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Ústav anatomie, histologie a embryologie

CTC u kolorektálního karcinomu

Disertační práce

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Poděkování

Doktorské stadium ovlivnilo moje budoucí směřování pravděpodobně více než pregraduální vzdělání. Zároveň to byla obrovská životní zkušenost, která mi umožnila rozšířit svoje znalosti a dovednosti a poznat inspirující a zajímavé kolegy z celého světa. V neposlední řadě jsem díky tomuto studiu poznal spoustu přátel, kteří mě zcela jistě budou pozitivně směřovat i po skočení doktorského studijního programu.

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Drahomír Kolenčík

Prohlášení:

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List of abbreviations:

AKT2	RAC-beta serine/threonine-protein kinase
BRAF	v-Raf murine sarcoma viral oncogene homolog B
CA-125	Cancer antigen 125
CD31	Cluster of differentiation 31
CD45	Cluster of differentiation 45
CDK4/6	Cyclin-dependent kinase 4 and 6
CDX2	Caudal-type homeobox 2
cfDNA	Cell-free DNA
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK	Cytokeratin
CRC	Colorectal Carcinoma
CTC	Circulating Tumor Cells
ctDNA	Circulating tumor DNA
DAPI	4′,6-diamidino-2-phenylindole
EGFR	Epidermal Growth Factor Receptor
EpCAM	Epithelial Cell Adhesion Molecule
Epi.CTC	Epithelial CTC
ESMO	European Society for Medical Oncology
ESR1	Estrogen Receptor Alpha Gene
HDSCA	High-Definition Single Cell Assay
HER2	Human Epidermal Growth Factor Receptor 2.
HR	Hormone Receptor
IF	Immunofluorescence
KRAS	Kirsten rat sarcoma viral oncogene homolog
mCRC	Metastatic Colorectal Carcinoma
Mes.CTC	Mesenchymal CTC
MRD	Minimal residual disease
MSI	Microsatellite instability
NCCN	National Comprehensive Cancer Network
OCULAR	Outlier Clustering Unsupervised Learning Automated Report
ORR	Objective response rate
OS	Overall Survival
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFS	Progression Free Survival
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PSA	Prostate Specific Antigen
SDOM	Standard deviation over the mean

SNAI1	Snail Family Transcriptional Repressor 1
t-SNE	t-Stochastic neighbor embedding
TWIST1	Twist-related protein 1
VIM	Vimentin
WBC	White blood cell

Abstrakt:

Úvod:

Přes celkově klesající incidenci představuje kolorektální karcinom (CRC) stále jednu z nejčastějších malignit s vysokou úmrtností. Zavedení fluidní biopsie do klinické praxe by zvýšilo množství informací o konkrétní nádorovém onemocnění, které by lékaři mohli využít při léčbě CRC. U jiných typů malignit pozorujeme rostoucí trend ve využívání tekuté biopsie, konkrétně circulating tumor DNA (ctDNA), v různých způsobech praktického využití, ze kterého by v budoucnu mohli profitovat i pacienti s CRC.

Metodologie:

Byly provedeny dvě studie, ve kterých byla k identifikaci a analýze cirkulujících vzácných struktur (cirkulující nádorové buňky a jiné cirkulující nebuněčné struktury) použita platforma HDSCA (High-Definition Single Cell Assay). V prvním šetření byla použita první generace platformy HDSCA sledující tři markery (CK, CD45 a DAPI). Následně byl stanoven počet nalezených podtypů cirkulujících buněk. Byla také provedena analýza dynamiky počtu těchto buněk mezi provedenými odběry. Tyto údaje byly následně korelovány s dostupnými klinickými údaji, včetně přežití pacientů bez progrese (PFS) a celkového přežití (OS). Při druhém šetření byly použity dva protokoly třetí generace platformy HDSCA. Protokol Landscape využíval pro čtvrtý imunofluorescenční (IF) kanál vimentin a CD31, zatímco protokol zaměřený na CDX2 pro čtvrtý kanál využíval CDX2 jako specifický marker pro colon a rektum. I zde byl stanoven počet cirkulujících vzácných struktur a analýza dynamiky počtu těchto struktur mezi odběry. Oproti první studii však byl kladen důraz na identifikaci a popis vzácných podtypů buněk a struktur nalezených díky novým markerům v protokolu.

Výsledky:

Naše první šetření prokázalo určitou korelaci hladin cirkulujících nádorových buněk (CTC) a přežití, která by mohla mít dopad na prognózu a predikci. V časovém bodě jeden měsíc po operaci koreloval vyšší počet CTC-CKtotal/ml s horším OS (p = 0,0492, HR = 1,02) a vyšší hladina HD-CTCs/ml naznačovala horší PFS (p = 0,0468, HR = 1,03). Podobná korelace byla zjištěna i pro dynamiku CTC mezi časovými body. Vyšší nárůst hladiny HD-CTC/ml než 49,77 mezi vzorkem před resekcí a vzorkem po resekci byl spojen s horším OS (P_BC = 0,0270, p = 0,8464, HR = 0,99). Zvýšení CTC-Apoptotic o více než 12,28 buněk/ml mezi vzorky před a

po resekci bylo spojeno se zkrácením PFS pacientů (p = 0,0024, HR = 1,01).

Druhé šetření dokázalo identifikovat a popsat specifické vzácné buňky a struktury. Nejprve byly pomocí protokolu Landscape kategorizovány dva buněčné podtypy nejen na základě přítomnosti nově přidaných markerů, ale také na základě morfologie. Předpokládáme, že populace buněk s tečkovaným vzorcem exprese CD45/CD31, která je morfologicky rozmanitá, zejména v různých tvarech a poměru jader a plazmy, je populací megakaryocytů.

Kromě toho předpokládáme, že buňky, které vykazují vláknitý signál vimentinu spolu s tečkovaným signálem CD45/31 a vyznačují se také vyšší mírou tvorby shluků, jsou populací cirkulujících endoteliálních buněk.

Další zjištěnou vzácnou strukturou byly onkosomy. Z morfologického hlediska se onkosomy vyskytovaly jak v kontaktu s blízkými jadernými buňkami (48,91 % všech onkosomů), tak jako oddělená vzácná událost (51,09 % všech onkosomů). Po zpracování a centrifugaci byly nalezeny v buněčné frakci periferní krve a z hlediska velikosti mohly být velké jako WBC nebo menší (~10 µm). Fenotyp onkosomů byl homogenní v celém tvaru s rovnoměrným rozložením IF signálu. Většina onkosomů také exprimovala signál CK.

Zajímavé poznatky přinesla také multi-assay analýza, při které jsme srovnávali preparáty z jednoho vzorku pacienta zpracované dvěma zmíněnými protokoly. Například nezjištěná korelace mezi populací CD45/CD31 z protokolu s barvením Landscape a populací CD45 z protokolu s barvením CDX2 (p = 0,645, τ = 0,12) znamená, že důležitým markerem pro odlišení těchto populací buněk je s největší pravděpodobností marker CD31.

Závěr:

Ukázalo se, že platforma HDSCA v sobě skrývá více informací pro potenciální využití v klinické praxi než jen prostý výčet buněk epiteliálního původu. Cirkulující buňky a struktury obsahují větší množství dat a informací, které lze dále analyzovat a korelovat s klinickými údaji. Pokud se to potvrdí v prospektivní studii s větším souborem a vyváženou a stratifikovanou populací, lze platformu HDSCA (a tedy i tekutou biopsii) využít pro výběr léčby/odpovědi na ni a prognózu u CRC. Také jsme ukázali, že neselekční přístup platformy HDSCA může také zvýšit porozumění nádorové patofyziologii rozšířením seznamu samostatných podtypů buněk, které mohou ovlivňovat maligní procesy.

Klíčová slova: tekutá biopsie, cirkulující nádorová buňka, bezbuněčná DNA, cirkulující nádorová DNA, CTC, cfDNA, ctDNA, kolorektální karcinom, CRC, HDSCA, high-definition single cell assay.

Abstract:

Background:

Despite the overall decreasing incidence, colorectal cancer (CRC) still represents one of the most common malignancies with a high mortality rate. Introducing liquid biopsy into real-world clinical routine would increase individualized actionable information that physicians could leverage before and during CRC treatment. In other malignancy types, we have been observing increasing trend in using liquid biopsy, specifically circulating tumor DNA (ctDNA), in various use cases, which may bring future benefits to CRC patients as well.

Methods:

We have conducted two investigations. In both cases, the High-Definition Single Cell Assay (HDSCA) platform was used to identify and analyze circulating rare events. In the first investigation, the first generation of the HDSCA platform analyzing three markers (CK, CD45 and DAPI) was used. Subsequently, enumeration of detected rare cell subtypes was done, together with the analysis of changing levels of cells between sample collection timepoints. This data was then correlated with available clinical data, including patient´s survival - progression-free (PFS) and overall (OS). In the second investigation, two protocols of the third generation of the HDSCA platform were used. The Landscape protocol utilized vimentin and CD31 for the fourth immunofluorescence (IF) channel, while the CDX2-targeted protocol for the fourth channel utilized CDX2 as a colorectal-specific marker. Also here, the enumeration of rare events was done and changing levels of cells were analyzed. However, in comparison to the first study, the focus was on identifying and describing rare subtypes of cells identified thanks to new protocol markers.

Results:

Our first investigation has shown some actionable correlation of circulating tumor cells (CTC) levels and survival. At the 1-month time-point after the surgery, higher count of CTC-CKtotal/mL is related to worse OS ($p = 0.0492$, $HR = 1.02$) and higher levels of HD-CTCs/mL was marker for worse PFS ($p = 0.0468$, $HR = 1.03$). Similar correlation was found for CTC dynamics between timepoints. A higher increase of HD-CTC/mL levels than 49.77 between pre-resection to post-resection sample was related to worse OS ($P_{BC} = 0.0270$, $p = 0.8464$, $HR = 0.99$). Also, if

CTC-Apoptotic increased levels by more than 12.28 cell/mL from pre- to post-resection samples, it worsened patients´ PFS prognosis (p = 0.0024, HR = 1.01).

The second investigation was able to identify and describe additional specific rare events. First, using the Landscape protocol, two cell subtypes were categorized not just on the channel-type classification, but also based on their unique morphological features. We hypothesize that the stippled CD45/CD31 population of cells that contains some level of variation, especially in different shapes and nuclear to plasma ratio, is a population of megakaryocytes.

Additionally, we speculate that the cells of interest that show threadlike vimentin signal together with a stippled CD45/31 signal and are also characterized with a higher rate of a forming clusters, is population of circulating endothelial cells.

Another detected rare event were oncosomes. From a morphological perspective, oncosomes were found both in the contact with nearby nucleated cells (48.91% of all oncosomes) and as a secluded rare event (51.09% of all oncosomes). After processing and centrifugation, they were found in the cellular fraction of peripheral blood, and in terms of size, they could be as large as WBC or smaller (~10 μm). The phenotype of oncosome was homogeneous across the vesicle, with IF signal distributed evenly. Most oncosomes were also positive for CK signal.

Multiassay analysis, crosschecking slides processed by both advanced protocols using the same sample, also brought interesting findings. For example, the non-correlation between CD45/CD31 population from the Landscape-staining protocol and the CD45 population from the CDX2-staining protocol (p = 0.645, τ = 0.12) implies that important marker distinguishing those populations is most likely the CD31.

Conclusions:

The HDSCA platform harbors more information for potential use in clinical routine than just simple enumeration of CK positive cells. There are nuances between the rare events that can be further analyzed and correlated with clinical data. If confirmed in a prospective study with a larger cohort and a balanced and stratified population, the HDSCA platform (and therefore liquid biopsy) can be used for treatment selection/response and prognosis in CRC. Also, we have shown that the unbiased approach of the HDSCA platform can increase the understanding of tumor pathophysiology by broadening the list of separate subtypes of cells that can influence malignant processes.

Key words: liquid biopsy, circulating tumor cell, cell-free DNA, circulating tumor DNA, CTC, cfDNA, ctDNA, colorectal cancer, CRC, HDSCA, high-definition single cell assay

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Introduction

For majority of countries, cancer is one the most challenging threats to health of population and financial stability of healthcare systems. If there is not enough treatment option for a cancer type, a term “high unmet need” can be used, and it signals high potential risk. Colorectal cancer (CRC) certainly belongs to a group of malignancies which poses significant risk to global population. CRC is ranking as the second leading cause of cancer related deaths, simultaneously it is the third most diagnosed cancer in the world[1], despite of the observed downward trends of mortality in various countries[2–4].

Recently, a study projecting changes in colorectal cancer mortality rates based on World Health Organization mortality 1989-2018 dataset has been published. For some regions, such as Europe, Asia, North America and Oceania, the study predicts continuous decrease of colorectal cancer mortality. On the other hand, for regions like Latin America and Caribbean countries, increase in mortality rates has been projected. Authors contemplate that differences between regions are due to the increasing implementation of screening programs and improved therapeutic procedures including patient management protocols. Finally, the study underlines that due to the population growth and ageing, the overall number of deaths are expected to rise globally for colon and rectal cancer by 60% and 70% until 2035.[2] This potential steep rise of deaths all over the world requires improvement in efficacy of treatment protocols and diagnostics. Also, with emerging new treatments in CRC, selection of subpopulation of patients that would benefit from targeted therapy is required to keep financial costs at bay. That is important, because if health care professionals have wide range of therapies to their disposal, there is high risk of financial toxicity[5,6], if the right patients population is not selected. Patients´ survival is not determined solely by the oncological disease but also by the socioeconomic determinants. The cost of treatment of CRC can have significant impact on its effectiveness.[7]

There is high need for introduction of mechanisms that would help physicians to identify optimal treatment for specific patient and liquid biopsy is one of the techniques that brings high hopes in delivering those crucial decision point

information.

Anatomical and clinical overview of CRC

CRC is classified from anatomical perspective into two subcategories: the proximal two-thirds of transverse colon, ascending colon, and caecum are described as right CRC (RCC) and the distal third of the transverse colon, splenic flexure, descending colon, sigmoid colon, and rectum are defined as left CRC (LCC)[8,9]. This categorization is used since differences in incidence and mortality between RCC and LCC were observed[10]. The most dangerous feature for patients is associated with RCC due to its wider lumen and more frequent diagnosis of flat tumor growths which relates to less apparent clinical symptoms and hence higher incidence of late-stage CRC cases[11]. The molecular difference between these two anatomical types can be found as well, as for example v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutation is significantly more often associated with RCC. To summarize, despite LCC and RCC being diagnosed as one oncological “unit”, they can differ in many aspects, patients´ prognosis included.

With respect to treatment choices, CRC represents a challenging opponent for patients and their physicians[12]. Besides the high proportion of late-stage diagnosis, the other burdensome feature of CRC is a wide intratumor heterogeneity and genomic instability that subsequently creates hurdles for optimal treatment choices. To summarize, three major molecular pathways associated with CRC are:

- Chromosomal instability (CIN)[13]
- Microsatellite instability (MSI)[14]
- CpG island methylator phenotype (CIMP)[15–17]

CIN is found in the most sporadic cases of CRC (above 85%)[18]. As one of the molecular pathways, it supports tumor progression and intratumor heterogeneity by increasing the rate of genetic aberrations. The status of CIN is caused by erroneous mitosis followed by spindle checkpoint activity[19]. MSI is the result of the inactivity of the DNA mismatch repair (MMR)[20]. It is a less diagnosed

status with 15% of CRC being associated with MSI[21]. MSI positive tumors are most likely focal and less differentiated[22] and located in RCC. Another distinctive aspect is extracellular mucin[23,24]. Finally, hereditary non-polyposis colon cancer (HNPCC) is also linked with MSI positive status since the most common mutations in HNPCC alter genes for MMR (MHS2 and MLH1)[25]. Last major molecular pathway recognized in CRC – CIMP – is characterized by inactivation of transcription of tumor suppressor genes caused by promoter CpG islands methylation. CIMP positive tumors are correlated with BRAF mutation[17,26], but it is still the most favorable group of patients in terms of prognosis[27]. Despite BRAF or MSI status, CIMP positive tumors tend to be less lethal for all stages. Closer molecular look shows that methylation only affect CpG islands in specific clones of CRC which is a distinctive mark from healthy tissue around the tumor[28].

Drilling down on the specific genes which plays dominant role in CRC evolution, according to current literature, there are several key driver genes[29]: APC, TP53 (exclusively found in advanced disease[30]), KRAS, BRAF, PIK3CA, and SMAD4.

To put those driver genes into a broader consequence, it is also necessary to mention signaling cascades in which they are involved. The WNT pathway shift is strongly associated with APC gene[31,32] and appears in both MSI positive and negative CRC (93% and 85%, respectively)[30]. Furthermore, evolution of CRC into more advanced stages is conditioned by WNT pathway alterations and signaling cascade linked with KRAS mutation[33,34]. Both KRAS and BRAF mutation are crucial alterations for further growth of a tumor via MAPK cascade activation[35], inducing aggressive proliferation in tunica mucosa[36]. Nevertheless, all these alterations need to be supported by changes in WNT pathway. Moreover, KRAS mutations play role also in PI3K cascade[37], that is integral for forcing healthy tissue to support tumor growth with escalated angiogenesis and growth of metastatic spots[38]. BRAF mutations are less frequent alterations which can be found only in 5-13% of sporadic tumors and overall are described to be a negative prognostic factor for overall survival (OS)[39–41]. As BRAF and KRAS are involved in the same signaling pathway, their mutations are rarely found simultaneously in the same patient[42, 39]. Finally, TP53 mutation and alteration in

PIK3CA cascade are another negative prognostic factors, especially in stage II/III patients treated with 5-fluorouracil[43].

Frequently diagnosed in the late stage, CRC represents not only therapeutical challenge for clinician but also diagnostics problem. In recent decades, many countries introduced screening programs to battle a high incidence of the late-stage CRC. For the most screening plans, as for standard clinical process, colonoscopy is defined as the most frequent choice of screening/diagnostic test[44]. Since 2000, there has been Czech CRC screening program in place. Fecal occult blood test was performed and followed with colonoscopy if the blood was positive. Additionally, since 2014, the screening program has transformed into population-based screening program when patients older than 50 years old were systematically invited by health insurance companies to participate. The results from a multicentric observational study in the Czech Republic showed that patients who were diagnosed via screening had better stage on average in comparison to patients diagnosed based on symptoms (11.9 % out of 23 669 patients were diagnosed via screening; 59% screening-diagnosed patients had stage I or II)[45]. The reason for delayed CRC diagnosis is mostly due to the nonspecific symptoms, i.e., weight loss, abdominal pain, anemia, rectal bleeding, changes in bowel movement or iron deficiency[46].

Definition of Liquid Biopsy

In general meaning, liquid biopsy refers to a method analyzing liquid tissue with the aim to identify and evaluate tumor remnants such as parts of DNA or cells. In the most cases, liquid tissue will be referring to peripheral blood, but liquid biopsy could analyze also other fluids (urine[47], saliva[48], stool[49], cerebrospinal fluid[50], pleural fluid[51], and ascites[52]).

In oncology research, a current trend is to use a term liquid biopsy to refer only to one specific modality of the liquid biopsy – analysis of circulating tumor DNA (ctDNA). As will be explained further, this association is simplifying and omits potential of additional modalities analyzed within other variants of liquid biopsy. It should be specified on all occasions what modality of liquid biopsy is referred to. Otherwise, there is risk for confusion in scientific community and most importantly among clinicians who could miss advantages of liquid biopsies platforms.

In this thesis, the focus will be on circulating tumor cells as one of the modalities of high-definition single cell analysis.

Circulating tumor cells

Cells or cell derivates in peripheral blood are easily accessible material for further analysis. Circulating tumor cells (CTCs) forms a small portion of oncological mass, which could be released either from a primary site of a tumor or from one of a solid metastases[53]. CTCs are distinguishable from white blood cells based on their size, with a physiological diameter ranging from 6 to 10 μm[54] – CTCs have generally larger size from 15 to 25 μm[55,56]. The size difference is leveraged for differential filtering mechanisms by some liquid biopsy technologies. For purpose of this thesis it is more important that CTCs are also characterized as cells with morphologically distinct shape of the nucleus[57] and by expression of different markers in comparison to white blood cells. Interestingly, CTCs were first described already 150 years ago[58], however for clinical purposes both detection technology of CTCs and practical applicability of analysis of these cells needed to mature. Generally, the greatest challenge with analyzing CTCs is their collection and identification. Therefore, only the

latest detection methods created the opportunity to use CTCs as a modality of liquid biopsy. Beside the morphological, genomic and proteomic data, latest literature showed that CTCs modality could offer even more than just cells. For example, Narayan et al. suggested that oncosomes, small vesicles with similar phenotype as surrounding CTCs, may be important for metastatic process, or that CTCs covered by platelets could be described in peripheral blood of oncological patients with metastatic disease. Despite CTCs minimal utilization in current clinical setting or at least in phase 3 studies of novel targeted therapies (in comparison to ctDNA), it is clear that given the variability and amount of information provided by this liquid biopsy modality, it could provide comprehensive description of individual oncological disease and therefore could be significant tool for future oncological tailored therapy.

Cell Free DNA

As it goes for whole liquid biopsy nomenclature, even the case of ctDNA requires clarification. Occasionally, the terms like cell-free DNA (cfDNA) and ctDNA are freely interchanged which subsequently may cause further confusion. In healthy donors, cfDNA is a physiological finding and could be defined as a remnant of cells which underwent apoptosis[59] or necrosis[59]. Moreover, literature also describes cfDNA which originates from lymphocytes via active release[60–62] as a possible response to infection or inflammation[63]. Same principles of releasing DNA parts are applicable in tumor cells as well. Malignant disease with neoplastic growth undergoes simultaneously rapid deterioration of cells causing shedding of mutated DNA into bloodstream, hence ctDNA[64]. It suggests that among all cfDNA produced by healthy cells of the body, there is also portion of mutated DNA originating from tumor cells[64]. Patients with malignant disease will also have higher amount of overall cfDNA (in mCRC patients was shown to be up to 209 ng/ml[65]) compared to healthy individuals (up to 100ng/ml[65]). Similarly, healthy individuals and patients with polyps had significantly lower levels of cfDNA to patients with primary CRC, according to published results[66]. The difference between cfDNA and ctDNA is then defined by the presence of tumor-associated mutations[64,67–69]. The well-known example which already made its way into a clinical routine is EGFR mutation in lung cancer. In this tumor type,

due to location of the tumor, pathologists often do not have necessary amount of solid biopsy for investigation[70]. Analysis of cfDNA can therefore substitute solid biopsy in those patients where analysis of tumor tissue is not the acceptable option. If EGFR mutation is found in patient´s peripheral blood, it is eligible for targeted therapy.

Liquid Biopsy Technologies

In liquid biopsy, specifically in CTCs realm, the key point to understand is technology behind the identification and collection of cells. In principle, knowing the basis of particular technology and the way it detects and analyze CTC predetermine limits and potential utilization of that technology in clinical setting. Technologies for collecting CTCs can be divided into four subgroups based on the approach to the enrichment or detection of rare events: methods using 1) size and density; 2) immunocapture; 3) without enrichment (software driven); or 4) combination of previous approaches. In all cases, enrichment could be either positive (detecting CTCs) or negative (removing white blood cells).

