,168,173] Contrary to this, Märkl (2016) found no prognostic significance of CTCs in MsV blood, and Sadahiro (2005) observed no significant survival difference based on CEA mRNA positivity.[153,159] A meta-analysis from 2010 of more than 3000 patients questioned whether the presence of POV- and MsV-CTCs influence their survival. In contrast to the well-elucidated PB-CTCs, the role of CTCs originating directly from DV is inconclusive because of the known heterogeneity of the respective detection methods, time points of blood collection, and the scope of clinical patient data. Despite the higher CTC yield enabled by DV sampling, the detected CTCs may have lower prognostic relevance as only a fraction of all CTCs trigger cancer dissemination, and most undergo apoptosis.[175] Applying downstream characterization at the genomic level can shed light on the biological characteristics of the CTCs.[156] CTCs can reflect the genetic changes characteristic of CRC, as was shown by our copy number analysis at a chromosomal level and could indicate the biological aggressiveness or potential to seed metastases [172].

Metastatic patients

Patients with liver metastases have significantly poorer survival compared to non-metastatic patients despite improved treatment options such as surgical hepatectomy, chemotherapy, or radiotherapy. Although surgery offers the best chance of cure, the 3-year recurrence rate is high at 60-70%, with early recurrence related to a worse prognosis.[181,182] Anatomically, blood flows from the liver directly into the systemic circulation via hepatic veins (HV) and can then flow to the lungs and more distant locations, potentially promoting CTC dissemination. An investigation of 105 CRC patients with liver metastases (CRC-LM) uncovered that CTCs were significantly more present in the hepatic outflow (HV) compared to the hepatic inflow (POV). The higher abundance, as well as the invasive phenotype of CTCs from the HV, suggested that CTCs derived from liver metastases might be involved in the further dissemination of cancer. CTCs entering the systemic circulation via the HV in the metastatic state can be considered drivers of new metastases and may explain the high recurrence rate in CRC-LM patients. In addition, the increased CTC shedding from large liver metastases might provide a biological explanation for the poor survival in these patients.[152] Of note, Weitz et al. also found that the invasiveness of the surgical procedure, i.e., a higher number of resected liver segments, was associated with increased tumour cell dissemination.[161] Aside from the systemic dissemination, the formation and progression of liver metastases might be promoted by HV-CTCs as implied by their co-occurrence with CTCs in the POV[152] and other studies that found elevated CTC levels in the hepatic inflow, including the POV and MsV.[151,156]

Connor et al. (2016) examined CRC-LM patients after liver mobilization and discovered associations between increased HV-CTC levels and advanced liver metastasis size, lymph node metastases, hepatic vein invasion, DFS, and OS. The higher predictive value of HV-CTCs compared to PB-CTCs cannot be fully explained by their (insignificantly) increased abundance. Rather, biological differences in CTC subpopulations between HV and PB blood could explain the different prognostic outcomes. In general, higher CTC counts must be viewed critically because they might get artificially elevated by the admixture of non-malignant CTCs derived from benign diseases (e.g. chronic inflammatory bowel disease). Artificially elevated counts would result in misclassification and diminished prognostics value.[154] Taken together, there is a pressing need for large-scale studies including clinicopathological and downstream data to evaluate the impact of DV-CTCs on prognosis and to determine their intrinsic biological role.[156,166]

4.1.3. Clinical Relevance of Tumour-Proximal EVs

Afroditi Nanou (University Twente) pioneered the term tdEVs and demonstrated that virtually all patients tested positive for CS-tdEVs and that their tdEV count was tenfold higher than their CTC count.[89] Because of the higher abundance and strong correlation to CTCs, tdEVs successfully complement CTCs and enable patient stratification into multiple risk groups. It has been proven in mCRC that an elevated tdEV load negatively impacts patient outcomes.[89,99] In our study, the predictive accuracy of tdEVs for OS was superior to CTCs, as reflected by higher AUC values and improved patient stratification by univariate, multivariate and Kaplan Meier analyses. Remarkably, higher tdEV counts constituted a significant and independent predictor for shorter OS in both (test and validation) cohorts of CRC patients. To the best of our knowledge, no other study has investigated CellSearch-enriched tdEVs in the DV. The present literature encompasses research on DV-EVs in the pulmonary vein blood of lung cancer patients.[177–180] These studies confirmed our observations of higher EV yield and correlation with unfavourable OS and PFS.[177–179] Furthermore, EVs were divided by size, and the results showed that the smaller ones were primarily present in the pulmonary vein and mainly found in patients experiencing recurrence.[180]

