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Circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA) as blood-based biomarkers for managing non-small cell lung cancer (NSCLC) patients

Dissertation

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1. Synopse

1.1.Fragestellung

1.1.1.Epidemiology of lung cancer

The global burden of cancer incidence and mortality is rapidly growing worldwide even though breakthrough development has been achieved in all fields of cancer research. Although Europe represent 9.7% of the global population, 22.8% of the total cancer cases and 19.6% of the cancer deaths in 2020 were registered in Europe, while America accounts for 20.9% and 14.2% of incidence and mortality, respectively. In contrast, cancer related death on the African and the Asian continent present with a higher share of death than the observed incidence (morality rate: 7.2% and 58.3%, respectively vs. incidence: 5.7% and 49.3% respectively). Simultaneously, the decline in mortality rates related to stroke and coronary heart disease, relative to cancer, in many countries has resulted in cancer as the leading cause of premature death with an estimated 19.3 million new cases and 10 million cancer death worldwide in 2020 [2, 3]. With an estimated 2 million new cases and 1.8 million deaths, lung cancer is the second most commonly diagnosed cancer and remain by far the leading cause of cancer related death worldwide in 2020. Incidence and mortality rates are much higher in men than in women and these numbers are even higher in transitioned countries than in transitioning countries. Germany for example diagnosed in 2019 23.546 women and 32.701 men with malignant tumors of the lung. 16.999 women and 27.882 men died of this disease the same year [4]. With about two-third of lung cancer deaths worldwide inherent to tobacco consumption, effective tobacco control and policies might largely prevent the disease. Nonetheless, Europe register around 10% of lung cancer deaths in patients with no smoking history and up to 20% of the cases in the US are seen in life-time never-smokers [2, 3, 5-8]. Still, since the introduction of the MPOWER package (consisting of 6 policy intervention strategies to reduce tobacco uptake) by the World Health Organization (WHO), progress has been substantial and directly associated with decline in lung cancer incidence in many western countries and this is particularly true for men in Germany where the incidence has been declining over the past two decades and now have come very close to those of women [4]. Unfortunately, progress is not even in countries across the globe. The survival of patients with lung cancer at 5 years after diagnosis is only 10% to 22%. For localized stage disease, the 5 years survival is 63%, but was drastically reduced to 7% once the disease spread beyond the lungs. The 5 years survival hardily evolved in a significant way for the inoperable lung cancer patients [9, 10]. The reason behind the high mortality rates observed is often the due to late-stage diagnosis of the disease, because of ineffective screening programs and late presentations of symptoms, but also due to many others factors like the acquired resistance to therapy or ineffective treatment options due to high tumor mutation burden and a great heterogeneity of lung tumors. In fact, lung cancer along with melanoma are by far cancers related to chronic mutagenic exposures (tobacco and UV lights, respectively) and exhibit consequently the highest tumor mutational burden (TMB).

1.1.2. NSCLC diagnosis and histological classification

Two main histological subtypes constitute lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) representing respectively 15% and 85% of lung cancer patients [11]. The main focus of this thesis work was set on NSCLC. While early diagnosis offers the best prognosis for NSCLC patients, 40% of NSCLC tumors will have spread beyond the lungs (stage IIIB and IV) by the time it is diagnosed and where the primary tumor is inoperable [9, 12]. The diagnosis is made, based on the 2015 WHO and endorsed by the 2021 WHO classification of lung tumors, by histopathological analysis of the surgically resected tumor and by the analysis of tumor biopsies when patients are not operable, which represent most cases [13, 14]. Specific subtypes of NSCLC display varying responses to different chemotherapeutic agents. Therefore, histological classification is important. NSCLC can thus be subdivided based on its histology: Adenocarcinomas (AD), squamous cell carcinomas (SCC), large cell carcinoma (LCC), large cell neuroendocrine carcinoma (LCNEC), adeno-squamous carcinoma, sarcomatoid carcinoma and carcinomas of salivary gland type [15]. AD and SCC are the 2 main histological subtypes in NSCLC and account respectively for 50% and 40% of all NSCLC cases [16, 17]. Adenocarcinoma, which arise from alveolar, bronchial, or bronchiolar epithelial cells, are more peripheral and retain a glandular histology [15-18].They generally express markers like thyroid transcription factor 1 (TTF1) or cytokeratin 7 [15-17]. Squamous cell carcinomas arise from the bronchial epithelium of the larger, more central airways, and are more strongly associated with smoking and chronic inflammation. They're generally characterized by expression of cytokeratin 6, p63 and SOX2 [16, 17]. Large cell carcinomas are diagnosed by exclusion of the two previously mentioned subtypes and can express markers for both adenocarcinoma and squamous cell carcinoma [15].

1.1.3. The metastatic cascade

Metastasis is a hallmark of cancer and remain the leading cause of cancer related mortality [19]. The paradigm of growing cancer cells detaching from the primary tumor to intravasate the surrounding tissue and blood vessels at late stage of disease progression has shifted to present with a new paradigm. In fact, the current knowledge of cancer evolution suggests that growing cancer continuously shed circulating tumor cells (CTC) into the bloodstream with the ability to evade immune defense mechanisms and colonize second sites to form overt metastasis [20, 21]. Tumor cells can thus be present in the circulation at early stages of the disease making their detection and analysis of a primary clinical importance. Active and passive shedding are the two main mechanisms responsible for the detachment of tumor cells from the primary bulk of tumor. Passive shedding of CTCs or CTC cluster can be triggered by e. g. shear forces or mechanical stress from surgery or growing cancer. While cancer cells are likely to keep their morphology and their expression pattern with passive shedding, active detachment of tumor cells require tumor cells to undergo EMT (epithelial to mesenchymal transition) to gain invasive capacities and contribute to a more aggressive phenotype in carcinomas [22]. Several changes occur during EMT: alteration of the cytoskeleton organization, changes in cell morphology, destruction of

epithelial cell-cell junctions, loss of apical-basal polarity and acquirement of front-rear polarity, acquisition of motility as well as ability to recognize and degrade ECM (extracellular matrix) allowing thus cell invasion. TGF-β-SMAD, the canonical and non-canonical Wnt, EGFR and ECM-integrin signaling pathways are the major signaling pathways responsible for triggering the EMT program. EMT-inducing transcription factors are then differentially activated, namely: the snail family which includes snail and slug, the ZEB transcription factor and the basic helix-loop-helix (bHLH) family of EMT transcriptions factors among which TWIST1, 2 and TCF3. Other EMT transcription factors might also step in the process such as YAP1/TAZ and SOX4 [22-24]. The last step of the metastatic cascade is the re-attachment of shed tumor cells to the second sites to proliferate and form overt metastasis, and this is achieved through the reverse EMT process: MET (mesenchymal to epithelial transition).

Disseminated tumor cells are cells that detached from the primary tumor and already migrated to distant organs. When they outgrow, they can from overt metastasis in any second site organ, theoretically. However, cancers of carcinoma origin are known to colonize specific target organs [25, 26]. It has even been proposed that metastasis patterns are not random and seem to be tributary to properties unique not only to the tumor cell but also to the target second site organ. These are what Paget refers to as cancer cell-intrinsic properties (seed) and the congenial microenvironment (soil): To sow the seed, the soil must be fertile [27]. Tumor cells that do not find the most conducive stain, won't generate metastatic foci. Therefore, the establishment of pre-metastatic (PMN) niche is a pre-requisite for the metastatic cascade. Peinado and coworkers extensively reviewed the PMN formation and reported that tumor-secreted factors like cytokines and exosomes shed by the primary tumor induce pre-metastatic niche formation in target organs before metastatic cell seeding occurs [28]. In contrast, cell seeding non-PMN lack a supportive tissue microenvironment and thus metastatic colonization fails. In NSCLC, the main sites of metastasis are bone, lung, brain, liver and adrenal gland [29]. Authors showed that also histology, age at diagnosis and sex have a significant impact on metastatic patterns in lung cancer [29]. For example, SCC patients have the lowest occurrence of metastasis while AD patients preferentially metastasize to the bone and lungs and metastasis in women occurs more frequently in the nervous system [29]. Even though great advances have been made into understanding the metastatic cascade, the exact molecular mechanism of organostropism and the consequent therapeutic answers require better understanding.

1.1.4. TNM stages and treatment options

NSCLC is a molecularly heterogeneous disease and therefore understanding its biology based on its specific subtype and staging is an essential prerequisite for an accurate assessment of the disease and the consequent treatment decision making.

1.1.4.1. Patients without distant metastases (Stage I-III)

For early stages NSCLC patients, surgical resection remains the recommended treatment by the European society for medical oncology (ESMO) [30]. Adjuvant

cisplatin-based chemotherapy in completely resected early stages NSCLC patients is the standard of care, offering a 5% overall survival (OS) benefit [30, 31]. Current ESMO guidelines recommends a two drugs combination with cisplatin (Vinorelbine, Gemcitabine, Docetaxel) for completely resected stage I-III NSCLC patients with no detectable genomic aberrations [30]. Though pre-operative chemotherapy is a primary importance to increase operability, more conclusive evidence favors adjuvant treatment rather than neo-adjuvant treatment [30, 32, 33]. Furthermore, nivolumab (anti-PD1) plus platinum-based chemotherapy has also been tested in the neoadjuvant setting. Nonetheless, no official approval of this regimen exists even though Checkmate 816 study showed promising benefits in terms of survival [30, 34]. If early stages NSCLC patients are EGFR mutants upon complete resection, more precisely patients harboring exon 19 deletions or exon 21 L858R point mutations, these should be given osimertinib in the adjuvant setting [30]. In this setting, osimertinib was approved by the US FDA (United States Food and Drug Administration) in 2020 while the EMA (European Medicines Agency) in 2021 recommended the extension of the therapeutic indication of osimertinib. In fact, according to a phase III ADAURA study, a clinically significant DFS (disease free-survival) benefit was demonstrated with adjuvant osimertinib versus placebo in EGFR mutant stage IB-IIIA NSCLC patients (DFS HR= 0.20; 99.12 CI, 0.14 to 0.30; $p<0.001$) [30, 35-37]. In 2021, FDA also approved atezolizumab (anti- PD-L1) alone in a randomized, multicenter, open- label, phase III IMpower010 study upon recovery from surgery and adjuvant platinum- based chemotherapy in IB-IIIA NSCLC patients [30, 38]. The cut-off of 1% PD-L1 tumor proportion score (TPS) was adopted by the FDA [39]. The EMA did not approve atezolizumab (anti-PD-L1) in this clinical setting until Mai 2022 with a main modification of the PD-L1 TPS cut-off from 1% to 50% [40]. For unresectable locally advanced NSCLC patients, chemoradiotherapy is the best option for medically fit patients. For patients with stable disease upon concurrent chemoradiotherapy and no progressive disease, PACIFIFC clinical trial showed that consolidation treatment with anti-PD-L1 monoclonal antibody durvalumab (anti-PD-L1) for 1 year has shown to improve prognosis of patients' whose tumor have express PD-L1 on ≥ 1% of tumor cells [41, 42]. Both FDA and EMA approved Durvalumab (anti-PD-L1) in such a clinical setting [43, 44] .

1.1.4.2. Patients with distant metastases (Stage IV)

The tumor is unresectable in stage IV patients. Therefore, tumors are categorized according to their histological subtype and further molecular profiling of the tumor will be a pivotal prerequisite since presence or absence of driver oncogene will guide therapy decisions making. It is of note that immunotherapy (IO) has become the primary treatment options for advanced stages NSCLC patients with no contraindications for immune checkpoints inhibitors' (ICI) owing to its long-term benefits in terms of survival. Therefore, assessing PD-L1 expression is mandatory for the best choice of care. Performance status (PS), age, comorbidities and patients' preferences should also be taken into account by therapy decisions making.

1.1.4.2.1. Stage IV without driver mutation

For newly diagnosed, metastatic squamous cell carcinoma patients without oncogenic driver mutations and no counterindications for ICI, ESMO guidelines recommend any platinum-based doublets with a third-generation cytotoxic agent (gemcitabine, vinorelbine, taxanes) as 1st line treatment option [45]. Regardless of PD-L1 expression, addition of anti-PD1 monoclonal antibody pembrolizumab to the platinum-based doublets yielded longer progression free survival (PFS) (hazard ratio for disease progression or death, 0.56; 95% CI, 0.45 to 0.70; P<0.001) and OS (hazard ratio for death, 0.64; 95% CI, 0.49 to 0.85; P<0.001) than chemotherapy alone [45, 46]. Similar treatment option is offered for newly diagnosed, metastatic non-squamous non-small-cell carcinoma patients, including non-oncogene addicted adenocarcinoma patients.

1.1.4.2.2. Stage IV with driver mutation

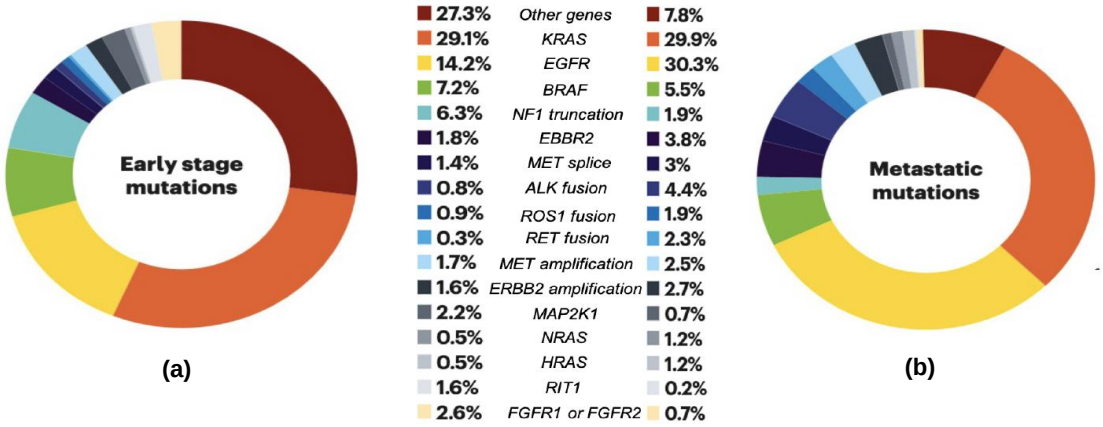

Figure 1: oncogenic driver alterations in lung adenocarcinoma patients according to disease stage; a) early-stage mutations; b) metastatic mutations (from [47])

Enhanced genomic sequencing technologies and the consequent comprehensive molecular profiling allowed for a variety of molecular alterations to be revealed in NSCLC [48]. This further supported a model whereby NSCLC depends on oncogenic "driver" alterations and as such facilitated discovery of genomic aberration such as chromosomal rearrangements, chromosome or copy number variations, insertions, deletions and mutations [49]. Those molecular alterations most commonly affect the tumor suppressor gene TP53 in about 50% of lung adenocarcinoma cases [50, 51] while TP53 mutation is present in more than 90% lung squamous cell carcinoma patients [49]. CDKN2A, involved in cell cycle control and mostly inhibited by homozygous deletions is also among the non-targetable genomic aberrations found in squamous cell carcinoma patients. Unlike squamous cell carcinoma, actionable mutations in many tyrosine kinase inhibitors are usually observed in adenocarcinoma patients. In fact, frequent activating mutations are found in driver oncogenes against which targeted treatment options exist including; epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma virus (*KRAS*), anaplastic lymphoma kinase (*ALK*), reactive oxygen species proto-oncogene-1 (*ROS1*) and B-raf proto-oncogene (*BRAF*) [18, 49]. Identifying these genomic aberrations constitutes a major stronghold in the

management of the patients' disease and thus guide treatment decisions for better outcome as targeted therapies directed against these specific molecular alterations have been developed, approved and incorporated into treatment guidelines [33, 52, 53].

EGFR: Ligand binding to *EGFR* receptor triggers dimerization and transphosphorylation of the tyrosine residues which leads to activation of downstream signaling pathways PIK3CA/AKT1/mTOR or RAS/RAF1/MAP2K1/MAPK1 [54, 55]. They will modulate transcription of genes involved in cell proliferation, survival (anti-apoptotic), angiogenesis, invasion and metastasis. The frequency of EGFR activating mutations varies considerably across regions and ethnicities. In Europe we observe 10-15% of AD NSCLC patients with *EGFR* activating mutation while in Asia it is observed in >20% of the cases [51, 56]. In a general population of AD patients, mutant EGFR is present in 14% of early-stage patients and 30% for late-stage disease (figure 1). Somatic activating mutation in *EGFR* gene include *EGFR* exon 19 deletion and the *EGFR* L858R point mutation in exon 21, both account for the vast majority of all *EGFR* mutation in AD NSCLC patients (90%) and confer high sensitivity to EGFR TKI [57].

Erlotinib and gefitinib are the first generation of non-covalent *EGFR* tyrosine kinase inhibitors (TKI) and are reversible competitive ATP inhibitors targeting *EGFR*. Afatinib and dacomitinib are second generation of covalent tyrosine kinase inhibitors and are irreversible inhibitors of the *EGFR* receptor [54, 58]. The most commonly acquired resistance mechanism to 1st generation TKI is the exon 20 codon 790 mutation of the *EGFR* gene (T790M). This is mainly induced through steric hindrance or by increased affinity of the tyrosine kinase domain for ATP leading to constitutive *EGFR* activation and the oncogenic transformation [59]. T790M mutation can be detected as a "second-site mutation" in more than 50% of *EGFR*-mutated lung cancers that have developed acquired resistance to first generation TKIs erlotinib and gefitinib. Osimertinib is a third generation TKI that's a selective inhibitor of the original sensitizing and T790M mutant form of EGFR while sparing the wild type (WT) *EGFR* allele [49]. C797S is the main mechanism for acquired resistance to third generation TKI osimertinib but not to gefinib and afatinib. However, sensitizing mutation, T790M, C797S lead to resistance to all three generation of *EGFR* TKI [55, 58].

ALK and ROS1: ALK encodes a transmembrane receptor tyrosine kinase with unclear function in humans [60]. ALK gene rearrangements result in overexpression and ligand-independent activation of ALK. Though many fusion partners exist, EML4 has been reported to be the most common ALK fusion partner [18, 49]. ALK-EML4 gene rearrangement is found in approximately 3-7% of NSCLC patients [49, 61]. In a general population of AD patients, ALK rearrangement is present in 0.8% and 4.4% of early and late-stage AD patients, respectively (figure 1). Similarly to *EGFR* mutations, ALK fusions are found more commonly in light smokers and/ or never smokers and are often associated with younger age. Crizotinib is the 1st generation ALK inhibitor which also functions as ROS1 and MET TKI. Ceritinib, Alectinib and Brigatinib are 2nd generation ALK TKI and they demonstrated increased potencies for ALK inhibition and

improved activity against multiple secondary ALK mutations that confer resistance to crizotinib [49, 55, 58].

ROS1 encodes a TK receptor, which become constitutively activated when a rearrangement leads to the fusion of its TK domain with a partner gene such as CD74 [62]. ROS1 fusion was observed in 1.9% of late-stage AD patients (figure 1) [47, 51, 58]. Due to the high homology between the kinase domains of ROS1 and ALK, drug used to treat ALK-positive tumors are the same one used to treat ROS1 tumors [52, 55].

KRAS: Even though not all KRAS alterations are driver mutations, activating KRAS mutations, occur in almost 30% of NSCLC patients [49, 63] (figure 1). 95% of KRAS mutations are found in codon 12 and 13 and rarely in codon 59 and 61 [61]. The KRAS protein is a GTPase and is an early player in many signals' transduction pathways involved in controls of cell proliferation [63]. Once mutated, negative signal is disrupted through MAPK activation which regulate cell proliferation and apoptosis, and PI3K pathway, crucial for antiapoptotic/ cell survival response, leading to cancer proliferation [63]. *EGFR* and KRAS mutations have strong specificity for tumors with glandular differentiation which might explain their prevalence on the mutational landscape in NSCLC patients [18]. Recently, an inhibitor of the "undruggable" KRAS have been approved for $KRAS^{G12C}$ mutated patients. Sotorasib, a covalent inhibitor that rapidly occupies $KRAS^{G12C}$ and extinguishes its activity, showed 32.2% of the patients with NSCLC having a confirmed partial response, and the majority (88.1%) had a stable disease for a few months or more with sotorasib, leading to a median progression- free survival of 6.3 months [58, 64].

BRAF: BRAF mutations in the G loop (exon11) or activation segment (exon15) of its kinase domain can lead to cancer cell proliferation and survival [65]. 7% of early stage patients and 6% of metastatic patients with lung cancer carry BRAF mutations, predominantly adenocarcinoma patients, most of which are heavy smokers [49, 61, 66] (figure 1). 50% of BRAF's genomic aberration are $BRAF^{V600E}$ mutation. Targeting V600E BRAF-mutated NSCLC patients showed clinical benefits. Although vemurafenib and dabrafenib showed improved clinical outcomes as a monotherapy, achieving an ORR of 33-42% and median PFS of 5-5 to 7.3 months, respectively, [67, 68], the addition of a MEK inhibitor further improves outcomes in $BRAF^{V600E}$ NSCLC patients. Though $BRAF^{V600E}$ NSCLC patients benefits of BRAF and MEK inhibitor they eventually end up developing therapeutic resistance. Other common BRAF mutation include the $BRAF^{G469A/V}$ and $BRAF^{D594G}$ occurring in 35% and 6% of NSCLC patients, respectively [61]. New RAF inhibitors mutant sensitive are under development with the primary task to reduce affinity for WT BRAF.

1.1.5. Immunotherapy (IO) in NSCLC patients

Drs. James Allison and Tasuku Honjo were awarded the 2018 Nobel Prize in Physiology for improving T cell mediated anti-tumor activity when inhibiting those

immune checkpoints [69]. The complex genomic landscape, the high tumor mutational burden (TMB) and associated high immunogenicity make NSCLC an obvious target for IO. The discovery and the description of the programmed death (PD) and the T-lymphocyte-associated antigen 4 (CTLA-4) pathway as crucial checkpoints controlling immune evasion led, within the CheckMate-017 study, to the FDA approval in March 2015 of nivolumab, a PD-1 inhibitor for second-line therapy in metastatic NSCLC patients upon progression on platinium based chemotherapy [70]. In October 2015, pembrolizumab (anti-PD1) was granted approval in a similar clinical setting as nivolumab with the additionnal recommendation of PD-L1 testing [71]. The development and approval of other immune checkpoints inhibitors and their clinical validation within large interventional studies: Atezolizumab (anti-PD-L1) [72, 73], durvalumab (anti-PD-L1) [42] and cemiplimab (anti-PD1) [74] showed significant survival benefits in NSCLC patients and have thus been approved for clinical practice as first and/ or second line IO alone or in combination with other anti-cancer treatment. It is of note that significant long term benefits in terms of survival for metastatic NSCLC have for the first time been observed when using IO [75]. The only predictive biomarker used as a companion diagnostic test for first line IO is PD-L1 expression assessed by the conventional and gold standard method: immunohistochemistry from formalin-fixed paraffin-embedded (FFPE) samples [76-78]. However, large differences in the response to IO have been revealed- with response rates ranging from 15% to 45% in patients having a high PD-L1 Tumor Proportion Score (TPS) [79]. Therefore, selecting patients that might benefit from IO is a determining priority. Current guidelines suggest that in the absence of detectable druggable driver mutation, PD-L1 is assessed and the 50% cut-off is to determine patients' suitability for IO. It is of note that pembrolizumab is the only ICI approved as monotherapy in first line NSCLC patients even though low levels of PD-L1 are expressed (1% cut-off) [80, 81]. Nonetheless, PD-L1's use as a predictive biomarker for IO treatment have several limits and remains up today a challenging task even for specialized pathologist, at the technical level, but also and mainly because of tumor heterogeneity, one of the leading causes of cancer therapy resistance, tumor progression, and metastasis. Yet, except for TMB assessment in a subgroup of patients treated with nivolumab (anti-PD1)/ ipilimumab (anti-CTLA4), it is the only cleared biomarker incorporated into clinical practice guidelines [33, 52, 53]. Another factor potentially affecting the predictability of PD-L1 TPS is treatment- induced changes in PD-L1 TPS, like resistance mechanisms, as observed in retrospective studies [82-85].