The most widespread technique for CTC detection is immunocapturing focusing on identifying cells with specific antigen(s) on their surface. Positive enrichment select cells with epithelial cell adhesion molecule (EpCAM) antigen. On the other hand, negative enrichment aims to find cells (typically with CD45 antigen to focus on leukocytes) and remove them from further analysis.

Size and density technologies, platforms based on physical properties, collect those cells which are somehow different in the physical categories from typical cells in the blood stream.

Technologies without enrichment or software driven technologies do not try to identify cells immediately after the collection. Eventually, even those technologies will select cells based on certain characteristics, but they do not discard any cells from the sample. Therefore, they carry lower bias and offer the opportunity to reanalyze cells which were not identified in the first run. More crucially, those technologies are mostly driven by software analysis (machine learning). In contrast to enrichment technologies which some of them already made their way into prospective randomized trials and therefore close to clinical utilization, software drive technologies contain many variables that need to be address before their clinical application. Short overview of CTC detecting and capturing platforms is listed below in **Table 1**

Name	Methods	References
AdnaTest	Antibody targeting EpCAM conjugated to magnetic beads for labeling tumor cells in sample	[71,72]
CanPatrol™ CTC	Filtration (and CD45+ depletion)	[73,74]
Cellcollector®	Nano guidewire inserted into patient cubital vein collecting cell expressing EpCAM	[75,76]
CellMax CMx	Blood passing through antibody-coated microfluidic chip targeting EpCAM	[77,78]
CellSearch® *	Antibody targeting EpCAM conjugated to magnetic beads for labeling tumor cells in sample	[79,80]
ClearCell® FX	Blood passing through microfluidic biochip with larger cells along the inner wall	[81,82]
Cytelligen®	Antibody targeting CD45 conjugated to magnetic beads for labeling tumor cells in blood sample	[83,84]
DEPArray™	Cell suspension loaded into microchip-based sorter using dielectrophoresis to trap cells	[85,86]
Easysep™	Antibody targeting EpCAM or CD45 conjugated to magnetic beads for labeling tumor cells in blood sample	[87,88]
Epic Sciences/HDSCA*	After processing, cells are plated on slide and subsequently characterized based on surface markers	[57,89]
Herringbone Chip	Blood processed through antibody-coated microfluidic chip targeting EpCAM	[90,91]
ISET® *	Filtration on pressure-controlled system	[92,93]
MagSweeper™	Antibody targeting EpCAM or CD133 conjugated to magnetic beads for labeling tumor cells in blood	[94,95]
MetaCell®	Capillary-action driven size-based separation	[96–98]
Oncoquick®	The denser blood compartment migrates through the porous barrier of the polypropylene centrifugation tube	[99,100]

Table 1 List of CTC platforms with short description of respective methods

The High-Definition Single-Cell Assay (HDSCA) is the technology used in the investigations described in this thesis. It belongs to software driven technologies that identify cells of interest based on certain immunocharacteristics and physical properties, but as already described, it does not discard any of the collected cells and therefore has a potential to identify various classes of elements that could be further analyzed or correlated to patients' outcome.

Clinical applications

Current pharmaceutical industry pipelines tend to produce novel targeted therapeutical options to tumors with particular characteristics, which further underline the need for precision treatment and individual patient approach. Traditional approach to diagnose a patient with carcinoma and treat him (possibly for several years) without reevaluating the status of their disease goes directly against the idea of individualized patient approach including intratumor and intertumoral heterogeneity. Liquid biopsy technologies are built specifically to close this gap.

Both CTCs or ctDNA technologies offer clinicians additional information to address possible new resistance mechanisms appearing due to the treatment induced selection pressure or tumor heterogeneity, both spatial and temporal. Moreover, the important unit for patients´ prognosis is minimal residual disease. In the area of solid oncology, there is still no standardized approach how to measure minimal residual disease, despite the fact of traditional utilization of blood biomarkers in several carcinomas (e.g., PSA, CA-125). The standardization of minimal residual disease detection across the carcinomas could be crucial for (de)escalation of treatment, especially in early stages of the disease. Late discovery of clinically undetectable disease progression is a major threat to curative attempts. Liquid biopsy has the potential to provide earlier discovery of disease recurrence. Similar to already established circulating protein biomarkers that proved their utility for both diagnostic and prognostic purposes (e.g., CEA), both ctDNA and CTC could be used to provide additional information about disease development.

In short, the clinical routine has been already trying to establish oncological biomarkers to reveal disease progression as soon as possible. The critical condition for such procedure is non-invasive approach with possibility to repeat the procedure in regular time frames. In theory, the liquid biopsy offers all of the above not only in one type of cancer but across all cancers. The clinical need does not constitute the main hurdle to introduce liquid biopsy into clinical day to day routine, however methodology represents the challenge - how to deliver

actionable tests with minimum costs to health care providers.

Use in diagnostics

Early discovery of malignant process plays a vital role in effective treatment. Implementation of screening in the Czech Republic increased number of patients with adenomas and lowered the number of detected carcinomas[101]. However, despite of this favorable trend, there are still patient groups experiencing the opposite dynamic. In young adults between 30 to 39 years of age, whose are not a target population for current screening schemes, there was an increase in CRC incidence by 4.9% per year between the years 2005 and 2016[102]. Also, CRC in this age group tends to have more aggressive histological profile and generally is diagnosed in advanced stages[103]. Liquid biopsy could offer a possible solution for early diagnosis and potentially be an alternative to standard screening methods for younger patients.

Keeping in mind that CRC in younger patients could have different histological profile, liquid biopsy would instantly provide molecular profile for those patients. If patients would be diagnosed in advanced stage, the liquid biopsy would provide updates regarding clonal mutational status. That is important due to relatively high difference (up to 20%) between KRAS mutation status of primary tumor and metastatic sites[104]. As already mentioned, the intratumor heterogeneity, temporal and spatial, is typical for CRC. That has been shown in multiple publications. In a study with 40 mCRC patients, the authors discovered 21 patients with more than three CTCs in a sample of peripheral blood (7.5 ml). Mutational analysis of KRAS status of 16 patients then found 50% agreement between the KRAS status analyzed from solid and liquid biopsy – low concordance in this study could have been caused by a longer time gap between the surgery and liquid biopsy sample analysis[105]. Temporal heterogeneity and therefore need for repeated biopsy is also supported by studies showing induced clonal heterogeneity by a targeted therapy. A study using cell cultures found that clones surviving EGFR and/or BRAF inhibition targeted therapy harbor more DNA damage, down-regulate proteins for DNA mismatch repair, homologous recombination proteins and subsequently increase chance for additional pathological mutation[106].

For diagnostic purposes, cfDNA analysis is a few steps ahead of CTC analysis. For example, a study with 801 patients with CRC at different stages showed

promising potential for usage of cfDNA for diagnosis. Pathological staging was compared to scoring system based on analysis of 544 methylation markers, that reached promising results with 87.5% sensitivity and 89.9% specificity[107].

To evaluate implementation of liquid biopsy for CRC detection and diagnostics, various platforms were tested using samples from CRC patients to correlate CTC detection with clinical data. Nevertheless, only limited number of studies using CTC platform for correlation between CTC enumeration and morphology with TNM staging (staging based on tumor size (T), spread of cancer to nearby lymph nodes (N) and metastasis (M)) has been done. High concordance between TNM stage and CTC enumeration was reported in a study using a size-based separation platform MetaCell®[96]. Cohort of 98 subjects had 88.89% positive (presence of CTCs in their peripheral blood) colon cancer patients while 77.36% positive rate was observed among rectosigmoid cancer patients. Noteworthy, there was positive correlation reported between CTC enumeration and size of the primary tumor[108]. Other platforms also published data related to this subject. Platform CanPatrol™ reported similar results showing correlation between observation of CTCs in peripheral blood and tumor stage. Similarly to our investigation, baseline peripheral blood samples were collected prior surgery with curative intent, prior treatment in patients with palliative care, or in the chemotherapy intervals in the case of advanced disease. Identified CTCs were sorted based on epithelial (EpCAM, CK8, 18, and 19) and mesenchymal markers (VIM, TWIST1, AKT2, SNAI1). Volume of detected cells and the mean of mesenchymal cells or cells with both type of markers (epithelial and mesenchymal) displayed positive correlation with pathological stages. Out of 1203 patients, 481 patients had at least one or more epithelial CTC in one sample of 5mL blood. At least one CTC with mesenchymal marker was found in 684 patients. Ultimately, 924 patients were positive for at least one CTC with both epithelial and mesenchymal markers. The most interesting finding, also for our research, was the fact that the most segregated distribution and positive correlation with metastatic status was found in CTCs which were positive for mesenchymal markers[73]. Another study using HDSCA platform that was used also in our research, investigated correlation between liquid and solid biopsy. From 43 mCRC patients, total of 1058 CRC cells were collected either from a pre-surgery blood samples or from a solid biopsy from hepatic

metastatic tissue. The study confirmed overall intra-patient similarity in cell morphology. Methodologically, authors used hierarchical clustering of certain features (i.e. shape, size or marker distribution) normalized within each patient[89].

The technological advantage of cfDNA to CTC platforms (correlation of pathological stages and cfDNA analysis) caused their frequent use in clinical trials (see Chapter Liquid Biopsy in current clinical studies). However, to use liquid biopsy platforms (cfDNA or CTC) on daily basis, there is still need to show clear benefit and specific place of liquid biopsy if introduced into clinical routine, early detection or screening programs[109,110].

Use in treatment selection

Development of novel targeted drugs in CRC but also in other carcinomas suggests that there will be a need for precise patient selection to increase probability of successful treatment response. For example, current development in breast cancer already offers more than few targeted options for physicians and patients even with advanced breast cancer can survive multiple years, which is undoubtedly a positive development. However, long survival time and high number of potential targeted drugs for each line brought unexpected problem – should physicians rely on results of diagnostic biopsy which could reflect profile of advanced disease only little or should they risk any potential complication with a new biopsy from some of more accessible metastasis. In breast cancer, that does not apply only to genomic profile but to HER2+ status, as well. Recently, new antibody drug conjugates (trastuzumab deruxtecan, sacituzumab govitecan[111,112]) redefined HER2+ status of patients with a new clinical category - HER2low. It is more than likely that substantial cohort of patients with advanced breast cancer will need up to date HER2+ status results to make an effective therapeutical decision[113,114].

Similarly in CRC, there are few targeted options which already requires genomic or immunohistochemistry testing – MSI (dMMR/MSI high), BRAF mutation, NTRK fusion, HER2+ status. The selection of therapy choices is still not wide, and only small group of mCRC patients will benefit from those options. Therefore, the practical impact of liquid biopsy for treatment selection is still not as substantial as in the breast

cancer. Nevertheless, CRC represents high unmet medical need, and it is more than expected that new innovative drugs will be soon on a horizon together with the need to differentiate the right patients in timely manner.

In the meantime, liquid biopsy offers the possibility to overcome typical limits of solid biopsy. Generally, KRAS and NRAS mutation status varies among CRC metastasis and therefore poses a challenge for a standard solid biopsy from the primary tumor[115]. Detection of KRAS mutation from ctDNA can improve selection of those patients which will likely not profit from anti-EGFR treatment – e.g. cetuximab[116]. Hence, precise patient selection based on the RAS mutational status has significant clinical impact. Based on the results of a prospective phase 2 study, close to the 50% of mCRC patients in the study, originally tested as KRAS non mutated, eventually had a clone of cells with RAS mutation. This high frequency of temporal heterogeneity decreased significantly patients´ chance for appropriate treatment[117]. Liquid biopsy can be used to follow those changes and multiple studies were performed, correlating mutation detection from solid and liquid biopsy samples with various success. A study with OncoBeam™ RAS CRC assay displayed results with 96.4% correlation between solid and liquid biopsy. 55 patients with a positive test for RAS mutation also tested positively for RAS in cfDNA[118]. Only moderate concordance between plasma and tumor tissue was reported in another study. In a cohort of 140 mCRC patients, a study evaluating utility of cfDNA presented 72-87% agreement of liquid biopsy with tissue testing. Surprisingly, higher frequency of KRAS mutation was found in the plasma samples than in the solid tumor tissue[119]. Similar moderate agreement of 78.8% between primary tumor and cfDNA was also found in a study with 52 patients who underwent surgical resection of the primary tumor and had a histologically confirmed CRC[120]. Another study with mCRC patients by Bettegowda et al. showed 87.2% sensitivity of ctDNA in comparison to primary tumor tissue. In the cohort of total of 206 patients, 69 of them had positive KRAS mutation status in ctDNA, while only 10 subjects were tested positive for KRAS in the primary tumor but not in the ctDNA[121]. These results pose an important question, how physicians can rely on liquid biopsy if the results show only moderate concordance or does not display required sensitivity or specificity. One answer can be given by a study by Klein-Scory et al. who reported the emergence of RAS mutated clones

during anti-EGFR therapy with monoclonal antibodies in three mCRC patients, originally tested only as RAS wild-type[122].

Liquid biopsy does not currently aim to be a stand-alone test for oncological patients. In all presented studies, liquid biopsy utilizing either ctDNA or CTC provides additional and complementary information about an oncological disease. While presented results does not provide the needed prove of reliability of liquid biopsy platforms for clinical daily routine and subsequent possibility to replace tumor tissue testing, it clearly shows that it can provide an invaluable additional information for treatment selection. In the real-world the decisions are often made with great uncertainty and a non-invasive, always accessible test can be crucial for the right treatment choice.

Use in prognostics

Prognostic use of liquid biopsy is nowadays one of the most reachable clinical applications. Several studies and meta-analysis[123–126] have shown potential of liquid biopsy in cancer prognosis, including several phase 3 studies which included mostly ctDNA[127,128]. Analyzing levels of ctDNA could provide important data for physicians and select patient population with higher risk of the progression. Literature suggests that patients with worse PFS and OS outcomes have significantly higher levels of cfDNA in comparison to those with absolutely lower levels of cfDNA[129]. Additionally, patients whose KRAS mutation was detected using cfDNA analysis also experienced worse PFS and OS. Mutational analysis of the cfDNA taken together with the level of cfDNA levels could increase prognostic value of liquid biopsy analysis[129]. In a breast cancer study I-SPY2 TRIAL assessing an efficacy of MK-2206 (AKT inhibitor) with neoadjuvant chemotherapy against a neoadjuvant chemotherapy alone, 291 plasma samples were collected from 84 high-risk early breast cancer patients. The samples were collected at four different timepoints. Patients with remaining ctDNA levels at T1 (second timepoint) had significantly higher chance for a residual disease after neoadjuvant chemotherapy (83% non-pathological complete response), in comparison with ctDNA negative patients (52% non-pathological complete response; odds ratio 4.33, P = 0.012). All patients with complete pathological

response after neoadjuvant chemotherapy were ctDNA negative. Moreover, those with non-pathological complete response and detectable ctDNA levels were in higher risk of metastatic relapse (hazard ratio (HR) 10.4; 95% confidence interval (CI) 2.3-46.6)[130].

Similarly, the prognostic value of ctDNA in CRC was shown in a study with 130 CRC patients. In a group of patients without metastatic relapse, 455 samples out of 456 had no detectable ctDNA. On the other hand, 14 patients (out of 16) with a relapse had ctDNA positive samples[131]. Equivalently to the I-SPY2 TRIAL, those patients with treatment response to chemotherapy with no detectable ctDNA in any of the follow up samples, were without relapse.

In the realm of CTC liquid biopsy platforms, the first FDA approved CTC assay CellSearch® aimed to select CRC patients based on the presence of three or more CTCs per sample. Patients below the threshold had more favorable outlook than those with three or more cells per sample[132,133]. Additionally, a study from 2019 using CellSearch® shown 31.3% positivity rate in 80 CRC patients in different stages of the disease. The peripheral blood samples were collected prior the surgery. Analysis of the samples shown that detection of three or more CTCs was significantly correlated with worse OS. Furthermore, in patients with local or locally advanced CRC, finding of at least one cell was related to a poor OS[134]. In comparison, it has been published that samples of patients without carcinomas or other malignant oncological diseases rarely contain any CTC. In a study with healthy (without any malignant disease) 344 patients, more than one CTC was found only in one sample[135]. Despite existing evidence and a metanalysis including 15 studies with several technological approach for collecting CTCs, cells are less frequently used in clinical trials for assessing patients´ prognosis[126].

Liquid Biopsy in current clinical studies

Current research produces multiple reviews each year to summarize liquid biopsy technologies, either ctDNA or CTC based. However, it is rare to find review which would provide comprehensive summary of studies or clinical cases in which liquid biopsy plays role in standard clinical decisions or is a part of

prospective randomized trial phase 2 or 3. In those studies, liquid biopsy provides usually explorative endpoint, nevertheless it hints how liquid biopsy may interfere with patients´ treatment choice.

In clinical setting, ctDNA is a more common modality of liquid biopsy. As already described, in lung cancer, not only it is part of majority of the studies due to the lack of potential solid tumor samples, but it is also already a part of treatment algorithm in case of missing solid biopsy.

To provide detailed overview, **Table 2**, **Table 3, and Table 4** contains studies which reported some liquid biopsy results for breast, prostate and colorectal cancer. The studies investigated the most innovative treatment options with some being already in regular patients´ treatment algorithm (e.g., CDK 4/6i). Major goal of following paragraphs is to demonstrate that liquid biopsy has become a standard tool in selected indications, while in other indications research still has not picked up liquid biopsy as either prognostic or treatment response tool.

Very often, choice of modality of liquid biopsy – ctDNA or CTC – is driven by the type of investigated compound. If the researchers aim to detect certain mutation which can select potentially resistant tumors to a study treatment, then ctDNA analysis would be presumably a better choice. However, if the study wants to evaluate patients´ response to a study treatment and molecular profile of tumor is not the priority then CTC would be preferred option.

Breast cancer

Liquid biopsy in breast cancer has been utilized frequently in clinical studies for years. As it is described below, in most cases the liquid biopsy was used for either prognostic goals or for testing mutations in specific genes for treatment selection. Moreover, ctDNA is the dominant liquid biopsy modality in current phase 3 and phase 2 studies.

One of the few examples of phase 3 randomized trial utilizing CTCs was SOLAR-1, investigating efficacy of alpesilib with fulvestrant against placebo plus

fulvestrant. The population of this study were HER2- HR+ patients with metastatic breast cancer, previously treated by endocrine therapy. Patients were enrolled into two cohorts based on their PIK3CA mutation status. Most importantly, one of additional endpoints was PFS according to the level of ctDNA[136].

Similarly, MONALEESA studies (MONALEESA-2, MONALEESA-3, MONALEESA-7) used ctDNA for PFS stratification based on genomic profiling. The studies investigated efficacy of ribociclib, CDK4/6 inhibitor, in patients with advanced HER2- HR+ breast cancer[137–139].

Another CDK4/6 inhibitor abemaciclib was investigated in MONARCH studies to provide evidence on efficacy in HER2- HR+ advanced breast cancer patients. In MONARCH-2, ctDNA was collected to evaluate patients´ status by droplet digital PCR for only four hotspot mutations of PIK3CA (E542K; E545K; H1047L; H1047R) and ESR1 (D538G; Y537C; Y537N; Y537S). Study MONARCH-3 included ctDNA biomarker analysis as one of the exploratory endpoints. Any genomic alterations were then correlated with PFS and objective response rate (ORR)[140,141]. That suggest that there is an increase interest to incorporate liquid biopsy into modern phase 3 trials.

Destiny-Breast01 was a phase 2 trial of 184 patients with HER2+ positive mBC[142]. It is one of the first studies with the molecule trastuzumab-deruxtecan (out of 12 studies). In the first stage, the right dosage was specified and in the second stage the primary endpoint was PFS and safety. However, after ESMO2021 where results of Destiny-Breast03[143] were presented, international guidelines were changed immediately even prior to FDA or EMA registration and the compound became highly demanded choice of treatment. Interestingly, in Destiny-Breast01 Modi et al. reported second data cut-off results in 2020 and provided information that ctDNA was collected prior to first dose, every 3 cycles of treatment, and at the end of treatment. The goal of collecting the samples was to discover single-nucleotide variation/insertion and deletion, amplification, and fusion of approx. 500 genes[144].

Finally, PALOMA-3 trial also analyzed efficacy palbociclib in HER2- HR+ advanced breast cancer patients. Plasma samples were collected at the beginning of the treatment and at the end. The panel of 17 targetable driver and CDK4/6-

related genes included all exons for RB1, CDK4, CDK6, CDKN1A, CDKN1B, and NF1; exons 5–8 of TP53, and hotspots for ERBB2, PIK3CA, AKT1, ESR1, FGFR1, FGFR2, FGFR3, KRAS, HRAS, and NRAS. The study subsequently analyzed correlation of “high and low” circulating tumor fraction (threshold was 10%) with clinical parameters PFS and OS. As expected, low circulating tumor fraction showed favorable PFS and OS for patients treated with palbociclib plus fulvestrant vs. placebo plus fulvestrant[145].

In breast cancer, there is unambiguous evidence for use of liquid biopsy in clinical trials phase 3. However, the clinical routine has not yet adopted none of the modalities which liquid biopsies offer to patients. International guidelines (NCCN or ESMO) mention existence of CTC and ctDNA and their potential predictive, prognostic or treatment response application, but there is no specific recommendation:

“The clinical use of Circulating Tumor Cells (CTC) or circulating DNA (ctDNA) in metastatic breast cancer is not yet included in the NCCN Guidelines for Breast Cancer for disease assessment and monitoring”[146]

“Genomic profiling and further diagnostic tests [e.g. on tumour tissue or circulating tumour DNA (ctDNA)] should only be carried out as part of routine clinical practice if the result will change the treatment approach, as guided by the ESCAT scale, or if the patient can access appropriate clinical trials”[147]

Nevertheless, the situation is also highly dynamic. Recently during St. Gallen Breast Cancer Conference 2023 which ends usually with a consensus session, the use of new potential biomarkes has been discussed. While Tumor Infiltrating Lymphocytes (TIL) were accepted as potential treatment biomarker, ctDNA *“have no role in the treatment of early-stage breast cancer”*[148]. Few weeks later, PADA-1 trial results were presented during ASCO 2023 showing practical utilization of ctDNA by monitoring presence of bESR1 mutation. Patients with advanced HR+ HER2- BC were enrolled and monitored for rising bESR1 mutation during first-line treatment, consisting of aromatase inhibitor and palbociclib. Once the mutation was detected or its amount increased and if there was no detection of synchronous disease progression, patients were either given fulvestrant or stayed with aromatase inhibitor

(palbociclib was given in both arms in unchanged dosing)**(Figure 1)**[128]. Results showed that switch of hormonal therapy was beneficial for patients with a detected increase of bESR1 mutation. Patients treated with fulvestrant and palbociclib in the second step had median PFS 11.9 months (95% CI 9.1-13.6) vs. 5.7 months (CI 95% 3.9-7.5) in the arm with aromatase inhibitor and palbociclib (stratified HR 0·61; 95% CI 0·43–0·86; two-sided p=0·0040).