Unfortunately, there is a paucity of research examining the role of tdEVs in curable CRC patients. For this reason, our study contributes significantly to this topic, as it provides compelling and novel evidence that DV-tdEVs outperform CVC-tdEVs and CTCs regarding

prognostic accuracy. Interestingly, the strong correlation between DV-tdEVs and tumour size supports the hypothesis that DV-biomarkers can complement conventional staging. The detection of all biologically relevant tdEVs remains a significant challenge. This challenge may be addressed by integrating plasma, advanced fluorescence microscopy technology, and antibodies targeting additional membrane markers. [89,97,183]

4.2. Qualitative Evaluation of the Combined Tumour Load

The observed prognostic relevance of CK-positive objects, especially of tdEVs, and the recognized broad phenotypic diversity within and between the blood collection sites prompted us to quantify the combined tumour load (CTCs and tdEVs) diversity and correlate it with OS. We hypothesized that higher diversity would be associated with poorer survival as we assumed that a higher number of tdEVs/CTCs should potentially include more phenotypic classes. We included a pre-processing step with Uniform Manifold Approximation and Projection (UMAP) before calculating the Shannon index according to the distribution of CTCs/tdEVs between clusters. Through this approach, we uncovered phenotypic differences within and between the samples and an association with adverse OS. Oeyen et al. (2020) similarly calculated the Shannon Index based on five k-means generated clusters without including a pre-processing step in a study of 170 mCRPC patients starting a new line of androgen receptor signalling inhibitors (ARSi). Analogous to our approach, the Shannon Index was determined by the number of CTCs per CTC cluster per blood sample. Diversity was associated with adverse outcomes, typically reduced during therapy and increased in cases of progressive disease.[94] Our initially introduced diversity index aligns with Oeyen's index as both indices were retrospectively calculated depending on the complete patient collective. This makes the approach particularly susceptible to outliers, which could result in significant discrepancies in the calculations, particularly in cohorts that do not follow a normal distribution. For this reason, we additionally tested a diversity index based on the mean of the standard deviations of fluorescence expression signals. This enabled the calculation of a prospective and sample-specific diversity index. This new diversity index achieved the highest AUC score, well-balanced sensitivity and specificity values, and a negative predictive score of 91%. Biologically, these results are consistent with the findings of Isebia et al., who included 104 CNPC patients before androgen deprivation therapy (ADT) and 66 CRPC patients. Isebia et al. demonstrated that CRPC patients exhibited more often every defined CTC subclass compared to CNPC patients. In addition, CTC clusters and heterogenous CK CTCs were more abundant in CRPC than CNPC, which supports the hypothesis that a more aggressive tumour stage might be associated with increased heterogeneity. Based on the strong correlation between tdEVs in CNPC patients and heterogeneous CK CTCs it became apparent that the tdEVs represent more than apoptotic byproducts on a biological level.[93]

A major limitation of CS diversity is that it only includes EpCAM-expressing particles. However, epithelial markers on CTCs get lost during EMT, so CS misses a relevant portion of EpCAM-negative and $EpCAM^{low}$-expressing CTCs.[184] De Wit et al. (2018) captured $EpCAM^{low}$ CTCs derived from CRPC patients by microfiltration of the $EpCAM^{high}$ CTC-depleted blood after CS enrichment. Although only $EpCAM^{high}$ CTCs were linked to poor survival, $EpCAM^{low}$ still represented an interesting subclass with its own cancerous characteristics.[129] The biological insights into the EpCAM-negative and -positive CTCs, in turn, contribute to the development of physical CTC enrichment technologies. A study of nearly 2,000 samples and 72,000 CTCs from prostate, breast, bladder, and CRC patients and cultured cell line cells investigated differences in CK size diameter and nucleus diameter. When comparing cells within the respective entities, patient-derived cells and, to a lesser extent, their respective nuclei were smaller than cell line cells. Beyond that, BC cells were the largest and CRC cells the smallest. Interestingly, differences in size between leukocytes and CTCs were relatively marginal. Park et al. also found that patient-derived cells were smaller and presented a larger nuclear-cytoplasmic ratio, a more elongated shape, and more variability regarding the nuclear-cytoplasmic ratio compared to cultured cells. These findings could help refine and establish size-dependent isolation methods.[185,186]

Our study reinforces that CellSearch combined with image analysis programs such as ACCEPT, can be successfully used to generate a diversity biomarker that increases the predictive accuracy in the adjuvant period of curable cancer patients. A deeper understanding of cancer diversity and its associated subclasses can be gained by implementing retrospective cohort-dependent and prospective cohort-independent diversity indices. In addition, the assessment of diversity and subclasses could help refine and establish physical CTC enrichment technologies.