1.1.6. Liquid biopsy

Tissue biopsy and the consequent tumor profiling combined with high resolution imaging technologies (example: Magenetic Resonance Imaging (MRI), Positron emission tomography (PET Scan), Computerized tomography scan (CT scan) have always been the gold standard method for providing significant prognostic and predictive information on patients' disease [86]. Though of a primary importance in the clinical routine, tissue biopsy or sequential re-biopsy might not be feasible, especially in advanced disease NSCLC patients. Many factors can influence the feasibility of

biopsy in patients. Technically, tissue from advanced lung cancer disease patients is sometimes difficult or even impossible to obtain and lack of sufficient and qualitative tissue material can be a limitation for downstream analysis [14]. Considering the invasive character of the procedure, it can also be associated with adverse effects such as bleeding, infection and pneumothorax. Postponing systematic treatment for patients to allow re-biopsy to occur and perform the consequent molecular analysis can be critical for the patients due to progressive symptoms and deterioration of the performance status (PS). Furthermore, a bulk of tumor in the tissue biopsy is not representative of the whole tumor caused by intra-tumoral heterogeneity, which might only yield a snap of the actual tumor alterations. The evolving mutational landscape of the tumor requires a real-time monitoring of the latter to unravel resistance mechanism and adapt treatment by identifying potential novel molecular therapeutic targets, a step forward towards personalized cancer care [87]. To overcome these challenges or complement the gold standard method that is tissue biopsy, liquid biopsy is an alternative. Liquid biopsy is a unique source of tumor material derived from a blood sample or a sample originating from other body fluids (e.g., cerebrospinal fluid, pleural effusion, ascites, saliva, bile, stool, and urine) [88, 89]. Blood based biomarkers can be diverse: circulating tumor cells (CTC) of the mononuclear cell fraction, extracellular vesicles, tumor educated platelets and circulating cell free nucleic acid (cfDNA or cfRNA) from the plasma fraction. The minimally invasive procedure presents with the characteristic of analyzing several features of the tumor in a dynamic setting and

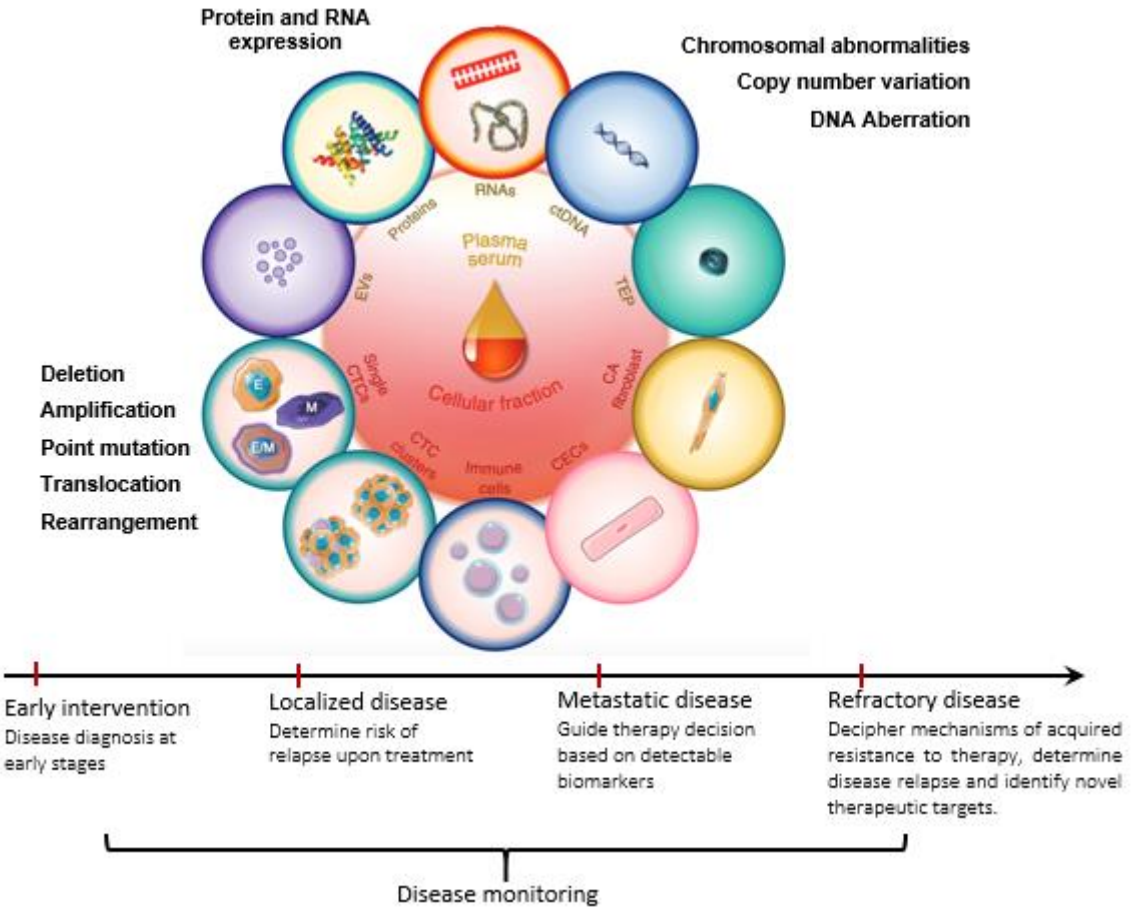

Figure 2: circulating biomarkers as liquid biopsy for personalized medicine (adapted from [90])

provide additional information about the tumor (figure 2). Analytes present in the blood could thus mirror not only the real-time state of the patient but sequential sampling of the tumor might give clues on presence of minimal residual disease (MRD) upon treatment foreseeing patients' relapse by adjusting to the right course of treatment [88]. As such blood- based analytes can be predictive factors of patients' clinical outcomes (PFS and OS) and these claims are extensively supported in an incredibly growing body of literature through the last decade [90].

1.1.6.1. Circulating tumor cells (CTCs)

Thomas Ashworth was the pioneering in discovering CTC existence in 1869 while reporting a case of cancer in which cells similar to those in the tumor were seen in the blood after death [91]. A finding that threw the light on the mode of hematogenous dissemination of tumor cells and provided first tracks for metastatic foci formation with cells retaining similar characteristics as the ones found in the blood. Since then, the light was shed on CTC biology. The current knowledge of cancer evolution suggests that growing cancer continuously shed CTCs into the bloodstream with the striking ability to colonize second sites and form metastatic foci as disseminated tumor cells (DTC), whose detection correlate to poor prognosis in most solid tumors, including NSCLC [92, 93]. CTCs, as they derive from the primary tumor, they can also detach from the existing metastatic sites [94]. Detection of these CTC within the bloodstream is thus of a primary importance in lights of its ability to provide a real-time snap of the tumor and/ or the distant metastasis through any potential molecular profiling. Example of clinical relevance of CTC is sequencing of the cells' genome to decipher genomic aberrations and adjust the course of treatment or unravel the presence of a predictive and/ or a prognostic marker ranging from the epigenomic to the post-translational proteomic features to the simple surface protein flag expressed on a CTC [88-90]. The broad scope of applications of CTCs in the clinical setting is large and has largely been reviewed and their clinical utility further supported. CTCs have a limited half-life (between 1 and 2.5 hours) in blood circulation [95]. They are considered a rare event: 1 CTC among an incredibly high background of leucocytes (10^6-10^8leucocytes/ mL of blood) [94, 96], which makes their detection in the blood for downstream analysis a true challenge that mostly first requires a pre-enrichment step with highly sensitive techniques [97]. Tremendous advances have been made in CTC enrichment techniques and cutting-edge technologies for tumor cell detection have proven their clinical relevance in several tumor entities including lung cancer.

Several different platforms and assays have been developed for CTC enrichment [97, 98]. They can be classified into two main groups: On the one hand, label-dependent, essentially relying on biological properties either by positive selection targeting the surface protein of interest expressed on tumor cells and/ or negative selection to deplete hematopoietic and immune blood cells by targeting e.g. CD45. Immunoaffinity-based technologies for positive CTC enrichment include e.g CellSearch®, AdnaTest®, MACS®, Isoflux, GILUPI CellCollector™, Herringbone Chip, Ephesia. Except for AdnaTest® that relies on an antibody cocktail, all these platforms above and many others depend on the most wide-spread cell surface protein used to achieve positive

CTC selection: EpCAM (Epithelial cell adhesion molecule) [98, 99]. EpCAM- based positive selection is the foundation of the FDA cleared CellSearch® system validated for CTC detection to monitor patients with metastatic epithelial carcinoma such as breast, colorectal and prostate cancer [100]. Although rather low number of CTCs is detected by CellSearch® system in the blood of NSCLC patients, due to CTC with very low or non-detectable EpCAM levels, presence of CTCs correlates with NSCLC patients' clinical outcomes [101]. Nonetheless, this poses a true limit of the CellSearch® system with NSCLC patients whose CTCs might undergo write out-EMT, downregulating *de facto* their EpCAM levels and switch to a more intermediate or mesenchymal phenotype. On the other hand, label-independent enrichments methods are primarily based on CTC's physical properties. Among these, the recently cleared FDA Parsortix™ cell separation system provides a size and deformability-based CTC enrichment. Though cleared only for breast cancer patients to date, the Parsortix™ system, which capture viable cells and allows for downstream analysis of any target of interest regardless of EpCAM status has also shown to be of clinical relevance in many other tumor entities including lung cancer [102-104]. Other technologies relying on other specific physical properties such as density gradient centrifugation (Ficoll-Paque®, RosetteSep™, OncoQuick …), inertial forces (ClearCell® FX, vortex …) or electrophoresis (DEPArray, ApoStream®…) have been developed. Other functional assays like the Epithelial ImmunSPOT Assay (EPISPOT) capture viable CTCs based on cells bioactivity such as protein secretion based on specific shed tumor-associated protein or cell adhesion [97, 99].

With the evolving understanding of our CTC biology, new technologies arise to address new challenges. Therefore, the studied tumor entity and the desired downstream analysis should guide the choice of the technology for CTC studies given the absence of a "one-size-fits-all" technology for such a purpose.

1.1.6.2. Circulating tumor cell-free DNA (ctDNA)

Circulating DNA fragments were first reported in 1948 in immune complexes derived from patients with systemic lupus erythematosus [105]. Somatic point mutations in cfDNA, however, were identified only in 1994 [106] and since then, a growing body of literature have investigated cfDNA biology and proved the clinical utiliy of ctDNA as a liquid biosource in several tumor entities, including lung cancer [107]. The total fraction of circulating cell free (cf)DNA in cancer patients consists of mitochondrial DNA (mtDNA) and circulating tumor cell-free DNA (ctDNA). The mean half-life of cfDNA in the blood is 16 minutes ranging from 4 to 30 minutes, reported around 2 hours elsewhere [108, 109], which makes it ideal for real-time monitoring. Standard serum tumor markers such as CA-125 and CEA have a half-life of several days even weeks [110, 111]. As such, changes in ctDNA can be more accurate in the prediction of treatment response than traditional tumor markers. In fact, the precise longitudinal tumor surveillance via serial ctDNA measurement empowers the apprehension of targetable molecular aberrations including somatic point mutation that drive cancer

progression and treatment resistance. Furthermore, cfDNA carries information about genetic and epigenetic tumor-specific alterations. We and others have extensively reviewed clinical applications of ctDNA in several tumor entities in different clinical setting and in NSCLC patients specifically (figure 3) [1, 112, 113].

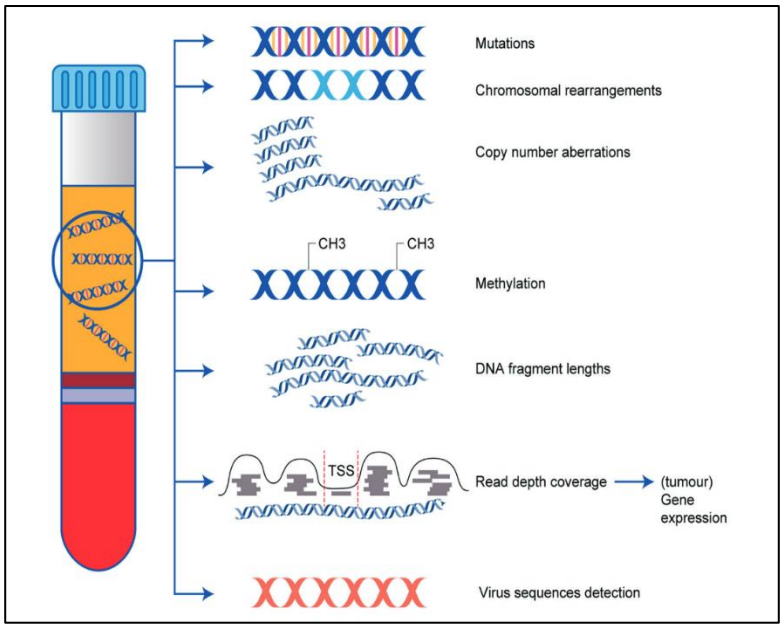

Figure 3: potential clinical application of ctDNA (from [1])

As for CTCs, cfDNA- based liquid biopsy approach may unlock the dilemma of intra-tumor heterogeneity and provide an extensive and more comprehensive picture of the clonal composition the metastatic or advanced cancer patient's malignancy. Though of clinical importance, the low cfDNA amounts found in cancer patients, particularly in early-stage diseases, remains a major challenge for cfDNA's downstream analysis. cfDNA concentration in blood varies significantly between healthy individuals and cancer patients ranging between 0 and 100 ng/mL for the first, and 0 to 5 and more than 1000 ng/mL for the latter. A significant part of total cfDNA consists of non-mutated DNA while the fraction of ctDNA constitutes approximately 0.01 to 89% of cfDNA according to several studies [114, 115]. It may however increase to higher levels with progressive stage disease but also by tissue damaging procedures that include radiotherapy, chemotherapy, surgery or even tissue biopsy …. Interestingly, ctDNA levels were shown to correlate to tumor burden in several tumor entities further supporting the prognostic utility of ctDNA as a liquid biosource [112, 116]. It is of note that the marked variation in ctDNA levels among patients is different among tumor entities as shown in the review from Mouliere [117]. Therefore, developing high- sensitive assays to decipher tumor- specific variants in cfDNA is a pre- requisite to enable the detection of genomic alterations. The latter were essentially identified over two different approaches: targeted (using pre-defined genes such as ddPCR) or non-targeted technologies (e.g. whole exome/ genome sequencing) [90]. Still, around 30% of NGS-based analysis using cfDNA fail for several reasons further highlighting the need to further improvement [118]. Nonetheless, the identification of the gatekeeper resistance mutation *EGFR* T790M in NSCLCA patients and the consequent validation by the FDA of the liquid biopsy assay Cobas *EGFR* mutation Test v2 on cfDNA is a major breakthrough and a perfect illustration of ctDNA use in therapy decision making [119]. Two other liquid biopsy tests have been approved by the FDA to detect mutations in the DNA from tumor cells in the blood of NSCLC i.e., the Guardant 360 CDx test [120] and the FoundationOne Liquid CDx [121].

Lately, the predictive capacity of ctDNA was demonstrated in urothelial carcinoma [122]. Patients with detectable ctDNA in the context of adjuvant IO were identified as a high-risk population who will benefit from ICI therapy. This, *de facto,* upgraded ctDNA

analysis to play an important role in clinical trial design and this evolved to the point of introducing the concept of ctDNA- based MRD detection [123].

Despite the high number of studies on the clinical utility of cfDNA, there's still room for improvement especially with regards to cfDNA biology. cfDNA is cleared by the liver and the kidney and yet, the clearance process is up to day not completely elucidated [107]. Moreover, cfDNA is thought to be predominantly released from apoptotic and necrotic tumor cells deriving from the primary tumor, the metastatic site or even CTCs [90]. However, the latter is argued since there's another postulate claiming that cfDNA do not derive from CTC. This postulate resides on the fact that there is a discrepancy between the number of CTCs and the quantity of cfDNA in the blood. As put forward by Crowley [124]"single human cell contains 6 pg of DNA and there is a median of 17 ng of DNA per ml of plasma in advanced-stage cancers; therefore, if CTCs were the primary source of ctDNA it would require over 2,000 cells per ml of plasma. In reality, there are, on average, less than 10 CTCs per 7.5 ml blood". Consequently, ctDNA offers a much higher analytical signal that CTC cannot offer. Nonetheless, the capacity of identifying a tumor cell from the blood as an entire entity with the complete genetic information remains of particular and unique advantage that ctDNA cannot provide. Future studies shedding the lights on the cellular and molecular process driving cfDNA release/secretion will aid to counteract the current challenges relative to cfDNA detection and downstream analysis.

1.1.7. Aims of this Thesis

The discovery of tumor associated genetic mutations and the investigation of cellular and molecular mechanisms driving these genomic aberrations has been the major focus of cancer research during the last decade. Major advances have been made in the field notably as a result of spectacular revolution of DNA sequencing technologies that helped understand the clinical impact of the detectable mutations. However, current clinical practices for molecular testing in NSCLC patients face key challenges of technical, logistical and tumor related nature.

In this context, liquid biopsy has gained considerable attention owing to its numerous advantages over conventional tissue-based methods. Investigating EGFR mutations and their association to clinical outcomes in response to EGFR TKI has paved the way for the avenue of ctDNA- based liquid biopsy analysis. This has been further supported by the FDA approval of the first ctDNA assay (Cobas, Roche) [125]. Therefore, ctDNA mutation detection might appear as a promising alternative to characterize in real-time patients' mutational landscape and adapt treatment decision accordingly. Targetable genomic aberrations can also be detected on CTCs: aberrations such as MET amplification have been detected in both CTC and ctDNA of.an ALK positive NSCLC patient [126]. Furthermore, CTC represent a powerful approach to detect a variety of different cancer-specific abnormalities such as proteins, miRNA or RNAs and is currently used as a potent prognostic tool mainly in advanced tumor settings. Nonetheless, the presence of low concentrations of both these two analytes especially

at early disease stages might still be challenging with regards to the current methods used and the sensitivities limitations they might present.

The main aim of this thesis was to investigate liquid biopsy-based biomarkers, cfDNA and CTCs in NSCLC patients, precisely with the following aims:

Aim 1: to provide a first proof of principle for the validation of a novel platform for detection of lung cancer associated mutations in NSCLC patients with high sensitivity using plasma derived ctDNA. Furthermore, we want to investigate whether this ctDNA assays is suitable for detection of mutations in patients with lower tumor burden such as oligo-brain metastatic patients. Validation of such an assay can provide treatment decision maker with further relevant clinical information of the disease without resolving to standard invasive methods in a short turnaround time and clinically relevant time-frame.

Aim2: to determine PD-L1 expression on CTCs and compare PD-L1 expression on CTCs and cytological specimen with paired tumor tissue in advanced NSCLC patients. With national guidelines recommendations with regards to PD-L1 assessment on patients, CTCs emerge as a promising alternative source of tumor for PD-L1 testing.

1.2. Material und Methodik

1.2.1. Study design

Patients' cohort

Our study consisted of advanced stage NSCLC patients or non-curatively treated locally advanced patients. Patients were treated at the University Medical Center of Hamburg (Manuscript I) and the LungenClinic Grosshansdorf (Manuscript II), Germany. We defined a limited number of in- and exclusion criteria:

Inclusion criteria:

- Male or female patient, age ≥ 18 years (Manuscript I and II)
- Signed informed consent (Manuscript I and II)
- Approval by local ethics committee (Manuscript I and II)
- Histologically or cytologically confirmed diagnosis of Non-Small Cell Lung Cancer (Manuscript I and II)
- Tumor blocks / tumor biopsy samples and cytological specimens have to be available (Manuscript II)
- 3 x 7.5 ml whole blood draw for CTC (Manuscript II)

Exclusion criteria:

- Other diagnosis than NSCLC (Manuscript I and II)
- Previous treatment with systemic chemotherapy or IO (Manuscript II)
- Contraindication for systemic therapy (Manuscript I and II)
- No signed informend consent (Manuscript I and II)

The study was approved by the the ethics review board of the University of Hamburg (Nr.PV-5392, 06/12/2016, Ärztekammer Hamburg) (Manuscript I) and by the ethics committee at the University of Luebeck (Az. 17-161) (Manuscript II).

Manuscript I describe 56 patients, half of them recruited before 2015 and manuscript II describes 76 patients recruited between February 2018 and April 2022 (figure 4). Study design of Manuscript I (Manuscript I, figure S1) was based on interdisciplinary collaboration between the departments of tumor biology, Neurosurgery and pathology of the university medical center of Hamburg, the department of personalized oncology in Heidelberg and Agena Bioscience. 56 patients had their blood analyzed retrospectively. Study design of manuscript II is illustrated in figure 4 and was based on interdisciplinary collaboration between the departments of tumor biology, pathology of the university medical center of Hamburg, department of respiratory medicine from "Agaplesion Diakonieklinikum Rotenburg", the institute of Pathology from the University of Luebeck, the pathology department from the Borstel research center and Grosshansdorf "Lung Clinic".

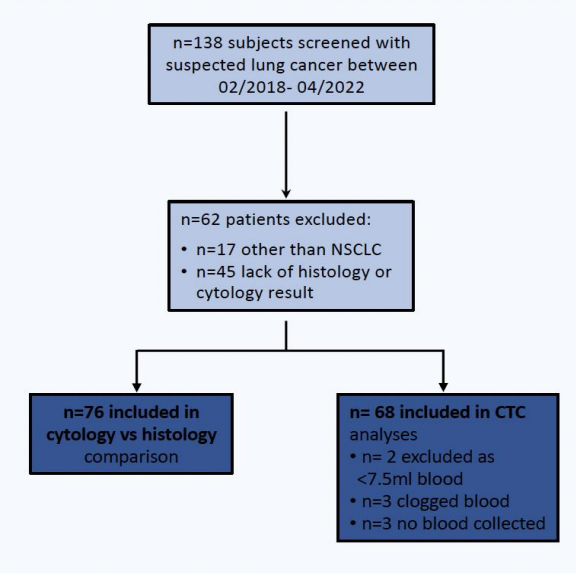

Figure 4: study design (From Manuscript II, figure 1)

1.2.2. Histological and cytological specimen analysis

Histologcial (Manuscript I and II) and cytological specimen (manuscript II) experiments were not performed by the PhD student.

The histological biopsies/ cell blocks performed at diagnosis (if feasible) or progression were formalin-fixed and paraffin-embedded (FFPE) for histopathological analysis to confirm diagnosis (Manuscript I and II). Tumor material was collected via fiberoptic bronchoscopy from primary tumor specimen. Other tumor materiel was obtained from surgical tumor tissue via ultrasound-guided percutaneous tumor biopsy (Manuscript II). Sections were cut at 4 µm thickness, placed on charged slides, and dried in an oven for 1 hour. Human tissues sections were dried at 56 to 60°C. The cut sections were stored in dark at 2 to 8°C and used for the PD-L1 IHC assay within 4 months (Manuscript II). Tumor specimens were smeared in a rapid on-site evaluation (ROSE) to obtain matched cytology imprints from the same tumor site. Cytologic evaluation was performed using the imprint, effusion and fine needle aspiration technology. Therefore, corresponding tumor tissue samples will be rapidly touched on appropriate glass-slides.

1.2.2.1. PD-L1 expression on histological and cytological specimen (Manuscript II)

PD-L1 IHC 28-8 pharmDx was used to stain for PD-L1 expression with the BenchMark ULTRA system (Ventana Medical Systems, Tucson AZ, and U.S.A). PD-L1 TPS was determined as the percentage of tumor cells with partial or complete membranous staining relative to total number of at least 100 viable tumor cells (Manuscript II). Based

on staining results, we determined 2 thresholds: positive PD-L1 expression (1% cut-off TPS) and high PD-L1 expression (50% cut-off TPS). TO avoid bias, different pathologists performed and analyzed PD-L1 staining from the site-matched cytology and histology specimen.

1.2.3. Liquid biopsy analysis

1.2.3.1. Blood Sampling (Manuscript I and II)

The blood sampling was performed at the department of tumor biology at the university medical center of Hamburg (Manuscript I). Samples received from the "LungenClinic" were scheduled in the laboratory system with the date and time of blood draw (Manuscript II). Three patients presented with serial sampling (Manuscript I). One patient had 9 follow-ups and for 2 other patients 2 blood samples were available for each.

1.2.3.2. Plasma and cfDNA isolation (Manuscript I)

The pre-analytical handing of blood samples might be determining for ctDNA purity and quality and can de facto bias downstream analysis. Peripheral blood (7.5mL) in ethylenediaminetetraacetic acid (EDTA) tubes (Sarsted, Nürnbrecht, Germany) was collected and processed within 2 hours by a first centrifugation (300g for 10 minutes) followed by a second centrifugation (1800g for 10 minutes). Plasma was then stored at -80°C for downstream cfDNA isolation. cfDNA purification was performed using QIAamp circulating nucleic acid kit (Qiagen). The ctDNA quantity was measured by Qubit Fluorometer (Thermo Fisher) and the quality was assessed by TapeStation (Agilent).

1.2.3.3. ctDNA analysis with the MassARRAY system (Manuscript I)

The UltraSEEK Lung Panel on the MassARRAY® System (Agena Bioscience, San Diego, CA, USA) analyzes 74 different hot-spot mutations in EGFR, KRAS, BRAF, ERBB2 and PIK3CA (Manuscript I, table S1)). Details on the MassARRAY system and the mutations detection are found in manuscript I.

1.2.3.4. CTCs isolation from patients' blood (Manuscript II)

Blood samples of 7.5 mL were collected EDTA tubes (Sarsted, Nürnbrecht, Germany). Samples were processed within 24h on the label-independent, microfluidic system (Parsortix^{TM}, ANGLE plc, Guildford, United Kingdom). Cytospin funnels were used to collect cell suspension and centrifuged onto a glass slide (1200 rpm, 5 minutes), dried overnight, and stored at -80°C until further processing for PD-L1 immunofluorescence.

1.2.3.5. PD-L1 expression on CTCs (Manuscript II)

PD-L1 expression on CTCs was assessed by immunofluorescence. Cells were fixed with 0.5 PFA for 10 minutes, washed, blocked and incubated with PD-L1 antbody clone HL1041 overnight. (GTX635975, 1:100). BD HorizonTM BV421 goat anti- Rabbit (BD Bioscience, 1:200) was used as a secondary antibody and incubated for 45 minutes. After additional washing steps, a multicolor antibody cocktail was added for 60 minutes:

pankeratine (eBioscience, 1:200), CD45 (Milteny Biotec, 1:200) and DRAQ5™ for nuclear staining (BioLegend, 1:5000). Table 2 illustrates the multicolor staining cocktail applied to patients' slides.

Target antibody	Clone/ host	Fluorophore
Nuclei	-	DRAQ5
CD45	HI130/ mouse	PerCP
Pankeratine	AE1/AE3/ mouse	eFluor560
PD-L1	HL1041/ rabbit	Uncojugated
PD-L1	Secondary/ goat anti-rabbit	BV421

Table 2: multicolor staining used for downstream immunofluorescence (Modified from Manuscript I, Table S1)

1.2.4. Statistical analysis (manuscript I and II)

Signal to noise ratio that corresponds to normalized intensity of the mutant allele has been performed by using Typer software version 4.0.26.74 (Agena Bioscience). The signal intensity of the mutant allele has been normalized against the capture control peaks present in the spectrum of each run (Manuscript I).

Receiver Operator Characteristic (ROC) was used to evaluate PD-L1 expression from cytology imprints and CTCs as predictors for positive PD-L1 expression (tumor cells expression score ≥1%) and PD-L1 high expression (tumor cells expression score ≥50%) from standard immunohistology specimen. We used Fisher exact test to identify differences in clinical variables between the study groups (manuscript II).

We used Pearson's test to examine the correlation between two continuous variables. Statistical analyses were performed using R (version 4.2.1, R Foundation, Vienna, Austria) to examine correlation. An alpha error of less than 5% was considered statistically significant (manuscript II).

1.3. Ergebnisse

Manuscript I

The main aim of this study was to provide a first proof of principle of the MassARRAY system for detecting cancer associated genes. Our cohort included 56 lung cancer patients with advanced stage disease.

1.3.1. UltraSEEK™ lung panel in advanced NSCLC patients allows mutations detection using cell free DNA

EGFR mutations were detected in 25.0% (14/56), KRAS in 21.4% (12/56), ERBB2 in 5.4% (3/56), PIK3CA in 5.4% (3/56), and BRAF in 5.4% (3/56) of the patients (Manuscript I, figure 1A).