Based on both recent released publications, it is difficult to precisely predict future use of ctDNA, but incorporating targeted therapy for specific mutations will most likely cause increasing importance of this liquid biopsy method, especially in later stages of the disease.

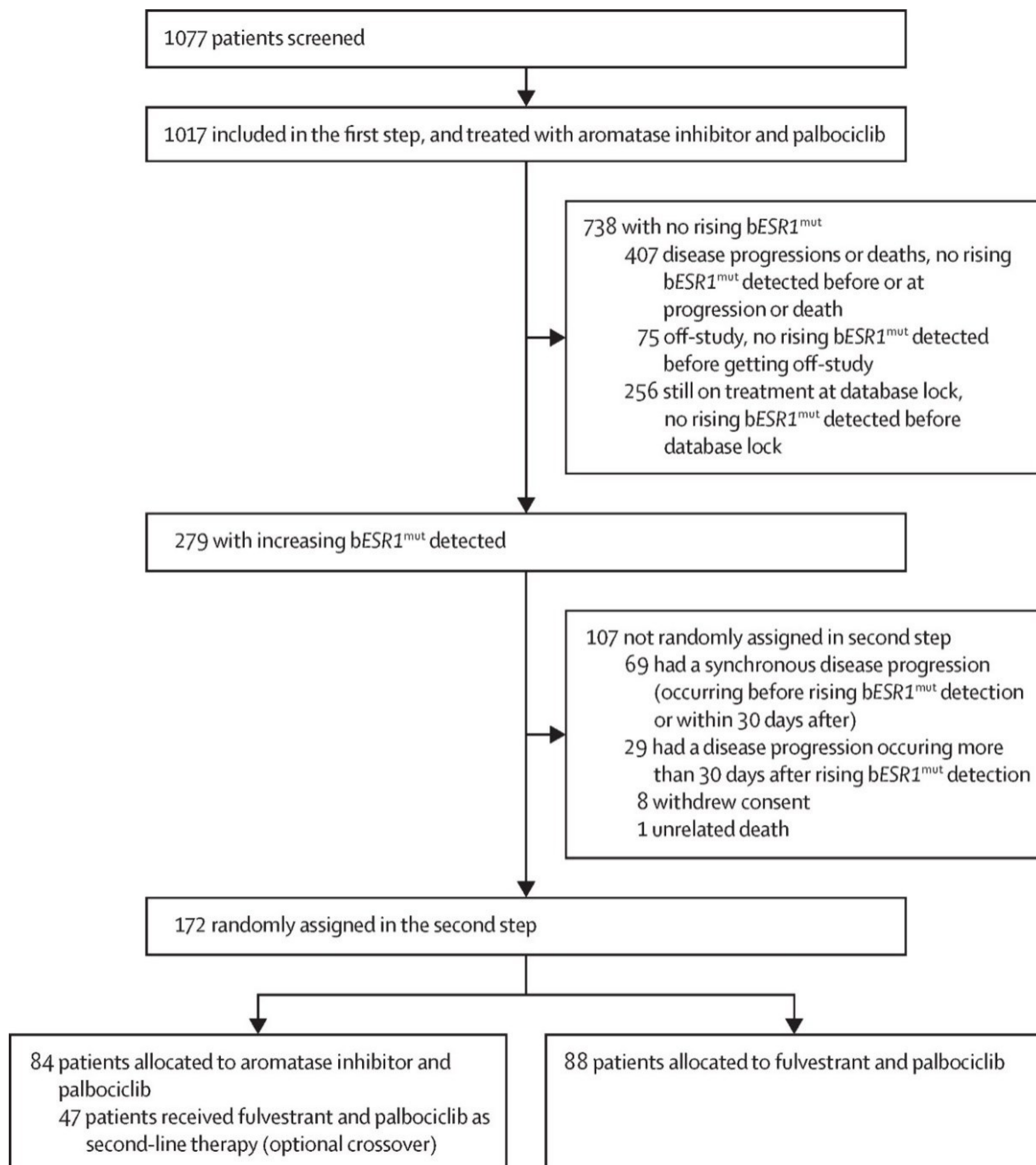

Figure 1 *PADA-1 study in which patients were selected based on detection or increasing levels of bESR1 mutation in ctDNA. Results of the study create a need to detect bESR1 mutation in patients for treatment optimalization.*

Study	**Modality**	**Use**	**References**
SOLAR-1	ctDNA	Prognostic in PFS (levels of ctDNA) and OS (mutational status from ctDNA)	149
MONALEESA-2	ctDNA	Prognostic via detecting alterations (PIK3CA, TP53, RTK) in baseline ctDNA and then compare PFS between mutated and wild-type cohorts	137

MONALEESA-3	ctDNA	Prognostic via detecting alterations (PIK3CA, ESR1, TP53, CDH1, FGFR1, FGFR1/ZNF703/WHSC1L1) in baseline ctDNA and then compare PFS between mutated and wild-type cohorts	139
MONALEESA-7	ctDNA	Prognostic via detecting alterations (PIK3CA, TP53, CCND1, MYC, GATA3) in baseline ctDNA and then compare PFS between mutated and wild-type cohorts	138
MONARCH-2	ctDNA	Prognostic via detecting alterations (PIK3CA, ESR1) in baseline ctDNA and then compare PFS between mutated and wild-type cohorts	140,141
MONARCH-3	ctDNA	Prognostic via detecting alterations (PIK3CA, TP53, EGFR, FGFR1, NF1, MYC, CCND1, ESR1) in baseline ctDNA and then compare PFS and OS between mutated and wild-type cohorts; Treatment selection pressure via detecting alterations in samples at end of treatment vs. baseline samples	150,151
Destiny-Breast01	ctDNA	Treatment response via detecting alterations of ≈ 500 genes (e.g. decrease of ERBB2 copy number reported and correlated with treatment response)	144
PALOMA-3	ctDNA	Prognostic via detecting alterations (ESR1, PIK3CA, TP53) in baseline AND at end of treatment ctDNA and then compare PFS between mutated and wild-type cohorts and between baseline and end-of-treatment samples	145
PADA-1	ctDNA	Treatment selection via detecting ESR1 mutation in ctDNA and consequently switching study treatment or continue with same therapy	128

Table 2 *Overview of clinical studies and purpose of use of liquid biopsy modality. In the breast cancer research, liquid biopsy based on analysis of ctDNA dominates.*

Prostate cancer

In prostate cancer, both liquid biopsy modalities are utilized, but in contrast to breast cancer treatment there is less clinical need for liquid biopsy. The main reason for that could be lower number of targetable treatment options than in breast cancer. It is also not coincidence that the most studies in prostate cancer utilizing some forms of liquid biopsy are those with PARP inhibitors. The experience with PARP inhibitors in ovarian or breast cancer is that the highest efficacy is present in mutated malignancies. In contrast, the studies with new hormonal agents (darolutamide, apalatumide, enzaltumide) did not utilize any form of liquid biopsy due to their high efficacy across population that there was simply no reason to distinguish responders from resistant patients[152–154].

A phase 3 study PROfound, testing efficacy of olaparib in monotherapy after progressing on novel hormonal agents, used as one of the explorative endpoints CTC enumerations, which it makes the first study on this list which used in clinical trial CTC modality of liquid biopsy for any endpoint. Specifically, trial used “the circulating-tumor-cell conversion rate” which was defined as the percentage of patients with a decrease in the number of circulating tumor cells from ≥5 cells per 7.5 ml of whole blood at baseline to <5 cells per 7.5 ml after baseline[155].

Similarly, phase 2 study TALAPRO-1 investigated efficacy of another PARP inhibitor talazoparib in patients with DNA repair defects and metastatic castration-resistant prostate cancer, previously treated with taxan-based chemotherapy and experiencing progression on novel hormone agents. One of the endpoints was again simple CTC conversion rate (to CTC = 0 and <5 per 7.5 mL of peripheral blood) between two timepoints (before and after the treatment)[156].

Another phase 2 study GALAHAD analyzed effect of next PARP inhibitor niraparib in patients with metastatic castration-resistant prostate cancer and with progression on taxan chemotherapy and androgen deprivation therapy. The study also used CTC conversion rate (only to < 5/7.5 mL blood) as one of its endpoints[157].

Recently, ctDNA was used in PROpel study, phase 3 study, to prove efficacy of abiraterone+olaparib combination treatment against abiraterone+placebo. Despite

the primary endpoint being progression free survival (PFS), patients´ mutation status was also assessed. Since part of the patients (~25%) had no tumor tissue available, ctDNA analysis for specific mutation was necessary. The results show high concordance between liquid and solid biopsy for patients with both analysis availlable[158].

Similarly, TALAPRO-2 study[159] enrolling similar population as PROpel study also utilized ctDNA for testing patients for potential mutation in selected genes. Interestingly, we see that in both drugs, olaparib and talazoparib, there is a clear shift in preferring ctDNA over CTC in recently designed studies which shift treatment to earlier stages.

Also, phase 3 study TRITON3 focusing on efficacy of rucaparib against physician´s choice of therapy in metastatic castration-resistant prostate cancer patients with homologous recombination deficiency (term mostly associated with ovarian cancer) disclosed as one of the key explorative endpoints an analysis of pretreatment blood samples collected from all patients for BRCA 1/2, and ATM gene mutations in ctDNA[160].

Study	Modality	Use	References
PROfound	CTC	Treatment response via CTC conversion rate - defined as the percentage of patients with a decrease in the number of circulating tumor cells from ≥5 cells per 7.5 ml of whole blood at baseline to <5 cells per 7.5 ml after treatment	155
TALAPRO-1	CTC	Treatment response via CTC conversion rate - defined as the percentage of patients with a decrease in the number of circulating tumor cells from ≥5 cells per 7.5 ml of whole blood at baseline to <5 cells per 7.5 ml and to CTC=0 after treatment	156

GALAHAD	CTC	Treatment response via CTC conversion rate - defined as the percentage of patients with a decrease in the number of circulating tumor cells from ≥5 cells per 7.5 ml of whole blood at baseline to <5 cells per 7.5 ml after treatment	157
PROpel	ctDNA	Treatment response via detecting alterations (ATM, BRCA1, BRCA2, BARD1, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D, RAD54L) in baseline ctDNA	158
TALAPRO-2	ctDNA	Treatment response via detecting alterations (CDK12, BRCA1, BRCA2, ATM, CHEK2, ATR, ANCA, MLH1, MRE11A, NBN, PALB2, RAD51C) in baseline ctDNA	159
TRITON-3	ctDNA	Treatment selection via detecting alterations (BRCA1, BRCA2, ATM) in baseline ctDNA	161

Table 3 *Overview of clinical studies and purpose of use of liquid biopsy modality. In prostate cancer research, we see mixed used of CTC and ctDNA. However, studies with later start date began to prefer ctDNA.*

Colorectal cancer

Interestingly, clinical research in CRC has not produced that high number of phase 2 or 3 studies utilizing liquid biopsy in comparison to breast cancer. In the case of CRC, the most plausible explanation is lower amount of novel targeted drugs which would see additional benefit in utilizing ctDNA.

One example could be BEACON CRC study. In this open label, phase 3 trial, 665 patients with BRAF V600E mutation were enrolled. Patients were then treated in ratio 1:1:1 with encorafenib, binimetinib and cetuximab (triple therapy vs. double therapy vs. control arm of investigators´ choice) to investigate efficacy of the triplet therapy against control arm. The primary endpoints in the study were overall survival and objective response rate[162]. Recently, authors of BEACON study have presented

results from ctDNA analysis. The main goal of this analysis was to establish whether detection of BRAF V600E mutation in the baseline sample ctDNA collected on day 1 of cycle 1 correlated with response. The patients were separated into high (>median), and low (<median) categories based on variant allele frequency (VAF) of BRAF V600E. Low category included the patients with no mutation or no ctDNA detected. Out of 631 patients with ctDNA, available samples of 544 patients were used in this analysis. The mutation was detected in 90.4% of cases (492 out of 544). The results have shown that objective response rate and overall survival in both categories have shown favorable results for triplet therapy against the control arm. The results have shown that high frequency of variant allele could be used also for patients´ prognosis, not only treatment selection. The patients in high category had decreased OS (median OS [95% CI]: triplet 7.2 [6.0–8.0] months, n = 99; doublet 5.4 [4.4–6.1] months, n = 88; control 4.2 [3.4–4.8] months, n = 85) compared with patients with low VAF (triplet 14.8 [10.2–19.8] months, n = 97; doublet 14.8 [11.7–23.0] months, n = 99; control 9.3 [7.5–11.3] months, n = 76)[163].

On the other hand, in comparison to prostate cancer there is higher unmet medical need in CRC based on mortality[1]. It is then expected that novel diagnostic and prognostic tools, same as novel drugs, will be introduced soon in CRC than, as well. Despite less novel targeted drugs which would need ctDNA in clinical practice, there has been reported results investigations into potential utility of ctDNA for minimal residual disease, treatment selection and prognosis. The most recent study done within MD Anderson INTERCEPT program showed that scanning patients for ctDNA positivity followed by imaging will result in 53% of ctDNA positive patients having concomitant radiographic findings. 1259 patients with stage II-IV CRC treated with curative intent were enrolled into INTERCEPT program with the goal to integrate minimal residual disease (MRD) testing into clinical routine. Out of those, 1049 patients were included into ctDNA analysis and 159 (15%) of them were tested positive for ctDNA. New metastatic sites were found in 46 patients (53%)[164]. In similar fashion, GALAXY is a prospective observational study which monitors ctDNA status. Based on the results, patients can be enrolled in investigator-initiated phase 3 trials VEGA (de-escalation) and ALTAIL (escalation)[165]. In the recent update during ASCO 2023, authors presented an updated analysis and the lead time interval of

ctDNA positivity to radiographic recurrence in patients with radically resected stage II-IV CRC. Authors reported that 286 (14%) out of 2083 patients were found to be positive for ctDNA at 4-weeks timepoint and experienced decreased disease-free survival with 12 times more likelihood of recurrence than ctDNA negative patients (HR:12, 95CI: 9.1-15%; $p < 0.0001$). Additionally, ctDNA positive patients with BRAF V600E mutation experienced significantly shorter disease-free survival in comparison to ctDNA positive BRAF wild-type patients ($p < 0.001$). Moreover the median lead time interval to radiographic recurrence was 142 days (IQR 43-189)[166]. Another studies that aim to investigate use of ctDNA (mainly for predictive purposes, similarly to the INTERCEPT and GALAXY studies) in local and locally advanced CRC are currently underway[167–170].

Study	Modality	Use	References
BEACON CRC	ctDNA	Treatment selection and prognostic via detecting BRAF V600E alteration	163
INTERCEPT	ctDNA	Disease progression via overall ctDNA positivity	164
GALAXY	ctDNA	Disease progression and prognostic via detecting BRAF V600E alteration and overall ctDNA positivity	165,166
VELO 2	ctDNA	Treatment selection and treatment response via detecting RAS wild-type mutation (and other) in baseline ctDNA and after disease progression	171

Table 4 *Overview of clinical studies and purpose of use of liquid biopsy modality. In CRC research, there is limited use of liquid biopsy in clinical trials.*

Liquid biopsy in breast cancer vs. CRC

Despite limited use of liquid biopsy in current studies in CRC, there is one clear trend across the whole development of novel treatments. There is a rapid increase of newly approved drugs every year[172].

Between years 2000 and 2022 FDA had approved 573 new indications for 206 various products. Out of those, 50 of them were cytotoxic, 277 were targeted drugs and 246 were targeted biologics. Even more impressive is the rate of approval which also increased over that period. The mean annual approval in a 5-year period increased from 7.4 indications per year in the period 2000-2004 to 56 (sic!) per year for 2017-2022. Unsurprisingly, the steep increase in the last decade was driven by targeted therapies and biologics, while cytotoxic molecules approval slightly decreased. In the last two years, that being 2020-2022, targeted biologics has overcome targeted drugs.

In the context of this subchapter, there is an interesting correlation to observe. According to the report, new approvals for breast cancer can be found throughout the period of twenty years. In the last 7 years since 2015, the most innovative treatments based on disease types, among others, were breast cancer and genitourinary cancers. Both had 35 and more approvals per year in that period. On the other side, novel therapies were scarce throughout that period for colorectal cancer, suggesting that there is still room for opportunities.

Coupled with the use of liquid biopsy platforms in clinical trials, we can see that breast cancer, with high number of approvals since 2000 **(Figure 2)** and therefore high number of clinical trials, is also a disease type with the most frequent use of liquid biopsy. The prostate cancer trials using liquid biopsy reported in this thesis were designed and published mostly after 2015. Therefore, we can hypothesize that with the increase of novel treatments (and clinical trials), we can expect an increase of use (and need) of liquid biopsy platforms also in CRC.

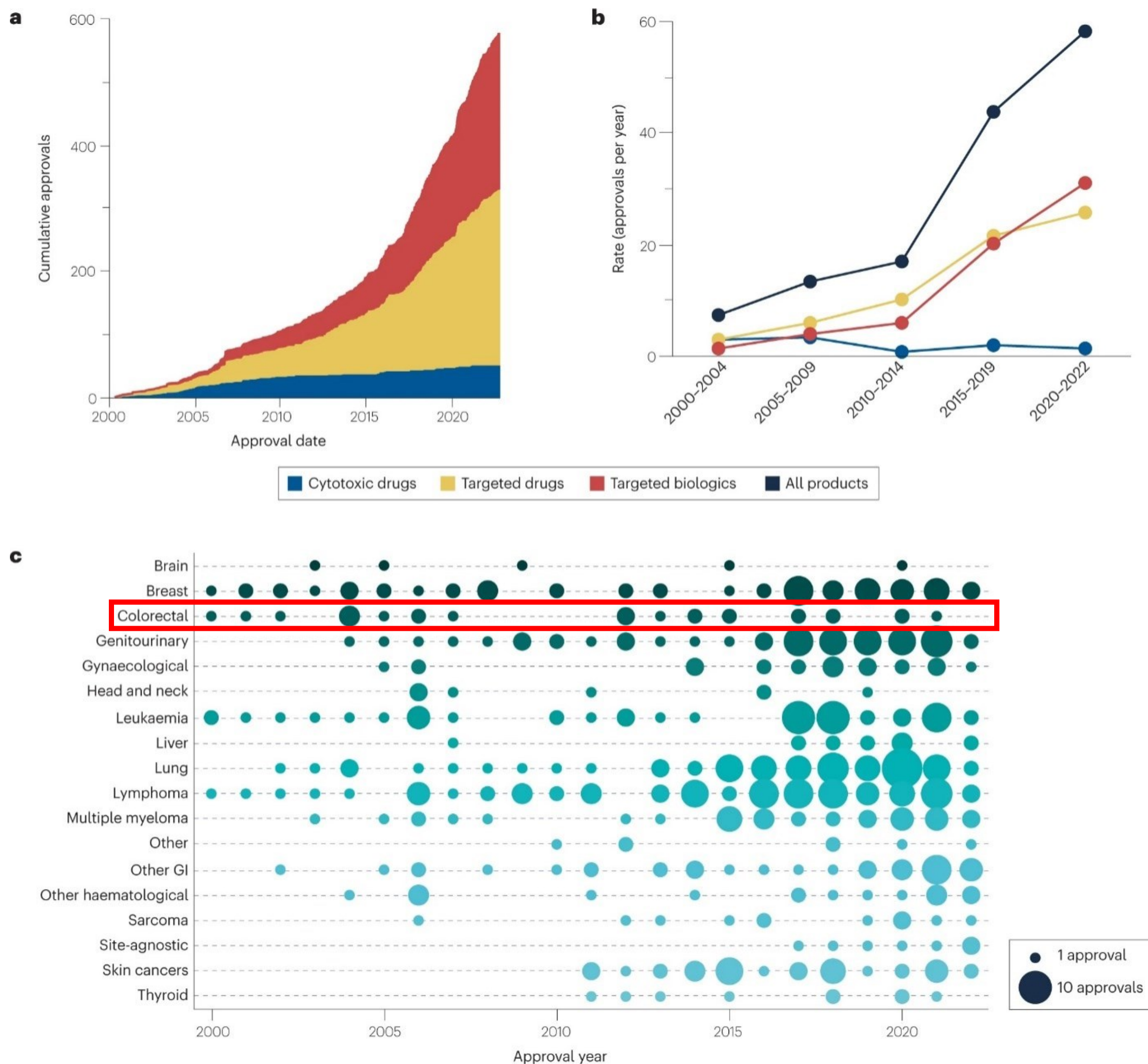

Figure 2 *Number of FDA approval in the last 20 years shows high innovative pace in breast or lung cancer. Number of CRC approval was significantly lower suggesting room for innovations in coming years, coupled with increasing number of phase 3 studies needed liquid biopsy.*

Goals and hypotheses

The research of CTCs in CRC is complex and most of the platforms rely on some kind of enrichment and subsequent quantitative analysis for CTC enumeration. This thesis heavily leverages software driven analysis of HDSCA which allows to concurrently exploit several characteristics of collected cells. Moreover, the technology of HDSCA experienced development over the years and results show that additional target markers can provide important data for further research.

In the first study, we aimed to analyze peripheral blood of 47 mCRC patients and employing HDSCA 1.0 platform to identify cells associated with the presence of tumor (see Chapter Methodology). The software allowed to characterize and classify large number of cells and final selection of CTCs is then controlled by an analyst. We have hypothesized that we could distinguish cohort of patients with better survival prognosis based on CTC levels. Additionally, we have expected to find similar survival difference based on results of CTC dynamics measured as changing levels of CTCs between pre-defined timepoints.

In the second investigation, we aimed to analyze 18 samples of peripheral blood from 10 patients, this time utilizing updated HDSCA 3.0 platform to identify cells associated with the presence of tumor. Methodologically, we have used two immunofluorescence protocols to provide comprehensive description of CTCs and oncosomes. We hypothesized that use of two distinct staining protocols will allow us to detect new types of rare events which were previously unidentified. Moreover, we also assume that adding CDX2 monoclonal antibody to the second protocol will help us to identify distinct cohort of CTCs with colorectal origin. Similar to first investigation, we also hypothesized that rare events dynamics between draws would show correlation with survival data.

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Methodology

Materials and Methods: High-Definition Single-Cell Assay

This thesis includes two studies using HDSCA platform for identification of rare events (cells and other non-cellular events). As HDSCA is still in a fast-paced development, various versions of the platform were used in individual presented studies. Briefly, the first part of the study was done using HDSCA 1.0 (analysis of 3 fluorescence channels), while the second study was using a HDSCA 3.0 (included 4th channel which allowed to capture more diverse phenotype of rare cells). In this subchapter, HDSCA will be presented in general terms, describing common features for both generations. In the respective results chapters, the differences between its versions will be highlighted. Especially, in the second part of the results, HDSCA 3.0 offer the opportunity to investigate rare events in more details and categorize CTCs into more precise categories, based on additional phenotype data.