4.3. Limitations and Potential of CTC Image Analysis

By validating the published ACCEPT gate settings in 510 (malignant, benign, healthy) samples, we noticed that ACCEPT enumeration resulted in a substantial increase in counts compared to the manual enumeration. This problem was addressed by implementing an R-pipeline with additional selection criteria that complemented the published gate settings, e.g. by selecting only CTCs with a CK size larger than the DAPI size. This CTC gate was created based on biological assumptions and experience in image analysis, although the settings do not sufficiently address the actual complexity of CTCs. Owing to the adapted gate settings, higher specificity and lower sensitivity values were achieved. The overall more stringent and improved performance of the R-adapted gate settings, compared to the already published Twente gate settings, served as the foundation for the subsequent analysis of CTCs. Yet, visual inspection of our more specifically chosen CTCs still pointed to the problem of misclassifying CTCs. A reliable CTC count is essential, as minimal count differences can lead to incorrect risk-group classifications. To facilitate CTC evaluation, it is necessary to undertake accurate and comprehensive segmentation.[87]

4.3.1. Limitations of the ACCEPT Software and CellSearch Platform

Insufficient segmentation by the CS system causes miscalculation of CTCs, erroneous merging of multiple CTCs into one CTC, and a space-occupying visualization.[83,87] Compared to the segmentation integrated into CS, the Bregman active contour method integrated into ACCEPT offers a more precise segmentation by capturing cell outlines and evaluating morphology and intensity levels. Further refined segmentation algorithms encompass even more complex classification rules which are not applicable in ACCEPT. Unfortunately, these complex algorithms become again more inaccurate when facing high sample densities, especially within DLA samples, as they join objects in proximity into a single event. Complex classification within deep learning (DL) encodes complex classification rules that are challenging and often not fully comprehensible (known as a black box). Although DL can improve automatic detection, it requires extensive training sets containing many images.[85,87,95]

4.3.2. Improvement of Image Analysis for CTC Identification

StarDist is a segmentation method based on DL and describes events in a star-convex shape. On average, it segments 10% more CTCs in peripheral blood samples and 20% more in DLA samples, misses less than 0.1% of the CTCs detected with CS, and detects all cell types as well as tdEVs. A limitation of this DL method is that certain cells cannot be described with a star-convex model. The second challenge is that certain cells with a low fluorescence signal next to strongly labelled cells go undetected, resulting in false-negative events. Third, objects are erroneously segmented at the edges of the cartridges due to autofluorescence. Although these three limitations could be addressed by human reviewers by evaluating a selection of problematic events, this would again introduce the problem of intra- and inter-reviewer variance. Due to the clear misclassifications and the difficulty in distinguishing between malignant and benign events, further training and refinement of this method was conducted.[87] A recently developed pipeline incorporated a DL platform and an additional refinement strategy, which provides optimized numbers of CTCs, tdEVs, and other particles. In this new pipeline, the contrast maximization method is used as a supervised refinement strategy to

differentiate between benign (non-CTCs) and metastatic CTCs (a mixture of CTCs and non-CTCs). Using a t-SNE map combined with a kNN algorithm, areas and, thus, cells with a high probability of representing a CTC can be identified. In the t-SNE map, areas of cells where non-CTC (benign) and CTC (malignant) overlap are classified as non-CTC. CTCs and tdEVs identified with the new pipeline performed better and had a stronger correlation to HR values compared to ACCEPT and operator identification. Future use of this pipeline will show whether it has the potential to fully replace human operators.[85]