1.3.2. Variant allele frequency (VAF) of detected mutations and concordance of EGFR mutational status

Mutations were detected in plasma NSCLC patients with a VAF ranging from 0.1 to 5.0%. The median VAF in oligo-brain metastatic patients was 0.4% (range 0.1–5.0%), 0.9% (range 0.2–5.0%) in multi-brain metastases patients and 1.2% (range 0.3–2.3%) in patients with other metastases (figure 5). An overall concordance rate of 86.4%

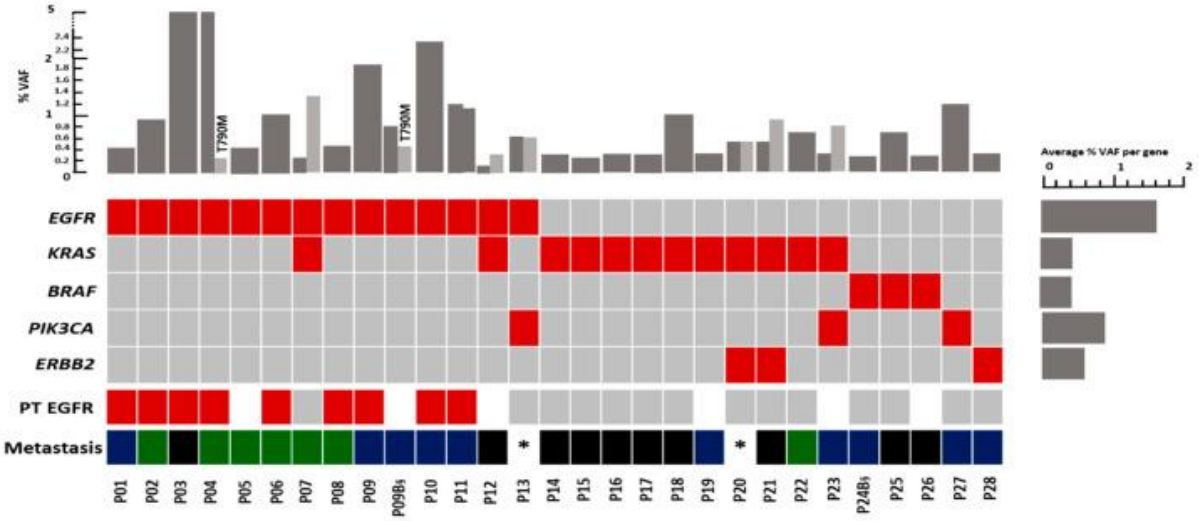

Figure 5: Overview of detected mutations with their variant allele frequency (VAF) and EGFR mutational status from primary tumor (PT) (from Manuscript I, Figure 2). Color cod; Red: Mutation detected; Grey: No mutation detected; Black: Oligo-brain metastases; Green: Multi-brain metastases; Blue: Other metastases.

(38/44) was observed between EGFR mutational status in plasma and matched histological specimen. Six discordant cases were identified (P02, P04, P07, P08, P11, and P13) (Figure 5).

1.3.3. cfDNA analysis with the MassARRAY system support patients management by monitoring response to therapy

We analyzed serial blood samples from an 80-year-old patient with a stage IV, multi-metastatic adenocarcinoma of the lung for mutations in ctDNA over a period of 22 months. The MassARRAY system allows to detect EGFR recurrence in ctDNA 6 months before CT scan exhibited a progressive disease (Figure 6). This further support the use of the MassARRAY ctDNA platform to monitor response to therapy in lung cancer patients using cfDNA.

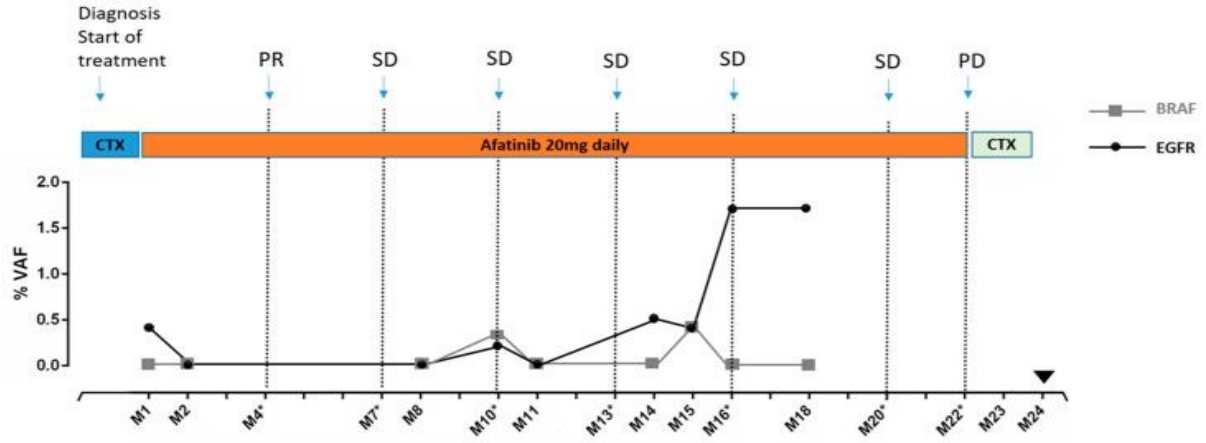

Figure 6: EGFR and BRAF mutations detection in ctDNA from on patients with serial blood sampling (from Manuscript I, Figure 3)

Manuscript II

The second aim was to investigate PD-L1 expression on CTCs. A specific part of this study was to compare PD-L1 expression on CTCs and cytological specimen with paired tumor tissue.

1.3.4. Optimization of PD-L1 clone HL1041 for CTC staining

The main part of my work was to develop a multicolor staining cocktail for CTC detection upon isolation with the label independent CTC enrichment system: Parsortix™. We started by optimizing the use of our new PD-L1 antibody clone HL1041, by assessing its performance on a cell line highly positive for PD-L1 expression (H1975 NSCLC) and a cell line negative for PD-L1 expression (MCF-7 breast cancer). Optimization of a new PD-L1 clone is due to unsatisfactory results with the traditional clones used for immunofluorescence staining: D8T4X or E1L3N. A positive PD-L1 cell line was used as control for primary and secondary PD-L1 staining as shown in figure 7 (Figure 7).

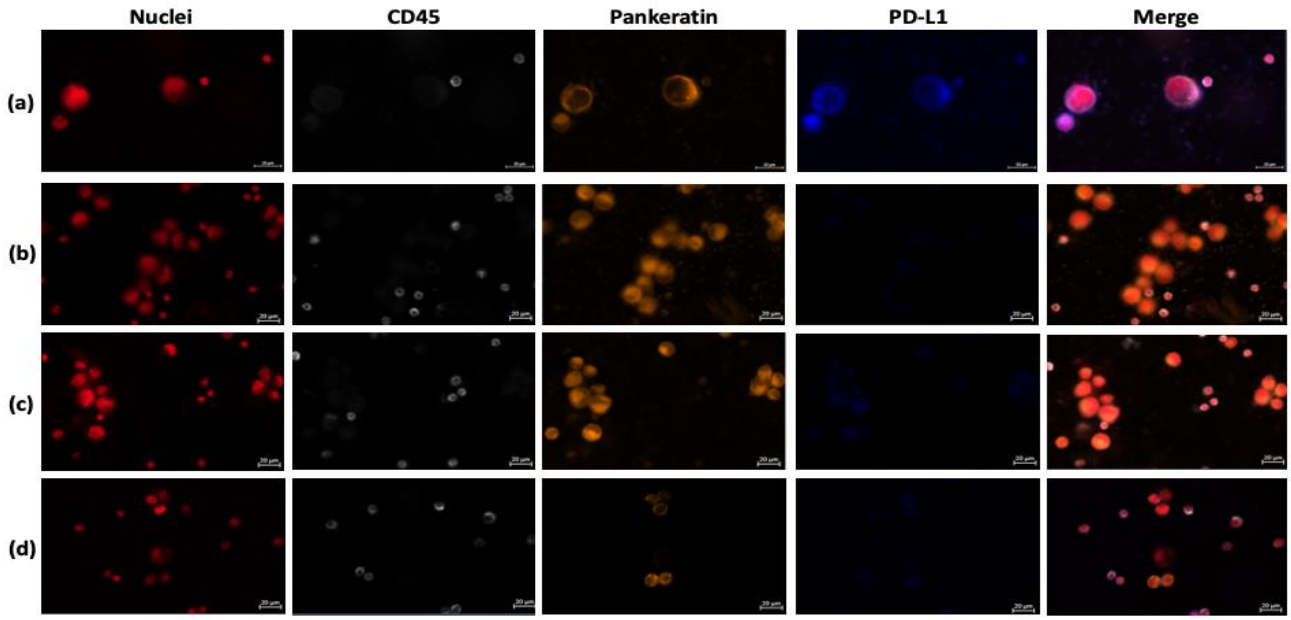

Figure 7: Optimization protocol of PD-L1 clone HL1041 using cell lines positive and negative for PD-L1 expression. a) H1975 NSCLC (PD-L1 positive) cells spiked in healthy donors (HD) peripheral blood mononuclear cells (PBMCs). b) H1975 NSCLC (PD-L1 positive) cells spiked in HD PBMC without primary antibody. c) H1975 NSCLC (PD-L1 positive) cells spiked in HD PBMC without secondary antibody. d) MCF7 breast cancer cell line negative for PD-L1. 40X magnification. 20µm scale bar is applied to all pictures

1.3.5. CTC detection using immunofluorescence on Parsortix™ slides

Once the CTC staining protocol was established, 68 out of the 76 samples were assessed for PD-L1 expression on CTCs. CTCs were detected in 27/68 samples (39.7 %). Examples of single CTC and CTC cluster staining with positive versus negative PD-L1 expression are shown (Manuscript II, figure 2).

1.3.6. PD-L1 agreement between cytology and histology

1% cut-off PD-L1 TPS: The predictive capacity of cytology imprints for PD-L1 positivity (≥1%) indicated a positive predicted value (PPV) of 91%, a negative predicted value (NPV) of 33%, AUC= 78% [95% CI: 65-90%]. A positive agreement rate of 91.0%, was seen in 61 cytology imprints out of 67 matched tissue specimen. Negative agreement was 33.3% and was only seen in 3 cytology imprints out of 9 matched histology specimens.

50% cut-off PD-L1 TPS: Considering high PD-L1 expression (≥50%), cytology imprints showed a PPV of 64% and a NPV of 85%, AUC= 79% [95% CI: 67-91%]. A positive agreement rate of 79%, seen in 23 cytology imprints out of 29 matched histology specimens. Negative agreement was 85%, seen in 40 cytology imprints out of 47 matched histology specimens.

1.3.7. PD-L1 agreement between histological specimen and CTCs

A relatively good overall agreement with regards to PD-L1 expression (1% TPS) between CTC-based liquid biopsy and histological specimen was observed: 66.7%. Three patients exhibited PD-L1^{+} CTCs while tissue was negative for PD-L1 (Table 4). With high PD-L1 expression (50%), the agreement rate dropped to 51.9% (Table 4). Of all patients with high PD-L1 expression in histology specimens, 90.0% had PD-L1^{+} CTCs; however, 70.6% of patients with negative PD-L1 on histological specimen displayed PD-L1+ CTCs (Manuscript II, Table 3).

1.3.8. PD-L1 agreement between cytological imprints specimen and CTCs

An overall agreement rate of 62.9% of positive PD-L1 expression was observed between cytology imprints and CTCs. Similar to histological specimen, the agreement rate dropped to 51.9% when high PD-L1 expression in the cytology imprints was considered (Manuscript II, Table 4). The addition of CTCs PD-L1 expression assessment significantly improved the predictive capacity of cytology imprints compared to standard immunohistochemistry for PD-L1 positivity at 1% PD-L1 TPS; AUC= 91% [95% CI: 79-100%] but also at 50% PD-L1 TPS; AUC=84% [95 CI: 69-100%].

1.4. Diskussion

The investigation of blood- based biomarkers have reshaped our approach of cancer patients' management in general, and NSCLC patients in particular. The introduction of several d targeted therapies directed against druggable molecular alterations in NSCLC patients helped develop ctDNA assays and optimize methods' sensitivities. Molecular testing became a gold standard for managing NSCLC patients. And with further establishment of IO in lung cancer patients as a primary treatment option owing to long terms benefits in terms of survival, PD-L1 assessment on CTCs is more than ever, of a clinically relevant importance. To date, tissue remains still the gold standard method for assessing NSCLC histology and considered as the keystone to guiding

therapy decision upon molecular characterization. However, tissue specimen analysis is often compounded by the specimen type with often a very low tumor purity owing to the high proportion of stromal cells, lymphocytic infiltration and necrosis. The development of several technologies with their specific failures due to differences in sensitivity/ specificity (different NGS platforms yields different results), the discordant results in a multitude of tests (e.g., immunohistochemistry vs fluorescence, tissue vs cytology …), absence of standard operating procedures (SOPs) for commonly used technologies … and many other reasons under different aspects hinder the accurate molecular diagnosis in NSCLC patients. The tumor biology related aspects are heralded by the intra- inter- tumor heterogeneity owing to the diversity of molecular subgroups within NSCLC patients and the consequent sampling bias remain a real clinical challenge. Tumor biology related challenges are exacerbated by clonal evolution of different metastatic sites and the acquired resistance in response to treatment. There's thus an unmet need to further characterize genomic aberrations in NSCLC patients. ctDNA could represent a valuable blood source to detect molecular alterations but remains limited by several challenges including assays' sensitivities to detect minor mutant allele and the lack of multiplexing assays. Both of which are feasible with NGS- based approach using ultradeep sequencing, not often accessible.

Part 1. Belloum* et al., Cells 2019: Discovery of Targetable Genetic Alterations in NSCLC Patients with Different Metastatic Patterns Using a MassAarray-Based Circulating Tumor DNA Assay

1.4.1. Driver mutation detection on cfDNA in advanced NSCLC using a targeted panel

Using a PCR based method coupled with Mass-spectrometer (MassArray) based ctDNA detection method, we detected genomic aberrations in cfDNA from 50% of the enrolled NSCLC patients (28/56). EGFR mutations were detected in 14 patients (25.0%) and the most common EGFR mutations were exon 19 deletion (found in five patients) followed by exon 21 L858R point mutation (found in four patients). KRAS was detected in 12 patients (21.4%), BRAF, HER2 and PIK3CA were all detected in 5.4% of the cohort. The mutation distribution and frequencies are in line with what was previously reported with regards to plasma genotyping [127-131]. Several meta-analyses have investigated ctDNA's clinical utility and the performance of the detection methods for EGFR mutational status [132, 133], and to a lower extend, for KRAS mutational status [134]. Rossel et al for example reported EGFR mutations in 16.6% of NSCLC patients with exon 19 deletion and L858R point mutation being the most predominant EGFR mutations which align with our findings [135]. KRAS were identified in 9% of plasma samples [136]. Both studies used PCR- based approaches to unravel genomic aberrations on plasma derived cfDNA.

With driver mutations detected in our cohort with a variant allele frequency ranging from 0.1% to 5%, the performance of our multiplex assay's sensitivity is satisfactory considering that low frequency genomic aberrations are hard to target. Not surprisingly, the highest allele frequencies were observed in patients with multiple metastases rather than oligo-brain metastases. This confirms previous finding claiming that tumor cfDNA shedding is the chief driver of assay sensitivity and the extend of the disease's

aggressivity is likely to determine, besides the technique' sensitivity *per se*, the limit of detection [90, 137]. The tumor burden thus highly conditions ctDNA release in the blood and the correlation between metastatic sites and ctDNA positivity observed in our study and others' support such a claim [138, 139]. Couraud et al even showed that ctDNA concentration in plasma is significantly associated with the number of metastatic sites [140]. Furthermore, several studies investigating primary or oligo-brain metastatic tumors support the claim that blood brain barrier can reduce ctDNA release into the bloodstream and subsequently limit the sensitivity of liquid biopsy approaches to detect targetable genomic aberrations [141, 142]. Still, we manage in our study to detect genomic aberrations in 50% of oligo-brain metastatic patients, a finding that is consistent with previous studies where mutations were detected in the plasma of 52% of oligo-brain metastases patients with lower VAF in oligo- compared to multi-brain metastasis patients [143] . Metastases progression to the central nervous system are frequently found in oncogene-driven NSCLC patients (50% for brain metastases and 10% for leptomeningeal metastases) [144], and in most of these cases, tissue based genomic profiling are mostly not available. Several other studies upheld the clinical utility of plasma ctDNA and cerebrospinal fluid (CSF) ctDNA to monitor NSCLC patients with brain metastatic tumors, including detection of genomic aberrations [143, 145-150].

The discovery of recurrent genomic aberrations in genes such as EGFR, ALK, ROS1, BRAF, MET, RET and NTRK led to the development of several approved targeted therapies in first line setting for advanced NSCLC patients. Tissue- biopsy based molecular testing is even recommended by the national guidelines even though plasma genotyping is preferred for its minimally invasive character. Studies have shown the importance of EGFR TKI inhibitors for conferring better outcomes for EGFR mutant patients while the development of Sotorasib (anti-KRAS G12C) showed durable clinical benefit further emphasizing the prominence of driver mutations detection on NSCLC patients [151]. Cumulative detection of these mutations is thus of a primary importance in lights of their therapeutic nature. Therefore, the validation of the multiplexing capacity inherent to the used MassArray system in our study is important in light of the therapeutic nature of the driver oncogene mutational status. Through detection of low-frequency driver mutation as low as 0.1% minor allele frequency along with therapeutic escape variants (e.g., T790M) by interrogating those variants within a single reaction, the MassArray system provide a clinically relevant time-frame.

1.4.2. Analytical validity of the MassArray system for ctDNA detection

The MassArray-based approach used in our present study is able to assess 74 mutations in one tube in a cost-effective manner, and it produces clinically relevant data within 2-3 days turnaround time. Sensitivity and specificity of the UltraSEEK method have been validated using plasma derived cfDNA from melanoma patients in previous study and further demonstrated in our study with a high sensitivity (> 0.1% ctDNA) and high specificity as demonstrated by the high concordance of results from tissue biopsy and liquid biopsy for EGFR mutational (almost 90%, as shown in the present manuscript) [152]. To the best of our knowledge, we were the first to validate the use of the mass-array based approach on a set of NSCLC plasma derived cfDNA.

Validation of such a sensitive method is further supported by scarcity of the mutant allele. The minor mutant allele versus the abundant wild type one can retain a one SNP difference making their detection a challenging task [107, 117]. There's thus an unmet need for highly sensitive methods and several of these have been developed and represent the foundation of our current knowledge: On the one hand, very sensitive targeted methods like digital droplet PCR (ddPCR) capable of providing enhanced sensitivity as low as 0.01%, are restricted to a few mutations and this remain unsufficient with regard to tumor heterogeneity [153, 154]. The multiplexing capacity of ddPCR is limited and often require NGS-based approaches. The latter can assess larger mutations panels and many NGS-based mutations panels for cfDNA analysis are being commercially available and more frequently applied to clinical practice [88, 89] complementing in some cases the conventional tissue- biopsy based techniques [155].However, NGS-based approaches for cfDNA mutations detection do not enrich for minor variants but rather rely on the sensitivity of the detection mechanism which often requires highly costly ultra-deep sequencing for minor allele detection [156, 157]. Unless deep sequencing is used, which is rare in clinical practice, NGS- based approaches are less sensitive and their reproducibility can be biased with ctDNA concentrations dropping below 1%, which is the case early-stage disease patients. This has been shown and further investigated in the technical validation study performed by EU/IMI consortium CANCER-ID [158]. This can thus explain the discordance of ctDNA measurements using different platforms [159]. An illustrative example of discordant results with regards to sequencing technology in cfDNA is the significant disparities found when two John Hopkins scientists submitted identical patient samples to two different commercial liquid biopsy providers: the FDA approved Guardant360 (Guardant Health, Inc) and PlasmaSELECT (Personal Genome Diagnostics) [160]. The reported results help prescribe patients with different treatment according to the commercial platform that was used, both, were accredited by the College of American Pathologists. NGS-based approaches are usually time consuming (turnaround time > 10 days).

1.4.3. EGFR mutational status: Tissue vs. plasma

The overall concordance rate of almost 90% (86.4%) observed between tissue vs liquid biopsy EGFR mutational state in our study is satisfactory and is consistent with previous studies supporting the use of plasma genotyping. EGFR's mutational status in tissue versus plasma varied from 58 % to 97% using different methods that yielded different sensitivities and specificities with regards to the driver mutation detection [128, 153, 161-163]. The discrepancy of EGFR mutational status observed between tissue versus plasma in our study could find its origin in the high rate of false negative detection in blood samples, up to 30% of cfDNA assays are false-negative [164] and therefore, EGFR negative ctDNA in plasma should be interpreted with caution and require further investigation and this is recommended by the national guidelines [165]. In fact, the national guidelines support the use of tissue biopsy in the absence of detectable targetable driver alteration in plasma derived cfDNA. The same holds true for T790M detection using tissue material in case of negative ctDNA results [166]. The evolving mutational landscape of a tumor, the development of novel resistance

mechanisms and the optimized high-throughput analysis often require a minimum amount of tissue material. The latter is hard to get especially at late stages of the disease. This statement is true for NSCLC patients knowing that more than 70% of NSCLC patients are initially diagnosed at an inoperable stage of the disease [18, 49]. Re-biopsy is thus often not an option, especially for each metastatic site. Though tissue is the gold standard method for assessing NSCLC histology and considered as the keystone to guiding therapy decision upon molecular characterization, it remains insufficient with regards to tumor heterogeneity, which is perhaps better reflected in liquid biopsy analyses.

1.4.4. T790M: the gatekeeper mutation

The current national guidelines advocate for plasma genotyping to detect acquired resistant mutation T790M in patients who progressed on first- or second-generation EGFR TKI and the consequent treatment adaptation with third generation EGFR TKI Osimertinib. Two EGFR-mutant patients in our cohort displayed T790M resistance mutation, that was not detectable on tissue material one month prior to plasma genotyping. This might explain the progressive disease observed in response to first-line erlotinib. A plethora of studies uphold the clinical utility of assessing plasma derived T790M gatekeeper mutation for advanced NSCLC patients under EGFR TKI treatment that consequently led to the development of many EGFR mutations detection kits that are commercially available and integrated into clinical practice [137, 167-169]. The most used is the cobas® EGFR Mutation Test v2 that has been approved by the FDA as a companion diagnostic test for treatment selection (gefitinib, erlotinib and osimertinib) [125, 170, 171]. There's a clear advantage of the mass-array approach over the conventional technologies with regards to the multiplexing capacity. This is essential in light of the presence of other resistance causing mutation in NSCLC patients such as C797S or G724S [172-174].

1.4.5. ctDNA monitoring presents with a clear advantage over CT-Scan: early detection of treatment failture

In my thesis, we performed serial plasma genotyping for a multi-metastatic NSCLC patient receiving first line chemotherapy. Unlike the primary tumor, the metastatic tumor profiling revealed EGFR exon 19 deletion and treatment was subsequently switched to afatinib, a second generation TKI. Upon second line afatinib, EGFR exon 19 deletion was detected in plasma derived cfDNA, concordantly with the metastatic site. A series of plasma genotyping in the following next months did not reveal EGFR mutations. Interestingly, this period coincided with patient's partial response and a stable disease in the following months. Subsequently, while CT-Scan was still showing stable disease, plasma genotyping revealed EGFR mutation. CT-scan showed progressive disease only 6 months after cfDNA analysis and 2 months before patients' death. It is true that the introduction of high-resolution imaging technologies such as low-dose computed tomography (also known as CT scan) played a pivotal role into reshaping patients' clinical diagnostic. CT-scan are capable of detecting even the small nodule in the lung and have been used to monitor NSCLC patients' disease in clinical routine for many years. However, CT-scan remains insufficient with regards to the

performance status. In fact, symptoms of progression and adverse effects are the main reason for the decline of the performance status (PS), further revealing an undetectable deterioration that actually finds its origin at the genomic level. This single case study showed a higher sensitivity in detecting MRD compared to CT scan, which is in line with other studies [175]. ctDNA- based MRD detection in different clinical setting is extensively reviewed here [176]. Hence the clinical relevance of plasma genotyping as a precise and reliable method to evaluate treatment efficacy and detect MRD and help *de facto* reduce long lasting ineffective treatments and spare patients form needless adverse events.

1.4.6. Study limitations and future perspectives

Our data support the use of ctDNA MassArray system to detect clinically relevant genomic aberrations and further uphold liquid biopsy as a robust tool to monitor patients' disease, especially at late stages where tissue material is hardly accessible because of patients' poor performance status or insufficient material for molecular testing. With clonal evolution driving different metastatic sites and consequently modifying molecular fingerprints, ctDNA- based liquid biopsy analysis could represent a more holistic view of the disease to better counteract tumor heterogeneity. Several other clinical applications supported the use of the MassArray system such as HPV detection [177] or tumor profiling [178]. In conclusion, ctDNA appears to be a useful technique for companion diagnostics, notably in cancer harboring druggable genomic aberrations. ctDNA analysis, however, could not be suited for analysis of tumors known to be poor cfDNA shedders (e. g. gliomas and sarcomas). We are aware that our study has several limitations. The probable presence of false- positive is a risk for every assay. Furthermore, only hotspot mutations in driver oncogene of interest were evaluated with the MassArray system. This makes this method unsuitable for other clinically relevant genomic aberrations such as ALK translocations or ROS rearrangement. The latter ones are known to be hardly detectable in plasma derived ctDNA unless using NGS- based approach with high sensitivity and specificity [107, 179]. Furthermore, it would have been interesting to associate the different molecular aberrations to patients' clinical outcomes. Unfortunately, survival data were not available for our cohort. Moreover, mutations can lead to alteration of epigenomic regulators resulting in an altered transcriptome, consequently worsening prognosis by further expanding the effect of a single molecular aberration [180]. Therefore, investigating the epigenomic and transcriptomic landscape could provide further clues on the tumor and consequently complement information obtained with the MassArray system. Associating this information to clinical outcomes could become of importance for patient´s management, especially with regards to IO [181-183]. Even though we were the first to validate the use of the MassArray system, more precisely, the lung panel in NSCLC patients, intending to move from validation to clinical implementations requires larger studies with matching mutations in tissue and plasma ctDNA along with the association to clinical outcome. For such a purpose to be achieved, it is compulsory to continue optimizing detection methods, defining and validating thresholds for ctDNA mutation detection as a surrogate marker to guide therapy decision, but mostly

designing an interventional study is essential with regards to the therapeutic nature of molecular aberrations in NSCLC patients [184].