HDSCA technology **(Figure 3)** is a unique way of identifying CTCs and other rare events as briefly described in Chapter Liquid Biopsy Technologies. Thanks to collaboration with Professor Peter Kuhn at Convergent Science Institute in Cancer, Michelson Center of University of Southern California, we were able to analyze cohort of 47 mCRC Czech patients and subsequently continue with the research on smaller cohort leveraging the HDSCA 3.0.

Software Analysis in HDSCA

Specific algorithm is used in HDSCA platform to identify potential rare events (those rare events are later confirmed by an analyst). The algorithm is named Outlier Clustering Unsupervised Learning Automated Report (OCULAR) and relies on extraction of predefined features, principal component analysis and subsequent ordering of those components in hierarchical manner. In the end, OCULAR aims to achieve four main assignments: extraction of image feature (based on fluorescent immunohistochemistry; 761 parameters), distinguishing rare events (common vs. rare DAPI+ events and DAPI- events), classification of the said events and finally producing reports. Analysis of image is done via visual segmentation of events and creating nuclear and cytoplasmic masks. In the next steps, the principal components are assigned to each mask to achieve dimensionality reduction. Ordering those

components hierarchically allows to distinguish common events and rare events. Following this computational algorithm, CTCs and oncosomes are then manually separated based on morphology and biomarker expression in the four fluorescence channels. Final classification of rare events is also conducted by hematopathologist-trained technical analysts.

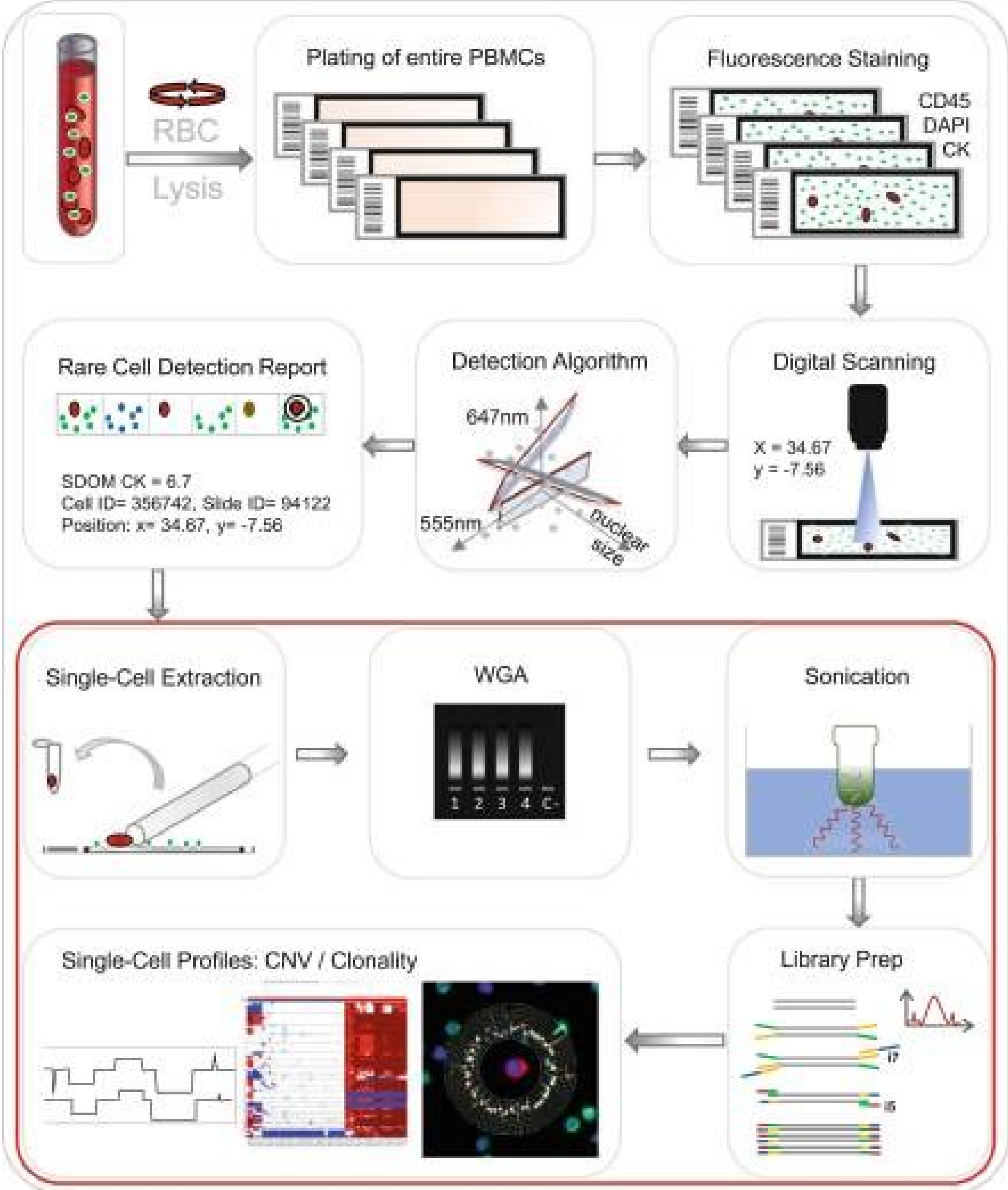

__Figure 3__ High level overview of HDSCA platform processing a peripheral blood sample: red blood cells after lysis are laid on a slide and stained (the staining differs between HDSCA 1.0 and 3.0), followed by full slide scanning and computational analysis of potential rare events. After manual check of a trained analyst, single-cell extraction, next-generation sequencing and CNV profiling is possible. (the red panel). However, the single cell analysis with genomic profiling is not part of this thesis.

HDSCA 1.0

Samples for the research using HDSCA 1.0 were collected in a cohort of 47 mCRC patient from the Czech Republic. One of the hypotheses was to investigate changing levels, dynamics, of CTCs over series of defined timepoints: 1–2 week prior to surgery, right prior the resection and post-resection on the day of surgery, and additional samples collected at regularly scheduled visits for up to 2 years. However, not all 47 patients have samples at every timepoint due to loss of follow-up or as deviation from the protocol. Peripheral blood was collected in Cell-Free DNA BCT® from Streck© (Streck; Omaha, NE, USA) and was processed in 48 hours latest.

Liquid biopsy specimen, peripheral blood in this case, collected from patient was processed within 24 to 48 hours to enable further cell characterization. During the procedure, erythrocytes were lysed and discarded, sample was stabilized and concentrated by centrifugation of the specimen. Specifically, blood sample was rocked for 5 minutes after which WBC count was established using Hemocue white blood cell system (HemoCue, Sweden). Subsequently, erythrocytes were lysed using ammonium chloride solution. After follow up centrifugation, nucleated cells were then again suspended in PBS. All nucleated cells in the sample were then plated on a custom adhesive glass slides (Marienfeld Adhesive slide, Paul Marienfeld GmbH & Co. KG, Germany) with a density of approximately 3 million nucleated cells per slide. In this state, slides were frozen and stored at -80 °C until further use.

After receiving slides in the Michelson Center, cells were stained with DAPI and antibodies against pan CK and CD45, and slides were prepared for scanning with immunofluorescent microscope. Specifically, the slides were first fixed with a 2% neutral buffered formalin solution for 20 min followed by permeabilization using 100% cold methanol for 5 min and blocking nonspecific binding sites with 10% goat serum (Millipore) for 20 min. This step was followed by antibodies used for staining and the staining protocol details were: anti-pan CK at a 1:100 dilution in 10% goat serum (anti-pan CK mix IgG1 [CK1, 4, 5, 6, 8, 10, 13, 18, 19], Sigma, C2562; anti-CK 19 IgG1, Dako, M0888) and anti-CD45 directly conjugated to Alexa Fluor® 647 at a dilution of 1:125 (mouse, monoclonal IgG2a, AbD Serotec, MCA87A647X). After primary antibody incubation, slides were washed in 1X phosphate buffered saline twice for 3

min each. A secondary antibody mix was applied containing goat anti-mouse Alexa Fluor® 555 IgG1 in a 1:500 dilution in 10% goat serum and nucleic acid stain 4′,6-diaminido-2-phenylindole (DAPI, Thermo Fisher Scientific (Waltham, MA, USA), 5 mg/mL in 1:50,000 dilution). Scanning of the slides was done in both generations of the HDSCA in a similar fashion. Slides were scanned automatically by high-throughput fluorescence scanning microscopy at 100x magnification creating 2304 frames per slide.

Selected CTCs by OCULAR and their images were evaluated by trained technical analysts with histology training for interpretation. In HD-SCA 1.0, detected cells were grouped into following categories (see also **Table 5**): cells positive for CK with nucleus (DAPI positive) and from morphological point of view, distinctly recognizable from surrounding white blood cells. Also, these cells, here called “HD-CTCs”, must be without any sign of apoptosis. CK positive CD45 negative cells with recognizable apoptotic changes in the nucleus were named as “CTC-Apoptotic”. Cells with morphology similar to HD-CTC, but without any expression of CK were defined as “CTC-NoCK”. Finally, cells with CK expression and negative for CD45 biomarker but with nucleus size similar to white blood cell in their proximity are called “CTC-Small”. In the effort to relate our research to previously published work that employed more standard methods for CTC enumeration without their further separation into distinct groups, we have also created a group “CTC-CKtotal” for all the cells positive for CK which were recognizable from white blood cells. Physical grouping of two or more HD-CTCs in a cluster is defined as CTC cluster or “CTCC”.

The CTC images from a 10× objective by a high-throughput automated fluorescence microscope were used for enumeration. WBC counts of peripheral blood were determined manually with a hemocytometer (Medonic M-series Hematology Analyzer, Clinical Diagnostic Solutions Inc., Fort Lauderdale, FL, USA) and the number of leukocytes detected by the assay per slide was used to calculate the actual amount of blood analyzed per test. Concentration of rare events per mL was calculated using the exact amount of analyzed blood. Therefore, fractional values of HD-CTCs/mL were possible to use in later analysis. To determine phenotypic differences of cells within a draw, cells were imaged by 40× objective and analyzed for cellular/nuclear shape and size.

For simplified workflow of HDSCA 1.0 see **Figure 4**.

Subtype	Markers			Size	Apoptotic
	DAPI	CK	CD45		
HD-CTC	+	+	-	>WBC	-
CTC-Apoptotic	+	+	-	N/A	+
CTC-NoCK	+	-	-	>WBC	-
CTC-Small	+	+	-	=WBC	-
CTCC	+	+	-	>WBC	-

Table 5 *Classification of subtypes in HDSCA 1.0; subtypes are not classified only based on marker positivity but also on morphological features.*

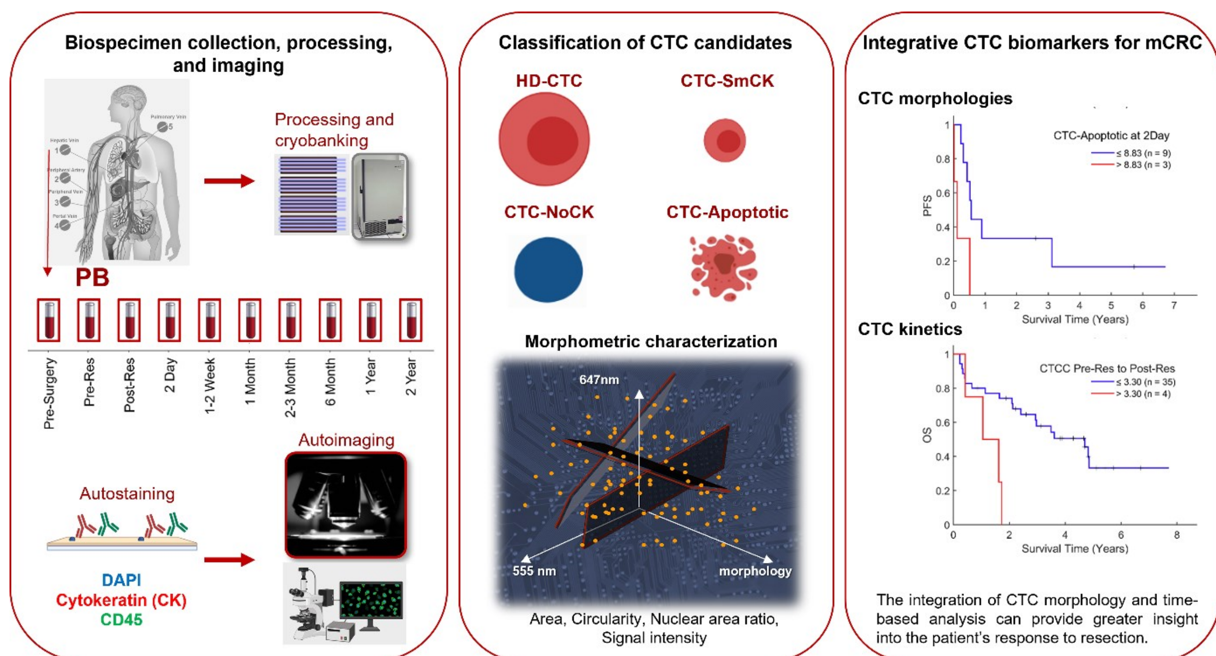

Figure 4 *Overview of process in the first investigation; slides prepared from patients´ samples were autostained and imaged (left panel), rare cells were classified based on marker positivity and morphological features (middle panel), combining enumeration and dynamics (kinetics) with clinical data provides Kaplan-Meier curves (right panel)*

HDSCA 1.0 Statistical Analysis

In the first research, demographic characterization of mCRC cohort was done by standard frequency tables and descriptive statistics. Additionally, analysis for correlations between categorical and ordinal or quantitative variables was done by Mann-Whitney U test or Kruskal-Walls ANOVA (defined by number of categories). Correlation between two ordinal or quantitative variables were tested by Kendall´s tau

nonparametric correlation coefficient.

For the purpose of survival analysis, survival was defined as time from the date of surgery to either date of clinically diagnosed disease progression or death (in the case of PFS) or to date of death (in the case of OS). Patients with no available data for events of PFS or OS were censored at the date of the last follow up.

Cox proportional hazards model was used to investigate the correlation between quantitative variable (CTC enumeration and number of metastatic sites). For all quantitative variables, at first Box-Cox transformation was performed in order to compensate for potential non-normal distribution and then were those variables tested. The p-value that indicated significance after the Box-Cox transformation was stated and is written down as P_BC.

For preparation of Kaplan-Meier plots, threshold value was set for each prognostic variable. Based on the threshold, two cohorts were defined. Threshold value was selected based on the lowest Cox-Mantel p-value in automated optimization process implemented in Matlab (2019a, MathWorks Inc., Natick, MA, USA),

All reported p-values are two-tailed, and the level of statistical significance was set at $\alpha = 0.05$. Statistical analysis was done in Statistica (ver. 12 Cz, TIBCO Software Inc., Palo Alto, CA, USA). Data were visualized using R (Version 4.0.3, Boston, MA, USA).

HDSCA 3.0

On top of the HDSCA 1.0, there was a development of the platform with CD31 and vimentin staining in the 4th fluorescence channel in order to provide deeper understanding of collected cells and to illuminate their potential origin.

In the study using HDSCA 3.0, cohort of 10 mCRC patients with total of 18 samples of peripheral blood was analyzed. Patients were part of the GS-US-296-0101 phase I clinical trial (#NCT01803282) with the aim to evaluate the safety and tolerability of a novel drug in combination with chemotherapy in two different mCRC indications. According to Institutional review board protocol, no other demographic or clinical data were at our disposal with the obvious exception of diagnosis and survival data. Patients were divided into two cohorts. The first cohort (patients 1, 3, 5, 6 and 9) was treated with the investigated drug in combination with mFOLFOX6 and bevacizumab and were in the first line of palliative treatment. The second cohort (patients 2, 4, 7, 8 and 10) was receiving the investigated drug in combination with FOLFIRI and bevacizumab while being in the second line of palliative treatment. There were two samples collected per patients, both on day 1 of either cycle 1 or cycle 3 therapy. Two samples are missing due to patients´ compliance. Unfortunately, all patients progressed. PFS is known for 9 out of 10 patients.

As described in the Chapter Result Part 2, the research required also healthy donors to control for significance of rare events levels. Peripheral blood of healthy donors provided by Epic Sciences (San Diego, CA, USA) was collected from 50 volunteers. At the time of sample collection, those volunteers did not have any history of neoplastic disease.

Similarly to HDSCA 1.0, blood samples were collected in 10 mL collection tubes (Cell-free DNA, Streck, La Vista, NE, USA) and were processed according to same protocol as HDSCA 1.0 until applying DAPI.

From the point of applying DAPI, the staining differs from the HDSCA 1.0. To be able define and characterize rare events, two staining protocols were used to produce data. The Landscape protocol was designed to find cells with epithelial, mesenchymal or endothelial phenotype. That was achieved by adding 100 ug/mL of a goat anti-mouse IgG monoclonal Fab fragments (115-007-003, Jackson ImmunoResearch, West Grove, PA, USA), rabbit IgG anti-human vimentin (Vim)

(clone: D21H3, 9854BC, Cell Signaling, Danvers, MA, USA) as a fourth color and mouse IgG1 anti-human CD31:Alexa Fluor® 647 mAb (clone: WM59, MCA1738A647, BioRad, Hercules, CA, USA) to the CD45 channel, hereafter referred to as CD45/CD31. The second protocol, CD2X-targeted was designed to reveal cells with CDX2 expression. That was achieved by adding colon specific CDX2 monoclonal antibody EPR2764Y: AlexaFluor 488 (Abcam, Cambridge, UK) as a fourth color for further characterization. CDX2 is a known transcription factor found in the cells of intestinal epithelium and was used to identify types of intestinal cancer. Samples from healthy volunteers were stained by the Landscape protocol.

The classification of cell subtypes is mostly done via description of channel positivity (see **Table 8** in Results Part 2). If a cell is described as CK|Vim then it is DAPI+, CK+, Vim+, CD45/CD31- (if the cell would be stained in the Landscape Protocol). In other publications utilizing HDSCA platform, there were described large extracellular vesicles[173–178], here referred as oncosomes. Similarly to rare circulating cells, also oncosomes are characterized by description of channel positivity with the difference of the “Onc” abbreviation that stands before the positive channel types. If an event is described as Onc CK|Vim then it is Oncosome DAPI+, CK+, Vim+, CD45/CD31- (stained in the Landscape Protocol).

Beyond the simple channel positivity description, there are two specific CTC populations reported in the second investigation: epithelial CTC (Epi.CTC) which is CK+, Vim- and CD45/CD31- with visible and distinctive nucleus (DAPI+), and mesenchymal CTC (Mes.CTC) which is CK+, Vim+ and CD45/CD31- with also visible and distinctive nucleus (DAPI+)

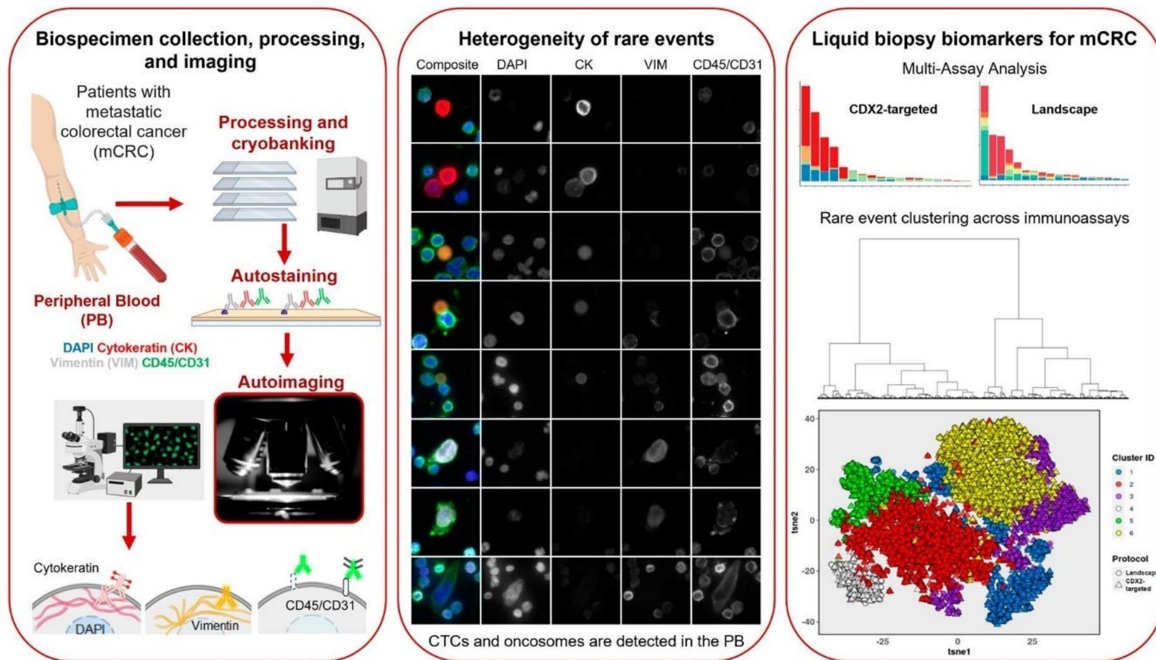

Figure 5 Overview of process in the second investigation; samples were processed similarly to HDSCA 1.0 with the difference of additional 4th IF channel (Vim, CD45/CD31) (left panel), rare cells were classified based on marker positivity and morphological features – adding 4th channel allow us to further analyze rare events (middle panel), using two distinct IF protocols gave us the opportunity to conduct Multi-Assay Analysis (right panel)

Multi-Assay Analysis

In the second investigation, there were two IF protocols (Landscape and CDX2-targeted) used to identify rare events in the slides from the same sample.

This setup allowed us to utilize so called multi-assay analysis. As described above, OCULAR uses 761 morphological parameters from 100x magnification images. For the multi-assay analysis 8 morphometric parameters were selected to find cells with similar features from both protocols. The 8 morphometrics parameters were following: median intensity for DAPI and CK channel, average distance of the cell outline to the center of the nucleus, the eccentricity and area of the cell and nucleus, and the ratio of nuclear to cellular area. In the multi-assay analysis, 5661 rare cells were evaluated using selected morphometrics parameters and categorized into groups based on similarity. An algorithm for clustering was imported from the scikit-learn library version 0.23.2 in Python. Additionally, Euclidian metric was used to find out the distance and the ward linkage criterion. After manual inspection, 111 rare events (cells

and oncosomes) were removed from grouping because of atypical nuclear and membrane masks. The ideal level of cell groups (between 2 and 16) was estimated based on quantitative group-separation metrics and selection of evident, rare cell types. Eventually 8 cell groups were established (via silhouette average method) as the ideal number of groups. Beside cell grouping, Spearman´s rank correlation was conducted between the two protocols for rare events enumerations.

For simplified workflow of HDSCA 1.0 see **Figure 5**.

HDSCA 3.0 Statistical Analysis

Spearman´s rank correlation coefficient was established for all analytes versus the PFS. Only 9 patients had available data for PFS analysis. The standard Kaplan-Meier plots were used for PFS. Patients were also stratified into cohorts based on levels of rare events per milliliter of peripheral blood and time until progression of disease or death. Specifically, median number of CTC per milliliter of peripheral blood was used for establishing a threshold, separating the patients into two cohorts. Scipy was used for the statistical work and the Kaplan-Meier plots were done by scikit-survival library in Python.