4.4. Future of Liquid Biopsy and Personalized Medicine

In the last decades, significant progress has been accomplished in the field of liquid biopsy. Despite continuous progress toward clinical application, no CTC test could be established in clinical practice. Envisioned clinical tests are most likely to be used initially in mCRPC or NSCLC patients, as substantially higher CTC yields are expected in these entities compared to CRC patients.[187,188] A promising blood test is the DefineMBC assay, which includes the combined analysis of CTCs and cell-free DNA (cfDNA). cfDNA is primarily formed during apoptosis or necrosis of healthy haematopoietic cells and secondarily through other mechanisms such as active secretion. Due to the short half-life in the circulation (between 16 minutes and 2.6 hours), it is assumed that circulating tumour DNA (ctDNA) can accurately reflect the current tumour burden quantitatively and qualitatively in real time.[119,189] The DefineMBC assay enables the assessment of protein expression, single-cell genomics, and the evaluation of ctDNA mutations and CNVs.[190]

Regarding CRC prevention, colonoscopy represents the gold standard for simultaneously detecting and removing benign and malignant lesions. However, participation rates for this invasive prevention measure are alarmingly low. Blood tests hold the potential to increase patient adherence.[191] A ctDNA-based blood test featured in The New England Journal of Medicine achieved a sensitivity of 83% in CRC patients and a remarkable specificity of 90% in patients with advanced neoplasia [192]. Even so, clinical tests usually require excellent predictive values. So, the currently documented sensitivity and specificity values of liquid biopsy tests need to be markedly improved to achieve positive-predictive and negative-predictive values sufficiently reliable for clinical decision-making.[192]

Regarding active CRC disease, liquid biopsy is expected to enable real-time monitoring assessing the patient's risk profile and tumour response, recurrence, and progression at an early stage. In UICC II colon cancer patients, it was shown that clinical decision-making based on ctDNA contributed to a decrease in chemotherapy usage, preventing adverse side effects in these patients. ctDNA-guided decision-making led to fewer chemotherapy recommendations than decision-making based on clinicopathological factors, while the 2-year recurrence-free survival remained consistent.[47,193] In mCRC patients, ctDNA could provide genomic data and aid in selecting the appropriate treatment, such as anti-EGFR or anti-VEGF therapy.[15]

Despite the promising results for ctDNA, several questions remain unanswered: (1) What is the actual influence of ctDNA positivity on recurrence-free and overall survival over an extended follow-up period? Does ctDNA positivity indicate a permanently elevated risk, or does the risk gradually minimize over time? (2) Could ctDNA-negative patients with an unfavourable pathological risk profile (T4) benefit from chemotherapy? (3) Who among the ctDNA-positive patients should receive chemotherapy and which chemotherapy regimen should be preferentially administered?[193] A disadvantage of ctDNA evaluation is that prior knowledge of the target of interest is essential since only a limited number of DNA mutations are expressed. Furthermore, cfDNA may not always originate from tumour cells as it can also result from the apoptosis of blood cells during treatment.[194,195]

On the other hand, it is important to highlight that CellSearch is a relatively standardized system that has been clinically validated across various tumour types. The use of CS-CTCs and CS-tdEVs provides additional and valuable information compared to ctDNA. CTCs allow for phenotyping, genotyping, and other investigations, such as the culture of primary cell lines.

By visualizing CTCs, we can draw conclusions about their malignancy and biological aggressiveness.[79,196,197] Another advantage lies in the ability to classify CTCs into subgroups and calculate diversity indices based on morphological information, for example using clustering tools.[195,198] Tumour heterogeneity represents an emerging next-generation hallmark of cancer and was a central element in our study. We believe that the phenotypic diversity of CTCs and tdEVs has the potential to personalize treatment as a biomarker in the adjuvant setting. Biologically, heterogeneity can be observed between different cancerous lesions and in the same lesion during the time of disease progression. The exposure of the tumour to treatments can modify its biological characteristics and might result in increased tumour heterogeneity, necessitating therapy adjustment. Such an adjustment of the therapy could involve combined therapy to target multiple therapeutic markers.[47,119,188,193,199]

In the future, the field of immuno-oncology might become of particular interest, including the evaluation of tumour mutational burden in ctDNA and immune checkpoint inhibitor proteins in CTCs.[200] A refined understanding of immunological processes in the context of cancer progression has led to the development of immune checkpoint inhibitors for treating tumours. Surface proteins such as programmed death-ligand 1 (PD-L1) are overexpressed in tumour cells, they deactivate the T-cell immune response and enable tumour immune evasion.[201] Checkpoint inhibitors targeting programmed cell death protein 1 (PD-1), Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4), and other markers have shown a robust therapeutic effect in cancer patients, especially in those with mismatch repair (MMR) deficient CRC.[202,203] An increased PD-L1 expression can be linked to a stronger response to regorafenib, an inhibitor of multiple protein kinases, suggesting that evaluation of the PD-L1 status might help to assess the therapy response.[204,205] In NSCLC and hepatic carcinoma patients, a decrease in the total CTC count can be linked to a positive response to immune therapy.[206–208] In addition, increased PD-L1 expression of CTCs might indicate a worse prognosis both pre- and post-therapeutically.[209–211]