Part 2. Belloum* et al., Molecular Oncology 2023: Comparative Evaluation of PD-L1 Expression in Cytology Imprints, Circulating Tumor Cells and Tumor Tissue in Non-Small Cell Lung Cancer Patients

1.4.7. PD-L1 testing on CTCs from NSCLC patients to predict response to immunotherapy

Immune escape defined by impaired immune system to remove transformed cells is the hallmark of carcinogenesis [75]. The only predictive biomarker used as a companion diagnostic test for first line IO is PD-L1 expression assessed by the conventional and gold standard method: immunohistochemistry from formalin-fixed paraffin-embedded (FFPE) tissue sections [77, 185]. PD-L1 expression is considered a key factor in selecting NCSLC patients who might benefit from IO treatment [186]. Immune checkpoint inhibitors, alone or in combination with other treatment options have revolutionized the treatment outcome of several tumor entities, including NSCLC, providing better clinical outcome for some patients with high PD-L1 expression. The antibody pembrolizumab (anti-PD1) has been FDA-approved for first line and second line therapy of NSCLC patients exhibiting high (50%) [76, 81] and low (1%) PD-L1 expression [187]. The combination of nivolumab (anti-PD1) plus ipilimumab (anti-CTLA-4) was also FDA approved for first-line treatment in NSCLC patients whose tumors had PD-L1 TPS≥ 1% [188]. Though of a primary importance considering its widespread use in the clinic, PD-L1's use as a predictive biomarker for IO treatment have several limits and remains up today a challenging task even for specialized pathologist, at the technical level, but also and mainly because of technical and spatial PD-L1 heterogeneity, another cancer hallmark and one of the leading causes of cancer therapy resistance, tumor progression, and metastasis [75, 186]. Consequently, even in operated patients with large tumor tissue blocks available, the tissue might still not be representative of the whole tumor constituting thus sampling bias responsible for attributing or not IO to patients. Even with a high PD-L1 expression studies have shown that IO might still fail, suggesting complex and incomplete understanding of the immunopathology of NSCLC [189]. PD-L1 expression is assessed in a considerable proportion of NSCLC patients with only biopsy or cytological specimen available. In fact, two third of lung cancer patients are presenting with advanced stages and their diagnosis is usually established based on small biopsies (bronchoscopy, needle, or core biopsies) and/or cytological specimen. CTCs appeared then as a potential alternative or additive for real time monitoring of the tumor and capable of alleviating the burden of tumor heterogeneity since these CTCs are derived from more than one tumor site and should be capable of representing a larger overview of a patient's tumor.

In the first step of our prospective study, we compared PD-L1 expression on tissue samples from standard immunohistochemistry with PD-L1 expression of the site-matched cytological specimen of the primary lesion. A second step of the study included analysis of liquid biopsy specimen, more precisely CTC where PD-L1 expression was assessed by immunocytochemistry.

1.4.8. PD-L1 assessment in clinical routine: histology versus cytology

The PD-L1 histology & cytology assessment was performed at “Großhansdorf Lungen Clinic” by specialized medical doctors where I did not take part of this.

Compared to matched histology specimen, cytology imprints in our study yielded a positive agreement of 91% on PD-L1 positivity (PD-L1 TPS>=1%) and yet, a poor negative agreement of 33.3%. According to literature, discordant cases typically show either higher score in cytology or in histology [77, 190, 191]. Our study suggests that with a low PD-L1 positivity (>=1%), cytology might overestimate PD-L1 positivity. When considering a high PD-L1 expression threshold (PD-L1 TPS>=50%), cytological specimen yielded a positive and negative agreement of 79% and 85%, respectively. The latter is capable of dropping patients not qualifying for first line IO. With regards to cytology- histology agreement, our data seem in line with what was previously demonstrated in literature ranging between 62-100% at 1% cut-off and 67-100% using 50% cut-off [77, 185, 192]. Many factors could contribute to the discrepancies including the different methods used for preparation of cytological materials and choice of AB. The commonly used PD-L1 clones 22C3, 28-8, and SP263 exhibit similar staining pattern and have been proved to be interchangeable [193]. SP142 clone on the other hand was described to have lower staining performance [77, 194]. It is of note that SP142, 28-8 and 22C3 were all FDA approved as a companion diagnostic tool for selecting patient with NSCLC for IO. Consequently, the different PD-L1 expression rates between paired samples and between studies is mainly to be attributed to spatial intra-tumoral heterogeneity which should be assumed to lead to misclassification of patients with regards to PD-L1 status. There is thus an unmet need to further characterize PD-L1 expression in patients, hence the second step of our article including CTC analysis.

1.4.9. PD-L1 expression on CTC detected with the Parsortix^{TM} device

In the first part of our study, we investigated PD-L1 status between paired histological versus cytological specimen with nearly 80% of the specimen collected via fiberoptic bronchoscopy. We investigated in the second part of our study PD-L1 expression on CTC isolated from the peripheral blood of the same patients. For cytological and tissue sections specimen, 28.8 antibody clone was used. PD-L1 clones 22C3, 28-8, and SP263 exhibited similar staining pattern and have been proved to be interchangeable [193]. Skov et al even demonstrated a high degree of agreement between 28.8 and 22C3. SP142 clone on the other hand was described to have lower staining performance [77, 194]. It is of note that, SP142, 28-8 and 22C3 were all FDA approved as a companion diagnostic tool for selecting patient with NSCLC for IO. Nonetheless, none if the cited antibodies were used for CTC immunofluorescence staining. Our data (not shown in this thesis) supports the use of rabbit HL1041 antibody clone for PD-L1 CTC analysis owing to better staining optimization results compared to E1L3N and D8T4x clones. We detected CTCs in 27/68 samples (39.7 %) with higher detection rates in patients with stage IV (41.9%) compared to patients with non-metastasized tumors (37.8%). A significant increase was found for stage IVB patients (64%, p=0.032). The average CTC number was 2.7 CTCs per 7.5 mL of blood ranging

between 1 and 13 CTC per patient. With 39.7 % of detectable CTCs, our data are in line with previous reports with a CTC detection rate ranging between 32% and 99% [104, 189, 195-204]. However, with regards to different CTC enrichment techniques, detection method, PD-L1 antibodies and patient cohorts used in different studies, comparisons between studies have to in interpreted with caution. Therefore, those parameters could potentially explain the large fluctuations of CTC and PD-L1^{+} CTC rate between studies. Most of the studies with high detectable CTC rate (range 75% - 93%) used label independent approach, the ISET© technology [195-197] or the FDA approved CellSearch® system [189]. Studies with Parsortix™-based CTC capture exhibited a lower CTC rate of 61% [104]. However, and as previously mentioned, there's an obvious discordance between CTC enrichment and detection platform for determining CTC count between studies. In contrary to previous studies, Papadaki et al reported a higher CTC counts in NSCLC patients when using the Parsortix™ system compared to the densitiy gradient centrifugation or ISET technology [205]. The hypothesis that the differences between label independent parsortix™ system and CellSearch® system is solely based on EpCAM expression have been argued Chudziak. Authors reported a higher EpCAM$_{low}$ CTCs and a higher CTC count using SCLC cell lines with Parsortix™ compared to CellSearch® system [206]. Studies have demonstrated that presence of CTCs in the peripheral blood of lung cancer patients is correlated to TNM stages [207] and more importantly, associated to poor clinical outcomes in terms of PFS and OS [88, 90, 208], regardless of the enrichment and/ or isolation method. The label-independent nature of the Parsortix™ device used here to capture CTC have been demonstrated in several studies and is further corroborated by the broad use of the device [102, 104, 209, 210]. The technology has been extensively evaluated including studies with NSCLC cohort as part of multicenter ring trials within our EU/IMI consortium CANCER-ID [102, 103, 209]. CTC detection in NSCLC patients using Parsortix™ device was comparable or even superior to most EpCAM-based enrichment approaches including the CellSearch® system and our data are in line with such claims [211]. In fact, the physical characteristics inherent to the microfluidic system (cell size and deformability) for the separation, capture and the subsequent harvest of rare CTC from the peripheral blood have the potential to bypass label- dependent bias in CTC positive selection and thus capture CTC regardless of the expressed surface antigen, including EpCAM^{low} CTCs or non-epithelial phenotype CTCs [97, 209]. Though the CellSearch® is FDA- approved for CTC enumeration and used as a predictive marker of poor prognosis in metastatic carcinoma [212-214], its wide clinical implementation is hampered by its shortcomings such as the manual process, specificity and sensitivity of the assay [215]. Furthermore, with the advantage of harvesting viable cells upon separation, the Parsortix™ device overcomes difficulties inherent to size-based enrichment strategies using size exclusion filters which requires fixation. Fixation makes downstream molecular analysis harder considering the arduous task of detaching CTCs. It's reported on the one hand that the Parsortix™ device, with its critical 6.5µm gap (10µm originally) for the ultimate separation, tend to enrich larger cell type more efficiently than the smaller one [102, 209]. On the other hand, the examination of cell size distribution used to confirm the previous assumption showed that no cell smaller than 10µm were present in these populations. This

suggests that the tendency to enrich for bigger cells cannot be fully attributed to cell size. Studies have shown that mechanical stress can alter CTC deformability and potentially explain such observed tendency. It is possible that our system does not enrich for CTCs smaller than 10µm like CTC from SCLC that might pass through the critical 10µm gap. The only alternative in this case would be epitope- based positive selection and/ or negative selection. Nonetheless, epitope dependent cell separation might fail to detect target of interest when presented with the task of capturing cells which have low or no expression of the target antigen [216].

1.4.10. Clinical significance of PD-L1 expression on CTCs

We reported in our study 77.8% of CTCs positive for PD-L1 expression. Several studies investigated the role of PD-L1$^+$ CTCs as a predictive biomarker for response to IO treatment. Though substantial differences between studies in terms of the cohort's choice and the clinical setting, most studies corroborated the clinical association between PD-L1 expression on CTCs and patients' response to IO and clinical outcomes [197, 202, 203, 217-221]. But the low numbers of participants in most of these studies make final conclusions regarding the prognostic value of PD-L1 expression difficult to achieve. PD-L1 expression on CTCs varied significantly between studies ranging between 8% and 95% [104, 189, 195-204]. With 77.8%, PD-L1$^+$ CTCs detected with the Parsortix^{TM} system is in line with previously mentioned studies even though caution I warranted when interpreting data considering that studies above used different antibodies targeted against PD-L1 and different enrichment methods for CTC isolation. If there are several studies upholding predictive utility of PD-L1, some others are reluctant to such assumptions owing to the lack of validity of PD-L1 TPS/ or CTC. A finding confirmed in Kulasinghe' study where authors demonstrated that neither CTC presence nor PD-L1 positive status were associated to PFS [198]. The presence of PD-L1 positive CTC in Guibert' study had no statistically significant association with advanced NSCLC patients' clinical outcome [197]. Nonetheless, a high proportion of patients with PD-L1$^+$ CTCs was associated to poor survival and all progressed patients had detectable PD-L1$^+$ CTCs [197]. Nicolazzo et al showed that PD-L1$^-$ CTC experienced clinical benefit from Nivolumab while NSCLC patients with PD-L1$^+$ CTCs had progressive disease [189]. Janning et al showed an upregulation of PD-L1$^+$ CTCs with disease progression with no obvious and statistically significant association to clinical outcome. The same holds true for downregulation of PD-L1$^+$ CTCs in responding patients [104]. The increase in PD-L1$^+$ CTCs is consistent however with previous reports where such an increase was observed in response to chemo-radiotherapy treatment and associated with poor prognosis, upholding the capacity of CTCs to monitor dynamic changes in PD-L1 expression [218]. Our data align with previous reports with regards to CTC count and PD-L1 assessment on CTCs. We analyzed our samples prospectively and did not focus on IO treated patients. As such, association to clinical endpoints are unfortunately not available as it would have been clinically interesting to assess the prognostic value of PD-L1 CTCs.

1.4.11. Concordance plasma vs. tissue

Using 1% cut-off, we showed 66.7% of concordance in PDL1^{+} expression between tissue and CTCs, with three patients showing PD-L1^{+} CTCs and yet, a negative PD-L1 expression in histology specimens. With a higher cut-off of 50% of PD-L1 TPS, the concordance rate dropped to 51.9%. With then not surprisingly 70.6% of patients having positive CTCs but negative tissue results. Similarly, PD-L1 expression on cytological imprints yielded a 62.9% and 51.9% concordance with CTC based PD-L1 assessment with the 1 % and 50% cut-off of PD-L1 TPS, respectively. The lack of a higher concordance of CTCs with paired tumor tissue have been corroborated in almost all studies discussed above [104, 197, 202]. Our results are satisfactory with regards to PD-L1 status concordance with histological specimen. This raises, however, a pivotal question inherent to the extent to which intra-tumor heterogeneity plays a role into PD-L1 assessment in tumoral tissue as in histological specimen as in blood- based liquid biopsy approach. Janning et al for example reported a considerable heterogeneity in PD-L1 status on CTCs in NSCLC patients, which might explain the lack of concordance of PD-L1 positivity on CTCs with tumor tissue. Dhar et al were the only ones claiming that positive PD-L1 expression on CTC and their matched histological specimen could be used as predictive biomarker for IO response in advanced NSCLC patients [222].

The decrease in concordance rates observed between tissue versus CTC and cytological imprints versus CTC while switching to a higher PD-L1 TPS seems coherent. A higher cut-off of PD-L1 TPS requires higher number of PD-L1^{+} cells on the analyzed tissue. Owing to the limitations inherent to the small and insufficient amounts of tissue biopsy material, it seems impractical to collect more tissue, especially at late stages of the disease. Interestingly, the addition of PD-L1 expression in our study has markedly improved the prediction capacity of cytology imprints for PD-L1 positivity and further demonstrate the complementary nature of CTCs for PD-L1 assessment. CTCs, in this context, are serious candidate capable of monitoring the dynamic of PD-L1 and further improve the predictive accuracy of PD-L1 to selection patients for IO.

1.4.12. Study limitations and future perspectives

The establishment of IO as a primary treatment option for NSCLC patients owing to its long-term benefits in terms of survival, is primordial to accurately select patients that might benefit from IO treatment. FDA and EMA approved drugs targeting PD1/PD-L1 pathways. This makes PD-L1 assessment elemental considering that selecting patients who might benefit from IO is tributary on PD-L1 expression. Our study first confirmed previous finding with regards to the feasibility of PD-L1 expression on CTC from NSCLC patients. We also supported the claim that tissue biopsy and the cytological smears at a single tumor site or time point are likely to represent a limited overall PD-L1 status. Adding CTC PD-L1 analysis in our study significantly improved the predictive capacity of cytological imprints for assessing PD-L1 positivity. This further confirms the capacity of PD-L1 CTCs analysis to complement standard methods and represent an alternative to unlock the obvious spatial and temporal heterogeneity of PD-L1 inherent to tissue specimen and cytological imprints. However,

there are few limitations to our study. The small size of our cohort and the low positivity rate could bias our data interpretation and underestimate PD-L1 expression. 80% of the specimens in our study were collected via fiberoptic bronchoscopy. The use of small biopsies underrates PD-L1 TPS in PD-L1^{+} tumors compared to surgically resected specimen [223, 224]. CTC PD-L1 was analyzed qualitatively and we did not quantify the proportion of PD-L1^{+} cells. We chose a binary description instead: PD-L1^{+} versus PD-L1^{-}. CTC detection methods remain challenging with regards to the diversity of enrichment method used ranging from the label- dependent ones to label-independent. Consequently, different methods enrich for different CTC populations, affecting *de facto* PD-L1 assessment on CTCs. Future studies should focus on determining a unifying conventional and efficient SOPs for CTC enrichment and detection and their cognate associated biomarkers in the different clinical setting. Interventional studies investigating these SOPs are of primary importance for better implementation of CTCs in clinical practice.

Part 3. Future perspectives: The relevant use of blood based liquid biopsy surrogates (CTCs and cfDNA) to unlock Intra-Tumor Heterogeneity (ITH)

Bert Vogelstein a pioneer of cancer metastasis research has said “The revolution of cancer research can be summed up in a single sentence, cancer is, in essence, a genetic disease” [225]. The DNA replication process of eukaryotic cells is not fully correct and errors can be introduced, even in the absence of external or internal mutagens. With the presence of mutagens such as tobacco, reactive oxygen species, or UV exposure as well as deficiency in DNA repair mechanisms associated to the outgrowth capacity inherent to tumor cells, those random errors are exacerbated. They accumulate and can hit genes with clinical relevance to tumorigenesis, leading de facto to driver oncogene activation or inactivation of tumor suppressor genes. The increasing tendency to DNA mutation is what characterize genomic instability and represent the engine fueling cancer progression and genetic, epigenetic and microenvironmental intra-tumor heterogeneity (ITH). The extensive ITH unraveled through large scale sequencing analysis have been shown to contribute to treatment failure and acquired resistance. Gerlinger et al showed that up to 70% of all the somatic mutations found with multi-regional tissue exome sequencing were heterogenous and thus indetectable in every sequenced region [226]. Authors concluded that a single tumor-biopsy reveals only a minority of genetic aberrations within the entire bulk of the tumor mutational burden. Conflictual clinical outcomes were drawn with regards to regional detected mutations. This is further supported at the RNA-expression level, where the expression profile was associated to good or poor prognosis depending on the sequenced region of the same tumor. To help addressing the issue of ITH, liquid biopsy-based biomarkers are a promising alternative capable of representing a larger picture of the tumor’s mutational burden. Longitudinal surveillance of the disease with accessible serial blood sampling allows to monitor ITH. Murtaza et al showed that ctDNA provides dynamic sampling of somatic mutations which can also be a better reflection of ITH than one single tissue biopsy [115]. The current knowledge of cancer evolution suggests the growing cancer continuously shed CTC in the bloodstream with the striking ability to colonize second sites and form metastatic foci. But multiple routes of

metastasis have been suggested that are not only essential to CTCs detaching form the primary tumor. The diversity of the mechanisms responsible for CTC entry in the bloodstream, invasion, survival, extravasation and dissemination uphold the use of CTCs to unlock ITH [21].

In conclusion, blood surrogates should complement conventional methods and the promise of resolving the dilemma of ITH require further functional studies(e.g., [227, 228]) to help understand mechanisms driving the clonal or subclonal nature of driver events in NSCLC patients, and these studies are still awaited and warranted.

2. Abkürzungsverzeichnis

AD: Adenocarcinomas
AKT1: AKT Serine/Threonine Kinase 1
ALK: Anaplastic Lymphoma Receptor Tyrosine Kinase
bHLH: basic helix-loop-helix
BRAF: B-Raf Proto-Oncogene, Serine/Threonine Kinase
CA-125: Cancer antigene 125
CDKN2A: Cyclin Dependent Kinase Inhibitor 2A
CEA: Carcinoembryonic antigen
cfDNA: circulating cell-free DNA
CSF: Cerebrospinal fluid
CT- scan: computerized tomography- scan
CTC: Circulating tumor cells
ctDNA: circulating tumor cell-free DNA
CTLA-4: Cytotoxic T-Lymphocyte Associated Protein
ddPCR: Digital droplet polymerase chain reaction
DNA: Deoxyribonucleic acid
DTC: Disseminated tumor cells
ECM: Extracellular matrix
EGF: Epidermal Growth Factor
EGFR: Epidermal growth factor receptors
EMA: European medicines agency
EMT: Epithelial to mesenchymal transition
EpCAM: Epithelial cell adhesion molecule
EU: European Union
FDA: The US. Food and drug administration
FFPE: formalin-fixed paraffin-embedded
FGF1: Fibroblast Growth Factor 1
HGF: Hepatocyte growth facto
HPV: Human papillomavirus
ICI: Immune check-point inhibitors
IF: Immunofluorescence
IHC: Immunohistochemistry
IMI: Innovative medicines initiative
ITH: Intra-tumor heterogeneity
KIT: KIT Proto-Oncogene, Receptor Tyrosine Kinase
KRAS: V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog
LCC: large cell carcinoma
LCNEC: large cell neuroendocrine carcinoma
MAP2K1: Mitogen-Activated Protein Kinase Kinase 1
MAPK: Mitogen-Activated Protein Kinase
MET: Mesenchymal to epiothelial transition
MET: Tyrosine-Protein Kinase Met
miRNA: micro- ribonucleic acid
MRD: Minimal residual disease
MRI: Magenetic Resonance Imaging

mtDNA: Mitochondrial DNA
mTOR: Mechanistic Target Of Rapamycin Kinase
NF-κB: Nuclear Factor Kappa B Subunit 1
NF1: Neurofibromin 1
NGS: Next generation sequencing
NSCLC: Non-small cell lung cancer
NTRK neurotrophic tyrosine receptor kinase
OS: Overall survival
PD-L1: Programmed cell death ligand 1
PD1: Programmed cell death protein 1
PET Scan: Positron emission tomography
PFS: Progression free-survival
PIK3CA Phosphatidylinositol 4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha Isoform
PMN: Pre-metastatic niche
PS: Performance status
RET: Proto-Oncogene Tyrosine-Protein Kinase Receptor Ret
RNA Ribonucleic acid
ROS1: V-Ros Avian UR2 Sarcoma Virus Oncogene Homolog 1
SART: Stereotactic ablative radiotherapy
SCC: squamous cell carcinomas
SCLC : small cell lung cancer
SNP: Single nucleotide polymorphism
SOPs: standard operating procedures
SOX2: SRY (Sex Determining Region Y)-Box 2
TCF3: Transcription Factor 3
TGF-β: Transforming Growth Factor Beta-1 Proprotein
TKI: Tyrosine kinase inhibitor
TMB: Tumor mutational burden
TNM: Tumor, Nodes, Metastases
TP53: Tumor Protein P53
TPS: Tumor proportion score
TTF1: thyroid transcription factor 1
TWIST: Twist Family BHLH Transcription Factor 1
UV: Ultra-violet
WES: whole exome sequencing
WHO: World health organization
YAP1/TAZ: Yes1 Associated Transcriptional Regulator
ZEB: Zinc Finger E-Box Binding

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4. Publikation 1

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Article

Discovery of Targetable Genetic Alterations in NSCLC Patients with Different Metastatic Patterns Using a MassARRAY-Based Circulating Tumor DNA Assay

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Abstract: Circulating tumor DNA (ctDNA) has shown great promise as a minimally invasive liquid biopsy for personalized cancer diagnostics especially among metastatic patients. Here, we used a novel sensitive assay to detect clinically relevant mutations in ctDNA in blood plasma from metastatic non-small cell lung cancer (NSCLC) patients, including patients with a limited oligo–brain metastatic disease. We analyzed 66 plasma samples from 56 metastatic NSCLC patients for 74 hotspot mutations in five genes commonly mutated in NSCLC using a novel MassARRAY-based lung cancer panel with a turnaround time of only 3 days. Mutations in plasma DNA could be detected in 28 out of 56 patients (50.0%), with a variant allele frequency (VAF) ranging between 0.1% and 5.0%. Mutations were detected in 50.0% of patients with oligo–brain metastatic disease, although the median VAF was lower (0.4%) compared to multi-brain metastatic patients (0.9%) and patients with extra-cranial metastatic progression (1.2%). We observed an overall concordance of 86.4% (n = 38/44) for *EGFR* status between plasma and tissue. The MassARRAY technology can detect clinically relevant mutations in plasma DNA from metastatic NSCLC patients including patients with limited, oligo–brain metastatic disease.

Keywords: lung cancer; ctDNA; mutations; liquid biopsy; brain metastases

1. Introduction

Non-small cell lung cancers (NSCLC), the most common cause of global cancer-related mortality, are diagnosed in around 40% of patients at late stages in which the primary tumor is inoperable (IIIB and IV) [1]. Knowledge about pathogenic driver mutations is crucial for therapeutic decision-making, since treatment with drugs targeting specific driver mutations improves outcome and quality of life for most patients [2]. However, in many patients with recurrent or progressive disease this information is not available because these patients frequently do not undergo re-biopsies, in particular if the brain is involved as the distant site of metastases. This is mainly due to risk complications associated with tissue biopsy especially at late stages of disease. The occurrence of brain metastases in NSCLC is an increasing clinical problem due to augmented extra-cranial disease control by systemic therapies. Around 40% of advanced stage NSCLC patients will be diagnosed with brain metastases, and the dismal prognosis underlines the urgent need to obtain brain-specific information on therapy targets and resistance mechanisms [3]. Currently, genomic information is most frequently obtained from analysis of the primary tumor or metastases at easily accessible sites. However, brain metastases show a divergent mutation profile from the primary tumor or other metastases [4–6]. Thus, future developments in personalized therapy of NSCLC patients will depend on new approaches to obtain tumor DNA from brain metastases for genomic analysis.

In recent years, liquid biopsy has gained importance as novel minimally-invasive source of tumor material for molecular diagnostics that can be complementary to invasive tissues biopsies and to date, cell-free DNA (cfDNA) is perhaps one of the most promising surrogate blood based biomarker candidates for tumor tissue [7,8]. cfDNA refers to extracellular DNA molecules found in body fluids and thought to be released from cells through apoptosis, necrosis and potentially through an active secretion [9,10]. The tumor-derived fraction of cfDNA is commonly referred to as "circulating tumor DNA (ctDNA)". Analytes in blood such as ctDNA are considered to represent the whole tumor burden at various sites, although different metastases located in different organs might have different shedding rates [11,12]. In fact, due to its noninvasive character, ctDNA might circumvent not only the problem of tissue biopsy but also tumor' spatial heterogeneity. The dilemma of intra-tumor heterogeneity represents a true limit for personalized medicine approaches because of the reliability on a single tumor tissue biopsy to profile the mutational landscape inherent to each tumor. Gerliner et al. suggested that multiregional biopsy analysis might be required in order to predict the therapeutic outcome and draw a more complete picture of the tumor burden [13]. It is proposed that ctDNA provides a dynamic sampling of somatic alterations capable of representing a larger clonal hierarchy and thus track different treatment responses even at metastatic sites [14,15].

The power of ctDNA analyses in detection of acquired resistance mutations after treatment with 1st and 2nd generation *EGFR* tyrosine kinase inhibitors (TKIs) in NSCLC has been demonstrated in several studies [16]. The detection of ctDNA *EGFR* p.T790M is recommended in current guidelines after progression with *EGFR* TKIs in order to guide treatment initiation with the 3rd generation *EGFR* TKI osimertinib [17].