From the perspective of the rare events levels, the separation of the healthy donors and mCRC samples was done by a Wilcoxon rank sum test. Statistical associations between rare events levels were calculated by the Spearman´s rank correlation coefficient. All reported p-values are two-tailed, and the level of statistical significance was set at $\alpha = 0.05$. The statistical work was conducted via scipy in Python.

Results Part 1

Study Design

In the first study, there has been 161 samples of peripheral blood collected from 47 mCRC patients. Samples were collected in several time-points, starting before patients' surgery and finishing up to 2 years after the tumor resection with the goal to analyze samples for CTCs. Whole cohort had histological results of CRC and radiologically confirmed metastasis. Patients' characteristic is enclosed in **Table 6**.
Samples from each patient were taken in the pre-specified time-points. However, not all patients have samples available for all time-points. Specifically, for 10 patients, which equals 21.28%, peripheral blood sample was acquired 3-7 days pre-surgery. Additionally, 45 patients (95.74%) had their pre-resection draw taken on the day of surgery (after anesthesia and before first section). Following post-resection sample was drawn for similar number of patients after removal of tumor tissue – 41 (87.23%). In following weeks, months and even years, number of patients who provided specimen and were not lost for follow-up or dead varies. At 2 days after surgery, 12 patients (25.53%) provided blood sample, at 1-2 weeks 27 patients (57.45%), 6 patients (12,77%) at 1 month. Additional 6 patients (12.77%) gave their sample also at 2-3 months and slight increase of samples was experienced at 6-month mark with 10 patients (21.28%) providing their sample. Furthermore, only 2 patients (4.17%) gave their blood samples at 1 year and 2 years. 2-year mark was a final term for sample collection and patients were then only followed for PFS and OS analysis.

Clinical Factor	n	%	Clinical Factor	n	%	Clinical Factor	median	range
Sex			**T Stage**			**Age at Resection**	53	34-82
Male	25	53.2	1	1	2.1			
Female	22	46.8	2	2	4.3			
			3	30	63.8			
			4	7	14.9			
			NA	7	14.9			
Synchronous Disease			**N Stage**			**Tumor Size**	5	1.5-9.0
Yes	28	59.6	0	12	25.5	NA	n=13	
			1	10	21.3			
			2	18	38.3			
			NA	7	14.9			
Pre-Op Chemotherapy			**M Stage**			**Metastatic Lesion Size**	3	0.4-18.5
Yes	16	34.0	0	12	25.5			
No	18	38.3	1	32	68.1	NA	n=7	
NA	13	27.7	NA	3	6.4			
Resection Type			**Grade**			**Number of Metastasis**	2	1-10
Primary	21	44.7	1	8	17.0	NA	n=13	
Metastasis	24	51.1	2	21	44.7			
Both	2	4.3	3	5	10.6			
			NA	13	27.7			
Primary Tumor Location			**KRAS**					
Descending	29	61.7	WT	13	27.7			
Transverse	7	14.9	Mutant	10	21.3			
Ascending	10	21.3	NA	24	51.1			
NA	1	2.1						
Liver Metastasis Location			**CEA_>5ng/ml**					
Left	14	29.8	Yes	23	48.9			
Right	21	44.7	No	16	34.0			
All Over	8	17.0	NA	8	17.0			
NA	4	8.5						
1_year_progression			**MSI**					
No	25	53.2	Stable	11	23.4			
Yes	21	44.7	Instable	1	2.1			
NA	1	2.1	NA	35	74.5			
			Necrosis					
			Yes	3	6.4			
			No	20	42.6			
			NA	24	51.1			

Table 6 *Overview of patients' characteristics including available demographic and clinical data for the first investigation*

HDSCA allows foremost to analyze cells from morphological perspective. Each CTC subtype was analyzed via several variables: nuclear circularity, nuclear area, nuclear area ratio to cytoplasm, and CK signal intensity standard deviation over the mean (SDOM). Each CTC subtype could be found in **Figure 6**, including values of descriptive variables.

Positivity of blood sample was established based on the presence of any of HD-CTC (≥1 CTC/mL). **Figure 7** displays graphical representation of CTC enumeration over time, while **Table 7** shows details of positive samples throughout the follow-up.

Concretely, at 1-2 weeks prior the surgery, 7 patients (out of 10) were analyzed positive (median 7.02; mean 93.48 ± 226.77 HD-CTCs/mL). In the samples from the day of surgery (pre-resection draws), we found 30 patients (out of 45) positive (median 6.61; mean 86.82 ± 196.96 HD-CTCs/mL). In the post-resection draws, 32 patients (out of 41) were evaluated as positive (median 4.81; mean 37.95 ± 78.35 HD-CTCs/mL). In summary, following numbers of positive patients were observed in post-surgery time-points: 8 of 12 patients at 2 days, 18 of 27 patients at 1–2 weeks, 4 of 6 patients at 1 month, 5 of 6 patients at 2–3 months, 8 of 10 patients at 6 months, and 2 of 2 patients at 1 and 2 years.

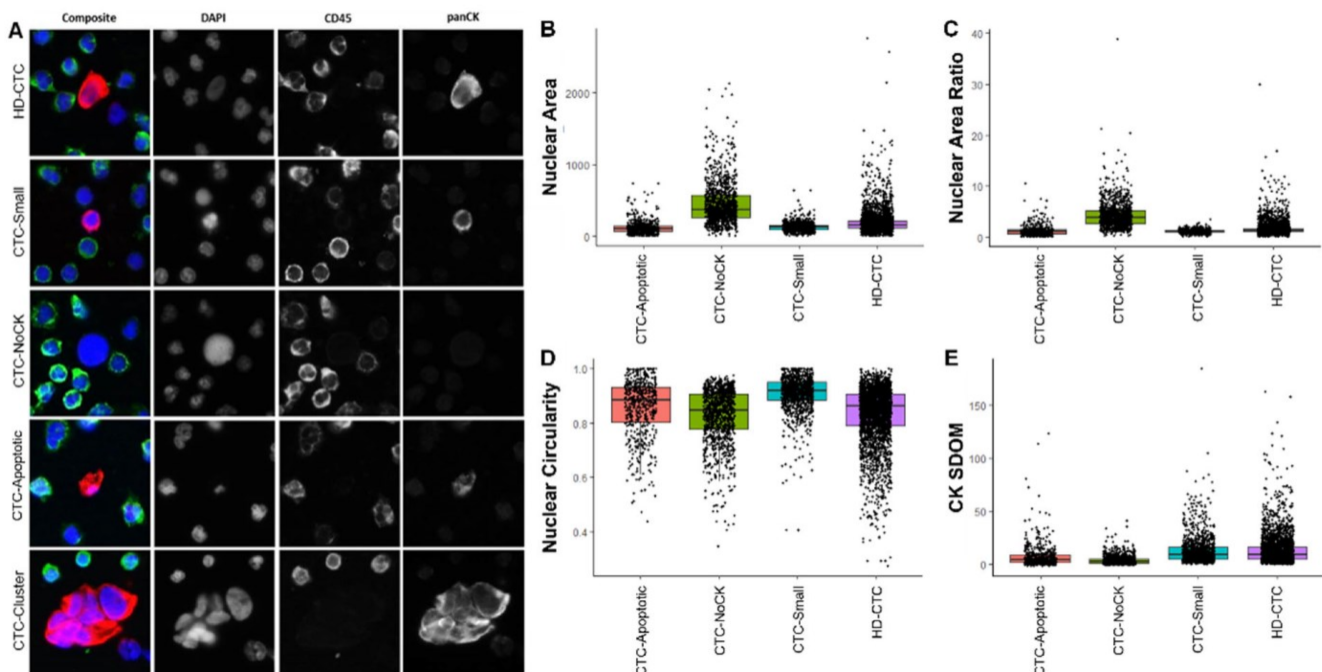

__Figure 6__ Quantitative and qualitative visualization of detected CTCs by HDSCA 1.0 in the first investigation; Image A shows

representative CTCs taken by 40x objective (Red: CK; Green: CD45; Blue: DAPI); Images B-E shows then quantitative metrics

Special interest of HDSCA analysis were given to cells clusters. Those clusters (CTCCs) consisted of at least 2 and up to 21 HD-CTCs cells in close contact.

As observed, clusters could be formed by various numbers of cells and also individual cells within the same clusters may have different morphological character. As it goes for CTCC positivity, patient´s sample was found positive if analysis discovered ≥1 CTCC/mL. Concretely, at 1-2 weeks prior the surgery, 5 patients (out of 10) were analyzed positive (median 0.39; mean 2.64 ± 5.02 HD-CTCs/mL). In the samples from the day of surgery (pre-resection draws), we found 17 patients (out of 45) positive. In the post-resection draws, 10 patients (out of 41) were evaluated as positive. In summary, these were the numbers of CTCC positive patients in samples from following time-points: 5 of 12 patients at 2 days, 5 of 27 patients at 1–2 weeks, 1 of 6 patients at 1 month, 1 of 6 patients at 2–3 months, 1 of 10 patients at 6 months, 0 of 2 patients at 1 year, and 1 of 2 patients at 2 years.

As already described, HDSCA platform offers not only HD-CTC enumeration but provides detailed analysis also for other CTC subtypes. These cells are defined as rare cells based on their morphological characteristics and biomarker status. For more information on these subtypes please see chapter Methodology.

In particular, 9 patients (out of 10) were interpreted as positive for cells named CTC-Small at 1-2 weeks prior resection draws (median 4.71; mean 28.77 ± 73.22 CTC-Small/mL). Immediately before surgery, 36 patients (out of 45) were determined as positive (median 9.32; mean 25.53 ± 63.89 CTC-Small/mL). In post-resection draw, 28 patients (out of 41 patients) were positive (median 6.73; mean 16.73 ± 24.01 CTC-Small/mL). Briefly, these were values for CTC-small positive patients in samples from following time-points: 8 of 12 patients at 2 days, 20 of 27 patients at 1–2 weeks, 4 of 6 patients at 1 month, 5 of 6 patients at 2–3 months, 7 of 10 at 6 months, and 1 of 2 patients at 1 and 2 years.

	HD-CTCs	CTC-small	CTC-NoCK	CTC-Apoptotic	CTCC
1-2 weeks prior surgery	median 7.02; mean 93.48 ± 226.77	median 4.71; mean 28.77 ± 73.22	median 13.30; mean 32.8 ± 63.87	median 5.96; mean 6.58 ± 5.44	median 0.39; mean 2.64 ± 5.02
Positive/Total	7/10	9/10	9/10	8/10	5/10
Day of surgery	median 6.61; mean 86.82 ± 196.96	median 9.32; mean 25.53 ± 63.89	median 7.82; mean 17.36 ± 22.56	median 3.41; mean 20.72 ± 54.59	
Positive/Total	30/45	36/45	36/45	34/45	17/45
Post-resection	median 4.81; mean 37.95 ± 78.35	median 6.73; mean 16.73 ± 24.01	median 11.29; mean 27.56 ± 43.80	median 4.81; mean 25.25 ± 59.86	
Positive/Total	32/41	28/41	35/41	29/41	10/41
2 days					
Positive/Total	8/12	8/12	9/12	9/12	5/12
1-2 weeks					
Positive/Total	18/27	20/27	26/27	20/27	5/27
1 month					
Positive/Total	4/6	4/6	5/6	5/6	1/6
2-3 months					
Positive/Total	5/6	5/6	3/6	2/6	1/6
6 months					
Positive/Total	8/10	7/10	9/10	5/10	1/10
1-2 years					
Positive/Total	2/2	1/2	2/2	1/2	1/2

Table 7 *Overview of positive patients out of all patients that had their sample collected at certain timepoint. In the first three timepoints, there is also information about median and mean of detected CTC subtype.*

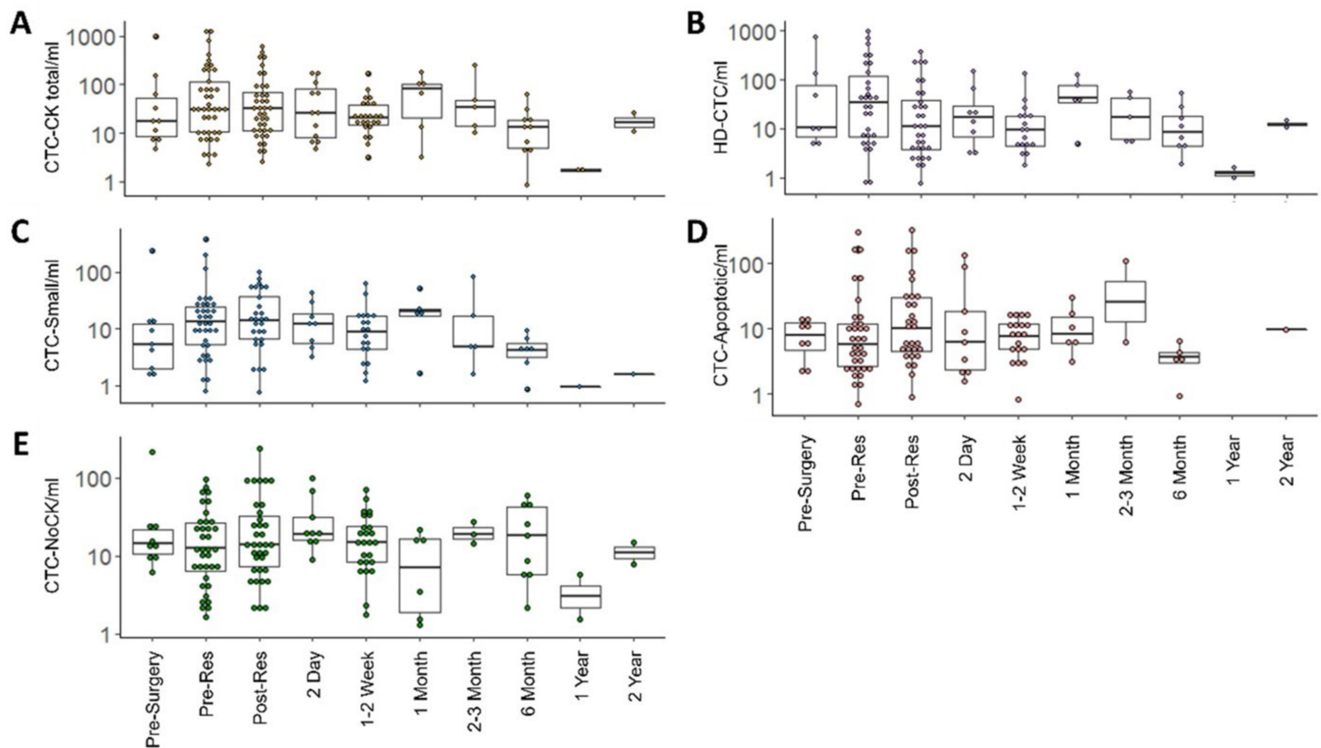

Figure 7 Enumeration of CTC subtypes throughout the whole follow-up of patients (A: CTC-CK Total/ml; B:HD-CTCs/ml; C: CTC-small/ml; D: CTC-Apoptotic/ml; E: CTC-NoCK/ml)

For CTC-Apoptotic cell type, 8 patients (out of 10) were found to be positive at 1-2 weeks prior resection draws (median 5.96; mean 6.58 ± 5.44 CTC-Apoptotic/mL). Immediately before surgery, 34 patients (out of 45) were determined as positive (median 3.41; mean 20.72 ± 54.59 CTC-Apoptotic/mL). In post-resection draw, 29 patients (out of 41 patients) were positive (median 4.81; mean 25.25 ± 59.86 CTC-Apoptotic/mL). Briefly, these were numbers of CTC-Apoptotic/ positive patients in samples from following time-points: 9 of 12 patients at 2 days, 20 of 27 patients at 1– 2 weeks, 5 of 6 patients at 1 month, 2 of 6 patients at 2–3 months, 5 of 10 patients at 6 months, 0 of 2 patients at 1 year, and 1 of 2 patients at 2 years.

Based on methodology description of HDSCA, it is also possible to localize cells which express little or any of CK signal (and are negative for CD45) but have distinctive features from morphological perspective. Those cells were categorized like CTC-NoCK.

For CTC-NoCK, 9 patients (out of 10) were interpreted as positive at 1-2 weeks prior resection draws (median 13.30; mean 32.8 ± 63.87 CTC-NoCK /mL). Immediately before surgery, 36 patients (out of 45) were determined as positive (median 7.82; mean

17.36 ± 22.56 CTC-NoCK /mL). In post-resection draw, 35 patients (out of 41 patients) were positive (median 11.29; mean 27.56 ± 43.80 CTC-NoCK /mL). Briefly, these were numbers of CTC-NoCK positive patients in samples from following time-points: 9 of 12 patients at 2 days, 26 of 27 patients at 1–2 weeks, 5 of 6 patients at 1 month, 3 of 6 patients at 2–3 months, 9 of 10 patients at 6 months, and 2 of 2 patients at 1–2 years.

CTC Subtype Correlation with Clinical Data

Demographical and clinical data of the cohort was then investigated for any potential positive or negative correlations or relations with CTCs values. Statistically significant relations were identified between HDSCA values and following clinical or demographical data or mutational status: KRAS mutation, tumor location, TNM stage, metastasis volume, metastasis location, synchronous disease, and if neoadjuvant chemotherapy was a part of the treatment plan.

Specifically, for those patients with known KRAS status, higher levels of HD-CTCs/mL in pre-resection samples (p = 0.0210, median = 18.56, range = 0.00–968.70, mean = 139.87) were discovered for mutated patients than for those with KRAS wild type (p = 0.0210, median = 0, range = 0.00–37.52, mean = 5.56). Similarly, KRAS mutated patients also shown higher number of CTCCs/mL in the same samples (p = 0.0290, median = 1.30, range = 0.00–63.40, mean = 9.11).

As mentioned in the Chapter Anatomical and clinical overview of CRC, connection between location of primary tumor and several tumor characteristics were described. Here, we analyzed if anatomical location of patients´ tumor (ascending, transverse, and descending) has any association with levels of any of the CTC subtypes. In pre-resection samples, the number of HD-CTCs/mL and CTCCs/mL was significantly higher (p = 0.0123 and 0.0436) in patients with the transverse location of the tumor (median = 193.76, range = 9.58–968.70, mean = 321.84 and median = 2.73, range = 0.00–63.40, mean = 13.37) in comparison to the ascending (median = 0.00, range = 0.00–694.89, mean = 82.89 and median = 0.00, range = 0.00–22.18, mean = 2.46) and descending (median = 5.38, range = 0.00–278.77, mean = 41.56 and median = 0.00, range = 0.00–15.87, mean = 2.23).

Based on the discovered relations of CTCs levels and specific anatomical locations, categorization of ascending, transverse, descending colon was here preferred prior to the RCC and LCC categorization, even though it is more often used nomenclature in clinical routine and corresponds with embryonic development.

Additional correlation was shown for post-resection samples, where we observed relationship between the levels of CTC-NoCKs/mL and tumor stage ($p = 0.0406$, $\tau = 0.2423$). Association between neoadjuvant chemotherapy and CTC-NoCK/ml in post-resection samples was also found. As expected, patients with neoadjuvant chemotherapy had lower count of CTC-NoCK/mL ($p = 0.0325$, median = 4.81, range = 0.00–27.28, mean = 9.25) than those that had only adjuvant therapy (median = 13.94, range = 2.05–234.84, mean = 37.73). This could be explained by cytotoxic effect on the cells not only in the tissue but also in circulation, which caused lower levels of this particular CTC subtype.

Moreover, the variables which are currently used for descriptive analysis of disease (metastasis volume, metastasis location, synchronous disease status) and that serve as a basis for prognosis and treatment mode, also shown associations with CTC subtypes. In the pre-resection samples, CTC-Apoptotic number displayed positive relationship with metastasis volume ($p = 0.0435$, $\tau = 0.2284$). In the same samples, metastasis location was positively correlated with levels of NoCK-CTC/mL, specifically the left side liver metastasis related to higher levels ($p = 0.0305$, median = 23.07, range = 1.67–96.10, mean = 30.94) in comparison to the right side (median = 7.42, range = 0.00–49.02, mean = 11.07). Again, in pre-resection samples, lower count of CTC-Apoptotic/mL was in the patients with synchronous disease ($p = 0.0256$, median = 2.52, range = 0.00–295.70, mean = 16.06) than in patients without (median = 5.79, range = 0.71–171.57, mean = 29.59)

Survival Analysis

As one of the potential clinical utilizations for CTC enumeration is either prognosis or treatment response (see chapter Liquid biopsy in current clinical studies), we performed survival analysis to identify potential correlations between numbers and types of CTCs and patients' survival.

Correlation of CTC subtype levels and survival of patients was done in two stages – *first*, following individual draws, and *second*, following changes of CTCs levels during observation period. First stage was standard analysis of any potential association between CTC subtype levels at certain time points and survival. The focus was to find any potential prognostic survival marker. Reporting only statistically significant associations, we found negative correlation for CTC-NoCK cells per mL with OS (p = 0.0213, HR = 1.05) in the sample collected 2 days after the surgery. In the same timepoint, higher CTC-Apoptotic levels also showed negative prognostic value for PFS (p = 0.0411, HR = 1.03; Figure 8C). Other correlations that were limited by number of samples are following: At the 1-month time-point, higher count of CTC-CKtotal/mL was related to worse OS (p = 0.0492, HR = 1.02) and higher levels of HD-CTCs/mL was marker for worse PFS (p = 0.0468, HR = 1.03). Unexpectedly, results for HD-CTC/mL and CTC-Cktotal/mL at the 6-month time-point sample showed that higher levels signalized better PFS prognosis (CTC-Cktotal/mL: P_BC = 0.0359, p = 0.0878, HR = 0.9; HD-CTCs/mL: P_BC = 0.0305, p = 0.0817, HR = 0.82).

These result from the first stage shows that additional layers of data for CTCs provide additional findings than just simple enumeration at one time-point.

For the second stage, the time perspective was further investigated. The focus was to analyze changing levels of CTC subtypes between different time points. This concept of dynamics is familiar for clinical physicians and has the potential to add information to clinical decisions. Following findings also underline the importance of repetitive (liquid) biopsy analysis in oncological patients to fully understand disease evolution and dynamics.

Similarly to the first stage of survival analysis, CTC subtype levels (dynamics between certain time points, calculated by subtracting the first sample levels from the second sample resulting into the difference between the two samples) was analyzed for any potential association with survival parameters. A higher increase of HD-CTC/mL levels than 49.77 between pre-resection to post-resection sample was related to worse OS (P_BC = 0.0270, p = 0.8464, HR = 0.99; Figure 8D). Alike, an increase of

3.3 CTCC/mL and more between the same samples also showed negative correlation with OS (P_BC = 0.0414, p = 0.3120 HR = 0.96; Figure 8E). Also, increase in CTC-Apoptotic by more than 12.28 cell/mL from pre- to post-resection samples negatively affected patients´ PFS prognosis (p = 0.0024, HR = 1.01; Figure 8F). From pre-resection sample to 2-day sample after surgery, significant dynamic for CTC-NoCK cells was observed, as an increase of more than 10.85 events of this subtype per mL of blood showed negative effect on OS prognosis (p = 0.0388, HR = 1.04; Figure 8G). Surprisingly, decrease more of than 10.92 CTCC/mL in 1-2 week sample in comparison to the pre-resection sample meant worse OS prognosis (p = 0.0478 HR = 0.94; Figure 8H).