As we could show in our study, the minimal CTC detection rates in organ-confined CRC could be addressed using tdEVs that increased the informative potential in virtually every patient sample. On top of that, blood sampling from DVs significantly increased the CTC detection rate. Nevertheless, most CRC patients continue to belong to the CTC-negative group despite the use of tumour-proximal liquid biopsies. This circumstance could be addressed by increasing the current blood volume of only 7.5mL. In the literature, the screening of several litres of blood by diagnostic leukapheresis (DLA) showed a remarkable rise in detection rates in multiple entities, and the DLA-enriched cells were shown to be intact, isolable, and genetically analysable.[82,199,212] Although leukapheresis (LA) is a routine clinical procedure, it is significantly more time-consuming and complex than a standard blood draw. In addition, the substantial enrichment of white blood cells results in a high cell density, posing technical challenges for cell separation and image analysis. Aside from DLA, wearable devices employ long-term blood sampling that has been validated in canine models. Like DLA, wearable devices were associated with only a few adverse side effects by avoiding cell loss due to heparinization and return of the blood back to the patient's venous system.[65,213] In addition to improving the CTC enrichment, the use of automated detection platforms and machine learning algorithms can enhance the accuracy and efficiency of particle detection. The improved pattern recognition, in turn, helps to identify particles that strongly impact patient outcomes.[85,87,95]

In light of a growing number of methodologies, technological platforms, and CTC definitions, the future challenge will be to work collaboratively on solutions in a standardized way through officially issued guidelines, for example, published by CANCER-ID.[214] Beyond that, the validity of many liquid biopsy studies is limited due to the low number of participants and often the restriction to one clinical centre. For this reason, extensive multicentre studies with a long follow-up period and clearly defined endpoints are crucial to assess the liquid biopsy's true clinical benefit. In parallel to clinical validation studies, biological and molecular mechanisms must be deciphered experimentally to integrate new findings into clinical practice.[65,119,215]

4.5. Conclusion

With the present study, we were able to demonstrate that DV blood has the potential to significantly increase the CTC yield in CRC M0 patients and may serve as an alternative to diagnostic leukapheresis. DV-derived CTCs and tdEVs were particularly associated with tumour size and improved patient risk stratification compared to central venous blood. A key novelty of our study lies in the finding that combining CTCs and tdEVs, while taking into account their phenotypic characteristics, allows for more accurate patient stratification.

But what is the actual clinical relevance of these findings for the treatment of CRC patients? As mentioned initially, there is an urgent need for sensitive biomarkers, particularly in UICC stage II and III patients, to support decision-making regarding therapy selection and the evaluation of treatment response. While the current findings are promising, they were obtained exclusively through retrospective analyses. In contrast, large randomized studies are essential to determine the therapeutic implications of these biomarkers. For biomarkers to be successfully translated into clinical practice, reimbursement of these assays and sufficient validation are critical to ensure their availability for all cancer patients.

Consortia such as the European Liquid Biopsy Society (ELBS) are working towards the shared goal of integrating liquid biopsy into clinical routine.[57] A notable example is a randomized trial that included patients with hormone receptor–positive, HER2-negative advanced breast cancer. In this study, patients classified into a high-risk group based on a CTC count ≥5 benefited more from chemotherapy than those who, despite having a high CTC count, were clinically assigned to a lower-risk group and treated with endocrine therapy. Although overall survival did not significantly differ between the standard (clinically guided) arm and the CTC-guided arm, the study impressively demonstrated that treatment decisions can be optimized in favour of prolonged survival.[216] Similar to how ctDNA-guided therapy has successfully reduced the use of chemotherapy without compromising overall survival, there is a clear need for corresponding studies evaluating the clinical utility of CTCs and tdEVs.[47] Despite continuous advances in screening, imaging, therapeutic strategies, and pathology, optimal outcomes in terms of patient survival and quality of life require a truly personalized approach to medicine—an approach that, it is hoped, can be achieved through improved CTC and tdEV technologies.

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