Although cancer patients generally have higher cfDNA levels compared to healthy individuals, the frequency of ctDNA varies extensively depending on tumor type and disease stage, described to range between 0.01% to 90% [18–22]. In NSCLC patients, the ctDNA levels are generally lower compared to other solid tumors [23]. Several different analytical methods with varying sensitivity and mutation coverage exist today. The sensitivity, specificity, and applicability of the numerous different published ctDNA assays have been reviewed extensively before [7,24]. Thus, highly sensitive and specific ctDNA assays are needed to accurately detect clinically relevant mutations in plasma DNA from brain-metastatic NSCLC patients. For implementation into clinical practice outside of academic institutions, these technologies need to be cost efficient and provide reliable results on a limited panel of druggable mutations within a short turnaround time. Single alterations such as the *EGFR* p.T790M mutation in plasma, can be carried out at very high sensitivity (<0.01% VAF) and cost efficacy by using Digital Droplet PCR (ddPCR) based methods [25–27]. However, these methods are restricted to the

analysis of a few single mutations, while multiplexing commonly requires NGS-based methods that show variable assay sensitivity and specificity [7,12]. Even though NGS based methods proved their relevance in the clinic and universal genomic sequencing is supported by the clinical community, its implementation in the clinical routine has not been achieved in many countries, mainly due to the high cost of such assays, technical expertise and bioinformatics infrastructure, making it less accessible for a plethora of medical laboratories.

In this retrospective study, we provide a first proof of principle for a validated ctDNA assay that can detect clinically relevant mutations based on a mass-spectrometry approach in advanced NSCLC patients, including patients with lower tumor burden such as oligo–brain metastatic disease. The MassARRAY system detects in a single multiplex assay 74 hot-spot mutations in five relevant and commonly mutated genes in NSCLC patients with high sensitivity. The present encouraging results qualify this NGS-independent technique as a cost effective, fast, sensitive ctDNA analysis and easily accessible for the medical laboratories.

2. Materials and Methods

2.1. Patients and Blood Samples

Blood was drawn from 56 patients with histologically confirmed metastatic (stage IV) NSCLC with a median age of 61 years for both genders. Smoking behavior was not recorded for this cohort. All patients of this retrospective cohort were treated at the University Medical Center of Hamburg, Germany (UKE) (Table 1). All subjects formally consented to the study. Only samples with at least 1.5 mL plasma available and no visual sign of hemolysis were used in this study. The study was approved by the ethics review board of the University of Hamburg (Nr.PV-5392, 06/12/2016, Ärztekammer Hamburg).

Table 1. Clinical characteristics of non-small cell lung cancer (NSCLC) patients' cohort.

Characteristics		Number	Percentage
Gender	Male	26	46.4%
	Female	30	53.6%
Histology	Adeno ca.	49	87.5%
	Squamous cell ca.	5	8.9%
	other	2	3.6%
EGFR in tissue *	Mutant	9	20.5%
	Wild type	35	79.5%
Disease stage	First diagnosis	39	69.6%
	Progressive disease	16	28.6%
	Complete response	1	1.8%
Metastases **	Brain metastases	37	66.1%
	Other metastases than brain	16	28.6%
	Unknown	3	5.5%
Brain- Metastases ***	Oligo-brain metastases	20	54.0%
	Multi-brain metastases	16	43.2%
	Unknown	1	2.7%

* *EGFR* tissue status was not assessed in 12 patients. ** For three metastatic patients, the metastatic spread within the brain was not documented at the time of blood draw. *** From one brain metastatic patient, the metastatic spread outside the brain was not documented.

In three patients, multiple blood draws were analyzed: From one patient (P01), 9 follow-up samples were available and in two other patients (P09 and P24), two blood samples were available (Figure S1 and Table S3). Figure S1 shows a flow chart on what type of samples and analyses were performed in this study (Figure S1). Thirty-seven patients (66.1%) had brain metastases, 20 of which (35.7%) had metastases only in the brain (oligo–brain metastatic disease) and 16 patients had additional extra central nervous system metastases (multi-brain metastases). For one patient with brain metastases, information of other metastases outside the brain were not recorded (Table 1). We defined oligo-metastatic disease as a purely localized metastases in a single organ, i.e., brain [28]. Therefore, oligo–brain metastatic disease refers to NSCLC patients with an isolated central nervous system (CNS) progression while no extra-CNS disease is recorded.

In this retrospective cohort, *EGFR* mutation status from tissue analyses was available for 44 patients. As more than half of the patients were collected before 2015 (Table S3), the standard testing was only including *EGFR* and *ALK* for adeno carcinomas. Due to the lack of clinical relevance, *EGFR* mutation status was not routinely assessed in squamous cell cancer patients during the recruitment period of our patients. Nine patients had sensitizing activating mutation in *EGFR* detected in tissue samples (Table S3). In 35 samples, the tissue biopsy was negative for *EGFR* mutations.

2.2. Plasma Isolation and cfDNA Extraction

Plasma was extracted from 7.5 mL of blood drawn in EDTA tubes using a double centrifugation protocol (10 min at 300× *g*, followed by 10 min at 1800× *g*). The cfDNA was extracted using the Circulating Nucleic Acid kit (Qiagen) according to the manufacturer's protocol. The ctDNA quantity was measured by Qubit Fluorometer (Thermo Fisher) and the quality was assessed by TapeStation (Agilent). 2.3. Mutations analysis using the MassARRAY system

The UltraSEEK™ Lung Panel on the MassARRAY® System (Agena Bioscience, San Diego, CA, USA) analyzes 74 different hot-spot mutations in *EGFR*, *KRAS*, *BRAF*, *ERBB2* and *PIK3CA* (Table S1). This panel was recently validated by using commercial standards in a ring trial comparing different ddPCR, MassARRAY and NGS based assays [29].

The assay consists of a single multiplex PCR reaction targeting specific regions of the five genes, followed by a single base extension relative to the specific mutation using chain terminators. Specific terminating nucleotides are then incorporated only when the mutant allele is present allowing for further enrichment of the mutant signal. The captured and enriched products are then identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as previously described [30]. Data analysis was performed using Typer software version 4.0.26.74 (Agena Bioscience). Normalized intensity was calculated for the signal intensity of the mutant allele, which had been normalized against the capture control peaks found in the spectrum. A value of one means the peak intensity of the observed mutant allele is equal to the peak intensity of the average of the 5 capture control peaks found in the spectrum. The capture control peaks are biotin-labeled, nonreactive oligos, which are added to the extension reaction and used as an internal control for the streptavidin-bead capture and elution of the mutant extension product steps. Mutant allele calls were returned by an automated software report specific for the UltraSEEK Lung Panel and signal-to-noise ratio ≥6 and a z-score ≥7 were considered significant. For allele calling, the reporter algorithm takes an instrument specific baseline for each mutation assay into account. Herein, the assay specific noise is assessed by analyzing a cohort of wild-type samples and mutant call significance was controlled by analyzing commercial mutations controls as a titration of mutant allele frequencies down to the limit of detection (LoD) of 0.1%.

3. Results

3.1. Overall Detection of Mutations in Cell Free DNA Using UltraSEEK™ Lung Panel in Advanced NSCLC Patients

Overall, our results showed that mutations could be detected in 28/56 patients (50.0%) using the UltraSEEK™ Lung Panel consisting of 74 different hotspot mutations in five NSCLC associated genes. *EGFR* mutations were detected in 25.0% (14/56), *KRAS* in 21.4% (12/56), *ERBB2* in 5.4% (3/56), *PIK3CA* in 5.4% (3/56), and *BRAF* in 5.4% (3/56) of the patients (Figures 1A and 2). The most common *EGFR* mutations were exon 19 deletion (*EGFR* p.E746_A750Del found in five patients) followed by *EGFR* p.L858R mutations of exon 21 (four patients) (Figure 2 and Table S2). In two *EGFR* positive patients, a resistance causing p.T790M mutation was also detected together with the activating *EGFR* mutation. In patient P04, a p.T790M mutation was detected in addition to exon 19 del (VAF *EGFR* p.E746-A750del 5.0%, VAF *EGFR* p.T790M 0.2%) (Figure 1B). The p.T790M was not observed in the tissue sample taken one month prior to the time of blood draw. However, the patient showed an extra cranial progression from erlotinib one month after the blood draw. From the second patient with a p.T790M mutation (P09), two blood draws were taken. In the first blood draw taken before the beginning of any systemic treatment, an *EGFR* p.E746_A750del was found in both tumor tissue and in the MassARRAY analysis. In a second blood draw 24 months after treatment initiation (patient received both erlotinib and osimertinib), we detected both the *EGFR* p.E746_A750del but also the p.T790M mutation. No tissue biopsy was taken but the p.T790M status was verified in plasma using the Cobas assay (Roche Diagnostics).

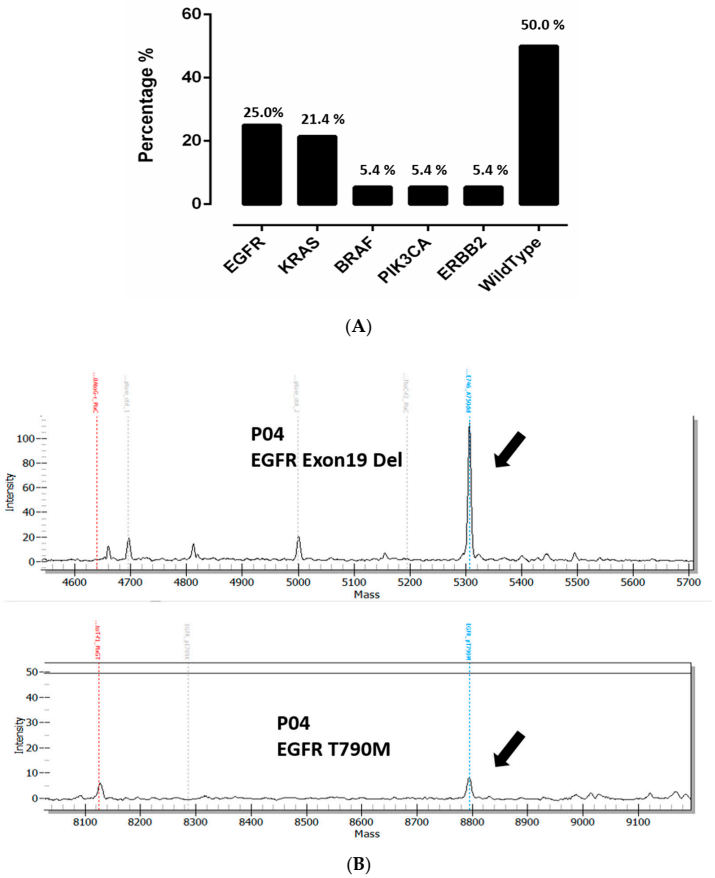

Figure 1. (**A**) Distribution of mutations detected in NSCLC ($n = 56$) patients cfDNA. (**B**) Two MassARRAY plots from patient P04 showing an *EGFR* exon19 deletion and a p.T790M resistance causing mutation.

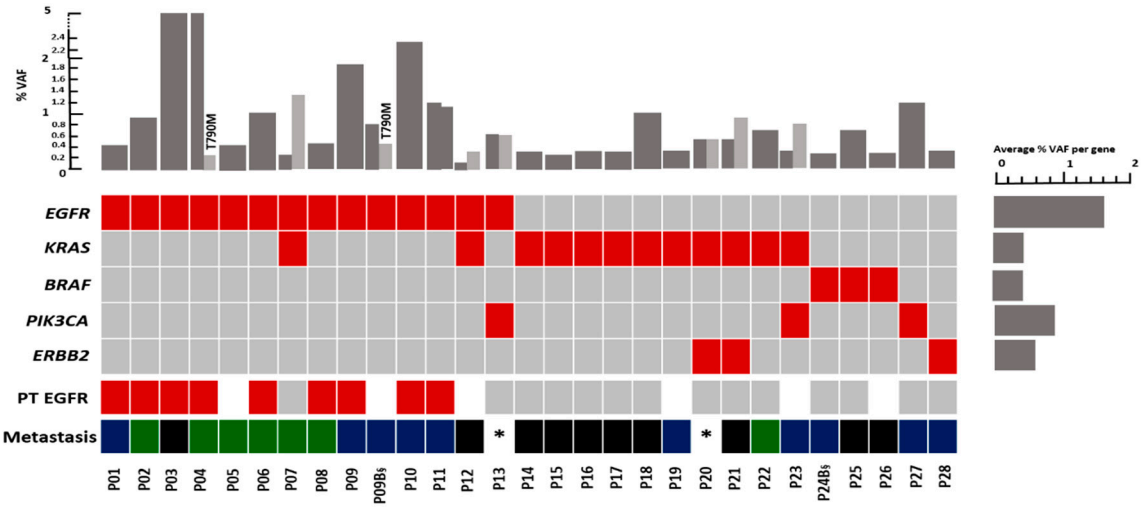

Figure 2. Overview of mutations detected (lower panel) and their variant allele frequency (VAF) (upper panel) in advanced NSCLC patients using the MassARRAY system. When double mutants are detected in one patient, the order of VAF bars are shown in the order of genes in the plot. Mutation detected; No mutation detected; Not determined; Oligo-brain metastases; Multi-brain metastases; Other metastases; VAF: Variant allele frequency, * Brain metastases in these patients was not documented. §: Follow-up sample, only the first mutation was considered.

The most commonly found *KRAS* mutations were *KRAS* p.G12A/p.G12V (found in five patients) and *KRAS* p.G12C (in four patients). Interestingly in patient P24, the first blood draw at first diagnosis did not show any mutation. Thirteen months later, however, during the second blood draw when the patient had a progressive disease, cfDNA analysis displayed a *BRAF* p.G469A/p.G469V mutation (P24B). Three patients had *PIK3CA* mutations. From these, *PIK3CA* p.H1047R was observed in two patients and *PIK3CA* p.E545K in one patient. *ERBB2* p.A775_G776insYVMA was reported in two patients while one patient displayed *ERBB2* p.G776 > VC mutation (Figure 2 and Table S2).

In eight patients, more than one driver mutation could be found including two patients (P04 and P09B) having *EGFR* activating mutations and the common gatekeeper resistance mutation p.T790M. Figure 2 shows that mutations in *EGFR* and *BRAF* as well as *EGFR* and *ERBB2* were mutually exclusive, which has also been described in primary NSCLC tumors [31]. Two patients (P07 and P12) had detectable *EGFR* and *KRAS* mutations, while *KRAS* and *ERBB2* mutations were identified in two other patients (P20 and P21). One patient (P13) had activating *EGFR* mutation and *PIK3CA* mutation and another patient (P23) displayed a *KRAS* and *PIK3CA* mutation.

Mutations were detected in plasma NSCLC patients with a VAF ranging from 0.1 to 5.0% (Figure 2 and Table S2). The median VAF in oligo–brain cases was 0.4% (range 0.1–5.0%), while the median VAF in patients with multi-brain metastases was 0.9% (range 0.2–5.0%). The highest median VAF was observed in patients with other metastases 1.2% (range 0.3–2.3%) (Table 2).

Table 2. Prevalence of detected mutations in circulating tumor DNA (ctDNA) samples.

	BRAF *n* (%)	EGFR *n* (%)	KRAS *n* (%)	ERBB2 *n* (%)	PIK3CA *n* (%)	Number of Pts with Mutation (%)	Median VAF of All Mutations
Oligo-brain metastases (*n* = 20) *	2 (10.0%)	2 (10.0%)	7 (35.0%)	1 (5.0%)	0	10 (50.0%)	0.4
Multi-brain metastases (*n* = 16) **	0	6 (37.5%)	2 (12.5%)	0	0	7 (43.8%)	0.9
Other metastases (*n* = 16) ***	0	4 (25.0%)	2 (12.5%)	1 (6.3%)	2 (12.5%)	8 (50.0%)	1.2

* Patient P12 had both an EGFR and a KRAS mutation, patient P21 had a KRAS and an ERBB2 mutation. ** Patient P07 had EGFR and KRAS mutations. *** Patient P23 had KRAS and PIK3CA mutations.

3.2. Distribution of Mutations in the Plasma of NSCLC Patients with Different Metastatic Patterns

Thirty-seven patients had brain metastases, 16 of which had additional extra-cranial metastases (multi-brain metastases) and 20 patients had the brain as the only site of metastases (oligo–brain metastases). From one patient, the metastatic spread outside the brain was not documented.

In 45.9% (17/37) of the brain metastatic patients (oligo–brain metastases and multi-brain metastases), a mutation in either *EGFR*, *KRAS*, *BRAF* or *ERBB2* ctDNA could be detected. 50.0% of the patients with oligo–brain metastatic disease (n = 10) had mutations detected in their blood with a median VAF of 0.4% (Table 2 and Figure 2). *KRAS* was the prevailing mutation present in seven out of the 20 patients with oligo–brain metastases (35.0%). *EGFR* and *BRAF* mutations were detected each in two patients with oligo–brain metastases (10.0%). Among the 16 patients with metastases sites other than in the brain, eight patients (50.0%) had mutations in their blood sample with a median VAF of 1.2%. Four of these patients had detectable *EGFR* mutations (25.0%). Two had *KRAS* mutations (12.5%) and two patients displayed a *PIK3CA* mutation (12.5%) and one *ERBB2* (6.3%). From the 16 multi-brain metastases patients, seven patients (43.8%) had detectable mutations in only *EGFR* and *KRAS* (Table 2). The highest median VAF was observed in the latter setting of brain metastases patients with 1.2%. The total cfDNA amount did not differ between the different groups.

3.3. Comparison of EGFR Mutation Status in Tumor Tissue and Plasma

Information about *EGFR* mutation status of the primary tumors was available for 78.6% (n = 44/56) of the patients. No other mutations included in the ctDNA analysis were assessed in tissue biopsy from this retrospective patient cohort. By comparing *EGFR* mutations status from plasma (MassARRAY) with matched tumor tissues, an overall concordance of *EGFR* mutational status of 86.4% (38/44) was observed. Six disconcordant cases were identified (P02, P04, P07, P08, P11, and P13 (Table S3)). In most of these cases, the differences in *EGFR* mutational status might have been influenced by the time and treatments between tissue biopsy and liquid biopsy analyses. As it was the case for patient P02, for instance. Here, the tissue biopsy analyses revealed an *EGFR* p.E746-A750del and a resistance causing p.T790M mutation. 5 weeks into treatment with osimertinib (an *EGFR*-TKI specific for p.T790M mutations), a liquid biopsy sample was collected which detected only the *EGFR* p.E746-A750del. The absence of the p.T790M clone in the liquid biopsy samples after 5 weeks into treatment may potentially indicate a response to p.T790M specific treatment with osimertinib. As explained before, a resistance causing p.T790M mutation was detected in the blood of patient P04 taken at extra cranial progression while it was not detected in tissue analysis a month before. In patient P07, the primary biopsy indicated a wild type for *EGFR*, while in the blood sample 5 months later, an *EGFR* mutation (p.L858R) with a low VAF of 0.2% was detected indicating possibly a subclonal origin of the *EGFR* mutation. In patient P13, tissue biopsy indicated a wild type *EGFR*. Two years later, the patient had a progressive disease and blood sample analysis detected a p.L858R *EGFR* and a *PIK3CA* p.H1047R mutation, both with VAF of 0.6%. The tumor tissue was not tested for *PIK3CA*. Unfortunately, confirmatory tissue biopsies for proof were missing for these patients. The concordances between variants found in tissue vs. liquid biopsy along with other clinical information are represented in Table S3.

3.4. Monitoring Patient's Disease by Tracking Mutations in Plasma ctDNA: A Case Report

We analyzed serial blood samples from an 80-year-old patient with a stage IV, multi-metastatic adenocarcinoma of the lung for mutations in ctDNA over a period of 22 months (Figure 3). At first diagnosis, the patient showed metastases at several thoraco–abdominal sites. Due to the high tumor burden, a systemic chemotherapy was started before results of the mutational analyses of tumor tissues were available. These analyses later revealed an *EGFR* p.E746_A750del mutation in the pleura even though in the primary lung tumor tissue analysis, no *EGFR* mutation was detected. Treatment was subsequently switched to afatinib, a 2nd generation *EGFR*-TKI. The first blood draw, performed two

weeks after beginning of afatinib treatment, also revealed the *EGFR* p.E746_A750del mutation with 0.4% VAF, concordant to the metastatic pleura cells. Two consecutive blood draws were carried out at months two and eight (M2 and M8), in which no *EGFR* mutation was detected. The decrease and absence of *EGFR* mutations in the blood coincided with a partial response that was detected in the first CT-scan 4 months after initiation of afatinib treatment (M4). The patient continued to have a stable disease based on CT-scans and clinical evaluation for a total of 21 months. During this time however, at month 10, the *EGFR* p.E746_A750del mutation reappeared in the blood sample with a lower VAF: 0.2%. An additional mutation was also detected at month 10: *BRAF* p.V600E with 0.3% VAF (which was not assessed in initial tissue analyses). However, 43 days later at M11, no mutation was detected in the blood. Figure 3 shows that while still having a stable disease based on CT-scans, the *EGFR* p.E746_A750del was detectable with a VAF of 0.5% at month 14 and then VAF decreased slightly to 0.4% at month 15, before increasing again to VAF of 1.7% at month 16. At this time, CT-scans still showed a stable disease and the patients did not experience any new symptoms. CT scans only showed a progressive disease for the first time 6 months after that substantial increase of *EGFR* VAF at month 16. However, at this time point (M22), the physical condition of the patient was deteriorating fast and the patient died shortly afterwards.

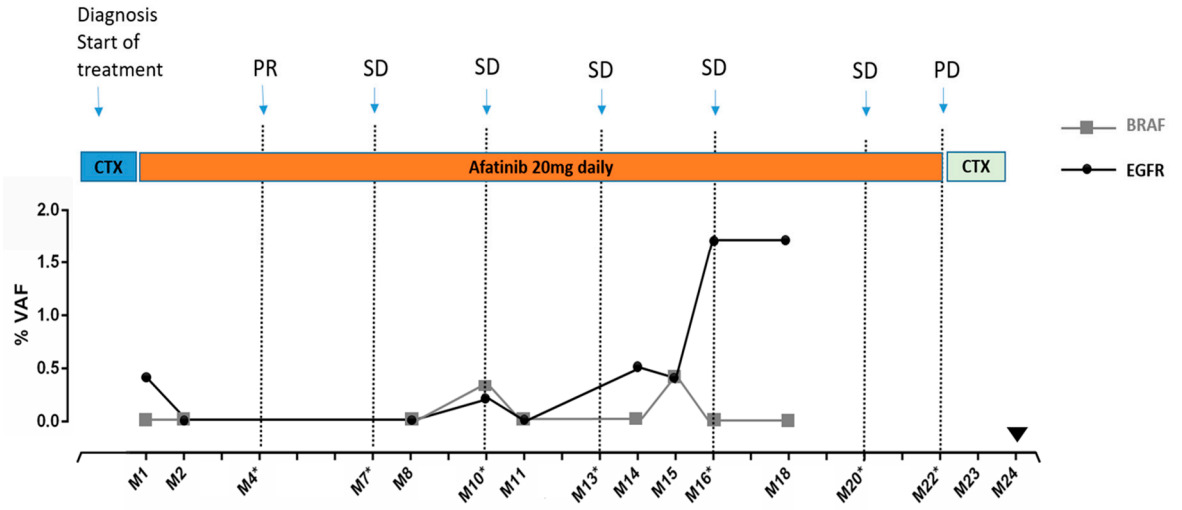

Figure 3. *EGFR* and *BRAF* mutations detection in ctDNA from one patient with known *EGFR* mutation followed at different time points. M: month; *: CT scan. PD: progressive disease. PR: partial remission. SD: stable disease. ▼: Death of the patient. CTX: chemotherapy. Afatinib: 2nd generation *EGFR* inhibitor.

4. Discussion

Exploring the mutational landscape of brain metastases in individual NSCLC patients is of primary importance for their clinical management. In these patients, biopsies of the brain metastases are seldom taken although a well-known dynamic mutational landscape in brain metastases has been described [5,6]. Here, we analyzed 74 different hotspot mutations in *EGFR*, *KRAS*, *BRAF*, *ERBB2*, and *PIK3CA* genes using a combination of a single multiplexed PCR reaction approach and mass-spectrophotometer based detection platform allowing a fast and cost-effective screening for a relevant number of mutations at high sensitivity. In fact, the sensitivity and the specificity of the assay have recently been validated in a ring trial using commercially available reference material [29], which showed that although sensitivity and specificity were comparable between the different used technologies, the MassARRAY was the assay with the lowest variability in intra-run variant calls. In our current study using patient material, mutations could be detected in 28 of the 56 (50.0%) analyzed metastatic NSCLC patient samples with the variant allele frequency ranging between 0.1% and 5.0%, including patients with oligo–brain metastatic disease. *EGFR* activating mutations in our study

were found in 25.0% (14/56) of patients, whilst 21.4% (12/56) of patients displayed a *KRAS* mutation. These data are in line with other studies in Caucasian populations, where, e.g., plasma cfDNA from 23.4% newly diagnosed metastatic NSCLC patients were mutated for *EGFR* and 22.6% had detectable *KRAS* [32,33].

Several reports have indicated that the blood–brain barrier (BBB) may inhibit the release of tumor cells or tumor cell products into the bloodstream [34–36]. Despite the sensitivity of the MassARRAY technology, we failed to detect ctDNA in approx. 50.0% of oligo–brain metastatic patients, and the VAF was lower compared to patients with multi-brain metastases. This finding is consistent with previous studies using NGS-based assays analyzing 37 genes [37], where 52.0% of oligo–brain metastatic patients had detectable mutations in ctDNA, and the median VAF was lower in patients with oligo–brain metastatic disease compared to patients with multi-brain metastases [37]. Similar obstacles have been also described for primary brain tumors, where somatic alterations in the plasma were also detected in only 50.0% of patients [36]. Besides blockage of ctDNA into the blood by the BBB, oligo–brain metastatic patients have a lower tumor burden than patients with multiple metastases, which might further lower their ctDNA concentrations in blood plasma.

Several studies have shown the superiority of cerebrospinal fluid (CSF) analyses compared to peripheral blood in primary brain tumors, supporting the barrier role of BBB [38–40]. A recent paper on NSCLC brain metastatic patients reported that *EGFR* mutations in CSF ctDNA were detected in 57.1% (12/21) of patients, while in only 23.8% (5/21) of paired plasma samples the same mutation could be found [41]. However, the detection rate for blood ctDNA was clearly below the rate found in our present study. In a second similar study, *EGFR* mutations in CSF ctDNA were detected in 63.6% (14/22) against 45.5% (10/22) of paired plasma samples [42]. Future studies using both CSF and plasma might be warranted. In general, obtaining CSF is more invasive than drawing blood, which might hamper the clinical use of CSF for sequential monitoring of tumor responses to therapy.