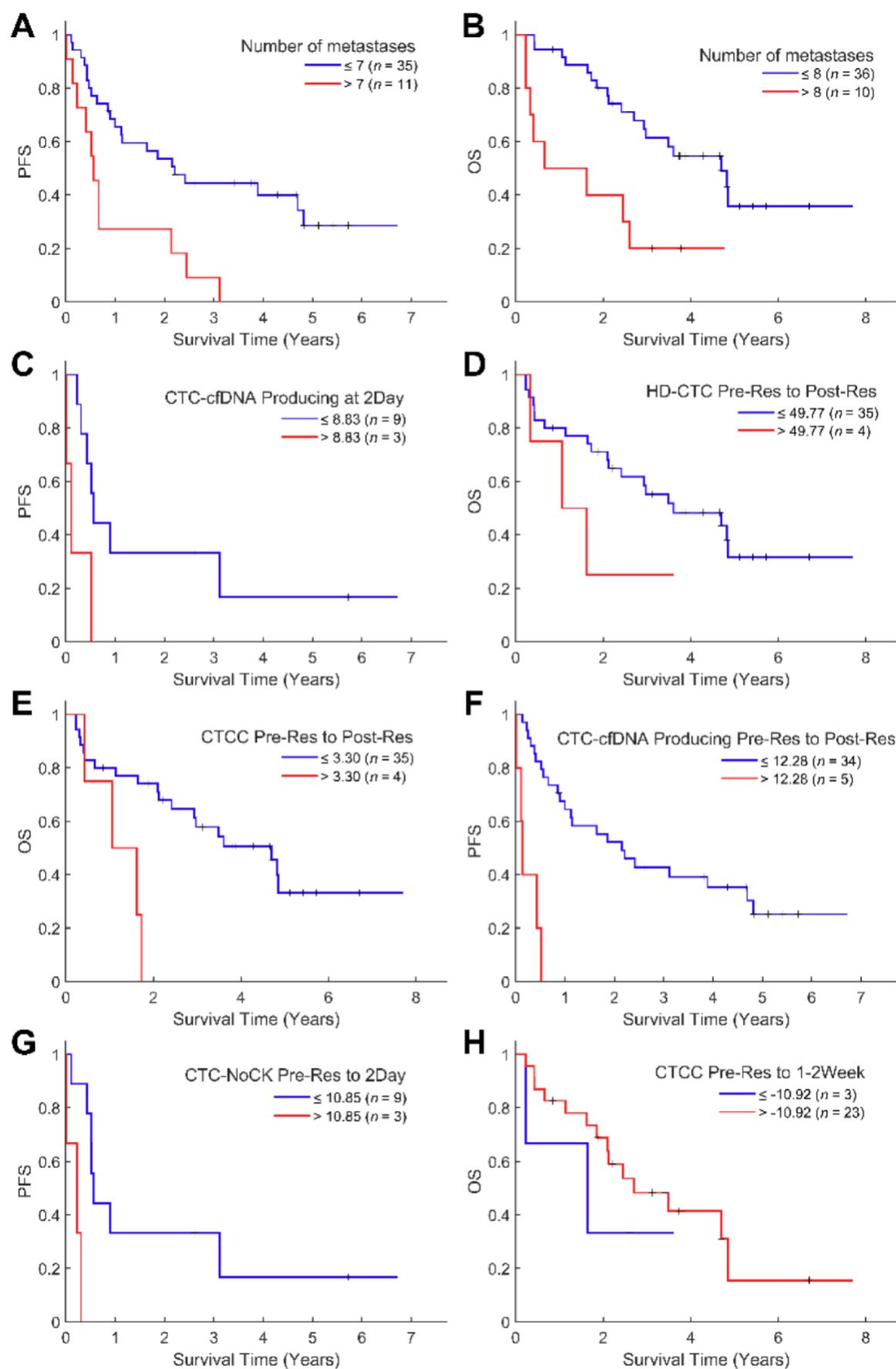

***Figure 8** Survival Kaplan-Meier curves of selected analytical results described in the subchapter; A: 7 or fewer metastasis => longer PFS; B: 8 or fewer metastasis => longer PFS; C: less than 8.83 CTC-Apoptotic/mL (here CTC-cfDNA Producing) in 2 days draw => longer PFS; D: change less than Δ49.77 HD-CTCs between pre-resection and post-resection => longer OS.; E: change less than Δ3.30 CTCC/mL between pre-resection draws and post-resection => longer OS. ; F: change less than Δ12.28 CTC-Apoptotic/mL between pre-resection and post-resection =>longer PFS. ; G: Change less than Δ10.85 CTC-*

NoCK/mL between pre-resection and 2 days post-resection draws => longer PFS. ; H: decrease more than Δ−10.92 CTCC/mL between pre-resection and 1–2 weeks post-resection draws => worse OS. .

Results Part 2

Landscape Rare-Event Detection: Rare Cells and Oncosomes

In the Landscape IF protocol, the samples were stained to find the rare events with epithelial, mesenchymal, endothelial or immune phenotype. Sample was considered as positive in case we observed ≥5 events/mL. Enumeration and frequency of rare events from the Landscape and CDX2-targeted IF protocols are reported in **Table 8**. On average, one test was done out of 0.53 mL of peripheral blood (standard error 0.05, median 0.53, range 0.22–0.97). Examples of cells and oncosomes found by the Landscape protocol are shown in **Figure 9**.

In all patients, median of any rare event (cells and oncosomes) was 287.46 (mean 387.74 ± 75.33) events/mL. The control group of healthy donors´ samples also contained some of the rare events with median of 40.05 (mean 49.96 ± 4.18) events/mL. Statistical analysis confirmed a significant difference between mCRC patients´ cohort and healthy donors ($p < 0.0001$).

Out of the total rare events, 58.25% were rare cells, which shows that by detection of oncosomes, new generation of HDSCA can find substantially more events by simply including events without nucleus (DAPI-). In HDSCA 3.0, rare cells were highly diverse in their phenotype and morphology. Median for all rare cells in mCRC cohort was 124.66 cells/mL (mean 224.80 ± 51.55). In similar comparison, median of healthy donors was calculated at 34.46 cells/mL (mean 43.21 ± 3.94). Statistical analysis confirmed a significant difference of rare cells between mCRC patients´ cohort and healthy donors ($p = 0.0112$).

IF Protocol	Event Classification	Sample Positivity	Mean (Events/mL)	Standard Error (±Events/mL)	% of Total Rare Events	Median (Events/mL)	Range (Events/mL)
Landscape	DAPI only	15/18	13.69	2.9	3.55	10.84	0.00–51.57
	CK (Epi.CTC)	7/18	57	36.36	14.77	3.55	0.00–549.63
	Vim	11/18	6.64	1.27	1.72	7.3	0.00–20.16
	CD45/CD31	10/18	10.29	2.44	2.67	8.05	0.00–33.16
	CK|Vim (Mes.CTC)	6/18	7.6	1.4	1.97	1.4	0.00–91.29
	CK|CD45/CD31	4/18	4.66	1.86	1.21	2.13	0.00–30.74
	Vim|CD45/CD31	11/18	24.52	8.73	6.35	7.79	0.00–121.00
	CK|Vim|CD45/CD31	15/18	100.4	35.02	26.01	12.89	0.00–453.13
	Onc CK	12/18	71.59	38.39	18.55	6.64	1.06–657.60
	Onc CK|Vim	10/18	32.72	13.64	8.48	7.38	0.00–217.65
	Onc CK|CD45/CD31	0/18	1.47	0.36	0.38	1.12	0.00–4.47
	Onc CK|Vim|CD45/CD31	14/18	55.37	16.22	14.35	34.32	0.00–268.32
CDX2-targeted	DAPI only	18/18	63.72	12.8	8.33	45.29	16.94–226.94
	CK	12/18	88.14	43.95	11.53	11.97	0.00–597.32
	CDX2	12/18	11.45	3.28	1.5	7.27	1.04–60.22
	CD45	3/18	5.44	3.35	0.71	0	0.00–59.22
	CK|CDX2 (CDX2.CTC)	14/18	19.95	6.71	2.61	11.34	0.00–124.08
	CK|CD45	12/18	36.66	13.71	4.79	10.02	0.00–203.63
	CDX2|CD45	12/18	29.37	12.46	3.84	9.8	0.00–185.68
	CK|CDX2|CD45	14/18	143.67	101.35	18.79	21.79	1.44–1843.34
	Onc CK	15/18	123.55	60.73	16.16	26	2.31–1035.65
	Onc CK|CDX2	18/18	222.4	65.04	29.08	114.87	15.55–1151.64
	Onc CK|CD45	0/18	0.06	0.06	0.01	0	0.00–1.06
	Onc CK|CDX2|CD45	10/18	20.27	7.64	2.65	14.5	0.00–1138.28

Table 8 *Enumeration and frequencies of rare events in the Landscape and CDX2-targeted IF protocol*

Focusing on specific cell phenotypes, CK-positive events were found in mCRC patient´s samples with a median of 45.41 events/mL (mean 169.66 ± 46.65). Again, the healthy donors´ median of CK-positive events, set on 12.39 CK-positive events/mL (mean 18.96 ± 2.70), significantly differ from the mCRC samples ($p = 0.0070$). Moreover, subgroup CK-positive cells formed 75.47% of all the rare cells. In more detail, Epi.CTCs subtype showed a significantly higher difference between median of mCRC samples (median 3.55 cells/mL; mean 57.00 ± 36.36) and healthy donors ($p = 0.0023$). Importantly, most of Epi.CTCs (92.58% of total count) were found in 2 samples, while 7 samples (out of 18 total) were positive for Epi.CTCs. Alike, there were only 6 samples (out of 18 total) Mes.CTC positive. However, statistical analysis did not confirm significant difference between median of mCRC samples (median 1.40 cells/mL; mean 7.60 ± 1.40) and healthy donors´ samples. Interestingly, the most often identified rare cells in mCRC samples were the CK|Vim|CD45/CD31 cells, but there was no statistically significant difference found between mCRC and healthy donors´ samples, as well. **Table 9** sums up the difference between patients´ and healthy donors´ samples

Subtype	All rare events	All rare cells	CK+ events	Epi.CTCs	Mes.CTC	CK|Vim|CD45/CD31
Median	287.46	124.66	45.41	3.55	1.4	
Mean	387.74 ± 75.33	224.80 ± 51.55	169.66 ± 46.65	57.00 ± 36.36	7.60 ± 1.40	
Healthy donors median	40.05	34.46	12.39			
Healthy donors mean	49.96 ± 4.18	43.21 ± 3.94	18.96 ± 2.70			
Significant difference	✓	✓	✓	✓	✗	✗
p value	< 0.0001	0.0112	0.0070	0.0023		

Table 9 *Overview of medians and means of CTC subtypes detected by HDSCA 3.0 Landscape Protocols that were found statistically significant from the healthy donors levels.*

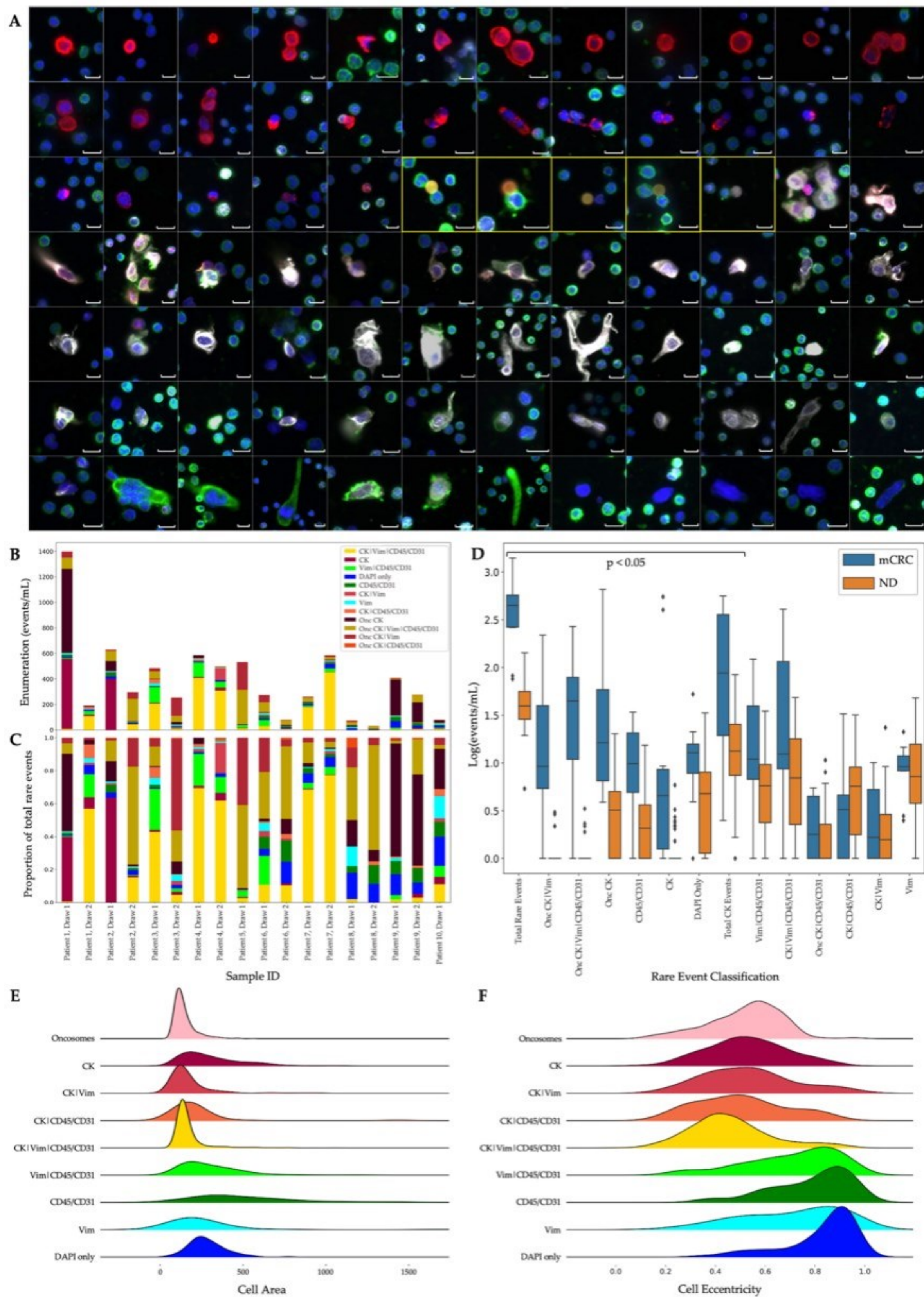

Figure 9 *Quantitaive and qualitative visualization of detected CTCs by the Landscape protocol of HDSCA 3.0 in the second investigation; Image A shows representative CTCs taken by 40x objective (Red: CK; Green: CD45/CD31; White: Vim; Blue: DAPI), oncosomes are highlighted in yellow frame, scale bar can be found in each cell image representing 10 µm; Image B: Enumeration by CTC subtype; Image C: Percentage of CTC subtypes in each draw; D: Comparison of Draw 1 and healthy*

donors (ND); Image E and F offer typical Cell Area and Cell Eccentricity per CTC subtype

Additional rare cells of the mCRC cohort were identified also via their noticeable morphology: CD45/CD31-only (median 8.05; mean 10.29 ± 2.44), DAPI-only (median 10.84; mean 13.69 ± 2.90), Vim-only (median 7.30; mean 6.64 ± 1.27) and Vim|CD45/CD31 (median 7.79, mean 24.52 ± 8.73) cells/mL. The only subtype with significantly higher levels in mCRC vs healthy donors´ samples were the DAPI-only cells ($p = 0.0047$).

Subsequently, two cell subtypes were categorized not just by using channel-type classification, but also based on their unique morphological features:

- CD45/CD31 large cells with specific morphological features (Figure 10A) were found in 50% of 18 samples (Figure 10B). The existence of these cells underlines the importance of complex approach in identifying cell of interests which cannot rely on straightforward methods or solely on phenotype classification. These cells display stippled structure of the CD45/CD31 phenotype, and the subset can be distinguished based on this morphological feature from typical homogenic signal associated with WBCs, which are regularly identified by a larger cell size, higher cellular eccentricity and larger nuclear size. The stippled CD45/CD31 population of cells also contain some level of variation especially in different shapes and nuclear to plasma ratio. Finally, based on the phenotype and described distinctive features, we contemplate them to be megakaryocytes.
- Vim|CD45/CD31 with fluctuating CK phenotype and recognizable morphology (Figure 10C) were found in 61% of 18 samples (Figure 10D). However, majority (81.37%) of these cells were collected in the patient 4 samples which limits the analysis. Again, this subset was defined not just using phenotypic characteristics but also based on their specific morphological features. The cells of interest show threadlike Vim signal together with a stippled CD45/31 signal. Additionally, there is fluctuating CK signal in terms of intensity and actual presence (only 45.60% of these cells are CK-positive). If the cells are CK-positive, the signal can be

manifested with different level of intensity as can be seen on Figure 10C. Another specific feature of this population is formation of clusters with other cells with the same phenotype. More than third (35.95%) of the Vim|CD45/CD31 collected cells were found in a cluster with other cells of the same population. Moreover, the cells can be differentiated from nearby WBCs by a large cells size and eccentric cellular membrane. Finally, based on the phenotype and described distinctive features, we contemplate them to be endothelial cells.

Beside rare cells, OCULAR also detected rare non-cell events: oncosomes. Amount of identified oncosomes represents considerable fraction of all rare events detected in Landscape IF protocol (41.75%).

First, from morphological perspective, oncosomes were found both in the contact with a nearby nucleated cells (48.91% of all oncosomes) and as a secluded rare event (51.09% of all oncosomes; Figure 10E). After processing and centrifugation, they were found in the cellular fraction of peripheral blood and in the question of size they could be as large as WBC or smaller (~10 μm). Phenotype of oncosome was homogenic across the vesicles with IF signal evenly distributed. Majority of oncosomes was also positive for CK signal (Figure 10F). Briefly, these were the frequencies for all subtypes of oncosomes in all patients:

All 18 samples were positive for Onc CK with a median of 6.64 (mean 71.59 ± 38.39) events/mL. The Onc CK|Vim|CD45/CD31 was positive in 16 of 18 samples and had a median of 34.32 (mean 55.37 ± 16.22) events/mL. The Onc CK|Vim was also present in 16 of 18 samples, with a median of 7.38 (mean 32.72 ± 13.64) events/mL. The Onc CK|Vim|CD45/CD31 and Onc CK|Vim counts were found to be highly positively correlated ($p = 0.002$, $\tau = 0.68$).

In summary, 6 phenotype specific subtypes of rare events were statistically different across the mCRC and healthy donors (Figure 9D). Three rare cells subtypes were found more frequently in mCRC samples in comparison to the healthy donors: Epi.CTC ($p = 0.0023$), DAPI only ($p = 0.0494$) and CD45/CD31 ($p = 0.0004$). Additionally, three subtypes of oncosomes were detected with higher rate in the mCRC

samples than in the healthy donors' samples: Onc CK ($p = 0.0001$), Onc CK|Vim ($p < 0.0001$) and Onc CK|Vim|CD45/CD31 ($p < 0.0001$).

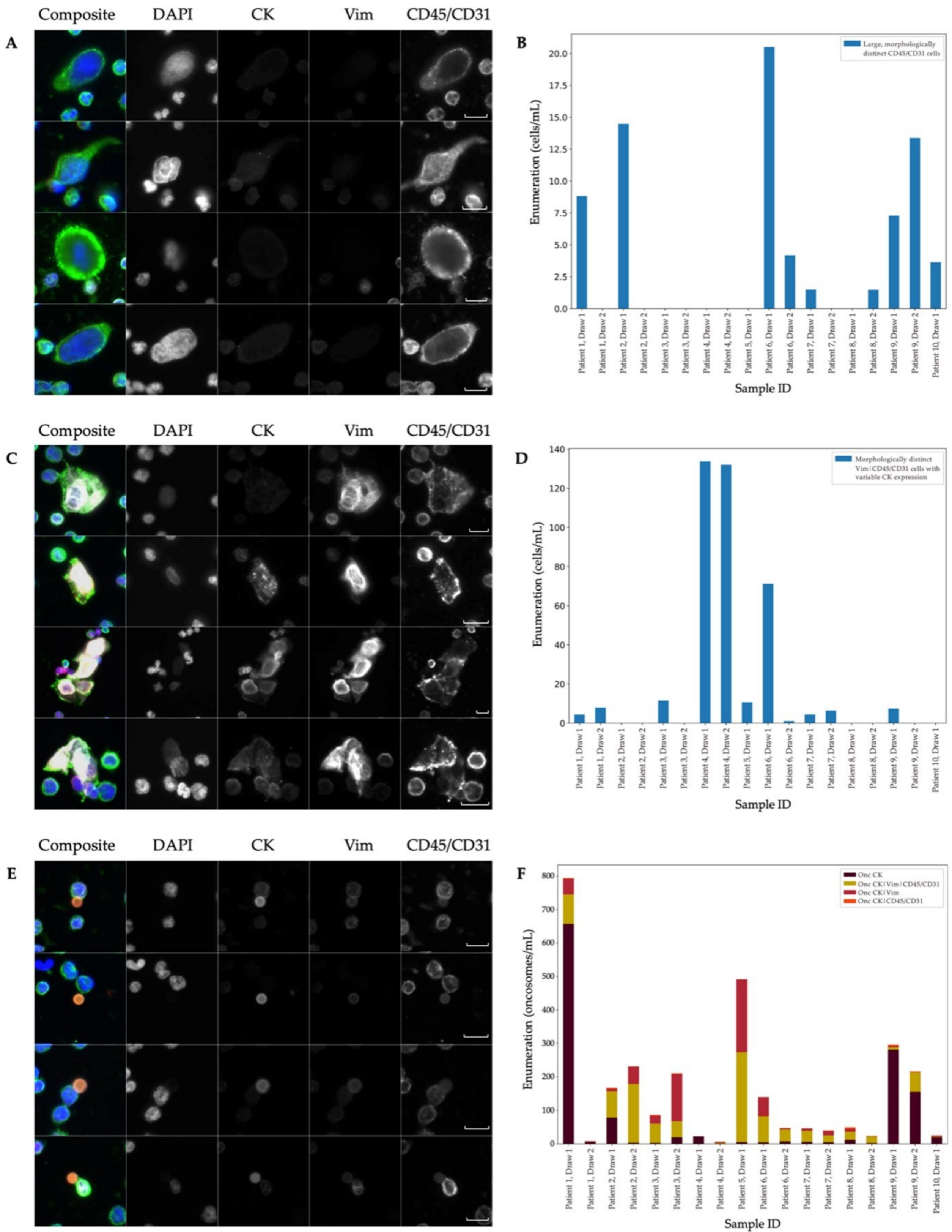

Figure 10 *Quantitaive and qualitative visualization of selected CTC subtypes detected by HDSCA 3.0 in the Landscape Protocol; Image A: CD45/CD31 large cells with specific morphological features, Image B: Enumeration of CD45/CD31 large cells per patient and draw; Image C: Vim|CD45/CD31 with fluctuating CK phenotype and recognizable morphology, Image D: Enumeration of Vim|CD45/CD31 per patient and draw; Image E: oncosomes, Image F: Enumeration of oncosomes per patient and draw. All images taken by 40x objective (Red: CK; Green: CD45/CD31; White: Vim; Blue: DAPI); scale bar*

representing 10 μm.