In three patients, follow-up samples were available and exemplified the power of longitudinal testing. In one patient, nine samples could be collected over a period of 22 months. Here the *EGFR* mutation was detected at initial diagnosis but then remained undetectable during a long stable disease phase. However, at month 16 when the CT-scan still did not show any progression, the *EGFR* mutation was again clearly detected in ctDNA. The CT-scan detected relapse 6 months later, two months before the patient's death. Our data thus support the use of ctDNA and sequential sampling to track upcoming resistance/relapse, and consequently upholds previous studies investigating the clinical relevance of blood based p.T790M mutation detection for NSCLC patients under *EGFR* TKI treatment [16,43]. Similarly, we recently showed, for the first time, using cfDNA plasma that a *MET* amplification can cause a resistance in *ALK* positive NSCLC patients receiving crizonitib [44]. Prospective clinical studies still need to show, whether tracking mutations on plasma DNA is as relevant as tumor biopsy analysis for making treatment decisions.

Some discrepancies between plasma and tissue DNA analyses are commonly observed in NSCLC and other tumor entities [25,37,45]. Here the MassARRAY based technology showed an overall concordance rate of 86.4% between the *EGFR* mutational status in tumor tissue vs. liquid biopsy. Besides technical issues, "private" plasma DNA mutations might support the overarching hypothesis of liquid biopsy: Blood functions as a pool of tumor cells and tumor cell-products released not only from the primary lesion but also from metastatic sites and therefore provides a more comprehensive information than a single tissue biopsy [11,12]. However, clearly larger studies including matched information of all mutations in tumor and plasma are needed to validate the ultimate sensitivity of this assay. Furthermore, although this study showed the feasibility of detecting point mutations at low VAF, it did not assess the clinically important *ALK*, *ROS*, *RET*, *NTRK*, translocations or *MET* exon 14 mutations. The mass-spectrometric approach is, however, adaptable to detect additional point mutations beyond the panel used in the present study, whereas translocations are in general harder to detect in plasma [46]. Despite these limitations, the benefits of this assay include its cost effectiveness and low turnaround time combined without the need for complex data analysis,

bioinformatics pipelines or large data storage capacity. Furthermore, the MassARRAY system is a flexible platform allowing a broad range of different clinical applications such as HPV detection [47], tumor profiling [48], pharmacogenetics (SNP) analyses [49], sample qualification [50], and SARS-CoV-2 testing [51], thus being well suitable and accessible for a various medical laboratories with clearly lower running costs compared to NGS based analyses.

5. Conclusions

Real-time monitoring of the changeable mutational landscape of metastatic patients by liquid biopsy approaches can be of great aid for their optimal clinical management. Here we show that the MassARRAY-based assay is a cost effective method that provided information on druggable mutations even in patients with limited, oligo–brain metastatic disease. We could furthermore show that by using longitudinal ctDNA monitoring we could track upcoming resistance and relapse before conventional imaging, showing that the MassARRAY-based assay is providing clinical meaningful results in an efficient and sensitive manner.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/11/2337/s1, Figure S1: Flowchart of the study cohort. Table S1: Targets genes in the UltraSEEK lung panel. Table S2: Detected mutations and the VAF in plasma cfDNA from NSCLC patients. Table S3: *EGFR* mutation status in matched plasma and tumor tissue of advanced NSCLC patients.

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5. Publikation 2

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SHORT REPORT

Comparative evaluation of *PD-L1* expression in cytology imprints, circulating tumour cells and tumour tissue in non-small cell lung cancer patients

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Keywords
CTCs; cytology imprints; NSCLC; PD-L1

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Alternative sources of tumour information need to be explored in patients with non-small cell lung cancer (NSCLC). Here, we compared programmed cell death ligand 1 (*PD-L1*) expression on cytology imprints and circulating tumour cells (CTCs) with PD-L1 tumour proportion score (TPS) from immunohistochemistry staining of tumour tissue from patients with NSCLC. We evaluated *PD-L1* expression using a PD-L1 antibody (28-8) in representative cytology imprints, and tissue samples from the same tumour. We report good agreement rates on PD-L1 positivity (TPS ≥ 1%) and high *PD-L1* expression (TPS ≥ 50%). Considering high *PD-L1* expression, cytology imprints showed a PPV of 64% and a NPV of 85%. CTCs were detected in 40% of the patients and 80% of them were PD-L1$^+$. Seven patients with *PD-L1* expression of < 1% in tissue samples or cytology imprints had PD-L1$^+$ CTCs. The addition of *PD-L1* expression in CTCs to cytology imprints markedly improved the prediction capacity for PD-L1 positivity. A combined analysis of cytological imprints and CTCs provides information on the tumoural PD-L1 status in NSCLC patients, which might be used when no tumor tissue is available.

1. Introduction

Lung cancer is the leading cause of cancer deaths worldwide [1]. Non-small cell lung cancer (NSCLC) contributes to more than 80% of lung cancer diagnosis. Most patients are diagnosed in advanced unresectable disease stages [2]. Immune checkpoint inhibitors (ICIs) targeting programmed cell death protein-1 (PD-1) and its receptor ligand-1 (PD-L1) have become a mainstay of treatment in NSCLC, particularly, in patients with advanced disease stages who lack druggable molecular alterations [3]. Tumoural

Abbreviations
AUC, area under the curve; CI, confidence interval; CTC, circulating tumour cell; EpCAM, epithelial cell adhesion molecule; ICIs, immune checkpoint inhibitors; ISET, isolation by size of epithelial tumour cells; NPV, negative predictive value; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; PPV, positive predictive value; ROC, receiver characteristic operator; ROSE, rapid on-site evaluation; TPS, tumour proportion score.

PD-L1 expression is still the most useful biomarker that predicts treatment response to ICIs [4]. Therefore, *PD-L1* expression is considered a key factor in selecting NCSLC patients who might benefit from a treatment with ICIs [5]. Based on their use in randomized clinical trials [6–8], the current standard of care is to quantify tumoural *PD-L1* expression in histology specimens. Nevertheless, there remains an unmet need to quantify tumoural *PD-L1* expression in a considerable proportion of NSCLC patients with only cytology samples available for diagnosis [9].

So far, several studies have investigated the feasibility of tumoural *PD-L1* expression in cytology samples [10]. Many were retrospective analyses or have compared the *PD-L1* expression between selected paired and matched histology–cytology samples [10]. However, data from prospective real-world studies elucidating the agreement on *PD-L1* expression of unpaired histology and cytology samples obtained from the same tumour lesion are still scarce. In addition, the relationship between the detection rate of circulating tumour cells (CTCs) and their *PD-L1* expression with the tumoural *PD-L1* expression remains uncertain. While active immune checkpoint receptors represent a potential mechanism of tumour immune evasion [11] and CTCs might be a surrogate marker of tumour immune evasion [12,13], an association between CTCs detection and tumoural *PD-L1* expression might exist.

In this prospective study, we sought to investigate the relationship between *PD-L1* expression on tumour tissue from standard immunohistochemistry with the *PD-L1* expression of site-matched cytology imprints of primary tumour lesions and the detection rate of CTCs and their *PD-L1* expression in patients with NSCLC. This investigation may provide the first evidence of whether alternative sources of tumour cells are informative for the assessment of PDL1 expression.

2. Materials and methods

2.1. Study design

In this prospective observational single-centre study, we recruited patients with suspected NSCLC who underwent routine procedures for lung cancer diagnosis at the LungenClinic Grosshansdorf. The analysis included subjects with NSCLC who were 18 ≥ year old. Exclusion criteria were diagnoses other than NSCLC or previous treatment with systemic chemotherapy or immunotherapy (Fig. 1). The written informed consent was obtained before enrolment. The study was approved by the ethics committee at the University of Luebeck (Az. 17-161) and conducted according to the declarations of Helsinki.

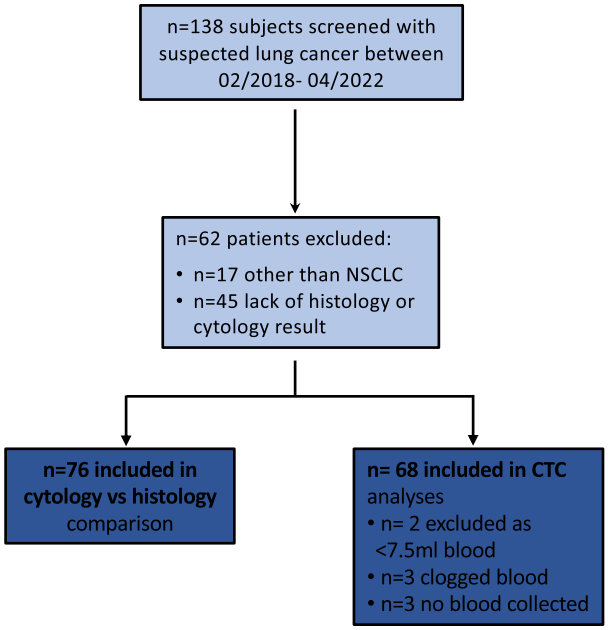

Fig. 1. Study cohort recruitment flowchart. A total of 138 patients were screened between 2018 and 2022 of which 76 patients were included in the histology and cytology analysis and 68 in the CTC-based liquid biopsy analysis.

Primary tumour specimens were collected via fibreoptic bronchoscopy; this comprises biopsies from endobronchial visible tumour, tumour mucosal infiltration or transbronchial biopsies. Further tumour specimens were obtained from surgical tumour tissue or via ultrasound-guided percutaneous tumour biopsy. Tumour specimens were smeared in a rapid on-site evaluation (ROSE), so matched cytology imprints were from the same tumour site. Different pathologists did further PD-L1 immunostaining on unpaired, yet site-matched, cytology and histology samples.

2.2. Tissue immunohistochemistry and immunocytochemistry

The immunohistochemical staining was performed on 4-µm-thick sections obtained from formalin-fixed paraffin-embedded tumour tissue. The quantification of *PD-L1* expression was done by estimating the number of PD-L1-positive tumour cells as a percentage of all tumour cells in both histology sections and cytology imprints. Tumour proportion score (TPS) was determined as the percentage of cells, which are PD-L1 positive to the total number of cells that included at least 100 viable tumour cells [14]. Staining of samples was done with the antibody clone Dako 28-8 according to standard operating procedures [15]. Although the 28-8 antibody clone was used in this

study as it is the standard antibody used for routine clinical diagnostics of NSCLC at the department of Pathology in Hamburg, a high degree of agreement between the 28-8 and 22C3 PD-L1 antibody clones for histological and cytological samples staining results have been described before [10,16]. A consistently lower positivity rate has been described for the SP142 antibody [17,18].

2.3. Circulating tumour cell-based liquid biopsy

We used the Parsortix® Technology (ANGLE plc, Guildford, UK) to detect CTC from 7.5 mL blood collected in Transfix tubes (CTC-TVT tubes, CYTOMARK, Buckingham, UK) as previously described [19,20]. The Parsortix technology has been extensively evaluated including several studies on NSCLC and also as part of multicentre ring trials (CANCER ID consortium) [21–23]. Cells enriched by the Parsortix® system were directly harvested into cytospin funnels, centrifuged onto a glass slide (RCF 190 ***g***), dried overnight and stored at −80 °C until further processing. For staining, slides were brought to room temperature and fixed with 0.5% PFA for 10 min. Cells were washed with 0.5 mL of 1× PBS three times for 3 min each. 10% AB- serum (BioRad, Rüdigheim, Germany) was applied for blocking (20 min). Unconjugated rabbit anti-human PD-L1 antibody, clone HL1041 (GTX635975, 1 : 100) was incubated over night at 4°, after which cells were washed with 0.5 mL of 1× PBS three times for 3 min. BD Horizon™ BV421 goat anti-Rabbit (BD Biosciences, San Jose, CA, USA, 1 : 200) was used as a secondary antibody and incubated for 45 min. Following the three additional washing steps, directly eFluor560 conjugated pan-keratin (AE1/AE3-eBioscience, San Diego, CA, USA, 1 : 200), PerCP-labelled CD45 (clone H130-Miltenyi Biotec, Bergisch Gladbach, Germany, 1 : 200) and DRAQ5™ for nuclear staining (BioLegend, San Diego, CA, USA, 1 : 5000) antibodies were incubated for 60 min. Subsequently, cytospins were covered with Prolong Gold Antifade Reagent (Thermo Fisher Scientific, Dreieich, Germany), sealed with a cover slip and examined by fluorescence microscopy. Keratin-positive, DRAQ5 (nuclear)-positive and CD45-negative cells with intact morphology were defined as tumour cells. H1975 was used as a positive control for *PD-L1* expression, while MFC7 was used as a negative control.

Rabbit anti-human PD-L1 antibody, clone 28.8 was optimized to detect cell surface PD-L1 in formalin-fixed paraffin-embedded human tumour tissue specimens [15] and its specificity was demonstrated by antigen competition and genetic deletion of PD-L1 in tumour cell lines. It is an approved companion test antibody. However, its use in the immune-fluorescence setting is poorly investigated as the antibody is mainly used for IHC approaches. Rabbit anti-human PD-L1 antibody, clone HL1041 (Genetex, Irvine, CA, USA) targeting PD-L1 cell membrane as well, was compared to other antibodies frequently used for immunofluorescence PD-L1 staining, including PD-L1 E1L3N clone and D8T4X clone (Cell Signalling Technology, San Diego, CA, USA, both). *PD-L1* expression was assessed using cell lines with known different *PD-L1* expression levels [12]. Although these clones worked alike, a slightly higher signal was observed for the newly released clone HL1041 and thus this antibody was used for the CTC assays.

2.4. Statistical analysis

We used a receiver operator characteristic (ROC) to evaluate the percent*PD-L1* expression from cytology imprints and the expression of *PD-L1* in CTCs as predictors for positive *PD-L1* expression (tumour cells expression score ≥ 1%) and *PD-L1* high expression (tumour cells expression score ≥ 50%) defined according to standard immunohistochemistry staining. We used Fisher exact test to identify differences in clinical variables between the study groups.

To examine the correlation between two continuous variables, we used Pearson's test. Statistical analyses were performed using R (version 4.2.1, R Foundation, Vienna, Austria). An alpha error of less than 5% was considered statistically significant.

3. Results

3.1. Study population

One hundred and thirty-eight subjects with suspected lung cancer were screened. We excluded subjects who had a diagnosis other than NSCLC (n = 17) or due to lacking histology specimens or evaluable cytology imprints (n = 45) as shown in the flow chart (Fig. 1). The final analysis included 76 patients, of whom the majority had non-squamous non-small lung cancer in locally advanced or metastasized disease stages, Table 1. Nearly 80% of the specimens were collected via fibreoptic bronchoscopy; this comprised biopsies from endobronchial visible tumour, tumour mucosal infiltration and transbronchial biopsies. Further tumour specimens were obtained from surgical tumour tissue or via ultrasound-guided percutaneous tumour biopsy (Table 1).

Table 1. Patients' characteristics. PD-L1 high expression, PD-L1 tumour cell expression score ≥ 50%; PD-L1 positivity, PD-L1 tumour cells expression score ≥ 1%; PD-L1, programmed cell death-ligand 1; UICC, Union for International Cancer Control.

Age, years	65.2 ± 9.0
Sex, male *n* (%)	50 (67)
Histological subtypes, *n* (%)	
Adenocarcinoma	40 (52)
Squamous cell carcinoma	29 (38)
Not otherwise specified	7 (10)
UICC staging, *n* (%)	
IA	7 (9.0)
IB	2 (2.6)
IIA	2 (2.6)
IIB	3 (3.9)
IIIA	9 (11.8)
IIIB	15 (19.7)
IIIC	2 (2.6)
IVA	18 (23.6)
IVB	18 (23.6)
Type of biopsy, *n* (%)	
Endoscopic	60 (79)
Surgical	11 (14.5)
Percutaneous needle biopsy	5 (6.5)
PD-L1 expression score, %	
Histology specimens	36.1 ± 36
Cytology smears	40.4 ± 33
PD-L1 positivity, *n* (%)	
Histology specimens	67 (88.1)
Cytology smears	67 (88.1)
PD-L1 high expression, *n* (%)	
Histology specimens	29 (38.1)
Cytology smears	36 (47.3)

3.2. *PD-L1* expression in the tumour tissue samples and in cytological imprints

We found a moderate correlation of percent *PD-L1* expression between cytology imprints and the matched histology specimens ($R = 0.58$, $P < 0.001$). Likewise, we observed a similar estimation for the number of patients with positive *PD-L1* expression (TPS ≥ 1%); yet, a higher estimation of patients with high *PD-L1* expression (TPS ≥ 50%) according to cytology imprints than in histology specimens (Table 1). Compared to percent *PD-L1* expression from standardized immunohistochemistry, the predictive capacity of cytology imprints of PD-L1 positivity (≥ 1%) indicated a positive predicted value (PPV) of 91%, a negative predicted value (NPV) of 33%, AUC = 78% [95% CI: 65–90%]. Considering high *PD-L1* expression (≥ 50%), cytology imprints showed a PPV of 64% and a NPV of 85%, AUC = 79% [95% CI: 67–91%].

The overall agreement on *PD-L1* positivity for the whole cohort was 84%. Positive agreement on PD-L1 positivity was seen in 61 cytology imprints out of 67 matched histology specimens; yielding a positive agreement rate of 91.0%. The negative agreement rate was 33.3% and was only seen in three cytology imprints out of nine matched histology specimens. The overall agreement on PD-L1 high expression was 82.8%. Positive agreement on PD-L1 high expression was 79%; seen in 23 cytology imprints out of 29 matched histology specimens and the negative agreement was 85%, seen in 40 cytology imprints out of 47 matched histology specimens.

Furthermore, the overall correlation of percent *PD-L1* expression between cytology imprints and histology specimens was higher in surgical specimens ($R = 0.67$, $P < 0.01$) than in non-surgical specimens ($R = 0.56$, $P < 0.01$). Moreover, specimens obtained from surgically resected tumour tissue yielded a greater cytology–histology agreement than non-surgical specimens, that is, those that were obtained via fibreoptic bronchoscopy or percutaneous tumour biopsy. The cytology–histology agreement on *PD-L1* high expression was 100% versus 75% in surgical versus non-surgical specimens respectively. Nevertheless, the cytology–histology agreement on PD-L1 positivity in surgical specimens (91%) was yet comparable to the agreement from non-surgical specimens (89%).

3.3. *PD-L1* expression in CTCs

Sixty-eight out of the 76 samples were assessed for *PD-L1* expression on CTCs. Eight samples were excluded due to clogged or not evaluable blood samples ($n = 3$), low blood volume (< 5 mL, $n = 2$) or missing liquid biopsy samples ($n = 3$). CTCs were detected in 27/68 samples (39.7%). The detection rate of CTCs was comparable between patients with: non-resectable versus resectable (OR 1.59 [95% CI 0.49–5.14], $P = 0.43$), non-squamous versus squamous (OR 0.64 [95% CI 0.20–1.94], $P = 0.45$), negative versus positive *PD-L1* expression (OR 0.86 [95% CI 0.13–6.44], $P = 1.0$) and non-high versus high *PD-L1* expression tumours (OR 0.92 [95% CI 0.29–2.78], $P = 1.0$), or M1 versus M0 disease stages (OR 1.18 [95% CI 0.40–3.50], $P = 0.80$). Yet, CTC detection rate showed a tendency to be elevated in patients with stage IVB versus patients with all other disease stages (OR 3.52 [95% CI 0.90–15.5], $P = 0.063$) and was significantly higher in patients with stage IVB than those with stage IVA (OR 5.48 [95% CI 0.98–37.6], $P = 0.032$; Table 2).

The average CTC number was 2.7 CTCs per 7.5 mL of blood (range 1–13 CTCs; Table 2). PD-L1$^+$ CTCs were detected in 21 blood samples (77.8%) with an average of 1.4 PD-L1$^+$ CTC per blood sample (range

Table 2. CTC cohort characteristics. CTCs, circulating tumour cells; PD-L1, programmed cell death-ligand 1; UICC, Union for International Cancer Control.

Characteristics	*n* (%)	*n* CTC$^+$ (%)	*n* PD-L1$^+$ CTC (%)
CTC patients' cohort	68 (100)	27 (39.7, 1–13[a])	21 (77.8)
Sex			
Male	43 (63.2)	16 (37.2)	12 (57.1)
Female	25 (36.8)	11 (44.0)	9 (42.9)
Histological subtypes			
Adenocarcinoma	36 (52.9)	18 (50.0)	13 (72.2)
Squamous cell carcinoma	27 (39.7)	9 (33.3)	8 (88.9)
Not otherwise specified	5 (7.4)	0	0
UICC staging			
M0	37 (54.4)	14 (37.8)	11 (78.6)[b]
M1	31 (45.6)	13 (41.9)[c]	10 (76.9)[d]

[a]CTC range in the cohort.
[b]Include one patient with keratin$^+$ and PD-L1$^+$ cluster.
[c]Include 1 patient with keratin$^+$ and PD-L1$^-$ cluster.
[d]Include two patients with keratin$^+$ and PD-L1$^+$ cluster.

1–6). In these 21 samples, the PD-L1$^+$ CTC subset represented 10.0% to 100.0% of all detected CTCs. One M0 (stage III) patient had PD-L1$^+$ CTC cluster of three CTCs, while two-stage IV patients had each one CTC cluster with all cells positive for PD-L1. Only one stage IV patient had a cluster negative for PD-L1 (Table 2). Examples of single CTC and CTC cluster staining with positive versus negative PD-L1 expression are presented in Fig. 2.

We assessed for agreement on positive *PD-L1* expression between histology specimens and CTCs in patients who had at least one CTC (n = 27). Here, we found a relatively good overall agreement of 66.7%, with three patients showing PD-L1$^+$ CTCs yet, a negative *PD-L1* expression in histology specimens (Table 3). When considering high *PD-L1* expression, the agreement rate dropped to 51.9%. Of patients with high *PD-L1* expression in histology specimens, 90.0% had PD-L1$^+$ CTCs; however, PD-L1$^+$ CTCs were also detected in 70.6% of patients who had negative *PD-L1* expression (< 1%) in histology specimen (Table 3).

Furthermore, the overall agreement on positive *PD-L1* expression between cytology imprints and CTCs was 62.9%. Similar to histology specimen, the agreement rate dropped to 51.9% when high *PD-L1* expression in cytology imprints was considered (Table 4). The addition of CTCs PD-L1 expression has markedly improved the prediction capacity of cytology imprints for PD-L1 positivity; AUC = 91% [95% CI: 79–100%]

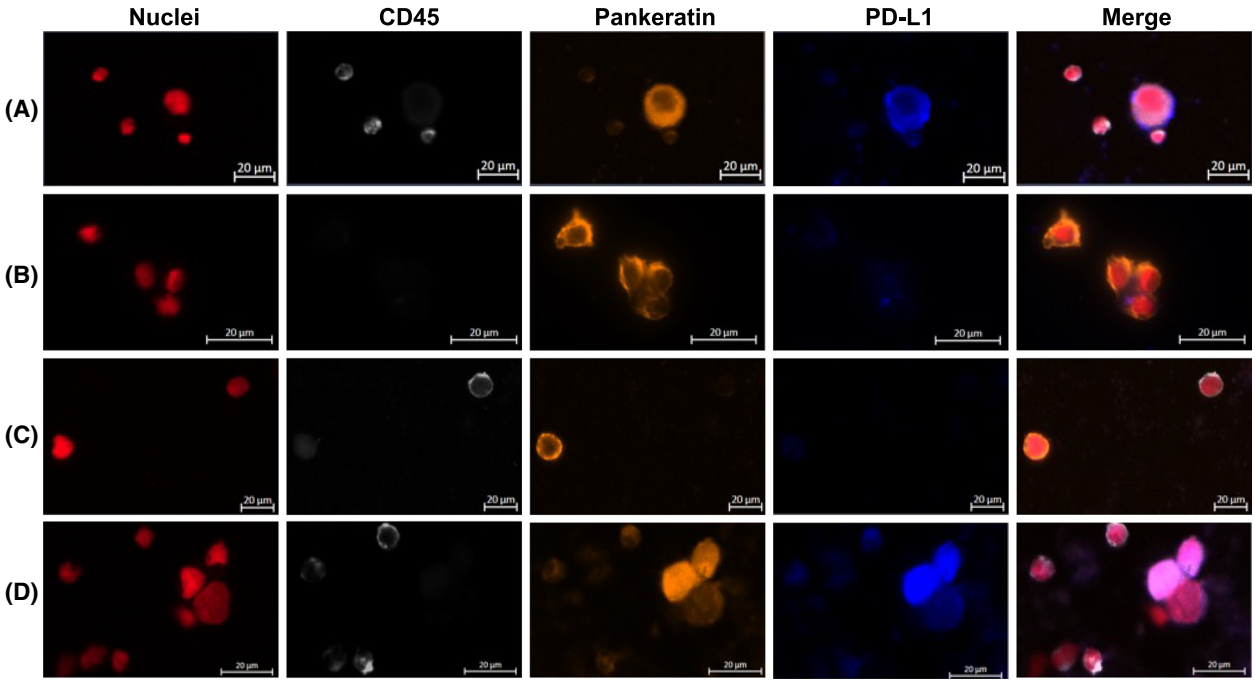

Fig. 2. Example of fluorescence microscopy of circulating tumour cells. Programmed cell death ligand-1 positive (A, D) versus Programmed cell death ligand-1 negative (B, C) circulating tumour cells. Red: nuclei. White: CD45. Orange: Pankeratin. Blue: Programmed cell death ligand-1. The scale bar of 20 µm applies to all pictures.

Table 3. Agreement between PD-L1 expression in tumour tissue biopsy (≥ 1 and ≥ 50% of tumour cells) and CTCs (n = 27). CTCs, circulating tumour cells; n.d., non-determined for absence of events (0); PD-L1, programmed cell death-ligand 1.

		PD-L1 expression in tumour biopsy (≥ 1% of tumour cells)		Positive; negative predictive value
		Yes, *n* (%) 24 (88.89)	No, *n* (%) 3 (11.11)	
Presence of PD-L1$^+$ CTCs	Yes, *n* (%)	18 (75.0%)	3 (100.0%)	85.7%; n.d.
	No, *n* (%)	6 (25.0%)	0	

		PD-L1 expression in tumour biopsy (≥ 50% of tumour cells)		
		Yes, *n* (%) 10	No, *n* (%) 17	
Presence of PD-L1$^+$ CTCs	Yes, *n* (%)	9 (90.0%)	12 (70.6%)	42.9%; 83.3%
	No, *n* (%)	1 (10.0%)	5 (29.4%)	

Table 4. Agreement between PD-L1 expression in cytological smears (≥ 1% and ≥ 50% of tumour cells) and CTCs (n = 27). CTCs, circulating tumour cell; n.d., non-determined for absence of events (0); PD-L1, programmed cell death-ligand 1.

		PD-L1 expression in cytological smears (≥ 1% of tumour cells)		Positive; negative predictive value
		Yes, *n* (%) 23 (85.19)	No, *n* (%) 4 (14.81)	
Presence of PD-L1$^+$ CTCs	Yes, *n* (%)	17 (26.1)	4 (100.0)	80.9%; n.d.
	No, *n* (%)	6 (73.9)	0	

		PD-L1 expression in cytological smears (≥ 50% of tumour cells)		
		Yes, *n* (%) 12 (14.44)	No, *n* (%) 15 (55.56)	
Presence of PD-L1$^+$ CTCs	Yes, *n* (%)	10 (83.3)	11 (73.33)	47.6%; 66.7%
	No, *n* (%)	2 (16.7)	4 (26.66)	

and for high *PD-L1* expression; AUC = 84% [95% CI: 69–100%] from standardized immunohistochemistry.

4. Discussion

The evaluation of tumoral *PD-L1* expression is essential for selecting patients with NSCLC who might benefit from treatment with ICIs. So far, the evaluation of tumoral *PD-L1* expression is only validated for histology specimens [6–8], excluding a considerable proportion of NSCLC patients for whom no tumour tissue is available [9,10]. In this prospective study, we therefore compared *PD-L1* expression from standard immunohistochemistry with the *PD-L1* expression of cytology imprints and CTCs. Tumour samples were obtained from the primary tumour site through various biopsy procedures and evaluated independently by different pathologists. Using this approach, we sought to avoid sampling bias and to represent real-world data on the potential use of cytology imprints and CTCs for examining *PD-L1* expression. Though *PD-L1* assessment was confirmed as a predictive biomarker for histological samples, the evaluation of *PD-L1* expression on paired cytological specimen has also shown comparable results [14,16,24,25]. However, to our knowledge, this is the first study comparing *PD-L1* expression on paired histological, cytological and CTC-based liquid biopsy specimen.

Overall, our data indicate a good cytology–histology agreement for both PD-L1 positivity and high expression. Furthermore, our study demonstrates the added role of CTCs-*PD-L1* expression as the combination of liquid biopsy and cytology has markedly improved the positive prediction capacity for PD-L1 positivity and high expression. Noteworthy, cytology imprints yielded excellent positive agreement (91%), yet a poor negative agreement, on PD-L1 positivity. This might indicate that cytology imprints overestimate PD-L1 positivity, that is, PD-L1 tumour cells expression score ≥ 1% in PD-L1 negative tumours as per immunohistochemistry. However, cytology imprints also yielded a good negative agreement on *PD-L1* high expression, that is, *PD-L1* tumour cells expression score ≥ 50% with a NPV of 85%, AUC = 79% [95% CI: 68–91%], indicating good capacity of these imprints in ruling out patients who might not qualify for a first-line monotherapy with ICIs. Our data also indicate that samples obtained from surgically resected tumour tissue might yield a greater cytology–histology agreement than those obtained via fibreoptic bronchoscopy or percutaneous tumour biopsy.

Our findings regarding the PD-L1 cytology–histology agreement are in line with the finding of other previous studies that reported an agreement rate of between 65–100% for both PD-L1 positivity and high *PD-L1* expression [10]. Many factors contribute to cytology–histology disagreement as well as to the heterogeneity in the reported agreement rates. This includes the intra-tumour heterogeneity of *PD-L1* expression [26], the number of tumour cells in cytology samples [27] as well as the discordance due to the

applied diagnostic tools in tumour sampling and staining procedures including used antibodies [28]. Further, the type of cytology specimens might have an impact as cytological cell blocks [29–31] which demonstrated better agreement with histology specimen than cytology imprints [14,32,33].

In this study, we also compared *PD-L1* expression between standard immunohistochemistry and a label-independent, microfluidic-based CTC enrichment system. As previously reported, our data confirm that the Parsortix system reliably detects CTCs in liquid biopsies from patients with NSCLC [20,23]. The detection rate of CTCs in our cohort was nearly 40%. Noteworthy was that CTCs detection rates were rather comparable between patients with resectable versus non-resectable as well as between patients with metastatic (M1) and non-metastatic (M0) disease stages. Nevertheless, the subgroup analysis has revealed that CTCs detection rate is significantly higher in patients with stage IVB (64%) than those with stage IVA (23%) or patients with non-metastasized tumours (37%).

Here, we also report that most CTCs (nearly 80%) showed positive *PD-L1* expression. *PD-L1* expression in CTCs of patients with NSCLC has already been assessed using various different enrichment techniques and PD-L1 antibodies [34]. Kulasinghe et al. [35] used the microfluidic-based ClearCell FX system to assess for CTC in a smaller cohort of patients with advanced NSCLC and reported a CTCs detection rate of 51% with 65% PD-L1-positive cells. In a further study, the same authors detected CTC in 60% of patients with stage IV NSCLC and 56% of selected patients with CTC were PD-L1 positive [36]. The absence of clinical relevance of *PD-L1* expression on CTCs prior to therapy has also been reported in Guibert et al. study [37]. The authors used the size-based separation ISET platform to yield a high CTC positivity of 93% at baseline (n = 89/96), with 83% of these patients expressed PD-L1 on at least one CTC. Sinoquet et al. [38] used the EpCAM-based CellSearch enrichment method, and reported a 43.4% of CTC positivity (n = 54 patients) with a low PD-L1$^+$ CTC rate of 21.7%. Janning et al. [19] reported a 68.5% CTC positivity rate and 81.9% of PD-L1$^+$ CTC in late-stage NSCLC patients using the same system as ours. Overall, with a CTC positivity of nearly 40% and PD-L1$^+$ CTC of 78%, our data align with what was previously reported in literature. We suggest that the observed variability could be attributed to the different enrichment techniques including the antibody that has been used. Still, the sample size and rather low positivity rate is clearly a limit of our study that could cause a bias in or data.

Furthermore, agreement rates between *PD-L1* expression on CTCs versus cytological imprints yielded similar results to CTCs versus histology specimen; 62.9% with PD-L1 ≥ 1% versus 51.9% with high *PD-L1* expression (≥ 50%). Notable was that the agreement rate between *PD-L1* expression on CTCs versus tissue was relatively low for almost all the previously described studies. Only Ilie et al. [39] reported a high agreement (93%) of PD-L1$^+$ CTC with matched tissue using the ISET platform. In our study, a higher agreement was observed compared to many other studies. This could be due to the use of a new more sensitive PD-L1 antibody that was shown to be very sensitive when used for immunofluorescence staining. By using this new staining protocol, a moderate to high agreement of 66.7% was observed when at least 1% of cells expressed *PD-L1*. However, the agreement drops to 51.9% when increasing the threshold of PD-L1 tissue positivity to ≥ 50%. Indicating that preferentially the PD-L1-positive cells enter or survive in the blood circulation.

For further refinement and in order to increase predictive accuracy, few limitations inherent to our study need to be considered. Though we followed standard procedure and kept the cell quantity as previously advocated (at least 100 viable tumour cells), the sample size might be a limit for which *PD-L1* expression might be underestimated. Nearly 80% of the specimens were collected via fibreoptic bronchoscopy making the chance of underestimating the PD-L1 content higher [40]. This limit further highlights the importance of a combined approach for PD-L1 assessment and suggests that the use of a liquid biopsy approach through CTC analysis might improve PD-L1 predictive accuracy.

5. Conclusions

Our study shows that the tissue biopsy and the consequent smear imprint at a single tumour site or a specific time point is insufficient to represent the overall status of PD-L1 on tumour tissue. There is obvious spatial and temporal heterogeneity of *PD-L1* expression on tumour tissue that cannot be unravelled by conventional tissue biopsy and cytological imprints which might explain the low agreement rate when considering a 50% positivity threshold. By assessing *PD-L1* expression on CTCs in a minimal invasive approach and a real-time detection using a label-independent, microfluidic system, may represent a complementary source for PDL1 immunostaining, which also makes the dynamic monitoring of PD-L1 during treatment more convenient for physicians and

less invasive for patients [41]. Still, future larger prospective studies assessing all these biomarkers in NSCLC patients receiving ICI are needed to be performed to assess the sensitivity and specificity of each approach.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MA, YB, HW, KP and MR were involved in study concept and design and drafted the manuscript. MA, YB, HW and MR carried out data analysis. MA, YB, MSc, NH-O, IW, MSz, JK, TP-V and HE were involved in patient sample collection. MA, YB, DH, LW, JK, SW and TP-V performed the experiments. MA, YB, DH, LW, SW, SP, SS, HE and HW carried out data interpretation. All authors have read and agreed to the published version of the manuscript.

Data accessibility

Data are available upon reasonable request form the corresponding author.

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6. Publikation 3

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REVIEW ARTICLE

Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond

Laura Keller[1], Yassine Belloum[1], Harriet Wikman[1] and Klaus Pantel[1]

Cell-free DNA (cfDNA) derived from tumours is present in the plasma of cancer patients. The majority of currently available studies on the use of this circulating tumour DNA (ctDNA) deal with the detection of mutations. The analysis of cfDNA is often discussed in the context of the noninvasive detection of mutations that lead to resistance mechanisms and therapeutic and disease monitoring in cancer patients. Indeed, substantial advances have been made in this area, with the development of methods that reach high sensitivity and can interrogate a large number of genes. Interestingly, however, cfDNA can also be used to analyse different features of DNA, such as methylation status, size fragment patterns, transcriptomics and viral load, which open new avenues for the analysis of liquid biopsy samples from cancer patients. This review will focus on the new perspectives and challenges of cfDNA analysis from mutation detection in patients with solid malignancies.

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BACKGROUND

Cell-free DNA (cfDNA) refers to extracellular DNA molecules (double-stranded DNA and mitochondrial DNA) originating from any cell type found in body fluids. cfDNA has been detected in the blood of diseased and healthy individuals already in 1948.[1] cfDNA analysis is currently applied in prenatal diagnostics[2] and its clinical use is also evaluated in several fields including cancer, organ transplant, autoimmune diseases, trauma, myocardial infarction and sepsis.[3–7] However, our understanding of the structure and origins, cell release mechanisms and clearance of cfDNA is still preliminary. Although the majority of cfDNA molecules originate from the haematopoietic system, there is a huge interest to determine the relative contribution of different organs in healthy and pathological conditions to the overall amount of cfDNA. Not only a multitude of release mechanisms including apoptosis, senescence, ferroptosis, NETosis, phagocytosis and necrosis, but also active secretion—including association to extracellular vesicles or induced by other mechanisms like expulsion of mature nuclei by erythroblasts, egestion of mitochondrial DNA or vital NETosis—have been described. On the other side, diverse parameters govern the degradation and elimination of cfDNA molecules: enzymatic cleavage in the circulation, elimination of nucleosome complexes by the liver and to a lesser extent removal of DNA fragments by the kidney. The description of these fundamental aspects of cfDNA biology is out of scope of this introduction, but has been discussed in excellent comprehensive reviews.[8,9]

The tumour-derived fraction of cfDNA, commonly named circulating tumour DNA (ctDNA), has received enormous attention during the last decade owing to its huge potential as a minimal invasive tumour biomarker in cancer patients. As for cfDNA, the correlation between tumour biology and ctDNA release is still not well understood and may not solely rely on the amount of dying cells. Not only the volume and metabolism of the tumour, but also its rate of proliferation, have been positively correlated to the amount of ctDNA in blood plasma.[10–12] Nevertheless, the proportion of ctDNA engulfed into extracellular vesicles actively released by tumour cells is still unclear and the effect of different therapy regimens on this active secretion mostly unknown.[13,14] Obviously, there is a huge need for more fundamental research on the kinetics of ctDNA in cancer patients.

The vast majority of published studies on the potential use of ctDNA in oncology deal with the detection of specific mutations detected in plasma or serum of cancer patients, and these studies have been reviewed in detail elsewhere.[7,15] Briefly, mutation detection in ctDNA has the potential to be used in early cancer detection, to determine the tissue of origin, prognosis, to monitor response and assess potential resistance to the treatment, or to detect minimal residual disease. However, epigenetic alterations are even more frequent than somatic mutation in cancer development.[16] Although mutation analysis of ctDNA shows a number of clinical applications, the assessment of cfDNA beyond the detection of point mutations, encompassing the study of chromosomal rearrangements, copy number aberrations, methylation, fragmentation and gene expression, is therefore also receiving increasing interest (Fig. 1).

Obviously, some tumour types and body sites release lower amounts of ctDNA into the bloodstream. Here, non-blood sources of ctDNA for molecular profiling have become valuable. Clearly in primary brain tumours, such as gliomas, central nervous system lymphomas and some paediatric solid tumours, cerebrospinal fluid (CSF) has shown higher sensitivity compared with peripheral blood.[17,18] Similarly, for some upper aerodigestive track tumours saliva, sputum or pleural effusions may also be good alternatives

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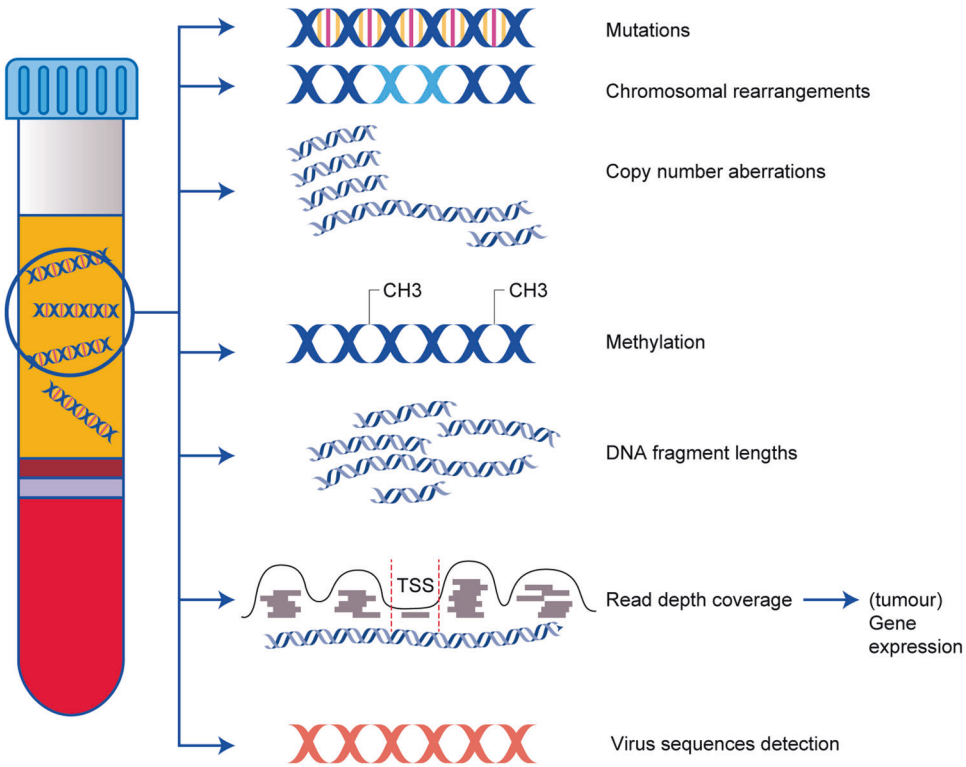

Fig. 1 Different features of ctDNA and potential clinical implications. This figure summarises the tumour-relevant clinical information that can be gained from the study of different features of cfDNA. Somatic genomic aberrations detectable on ctDNA include mutations, chromosomal rearrangements and copy number aberrations. Additional features characteristic for ctDNA are specific epigenetic aberrations like methylation patterns or different DNA fragment lengths. Information on tumour-specific transcription can also be obtained from ctDNA analysis by reading the inter-nucleosome depth coverage. In virus-induced tumours (e.g. EBV-related nasopharyngeal carcinomas or HPV-related head and neck tumours), the quantitative assessment of virus sequences have diagnostic validity. TSS transcription starting sites.

to blood, with recent reviews published elsewhere.[19,20] Urine, stool and seminal fluid are other examples of body fluids that have been used in different liquid biopsy approaches.[21]

Here, we will focus on ctDNA detected in blood plasma of cancer patients. We begin this review by providing an overview of the main methods used to detect mutations in ctDNA before discussing some of the associated challenges; it is not our aim, however, to comprehensively cover this topic within this review. We will then outline additional features of cfDNA beyond the detection of point mutations that can be assessed using liquid biopsy samples from patients with solid tumours.

MUTATIONS IN CTDNA

Approaches for the mutation analysis of ctDNA

Mutations in ctDNA from liquid biopsy samples can be detected via two different approaches. In the first approach, single, or low numbers of, mutations can be detected using highly sensitive techniques with high specificity and at a rather fast and cost-effective rate.[22] In 2016, the Cobas EGFR mutation Test v2 that interrogates by RT-PCR several mutations in exons 18, 19, 20 and 21 of epidermal growth factor receptor (*EGFR*) gene was the first liquid biopsy-based companion diagnostic to be approved by US Food and Drug Administration (FDA) and the European Medicines Agency for the prescription of EGFR inhibitors in patients with non-small-cell lung cancer (NSCLC) in cases when tumour biopsy tissue is not available.[23] Other targeted approaches, based mainly on digital PCR (droplet digital [ddPCR] or BEAMing dPCR), have been demonstrated to be able to detect specific known mutations, such as the main driver mutations of the primary tumour or variants associated with response to drugs in individual tumour types, and usually show high concordance with results obtained in tumour tissue[24–26] and reach a variant or mutant allele frequency detection (VAF/MAF) as low as 0.001% for the most advanced technologies[27] (i.e. the frequency of a particular genetic variation of a specific sequence [e.g. allele/mutation] relative to the other genetic variations of the same sequence). The detection and comprehensive molecular characterisation of minimal residual disease (MRD) is of particular importance in the adjuvant setting to improve clinical outcomes;[28] ctDNA detected via such targeted, highly sensitive approaches in the early stages of melanoma was reported to predict the relapse risk,[29,30] and might therefore be useful in the process of patient stratification for adjuvant therapy. Next step in the implementation of ctDNA in clinical routine is to demonstrate its utility in patient treatment selection. For instance, in the recently published TARGET study (registered in NIHR Central Portfolio Management System under the reference CPMS ID 39172), the primary aim was to match advanced stage patients to early phase clinical trials on the basis of plasma ctDNA analysis of both somatic mutations and copy number alterations in 641 cancer-associated-genes.[31] Another example is the Circulating Tumour DNA Guided Switch (CAcTUS) study (NCT03808441), which determines whether switching from targeted therapy to immunotherapy based on a decrease in levels of ctDNA in the blood will improve the outcome in melanoma patients.

Broader approaches have also been developed to interrogate multiple mutations in parallel and range from the analysis of several tens of mutations, to a genome-wide analysis of cfDNA by whole-exome sequencing (WES) or whole-genome sequencing (WGS). Most of these approaches use next-generation sequencing (NGS) but mass-spectrometry-based detection of PCR amplicons is also becoming available.[32] Besides increasing the probability of detecting a mutation in cfDNA, these broader approaches allow a more complete genotyping of the tumour, which can be used to

assess tumour heterogeneity or to follow clonal evolution of the tumour under treatment, as well as to identify potential resistance mutations before clinical progression is observed.[10,33,34] Another example of the application of nontargeted approaches also relates to cancer patients treated by immunotherapy, for whom mutation load (i.e. the number of nonsynonymous mutations found in a tumour) has emerged as a putative biomarker of the response to the treatment. Assessing mutation load and measuring its evolution through plasma analysis has also been evaluated as an alternative approach to tumour tissue determination.[35,36] More generally, comprehensive reviews have discussed the clinical utility of ctDNA in the new era of immunotherapy.[37,38]

However, one should be aware that the larger the panels, the more expensive and difficult it is to obtain high sensitivity for mutation calling.

Challenges associated with mutation detection in cfDNA

A key issue in the analysis of ctDNA is still the extent to which the information gained from the liquid biopsy sample reflects the tumour tissue. Both technical and biological factors can affect the concordance between tumour and plasma, generating false-negative and false-positive results in ctDNA analysis.

False-negative results might be explained by the low volume of plasma yielded (4–5 ml) from a typical blood sample of 10 ml, which limits the total number of available genome copies to be analysed: mutations within a tumour can be clonal or subclonal, and the amount of available genome copies is a limiting factor for the detection of variants of low allele frequency.[39] Moreover, the tumour fraction of cfDNA varies between cancer types as well as between patients affected by the same cancer type.[40] Even at the metastatic stage, some patients can yield a low amount of ctDNA,[41,42] and the question of why some tumours undergo limited shedding of ctDNA is still not completely resolved. In this regard, detection of mitochondrial tumour-derived DNA, as an alternative source of ctDNA might be a promising approach, owing to the thousands of copies of mitochondrial DNA per cell.[43] Proof of principle for this apporach was provided in patient-derived orthotopic xenograft models of glioblastoma in 2019.[11] Considerations about technical improvements for the methods used to analyse cfDNA could also help to overcome the limit of detection. Ultra-deep sequencing methods can lower the percentage of false negative and are currently under evaluation across different cancer types.[44–47] The size selection of cfDNA fragments (see below) or the choice of an alternative method for library preparation like single strand DNA libraries for NGS are additional solutions.[48]

False-positive results are another concerning issue when multiple mutations are interrogated by NGS platforms. The risk of introducing errors during library preparation and subsequent sequencing steps has led to the implementation of multiple mutation-enrichment methods and error-suppression strategies such as the introduction of molecular barcodes or bioinformatic analysis pipelines of the data.[22,39,49] The extensive comparison of paired tumour and plasma samples therefore represents an important prerequisite to evaluate the diagnostic accuracy of analytical platforms, especially for variants with allele fractions that are close to the limit of detection.[50–52] Different commercial NGS platforms might not have the same limit of detection or interrogate the same genomic regions as each other, and the field would benefit from rigorous cross-assay comparisons, as carried out between 2015 and 2019 by the EU Innovative Medicines Initiative (IMI) consortium CANCER-ID (www.cancer-id.eu) and sustained by the new European Liquid Biopsy Society (ELBS; www.elbs.eu) and other networks (the US Blood Profiling Atlas of Cancer; www.bloodpac.org). A cross-comparison of four commercial NGS platforms, all certified by the US-based college of American Pathologists-Clinical Laboratory Improvement Amendments, was carried out in 2019 with plasma–tumour-matched samples of early stage cancers that present a limited ctDNA amount.[53] Substantial variability in terms of sensitivity (38–89%) and positive predictive values (36–80%) was identified among the different platforms. Low predictive positive values were mainly associated with variants with an allele frequency below 1% and could be explained by technical factors (limited sensitivity, bioinformatic filtering of the data or even plain error of identification). Nonetheless, germline variants shed from normal cells and during clonal haematopoiesis (e.g. the presence of somatic variation in some cancer-related genes like *TP53* that do not necessarily lead to cancer) constitute another source of confounding factors that have to be considered when interpreting the data. By applying a highly sensitive and specific ctDNA sequencing assay on a cohort of 124 metastatic cancer patients and 47 controls without cancer, with matched white blood cell DNA, Razavi et al. found that 53.2% of mutations found in cancer patients had features consistent with clonal haematopoiesis.[47] This study highlights therefore the risk of false findings and the need to integrate white blood cell DNA as control when applying ultrasensitive ctDNA sequencing methods. Overall, it appears necessary that laboratories should comment on these different limitations in their reports.[54]

If these technical and biological factors could be ruled out, then ctDNA could be used to evaluate intratumour heterogeneity, as it is now well accepted that a single tumour biopsy procedure generates a limited representation of temporal and spatial heterogeneity, whereas ctDNA in plasma would represent a pool of the entire tumour or of the metastatic sites.[55] Up until now, clinical studies that have compared plasma analysis with multi-regional tissue biopsies are rare and limited to few patients, due to an increase risk of clinical adverse side effects linked to this invasive procedure (see Table 1). In this sense, studies conducted utilising rapid autopsy programs are of particular interest.[26] Some studies have shown that the quantitative level of mutations found in ctDNA reflects the architecture of the mutational landscape in tumour tissue, with truncal mutations more readily detectable than private mutations.[10,56–58] In the context of acquired resistance in gastrointestinal cancers, mutation analysis of ctDNA taken at progression was more informative than the corresponding analysis of tissue biopsies.[34] However, in some cases of melanoma patients ctDNA analysis only partially reflected heterogeneity, with under-representation of certain anatomical metastatic sites like brain or subcutaneous metastases.[12] A better understanding of the parameters that govern ctDNA release (i.e. proliferation/turnover, active secretion, type of cancer, location or tumour vascularity) is therefore needed.

COPY NUMBER AND STRUCTURAL DNA ABERRATIONS

As well as mutations, other cancer-related alterations in DNA (such as copy number aberrations [CNA]) and genomic rearrangements (inversions, translocations, insertions and deletions) can be studied using cfDNA. CNA can now also be easily detected by massively parallel sequencing methods thanks to the development of diverse analytical tools based on different features that can be extracted from NGS data (reviewed in ref. [59]). CNA are estimated to be present in almost all cancers of most histopathological types, so that the detection of CNA in cfDNA could potentially facilitate noninvasive diagnostic applications. However, the identification of CNA in cfDNA has proven challenging due to the prevalence of copy number variation in the healthy population,[60] the variable level of the tumour fraction in cfDNA, tumour ploidy and tumour heterogeneity. Currently, CNA in cfDNA can be detected using low-coverage (0.1×) sequencing of the genome followed by normalisation algorithms; this approach necessitates a ctDNA fraction above 5% to achieve good specificity and sensitivity,[56,61–63] although targeted approaches and new algorithms to detect CNA in a lower amount of ctDNA

Table 1. Studies evaluating the capacity of ctDNA to recapitulate intratumour heterogeneity.