Analysis of the CDX2-Targeted Protocol

To further investigate different cell subsets of the Landscape protocol, we have implemented another protocol with the aim to explore possible colorectal origin of circulating rare events found in the first protocol.

CDX2 protocol is further described in Methodology. The peripheral blood samples on the slides were stained by the antibodies including CDX2 antibody to confirm possible colorectal origin. After OCULAR analysis of the stained slides, the comparison test was conducted between same sample slides with either Landscape or CDX2 protocol. Latter protocol details including rare events rates, sample positivity (defined by ≥5 events/mL) and overall enumeration can be found in **Table 9**. Illustrative cells of described rare cell or oncosomes subsets are in **Figure 11A**. Furthermore, enumeration and rates of the displayed subsets defined by their phenotype are in **Figure 11B/C**.

Out of all rare events in the CDX2-targeted protocol, 52.10% were rare cells. OCULAR analysis identified that a median for CK positive cell population for all 18 samples was 108.74 (mean 288.43 ± 119.17) cells/mL. The subsequent analysis of collected images showed broad variability both within the cell groups that are positive for a certain IF channel and of course also between the groups defined by IF channel positivity, as morphology and the consistency of the IF signal display significant variations within the same channel type.

The importance of addition of CDX2 marker was to identify CTCs with the potential origin in colorectal tissue. Cells positive for CDX2 marker (either CDX2-postiive or CDX2.CTCs) represents only small fraction of rare events (2.52%) but were found in 14 samples. Representative images of the cell type can be found in Figure 11. Interestingly, in some cases CDX2.CTCs were clustered together.

As usual, not only CK-positive population carries noteworthy results. Following channel cell types were most frequent in CDX2-stained samples: DAPI-only, CDX2-only, CD45-only and CDX2|CD45 classifications (see **Table 7**). The non-CK rare cells were found in all 18 samples, OCULAR analysis then established their median at 87.13 (mean 109.98 ± 18.58) cells/mL. A notable subgroup of sizable and eccentric cells with

stippled CDX2 phenotype and large nuclei (the last row of Figure 11A) was identified in 11 samples. Finally, DAPI-only rare cells were unsurprisingly detected in all 18 samples and their median was 48.29 (mean 63.72 ± 12.80) cells/mL if stained in CDX2-targeted protocol.

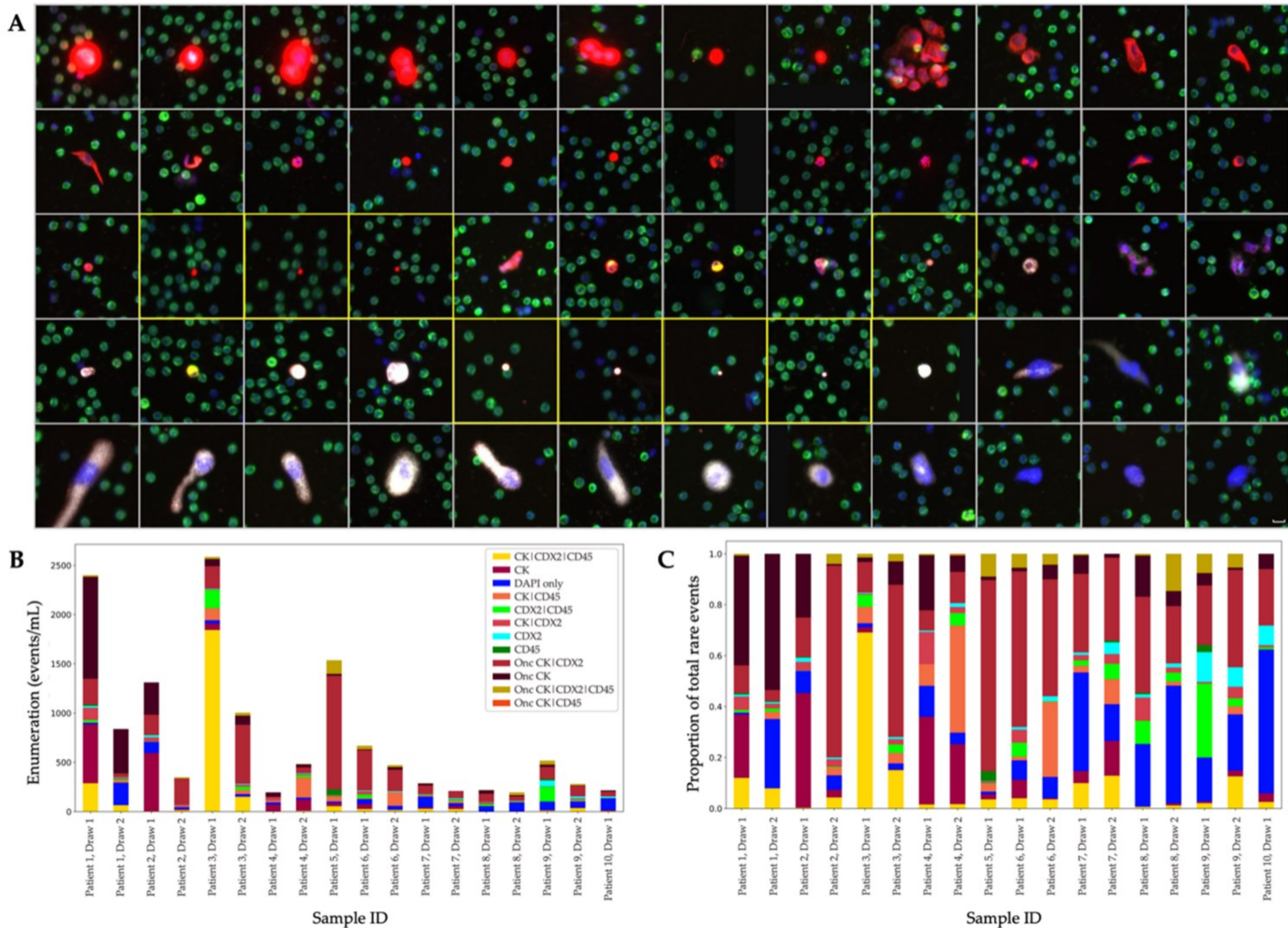

__Figure 11__ Quantitaive and qualitative visualization of detected CTCs by the CDX2 protocol of HDSCA 3.0 in the second investigation; Image A shows representative CTCs taken by 10x objective (Red: CK; Green: CD45; White: CDX2; Blue: DAPI), oncosomes are highlighted in yellow frame, scale bar representing 10 µm; Image B: Enumeration by CTC subtype; Image C: Percentage of CTC subtypes in each draw;

Multi-Assay Analysis

Multiple Assay Analysis refers to a process when we cross checked slides from one sample, however stained with both CDX2-targeted and Landscape IF protocols. The aim of this crosscheck was to find potential relation in the presence of rare events on

both slides. Some of the cell types observed in both protocols were, as suspected, positively correlated - the DAPI-only cells ($p = 0.032$, $\tau = 0.51$) and the CK-only cells ($p = 0.018$, $\tau = 0.55$). Consequently, positive relationships were exposed in the aggregate classifications, e.g. the oncosomes ($p = 0.003$, $\tau = 0.65$) and total CK-positive rare-event population ($p = 0.006$, $\tau = 0.62$). By implication, if one of the stains did not share the other biomarker but there was positive association between the rare events with exception of that one unshared biomarker, we assume that it is actually the same rare even type. Then, CK-only cells in the Landscape protocol correlates with CK|CDX2 cells from the CDX2 IF protocol ($p = 0.029$, $\tau = 0.51$). Similarly, positive correlation was also detected between the Onc CK|Vim and the Onc CK|CDX2 ($p < 0.001$, $\tau = 0.82$). A significant negative correlation was between the DAPI-only cells from Landscape-staining protocol and the CK|CD45 cells from the CDX2 IF protocol ($p = 0.029$, $\tau = -0.51$). Also, thought-provoking discovery was non-correlation between CD45/CD31 population from the Landscape-staining protocol and the CD45 population from the CDX2-staining protocol ($p = 0.645$, $\tau = 0.12$), that implies that important marker which distinguished those populations is most likely the CD31.

A unique advantage of comprehensive HDSCA platform and subsequently of OCULAR analysis is the ability to investigate morphological features of cells in relation to the size and shape of the whole cell and its nucleus. This morphological analysis based on the cell image was applied to enhance our understanding of detected rare events in the samples. All rare events across both IF staining protocols were characterized based on their morphological features and subsequently hierarchically clustered into eight groups with the goal to create clear separation of the assumed megakaryocytes and endothelial cells. Even though groups are defined based on their morphological features and marker phenotype, the rare cells still display variations in some degree. That implies heterogeneity within the cell group which can lead in the future into further investigation to determine origin of the cells in each group. The distribution of rare cells from the two IF staining protocols into the eight clusters together with representative images are in **Figure 12 & 13**

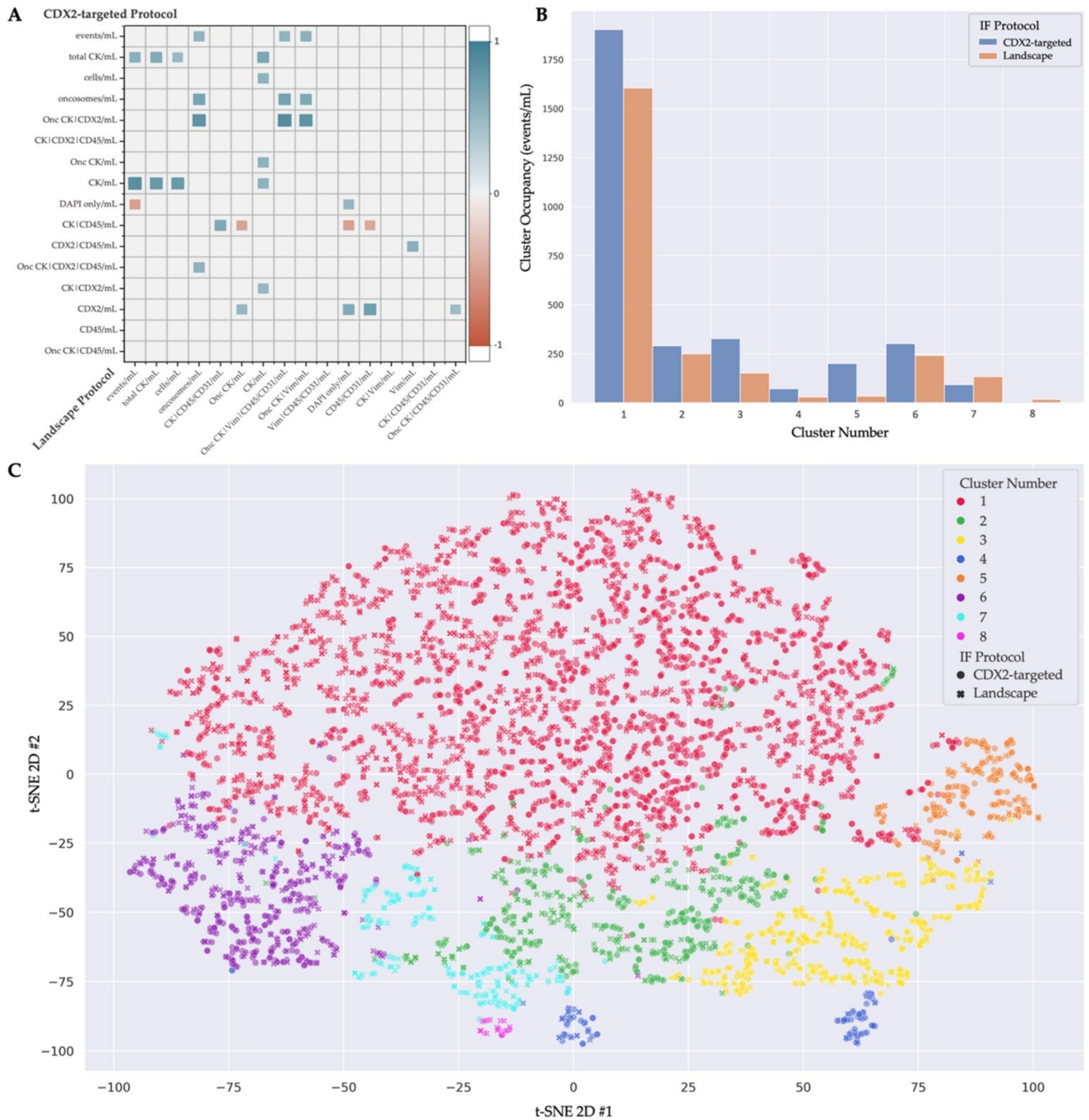

***Figure 12** Overview of the Multi-assay analysis of the Landscape and CDX2- targeted IF assay; Image A: rare events correlations between the assays, scale indicate positive (blue) or negative (red) correlation; Image B: Group (here Cluster) Occupancy of the rare cells when using the 8-group hierarchical clustering model; Image C: t-Stochastic neighbor embedding (t-SNE) plot of the 8 cell groups comprised of rare cells from both assays.*

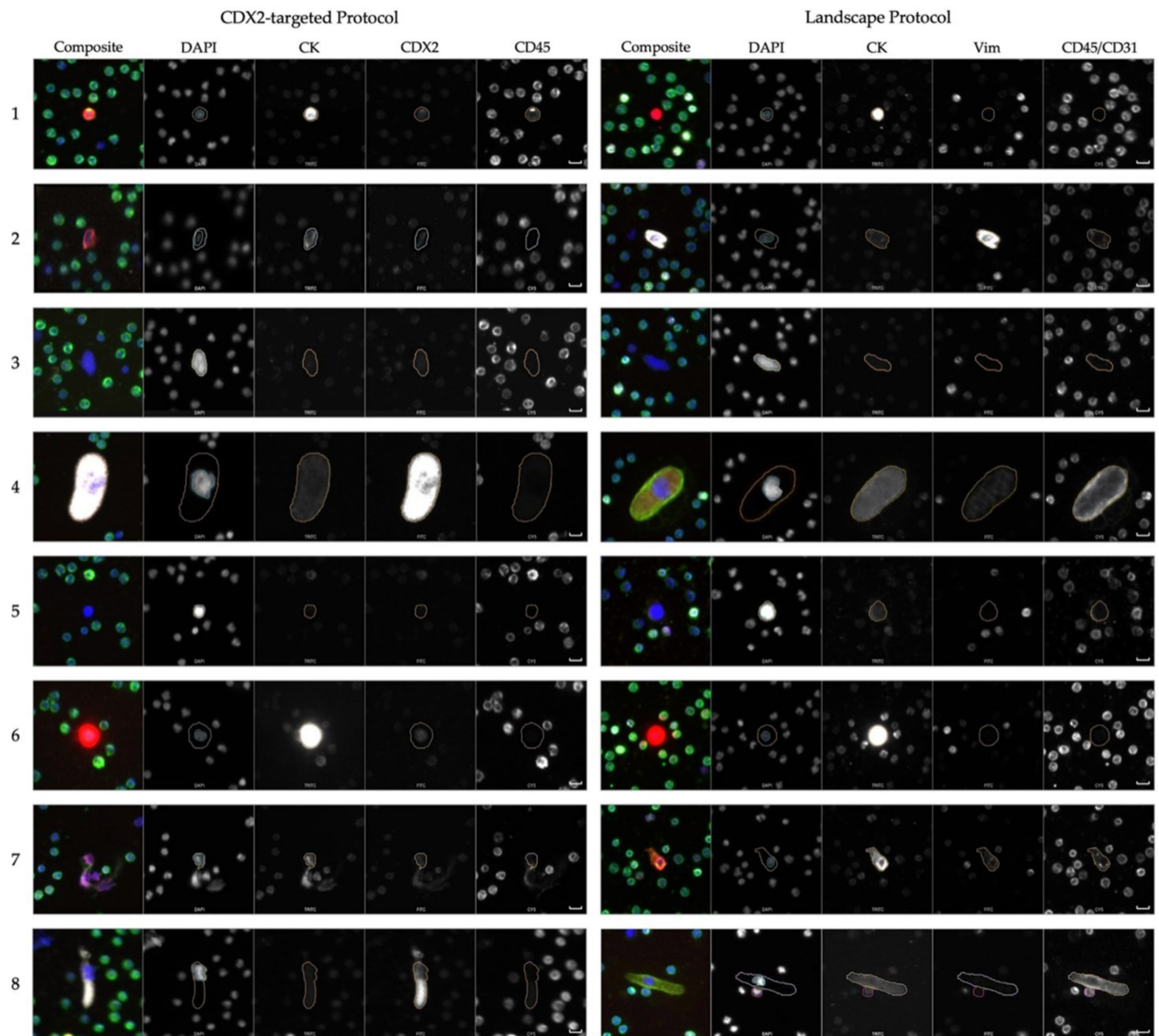

Figure 13 Representative images of the 8 groups (both assays included). Images taken by 10x objective, scale bar representing 10 µm; Channels in the Landscape Protocol: Red: CK; Green: CD45/CD31; White: Vim; Blue: DAPI; Channels in the CDX2-targeted Protocol: Red: CK; Green: CD45; White: CDX2; Blue: DAPI

Clinical Correlation of Liquid-Biopsy Data

Following an example of the HDSCA 1.0 investigation, an analysis of correlation between the rare events detected by both IF staining protocols and clinical events was performed. PFS was reported in nine patients (out of ten) (mean PFS = 6.98 months) and a positive correlation was found between the number of Onc CK|CD45/CD31 detected by the Landscape protocol in Draw 1 samples and PFS ($p = 0.0372$, $\tau = 0.70$) and length of PFS. Specifically, patients with ≥2.21 oncosomes/mL lived longer without

progression. On the other hand, a negative association between two types of events and PFS was found in an analysis of CDX2 stained rare event population in Draw 2: CK|CD45 cells ($p = 0.0362$, $\tau = -0.77$) and Onc CK ($p = 0.0068$, $\tau = -0.89$). Patients with ≥20.61 CK|CD45 cells/mL or ≥11.57 Onc CK/mL had shortened survival.

Levels of CTC change between timepoints can also play significant role in prediction and prognosis of patients´ survival, as was shown in the HDSCA 1.0 investigation. Dynamics of rare events (**Figure 14**) were significantly associated with PFS. As already mentioned, survival data were available only for nine patients, and two additional patients have not provided Draw 2. Hence, the analysis of dynamics of CTCs between the timepoints was conducted only in seven patients. The changes in the CK|CDX2|CD45 cells ($p = 0.0068$, $\tau = 0.89$) and the Onc CK|Vim|CD45/CD31 ($p = 0.0025$, $\tau = 0.93$) were positively associated with PFS (**Figure 14**)

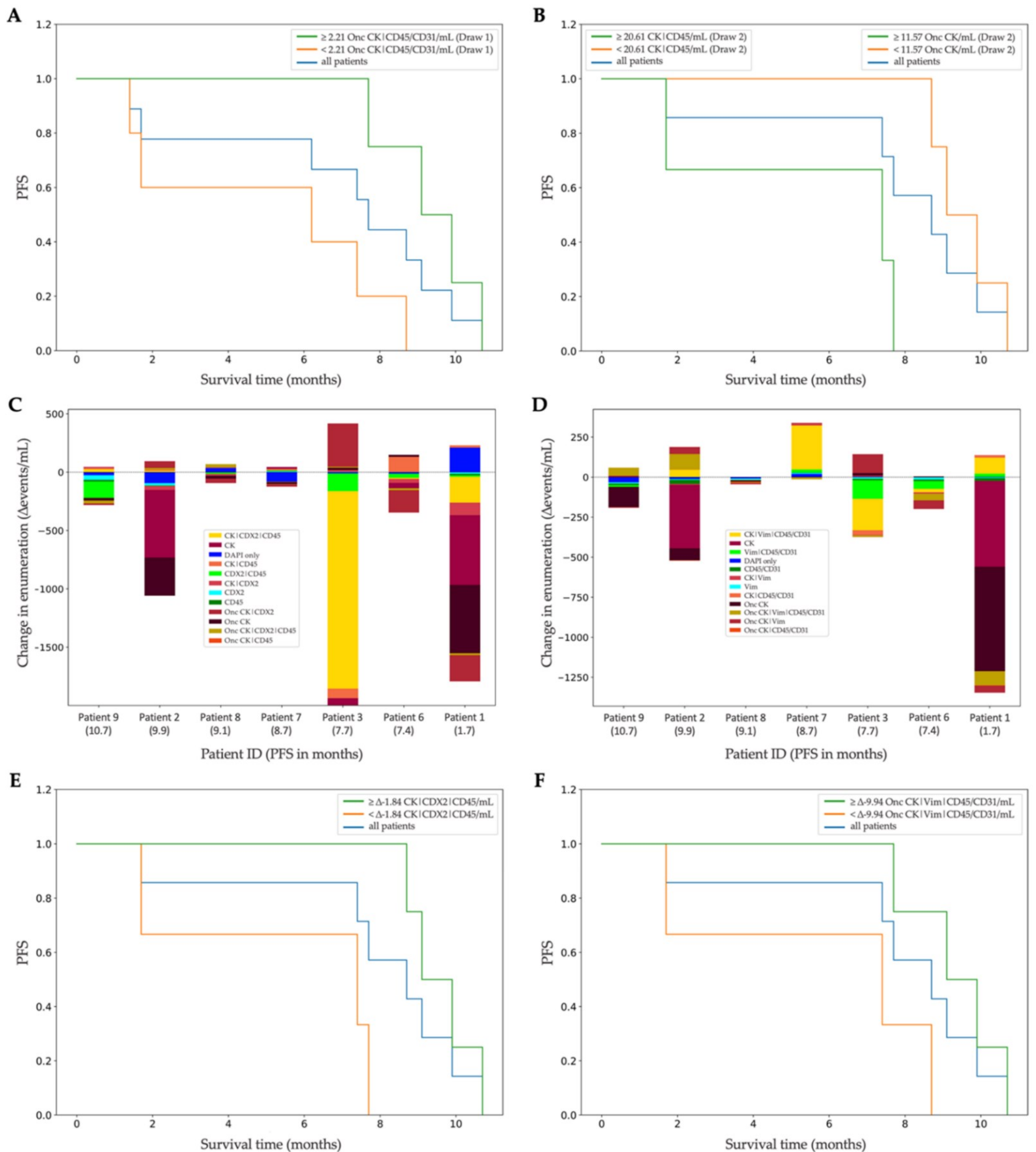

Figure 14 *Survival Kaplan-Meier curves of selected analytical results described in the subchapter; A: more than 2.21 Onc CK|CD45/CD31 in Draw 1 => longer PFS; B: 20.61 CK|CD45 cells/mL and more and 11.57 Onc CK/mL and more in Draw 2 => shorter PFS; C: Changes in rare events detected by the CDX2 protocol between draws in patients with available PFS and Draw 2; D: Changes in rare events detected by the Landscape protocol between draws in patients with available PFS and Draw 2.; E: decrease more than Δ-1.84 CK|CDX2|CD45/mL => shorter PFS.; F: decrease more than Δ−9.94 Onc CK|Vim|CD45/CD31/mL => shorter PFS. .*

Discussion

Liquid biopsy: Future and Challenges

Liquid biopsy has made its way into the clinical setting. As it was shown in the first chapter, there are numerous phase 3 clinical studies using ctDNA mostly as exploratory endpoints to assess either treatment response or to detect specific mutation in patients´ blood. However, this may be only the beginning of the list of benefits that can play a role in treatment selection or as a comprehensive prognostic/predictive marker.

There are some obstacles that need to be addressed to fully implement ctDNA and to start to introduce CTCs as another modality of liquid biopsy into the routine clinical practice. Specifically, pre-analytical variables, CTCs and ctDNA infrequency in peripheral blood, analytical validity and clinical utilization are some of the examples of the issues that needs to be solved by the liquid biopsy platforms. To address pre-analytical variables and to assure that healthcare providers are able to routinely collect liquid biopsy samples, standardized sample collection protocols need to be adjusted to challenges of clinical routine – how to collect, store and process samples in a way that is feasible and cost-effective for the clinical departments[179]. Specifically, improper handling of liquid biopsy in order to conduct cfDNA testing can result in compromising the quality of such DNA and can produce false positive/negative results in further mutational analyses[120]. Additionally, collection tubes, timeframe between collections, time to processing[180], and appropriate timeframe of sample collection and analysis in the context to other major patients‘ procedures (e.g. surgery) may influence CTC detection[126]. There have been efforts on international level to streamline pre-analytical procedure in the context of the European Union’s Horizon 2020 SPIDIA4P consortium project. That and actual need of clinical study investigators or clinicians show objective need for a standardized procedure[181].

Scarcity of CTCs or ctDNA poses rather a methodological and technological hurdle. Platforms and assays for detecting, collecting and analyzing circulating rare events, especially CTCs, must solve how to repeatedly and reliably analyze patients´ samples with a low number of said events[89,182,183]. Moreover, volume of the peripheral blood (or any other body fluid) is one of the pre-analytical limitations. On the other hand, the needed volume for analysis can differ from platform to platform as was shown

in the study of Dizdar et al.. The CellCollector® device, a wire inserted in cubital vein through a venous catheter and covered with anti-EpCAM antibodies, was designed to overcome the necessity of collecting any sample. This device aims to capture CTCs straight from the peripheral blood. The publication showed results of direct comparison of the CellCollector® device against standard of CTCs collecting platform in that time - CellSearch®. Finally, the frequency of detected CTCs was comparable between the platforms. In a cohort of 80 CTC patients, CellCollector® detected at least one CTC in 33 patients (41.3% positivity). In this particular case question remains, if the CellCollector® device will bring enough benefits for patients to overcome the invasive nature of collecting samples[134].

In CTC platforms, specific challenge to reliably detect CTC exist for positive enrichment techniques, such as EpCAM dependent platform, which may result in a selection bias of CTCs and miss distinct portion of CTCs expressing no or low levels of particular marker. Additionally, EpCAM dependent platforms will inherently miss any relations to other non-EpCAM cells[184] and therefore limit accessible information about a disease. Finally, platforms based on positive selection using cell size or marker expression will not capture those cells that underwent some changes in their phenotype, such as epithelial-to-mesenchymal transition[185].

In clinical setting, ctDNA platforms have been more established than platforms utilizing CTCs. However, even ctDNA platforms face some challenges regarding the analytic validity. That was shown in a study comparing two commercially available single-nucleotide variant panels for ctDNA analysis. PlasmaSELECT™ and Guardant360™ panels were compared in terms of correspondence of results in the samples collected from the same patients. The results showed low correlation of positive/negative results[186]. In addition, a publication comparing detection of KRAS mutation in the same patient plasma samples between OncoBEAM™ RAS CRC and Idylla™ ctKRAS Mutation Test showed that mutant allelic fraction plays significant role in sensitivity of the platforms and concordance between platforms improves only for patients with higher mutation fraction levels (>5%)[187]. Despite these publications, ctDNA testing for mutations is currently the most utilized approach of liquid biopsy in clinical settings. That is also supported by number of phase 3 studies, preferring ctDNA over CTC. That has been shown in subchapter Liquid Biopsy in current studies

Day to day routine in outpatient and inpatient providers require robust standardization which would allow them to rely on liquid biopsy results. Continuing implementation of liquid biopsy assays into oncology demands analytic validation and clear clinical benefit. Moreover, the patient survival results, or health quality is not the only clinical endpoint which needs to be improved by utilizing liquid biopsy. There are more “practical” questions regarding context of analyzing samples, such as clinical stage, type, line of treatment. While some forms of liquid biopsy have already found their place in clinical settings (i.e. lung cancer and EGFR mutation), clinicians still may miss bigger idea of minimal invasion diagnostic tools[188]. The challenge is in a dialogue between translation science and clinical setting to find reasonable and meaningful context for liquid biopsy application, for CTCs and ctDNA (separately or combined). The first step for such clinical utilization is full analytic validation in order to provide evidence that CTCs or ctDNA (or both) accurately and reliably deliver results for clinicians’ decision making. Clinical validation then means to show that application of liquid biopsy actually correlates, positively or negatively, with patients´ clinical outcome[189–191].

Finally, cost effectiveness is most likely the biggest hurdle for liquid biopsy to become routine part of clinical tests. In the Chapter Results Part 1, we have shown that dynamics of CTCs levels may bring valuable prognostic information. Therefore, only one sample and its testing may not bring enough information for prognosis or treatment decision. Number of necessary follow-up tests may increase cost of liquid biopsy in real world[192]. Furthermore, one of the major advantages of the liquid biopsy is continuous monitoring of the disease to promptly react to tumor clonal evolution and to early find cells with genotype or phenotype most likely resistant for conceivable treatment choice. Currently, even the forms of liquid biopsy which are already used in clinical settings are not used widely and not for every patient. One of the reasons is that ctDNA needs to be tested by next-generation sequencing which, in great number of patients and samples, would cause financial toxicity of such testing. A comprehensive and cost-effective platform which would integrate CTCs and ctDNA assays could overcome the financial uncertainty. However, such comprehensive platform would have to be clinically, analytically reliable and clinicians would have to see a clear patient benefit. Design of such platform therefore lies somewhere between

the needs of clinicians, patients and scientists developing these platforms.

HD-SCA platform: one platform for all

The HDSCA platform has already solved some of the forementioned issues. It is a validated rare event detection system for analysis of rare events in solid tumors[57,89,193,194]. In the Chapters Results Part 1 and Results Part 2, we have displayed results from CRC cohorts, but there are other publications describing utilization of the HDSCA platform in other solid tumor types[193–197]. The HDSCA technology offers enumeration of unique CTC subtypes, as well as description of cellular morphometric. In our research, we have combined not just enumeration or morphology but also time aspect with respect to a surgery and correlated these values with numerous clinical factors including patients´ survival. Chapters Results Part 1 and Results Part 2 warrant further research into the clinical utilization of CTC in CRC, but in larger cohorts.

Signature characteristic of CRC tumors is temporal intra and intertumoral heterogeneity demonstrated by diverse CTC morphology shown in the results of this thesis but also by other publications describing mCRC and other carcinomas[193–197]. The heterogeneity led us to categorize CTCs into subtypes as previously described. This process of categorization enabled subtype specific survival analysis with noteworthy observations. First of the actionable findings, CTC-NoCKs count increased at the 2-day after surgery was related with poor OS, pointing at a possible importance of such subtypes in pathological processes of mCRC. The fact that cells without or with limited expression of CK are connected with worse OS prognosis may indicate that these cells may play a role in spreading of tumor growth into secondary or tertiary locations and may represent transitional cell phenotype between epithelial and mesenchymal cells. Additional research into this phenotype with immunofluorescent assay including mesenchymal marker(s) further investigates the role of these cells and if their mesenchymal phenotype is part of the metastatic process. This direction was followed in our second study. Furthermore, investigation into genotype of these single cell should also aid to solve the question of the cells´ origin and elucidate their potential role in the disease transformation. These findings create grounds for another study which would investigate role of these subtypes in CRC pathophysiology.

Another subtype of CTCs which may elucidate tumor related processes at the time around the surgery is CTC-Apoptotic. Levels of this subtype in the 2-days after surgery samples suggest close relation of ctDNA and CTC. The finding of CTC-Apoptotic

implies apoptosis of the cells and most likely loosening their plasma composition into the bloodstream and subsequently increasing cfDNA. This discovery is in line with other publication investigating CTC in metastatic breast cancer on HDSCA platform[193]. Similarly, positive correlation between CTCs and cfDNA was found. Analysis of cfDNA in CRC was tediously researched since there is a potential benefit for patient in terms of treatment response, metastatic disease relapse and other clinical decisions[131,195,198]. Here can be found potential utilization of comprehensive liquid biopsy platform where positive results for CTC-Apoptotic cells could be further send for genomic analysis (CNV) to find any patients´ MSI/MMR status, KRAS mutations or any treatment or clinically relevant information. Overall, results from our research suggest that not only number of CTC-Apoptotic cells should be further investigated for potential clinical utilization, but also if levels, fragment size or CNV of cfDNA corresponds to dynamics of CTC-Apoptotic cells.

Similar to analysis of CTCs at certain timepoint, it appears that the correlation of PFS and OS to dynamics of CTC levels between two or multiple timepoints could also give us more information about a disease. Simply, not only certain levels of CTCs could be important for prognosis and treatment response evaluation but also slope of the change could be of importance. Correspondingly, a recent publication made similar findings in a cohort with 54 mCRC patients. Via a platform based on size of epithelial tumor cells authors were able to find that patients with increasing levels of CTCs between timepoints had PFS shorter by 7.8 months in comparison to patients with non-increasing CTCs[199]. In our investigations, there has been similar results for HD-CTCs (in the sample from pre-resection to pos-resection) and for CTCCs (in the samples from pre-resection to 1-2 weeks samples). An increase in both of those categories meant worse OS for patients. Finally, the general observation that the most significant CTC dynamics for patient outcome lies around the surgery is substantial for further investigation. As has been already suggested, the advantage of liquid biopsy platforms is hidden in the continuous disease monitoring or at least in several timepoint follow-ups. Here it can be seen that the reason is not just searching for MRD but also for dynamics of CTC levels and possibly for further ctDNA analysis. On the other hand, that brings again the question of cost-effectiveness to the equation.

Unexpected and remarkable finding was observed in dynamics of CTCCs

between the pre-surgery samples and 1-2 weeks samples. Apparently, a decrease of the CTCCs (∆−10.92 CTCC/mL) was correlated with worse outcome for patients. Conversely, raising levels of CTCCs (∆3.30 CTCC/mL) around the day of surgery related to worse survival. The latter observation was obviously expected and in line with other publications. In a study using a microfluidics-based self-assembled cell array (SACA) chip, relation between a presence of CTC clusters and higher risk of the recurrence of metastatic CRC was identified. Keeping in mind that the study contained samples from 166 patients and that our investigation suggests similar findings, it is reasonable to expect that cell clusters indicate higher chance of relapse rather than the opposite[200]. In this particular case, we hypothesize that surgery causes the shedding of cells into blood and therefore there is increasing number of CTCCs to be found due to removing tumor mass. However, at 1-2 weeks after the procedure causing damage to healthy and tumor tissue, a decrease of the cells can be observed due to the healing. This theory may hint that slower negative dynamic of CTCCs levels can be a sign of better response to the surgery because tumor keep shedding cells after period of time. Of course, the theory requires another investigation.

Plainly, to understand the dynamics of CTCs and CTCCs may result into the understanding of the actual pathophysiological process. A similar unintuitive observation was also seen in an analysis of Stage IV non-small cell lung cancer (NSCLC) patients with HDSCA platform. Alike, there was also several follow up samples collected to investigate levels and dynamics of CTC subtypes over the time. Analyzing dynamics and their relation to worsening or improving survival of patients, some of the dynamics also imply that decreasing number of CTC could be sign of worse survival[194].

All the findings from dynamic analysis between various time points suggest that liquid biopsy harbors more data than currently being utilized. Mutational analysis of ctDNA is currently actionable and easily translated into treatment decision. On the other hand, there is more potential actionable information beyond ctDNA mutational analysis or simple CTC enumeration (often biased by positive enrichment). Further research could reveal distinct biological processes with potential applicability into the clinical routine.

Results from our first investigation suggested that there is more to be discovered

than just the typical CK positive CTCs. Liquid biopsy platforms generally aim to find CTCs based on a particular criteria and then do CTC enumeration. This approach may seem to be appealing with its simplicity and lower cost compared to a more complex analysis. However, the main question remains: Will liquid biopsy CTC platforms find its clinical utilization as a biomarker if the ambition remains only to find and count EpCAM or CK positive cells? First, our review of phase II and phase III studies suggest that current research prefers ctDNA, as it offers clear clinical use in establishing mutational status, e.g. EGFR mutational status in non-small cell lung cancer[201–203]. Second, there are publications which shows that better than simple enumeration of CTCs[204] is to embrace the complexity, either by monitoring levels of CTCs[205], as we have shown here, and/or to combine CTC and ctDNA[193,206] to increase the accuracy of liquid biopsy.

Our first investigation underlined the necessity to examine morphology of detected CTCs and analyze changing levels of CTCs between time-points, especially with respect to therapeutic interventions. Keeping in mind that there could be more information within further CTC analysis and singular position of HDSCA platform for single-cell genomics[55,207–209], proteomics[173,210,211], and cfDNA genomic analysis[193,212], we have decided to conduct further research by adding new markers (Vim and CD31) to our IF staining protocols.

The results from our first investigation with a cohort of 47 patients despite its promising nature needs a larger study with precisely defined time-points of sample collection to confirm the findings we received in the smaller group of patients. We have also reported some exploratory findings which should be investigated in a prospective study with a stratified and balanced subgroups. Finally, our research creates a question of how much of platform complexity should be sacrificed for accessibility and lower cost.

HDSCA: Beyond standard CK positive CTCs

Not only the first investigation shown an importance of appropriately choosing timepoints and subsequently analyze dynamics of CTC levels, it also hinted a necessity of research of CTC subtypes and their differentiation, down the line allowing explanation of the pathophysiological processes of the malignant disease.

Unsurprisingly, the results of the second investigation confirmed minimal presence of CK-only positive CTC cells in mCRC[80,89,213]. However, results of the first investigation conducted in HD-SCA 1.0 displayed cells which differ from typical CK only positive cells and results from the secondary investigation aim to describe those cells and potentially provide more information on their origin. Additionally, results also showed some actionable clinical utility of these rare events. One of the rare events, Vim|CD45/CD31 cells, could be compared to previously described circulating endothelial cells (CECs). Interestingly, correlation between treatment response to the first line treatment[214] or survival[215,216] and CECs was already reported. The Vim|CD45/CD31 cells found in our cohort of mCRC patients still had some remaining expression of CK and were found individually or in clusters. Based on the morphometrics and the phenotype of these cells, we assume that Vim|CD45/CD31 cells are in reality CECs. The main argument for this comparison is a presence of Vim signal and punctate CD45/CD31 signal. Nevertheless, there is still need for genomic analysis to prove this claim, although other evidence (filamentous Vim and stippled CD45/CD31 signal) gives us enough confidence to refer to them as CECs[217,218]. Noteworthy, the cells with similar morphology and available biomarker profile were identified in previous studies[219], including our first investigation reported here.

Another type of rare cells described by using HD-SCA 3.0 were large CD45/CD31 positive cells with a specific morphology. These sizeable cells have numerous lobules per cell, large nucleus which takes most of the cytoplasm and punctate biomarker signal, similar to the previously described CECs. Again, based on this characteristic – up to 160 µm large CD31 positive cells with granular cytoplasm[220] - we assume that these cells are megakaryocytes. While there are still questions regarding their role in the evolution of malignant disease and potentially in the metastatic process, there was observed correlation between the presence of megakaryocytes in tissue surrounding tumor or in circulation and survival of patients, specifically in prostate[221] and non-small cell lung cancer[222]. In CRC, potential diagnostic significance of platelets is reported, as well[223]. As known, megakaryocytes are producing platelets and could represent a surrogate for platelets as diagnostic biomarker. While further investigation into origin of these cells combined with genomic analysis is warranted, based on these two cell types (here assumed megakaryocytes and CECs), there is the evidence that there are

more different rare circulating cells related to the oncological disease than just CK positive cells. Hence, a platform with a scope and ability to collect and detect those cells is needed for thorough sample analysis within potential clinical routine application.

Finally, not only rare cells can be collected and analyzed. Even non-cellular vesicles could play important role in the possible prognostic or treatment response function of the liquid biopsy platforms. Classification and characterization of vesicles was published according to their origin and size[175,224] and even here it is necessary to emphasize that positive or negative enrichment methods apply for these rare events, as well. Vesicles derived from a cell with a potentially oncological origin are called oncosomes and were found frequently among the rare events in the CDX2-targeted and Landscape assays in CRC cohort. Moreover, similar rare events were also reported in other types of cancer – prostate[173], bladder[174], upper tract urothelial carcinoma[176] – also using HDSCA platform, which implies that oncosomes are not CRC specific event. Coupled with the observation that simple enumeration in both assays for CRC patients shows correlation between oncosomes and PFS, oncosomes could be an important biomarker for patients and physicians to provide prognosis or to select treatment-responding population of patients. Interestingly, also dynamics levels of oncomes over time showed significant relation to PFS. Specifically in CRC case, these findings are important because of inconsistent findings of CK only positive CTCs in all CRC patients.

In our investigation we tried to leverage two immunofluorescent assays to collect more data from one sample and increase information regarding specific rare events. If we would not combine data from CDX2-targeted and Landscape assay, circulating cells or vesicles were missed or we had not enough data to hypothesize their origin or significance. Megakaryocytes or CECs represents a result which shows that image-based detection in larger cohort of patients or even in clinical practice could eventually avoid routine genomic tests and therefore decrease cost of patient´s testing, at least relatively to single-cell genomic or proteomics. Particularly, combining two or even more immunofluorescent assays is a significant advantage of HDSCA platform thanks to its non-enrichment approach described in Chapter Methodology.

For patients with an evolving oncological disease like CRC, especially in its advanced stage, it is crucial to not just correctly assess prognosis but also the most

effective non-refractory treatment. Both goals can be done by identifying patient´s cellular profile using multiple assays in HDSCA platform can be subsequently completed by genomic analysis (SNV or CNV), for either single cells or cfDNA.

Overall, HDSCA platform offer to a physician and health care system several tests which provide additional information about the disease and at the same time try to avoid the most expensive tests. Assessing morphometrics and phenotype of cells with immunofluorescent assay would serve to identify patients with a risk of relapse or disease progression. If treatment would cause absolute clearance of ctDNA and CTCs (or other rare events) under certain threshold, genomic analysis will not be necessary. Of course, if sample would show ctDNA load or presence of circulating rare events, the genomic analysis would follow to provide mutational status for the most frequent actionable mutations of specific disease.

As has been already discussed, CRC is frequently described with high intratumor temporal heterogeneity and phenotype of a tumor can change[225–227]. Based on experience from other malignancies and clinical research, especially breast cancer, there is high probability that there will be demand for predictive and prognostic tools which (see Chapter Liquid biopsy in current clinical studies), if necessary, will be able to provide physician with up-to-date information about the current CRC phenotype. HDSCA platform has the ability to not just provide real-time clinically actionable information, but also further investigate the role of the rare events found in our second investigation – specific cell types and oncosomes - in pathophysiology of CRC metastasis. The platform has the potential to provide results of genomic analysis, single-nucleotide variation and copy-number variation, in both types of liquid biopsy analytes – cfDNA and CTC[55,207,210]. Additionally, HDSCA is uniquely positioned for sequential multiplexed proteomic analysis[210,211] of the same slides that were used for morphological analysis with IF staining.

Same as our first investigation, the second investigation, especially the finding of non-cell rare events, warrants further research with larger cohort of patients in a prospective study with focus on mutational status of patients and its correlation to survival endpoints.

Conclusion

Our studies do not answer all the questions that need to be addressed before liquid biopsy platforms (and especially those based on collecting CTCs) can be fully introduced into real-world CRC treatment algorithm. Nevertheless, we have shown that identifying and collecting only CK positive CTC is not the only way how to employ CTC based liquid biopsy platforms. Moreover, HDSCA platform represents an unbiased rare-event approach to liquid biopsy, which can in the end also combine CTC and ctDNA analysis.

In our first investigation, we have demonstrated that CTC subtypes characterized based on their morphology and analyzed for correlation with patients´ data reveal another level of information which can further increase prognostic and predictive value of liquid biopsy. We were able to distinguish patients´ cohorts based on levels of certain CTC subtypes. Similarly, we have shown that changing CTC levels if introduced into the clinical routine brings additional value in terms of survival prognosis.

The second investigation build up on the first study's findings to further analyze certain identified cell subtypes. HDSCA platform proved to be a robust comprehensive platform which can detect not only rare cells but also additional rare events, oncosomes. The second investigation even more demonstrate invaluable benefit of unbiased inclusive approach not only for real-world use of liquid biopsy but also for additional research of tumor pathophysiology. Specifically, using two IF protocols in the same patients´ sample, we have identified two rare cell types, circulating endothelial cells and megakaryocytes, broadening specific CTC subtypes for further analysis in larger patients' cohort. Additionally, in the second protocol utilizing CDX2 monoclonal antibody with the aim to identify rare circulating cells with colorectal origin, we were able to find cells positive for CDX2 in majority of the samples, despite of the fact that these cells only represent small fraction of all rare cells. The CDX2 targeted protocol was valuable for characterization of previously unidentified rare events, but it seems that it will not drastically increase overall detection rate of rare events in the CRC. Lastly, we have hypothesized that rare circulating events will help us to correlate survival data for the patients, similarly to the first investigation. Obviously, the immediate limitation is lower number of patients with available draws and survival data. Despite those circumstances, we were able to find positive correlation in two cases for

CK|CDX2|CD45 cells and the Onc CK|Vim|CD45/CD31.

All these findings warrant additional research to find and establish specific clinical need for liquid biopsy in CRC treatment algorithm. That needs to be done in larger cohort in a prospective study with stratified and balanced population. With increasing number of use cases, we also expect to decrease cost per one sample (per one slide) analyzed by HDSCA platform, avoiding in the end financial toxicity of liquid biopsy.

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Reviews

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