Cancer entity	No. of patients	No. of tumour biopsies per patient	Tissue sequencing technique	cfDNA sequencing technique	Time of plasma collection	Concordance and conclusions	Reference
Metastatic serous ovarian cancer	1	8 collected at initial diagnosis/ surgery	Tam sequencing	Tam sequencing	Plasma samples were collected 15 and 25 months after initial surgery	TP53 was identified in 8/8 tissue biopsies at initial surgery. EGFR and TP53 mutations were found in plasma samples. Trace signal of the EGFR mutation in 2/8 tumour biopsies obtained from same metastasis, using a lower-specificity criteria defined for mutation detection.	[143]
Metastatic breast and ovarian cancer	1	1 tissue sample from breast and 4 ovarian tissues	Shotgun massive parallel sequencing	Shotgun massive parallel sequencing	Plasma samples were collected at diagnosis and 1 day after the operation.	SNV found in tumour were classified into seven different groups according to the degree of sharing these mutations between the four regions. Mutations that were shared by all four regions contributed the highest fractional contribution of tumour-derived DNA to the plasma. Mutations that were more region specific had a reduced contribution to plasma.	[56]
Metastatic breast cancer	1	8 tumour biopsies obtained at diagnosis from primary tumour and an LN; after 19 months from the brain metastasis; at autopsy breast, chest, liver, ovary and vertebrae.	WES confirmed by deep sequencing	WES confirmed by deep sequencing.	9 serial plasma samples collected during the last 500 days of clinical follow-up.	In plasma, trunk mutations from tumoural tissues were highest in abundance whereas metastatic-clade mutations were lower in abundance Plasma DNA captured differential response across distinct metastatic sites during targeted treatment 11 nonsynonymous high-confidence SNVs were identified and validated in plasma but not detectable at >2% AF in any of the analysed tumour biopsies. Among these, one was associated with resistance to treatment	[57]
Metastatic breast cancer	1	Primary tumour and 1 synchronous liver metastasis	NGS panel of 300 genes known to harbour actionable mutations	NGS panel of 300 genes known to harbour actionable mutations	Plasma samples were collected before therapy, and during at 2 and 6 months and at progression.	All plasma samples captured the entire repertoire of mutations found in the primary tumour and/or metastatic deposit	[144]
Metastatic colorectal cancer	1	primary sigmoid tissue and 2 liver metastases	Amplicon based sequencing (17 mutations)	ddPCR on RAS pathway hotspot mutations	Plasma was collected every 4 weeks until disease progression.	4/7 of tumour tissue mutations were identified in plasma	[145]
Metastatic gastro-intestinal cancer	5	Between 3 and 17 biopsies/ patient	Targeted exome sequencing	Targeted NGS panels (70 genes or 226 genes) Some SNV were confirmed with ddPCR	Plasma and tissue were obtained in parallel at progression and at rapid autopsy	Tumour biopsy identified resistance alterations less frequently than cfDNA. cfDNA detected multiple resistance alterations residing concurrently in distinct tumour subclones and different metastatic lesions.	[34]
Metastatic NSCLC	1	12 (7 metastatic and 5 primary tumour regions)	WES	Bespoke targeted NGS panels (103 variants)	5 PT regions were obtained at diagnosis, 1 metastasis during treatment (day 467) and 6 metastases at autopsy. 9 plasma samples were analysed during follow-up (day 151, 242, 340, 431, 466, 627. 662, 767).	At day 466, 18 out of 20 SNVs were detected in ctDNA; these subclonal clusters were shared between six out of seven metastatic sites. Single SNVs from two private subclones were also detectable in but were not identified vertebral biopsy. ctDNA analysis also identified 90 days before death subclones private to one metastatic site that was not identified in CT scan.	[10]
Surgical resectable NSCLC	32	181 multi-region tumour tissues in total were analysed	Targeted capture sequencing (1021-gene panel)	Targeted capture sequencing (1021-gene panel)	Not mentioned	Much easier to detect trunk mutations than branch mutations in ctDNA	[58]
Stage I–III NSCLC	4	Between 2 and 3 biopsies/patient	50 SNV Multiplex PCR-NGS	50 SNV Multiplex PCR-NGS	Plasma samples were collected prior to surgical resection of tumours.	43% of the selected mutations were detected in both cfDNA and tumour DNA, 25% of which were variants occurring late during tumour evolution and predicted to be subclonal in origin.	[146]
Metastatic gastric cancer	5	5	Customised 483 genes panel	Customised 483 genes panel	Blood samplesand tumour tissue samples were collected simultaneously.	The numbers of somatic SNVs and InDels in the plasma samples differed from those of the biopsies. The mutated genes identified in the plasma were all detected in one or more biopsy, which demonstrated that plasma ctDNA could partially overcome tumour heterogeneity	[147]
Metastatic melanoma	3	3 or 4 biopsies/patient	WES	WES	Plasma samples were collected at disease progression, and tissue samples were collected at death	99% ubiquitous mutations (present in all tumours), 64% shared mutations (present in two or more tumours), and 14% private mutations (present in only one tumour) were identified in plasma. Under-representation of ctDNA from subcutaneous disease sites and brain. Limited ability to detect private mutations in plasma was a result of the low mutant allele frequency.	[12]

ddPCR droplet digital PCR, *EGFR* epidermal growth factor receptor, *LN* lymph node, NGS next-generation sequencing, *SNV* single nucleotide variant, WES whole-exome sequencing.

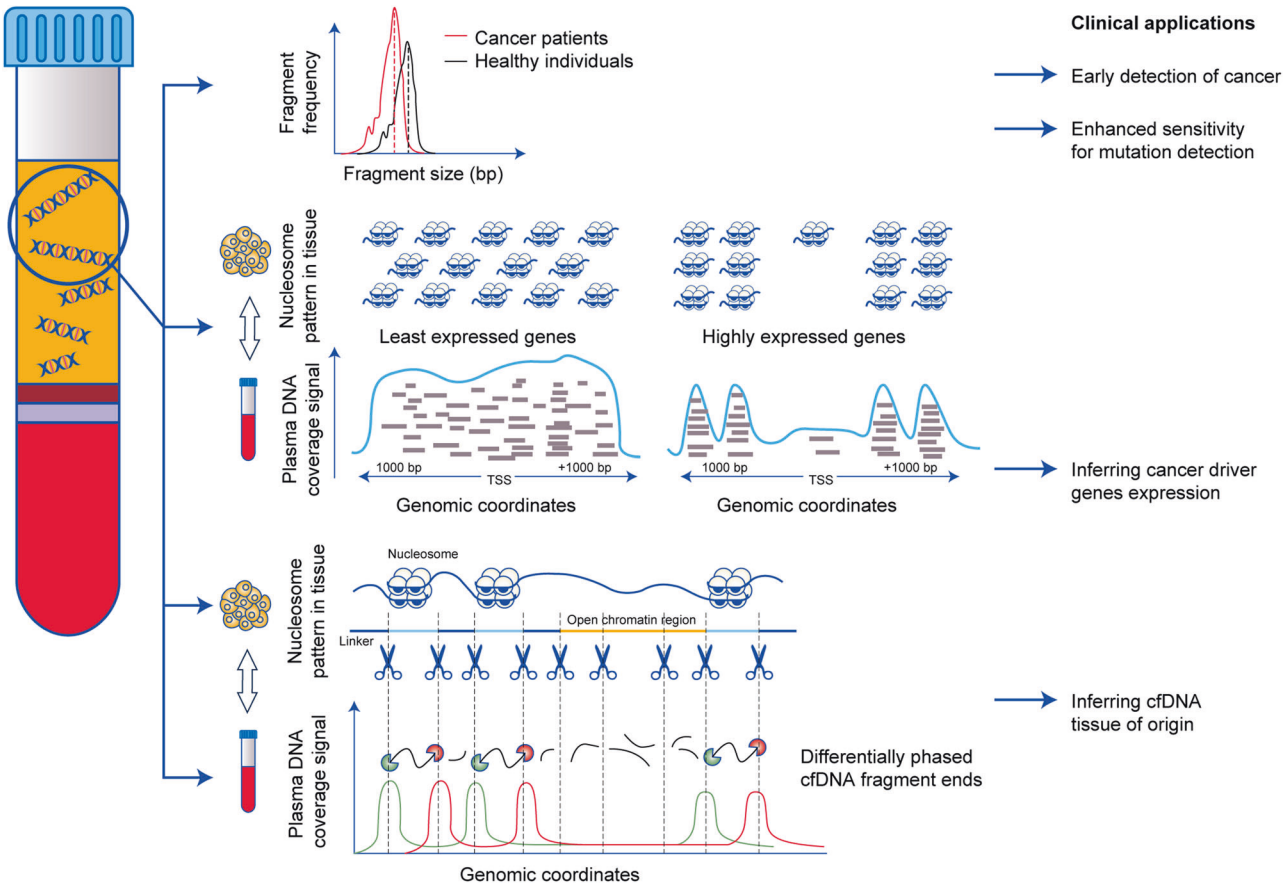

Fig. 2 Clinical applications of genome-wide fragmentation analysis of cfDNA in cancer patients. Analysis of length distribution of cfDNA has revealed that cancer patients present a more fragmented pattern (and consequently shorter fragments) than healthy donors. This feature can be leveraged to detect cancer without previous knowledge of genomic aberration but also to enhance sensitivity of mutation detection when monitoring tumour evolution. cfDNA coverage signal around TSS correlates with gene expression. Actively transcribed promoters at TSS display low nucleosome occupancy (that is translated in very low read numbers of cfDNA fragments) flanked by well-phased nucleosomes (translated in relatively high and well-phased read numbers of cfDNA fragments). Nonetheless, the region around an active TSS exhibits an overall lower coverage in comparison to inactive TSS promoters, which exhibit an increased coverage signal indicative of denser nucleosome packaging. Therefore, unravelling nucleosome occupancy at promoters from plasma DNA sequencing might help inferring expression levels of genes in the contributing cell types. cfDNA fragment ends pattern reflects nucleosome-depleted region and well-phased nucleosome arrays around the tissue-specific open chromatin region. This analytical approach allows by comparison of nuclear DNA from tissues for the determination of the relative contributions of various tissues in plasma DNA. For the design of our Figure, we were inspired by the figures in the publications of Van der Pol et Mouliere[8] and Murtaza et Caldas.[142]

(below 1%) have been developed within the past 5 years.[64,65] Again, whether CNA detected in plasma are representative of the tumour tissue is still a subject of investigation. In patients with hepatocellular carcinoma, CNA in plasma were comparable with respect to their size profile, with those found in tumour tissue in 63% of the chromosome arms analysed.[66] In 2018, a new algorithm for aneuploidy detection based on the amplification of long interspersed nuclear elements (LINEs) was evaluated on a large cohort of plasma samples from early and late stages of eight different cancer types that presented with a variable neoplastic cell fraction. Fifty-four percent of plasma samples had a concordant gain or loss in the primary tumour.[65] The presence of CNA in plasma has also been associated with clinical outcome, and their analyses have revealed new resistance mechanisms in patients with prostate cancer or NSCLC such as androgen receptor (AR) amplification and TMPRSS2-ERG fusion or MYC amplification, respectively.[67,68]

Genomic rearrangements, notably those involving the genes encoding the kinases ALK or ROS, or the presence of the fusion TMPRSS2-ERG, are potential therapeutic targets in lung cancer or a sensitivity biomarker for abiraterone acetate treatment response in prostate cancers, respectively.[69,70] These structural genomic abnormalities have the potential to be detected via NGS techniques with the additional benefit of detecting a large number of gene fusions with known and unknown partner genes, compared with previous targeted PCR assays. Indeed, data obtained over the past 1–2 years have shown that plasma genotyping using hybrid-capture NGS technology can reliably detect ALK or ROS fusions in NSCLC patients,[71,72] although these results need to be confirmed in larger patient cohorts.

DNA FRAGMENTATION PATTERNS

Several different studies published over the past 20 years have focused on the size fragmentation pattern of cfDNA, i.e. the length distribution of cfDNA fragments, which reveals relevant genetic 'non-coding' clinical information (Fig. 2). cfDNA size profiling is a fundamental parameter that can contribute to the better definition and detection of ctDNA. Not only does cfDNA size profiling provide clues about the origins of ctDNA, but it can also provide further clues about how to improve the analytical methods.

cfDNA fragmentation pattern analysis for better definition and detection of ctDNA

Gel electrophoresis and electron microscopy were first used to analyse the length of cfDNA in the plasma or serum of cancer patients and healthy donors and revealed that plasma DNA is not randomly fragmented. Fragments equivalent to whole number multiples of 180 bp[73,74] were first observed in both cohorts; however, different size distributions between healthy donors and cancer patients were already observed.[73] This figure was further refined to ~160 bp using NGS methods,[66] a result that inferred the existence of a nucleosome footprint and suggested that the release of DNA by apoptotic caspase-dependent cleavage was a major contributor to cfDNA presence in blood of both cancer patients and healthy donors—caspase-induced DNases periodically cleave DNA within the internucleosomal linker region (the exposed DNA that is not wrapped around histone octamers [147 bp with a DNA linker of 20–90 bp, mainly 20 bp]).[75] Despite other conflicting reports,[76] there is now a growing body of evidence that cfDNA in cancer patients is even more fragmented compared with cfDNA from healthy donors, with a significant proportion of fragments shorter than 145 bp occurring with a 10 bp periodicity.[66,77–79] The 10-bp periodic oscillation observed might correspond to the wrapping and protecting of the DNA from enzymatic cleavage around the nucleosome or a protein complex.[8] Consequently, whether ctDNA is effectively shorter than nontumour cfDNA is a pivotal question. The detection of tumour-specific genetic alterations (including CNA and mutations) in human plasma and in the plasma of mice bearing human cancer xenografts revealed that mutant ctDNA is generally more fragmented than nonmutant cfDNA, with a maximum enrichment in fragments between 90 and 150 bp,[66,78] an observation that was harnessed to enhance mutation detection using either in vitro or in silico size selection.[78] Low-coverage WGS used to analyse the fragmentation pattern of cfDNA on a genome-wide scale showed overall that the lengths of cancer-derived cfDNA molecules were more variable than those of wild-type cfDNA, ranging from 30 bases smaller to 47 bases larger.[80] Furthermore, the inclusion of cfDNA fragmentation in machine-learning algorithms can contribute to improving cancer detection, as the combination of cfDNA fragmentation pattern and somatic alteration analysis was shown to efficiently separate healthy subjects from cancer patients.[78,80] In particular, this low-pass WGS approach called DELFI (DNA evaluation of fragments for early interception) is able to analyse minute amounts of cfDNA, therefore opening up new avenues for early cancer detection, especially promising because of the prevalence of clonal haematopoiesis.

Interestingly, some studies have reported the presence of large DNA fragments of several kilobases in the blood plasma of human cancer patients,[74] but cfDNA over 350 bp was estimated to represent less than 2% of genome equivalent copy number in cancer patients.[48] These long fragments might also indicate a necrotic, rather than apoptotic, release mechanism[66,73] or might originate from active secretion.[81] However, these fragments could also derive from lysed blood cells and may be a preanalytical parameter to assess as quality control of the cfDNA extract.[48] Third-generation sequencing methods based on long reads sequencing would be helpful to investigate the biological significance of these long DNA molecules.[82]

Significantly, the fragmentation pattern of cfDNA can also be studied in other biological fluids such as urine and cerebrospinal fluid (CSF). Notably, however, a matched comparison of cfDNA in plasma, urine and CSF from glioblastoma patients revealed a different fragment distribution in CSF to that in plasma and urine, with a specific enrichment for tumour-derived cfDNA of fragments around 145 bp and a substantial proportion of fragments smaller than 145 bp.[83] This fragmentation signature could provide an alternative way to detect the presence of ctDNA in CSF that requires no prior knowledge of point mutations or SCNAs within the tumour. The fragment distribution is also different between plasma and urine, with smaller fragments in urine centred around 82 bp.[84,85]

The ability to extract and analyse small fragments of cfDNA therefore appears to be a critically important parameter in the detection of ctDNA. Importantly, wide variability in yield and fragment size across different extraction kits has been reported, making the choice of appropriate isolation method an important analytical parameter.[86,87] Moreover, single strand DNA template analysis revealed a higher proportion of shorter cfDNA fragments (below 80 bp) that are not readily detectable by standard double-stranded DNA library preparation protocols[48,88] implying careful consideration when choosing the method to analyse ctDNA.

cfDNA fragmentation in the analysis of the tissue of origin

Importantly, the results of the two large surveys of cfDNA fragmentation[78,80] have highlighted that both the overall size distribution and the fragmentation pattern throughout the genome varies across different cancer types, suggesting the potential for cfDNA size profiling to reveal the tissue source of cfDNA.[80] Indeed, the degree and diversity of the size fragmentation profiles reflect the different molecular structures that contain DNA (e.g. mononucleosomes, oligonucleosomes, hemi-nucleosomes, short sized transcription factors binding double strand DNA and so on) that are released from the cells and that undergo dynamic degradation in blood by endonucleases or exonucleases. Of note, the identification of the nucleases implicated in the fragmentation process in blood is still a subject of investigation.[89] It cannot be excluded that shorter cfDNA fragments could result from the degradation in blood of longer cfDNA originating from necrosis, phagocytosis, micro-particle-containing DNA, or active release from lymphocytes.[48] Nevertheless, nucleosome positioning, which defines DNA accessibility to nucleases, appears to play a significant role in shaping such cleavage patterns. As nucleosome positioning is an epigenetic determinant of gene expression that is cell- or tissue-specific,[90] it has been hypothesised that the tissue-of-origin of cancer could be inferred from nucleosome positioning.

The location of nucleosomes along genomic DNA can be uncovered by cfDNA deep sequencing features such as the number and distribution of fragments or the distribution and/or orientation of their endpoints.[88,91–93] Indeed, the number of fragments across the genome defining a depth coverage pattern reflects the nucleosome protection of DNA, and correlates with the results of nuclear chromatin micrococcal nuclease (MNase) sequencing assays in cell lines.[91,92] In MNase assays, digestion with the endonuclease allows the periodic spacing of assembled nucleosomes to be unravelled as the enzyme preferentially cleaves the exposed internucleosomal linker region of the chromatin. Therefore, it was hypothesised that the cfDNA cleavage pattern, which retains the characteristics of chromatin structure, can be exploited to infer tissue of origin and estimate gene expression. cfDNA read depth data from the plasma of healthy donors demonstrated peak patterns that correlated closely with those found in the micronuclease map of a lymphoblastoid cell line, further confirming the consistency of nucleosome positioning between cfDNA and its cognate tissue of origin and that cfDNA shed in the bloodstream of healthy donors mainly originates from the haematopoietic system[91,92] a finding further confirmed by a genome-wide map of nucleosome occupancy in cfDNA.[88]

Open chromatin regions are recognised as regulatory elements with well-positioned nucleosomes arrays flanking a depleted nucleosome region in the centre. This region of the chromatin is tissue specific.[94,95] Sun et al. introduced differentially phased fragment end signals, which represent differences in the read densities of sequences corresponding to the orientation of the upstream and downstream ends of cfDNA molecules in relation to the reference genome.[93] The

quantification of differentially phased cfDNA fragment ends allowed to unravel specific fragmentation patterns within the cfDNA molecule. These cfDNA patterns were identical to nucleosomal signatures found in tissue open chromatin region. Using this analytical approach, authors could identify lymphoblastoid cells as well as the liver as important contributors to the plasma DNA pool in healthy individuals.[93] Such a finding confirmed the hypothesis that cfDNA would only show the characteristic fragmentation patterns at open regions of chromatin where the corresponding tissues contributed DNA in the plasma. It appears that elucidating nucleosome positioning opens promising new perspectives to identify the tissue source of origin of cancer from cfDNA, with an important clinical value to classify cancers and, to a further extent, to characterise cancers of unknown origin, for example. The quantification of differentially phased cfDNA fragment ends applied to the plasma DNA from hepatocellular carcinoma and lung cancer patients correlated with the tumour DNA fraction (measured by CNA) in plasma and could identify the contribution of the corresponding tumoural tissue of origin.[93] Using another approach, Snyder et al. showed that nucleosome spacing inferred from cfDNA could also correctly identify the contribution of tumoural lineages in cfDNA from four metastatic cancer patients who presented with a high proportion of tumour-derived cfDNA.[88]

cfDNA fragmentation for the analysis of gene expression

It seems that cfDNA fragmentation could also reflect a general picture of gene expression. By focusing on short cfDNA fragments, Snyder et al. showed that nucleosome positioning directly harbours footprints of the in vivo occupancy of DNA-bound transcription factors.[88] Indeed, the loss of nucleosome positioning on both sides of transcription starting sites (TSS) is necessary for proper gene expression, to create a nucleosome-depleted region over the promoter that allows transcription factors to bind. Ivanov et al. used whole-exome sequencing data to demonstrate that cfDNA coverage downstream of TSSs reflects the classic silenced and highly expressed gene patterns.[91] The data did not cover the region upstream of TSS, including the nucleosome-depleted regions, as the capture of cfDNA during library preparation targeted only the exome and the untranslated region (UTR), enabling the prediction of expression possible for only a limited number of genes. Ulz et al., however, used whole-genome sequencing data to cover the entire promoter region in their analysis.[92] Two different regions were identified within TSSs at which different read depth coverage patterns for expressed and silenced genes were determined by nucleosome occupancy. Accordingly, a reduction in nucleosome occupancy for expressed housekeeping genes corresponded to decreased coverage.

A key point to address would be whether cfDNA datasets from cancer patients could predict the expression of the corresponding genes in their tumours. However, this represents a challenging task due to the various proportions of DNA released from tumour and nontumour cells, and preliminary in silico simulations showed that more than 75% of cfDNA fragments for a given TSS must be released by tumour cells to be able to infer expression status. In two patients with metastatic breast cancer presenting a high proportion of ctDNA, isoforms of cancer driver genes were identified in regions with somatic CNAs from cfDNA analysis and determination of their expression was confirmed by RNA sequencing of the matching primary tumour.[92] Fragmentation patterns from WGS data of plasma DNA have been used to infer the accessibility of transcription factor binding sites, and this approach has enabled tumour subtypes to be predicted in prostate cancer patients, as well as the detection of early stage colorectal carcinomas,[96] emphasising the clinical potential of this minimally invasive approach. Application of this method to track and decipher tumour resistance mechanisms driven at the transcriptional level (like tumour phenotype switching upon targeted therapies or immunotherapy) would be of high interest. However, these studies traditionally require a high content of bioinformatics analysis that is not readily amenable to routine diagnosis.

DNA METHYLATION

Understanding how other epigenetic phenomena such as methylation patterns or histone modification can affect cfDNA fragment size could also contribute to the improved identification of cancer patients. CpG islands are regions of DNA of at least 200 bp that contain a large number of CpG dinucleotide repeats; they are usually found within the promoter region and/or within the first exon of more than 60% of human genes. Under physiological conditions, CpG islands are usually unmethylated, whereas most CpG dinucleotides outside CpG islands are methylated. During cellular transformation, however, methylation profiles are reversed, with hypomethylation of CpG dinucleotides outside CpG islands and hypermethylation of CpG islands.[97]

Approaches to analyse methylation

In tissue, three major methods have been developed to differentiate methylated from unmethylated DNA. The most widely used technique for mapping DNA modification involves bisulphite treatment, during which unmethylated cytosine is deaminated to uracil while leaving methylated cytosine unchanged. The bisulphite-treated DNA can then be analysed by methylation-specific PCR (MSP) or sequencing, for example. Another popular method uses methylation-sensitive restriction enzymes prior to DNA amplification and detection: the methylation-sensitive enzymes digest only unmethylated CpG-containing motifs, generating digested DNA fragments that are enriched for unmethylated CpGs at their ends. Finally, affinity-enrichment-based methods have also been used in methylation status profiling. The methylated DNA immunoprecipitation (MeDIP) approach relies on anti-methylcytosine antibodies whereas a similar approach uses methyl CpG-binding-domain proteins to enrich for methylated DNA.[98,99] All these methods can be combined with high-throughput analysis such as NGS. As such, a large number of differentially methylated genes can be identified in a single experiment.

The analysis of methylation in liquid biopsy samples from cancer patients, however, is much more challenging due to the minimal amounts of tumour-derived cfDNA in plasma.[100] Consequently, affinity-based enrichment approaches such as MeDIP are relevant in the detection of cfDNA methylation.[101] Despite bisulphite treatment being harmful for cfDNA, as it leads to damage and loss of the starting material, it remains the gold standard method for deciphering methylation in cfDNA. A 2019 study adapted the reduced representation of bisulphite sequencing (RRBS) method for the analysis of cfDNA methylation in liquid biopsy samples (called cf-RRBS); this approach avoids the high cost of whole-genome bisulphite sequencing (WGBS), which requires deep sequencing for a reliable cfDNA methylation analysis and is not suitable for routine use.[102] In cf-RRBS, all 'off-target' cfDNA fragments not generated by the methylation-sensitive enzyme (MspI) are specifically degraded, thereby focusing the analysis on the 'on target' regions.

Potential clinical application of cfDNA methylation analysis

The clinical potential of cfDNA methylation analysis in cancer has been demonstrated in numerous studies investigating mainly single gene methylation profiles in different cancer entities (reviewed extensively elsewhere in refs. [103–105]). These studies have shown that methylated cfDNA derived from plasma or serum was associated with several clinical applications ranging from monitoring treatment and predicting response to therapy to indicating prognosis and detecting neoplastic lesions. A very recent study explored plasma methylome of metastatic castration-

resistant prostate cancer patient and revealed hypomethylation of AR binding sequences associated with AR copy number gain. Patients with such methylation pattern were shown to have a more aggressive clinical course.[106] Notably, methylation status evaluation of diverse genomic elements in cfDNA will become of high interest in the context of the emergent promising concept of epigenetic therapy combination with immune oncology drugs in the next future.[107,108]

Furthermore, other studies have generated prediction models for tumour burden based on the methylation profile of plasma cfDNA.[109]Methylation patterns are unique to each cell type and remain highly stable under physiological and pathological conditions such as cancer.[110] As such, plasma DNA methylation analysis might have the potential to detect tissue of origin for cfDNA, thereby aiding in cancer classification and characterisation. The application is not restricted to cancer, with Poon et al.[111] and Lun et al.[112] reporting differential methylation in cfDNA from foetal and maternal blood during pregnancy. Similarly, Lehmann–Werman et al.[113] used targeted sequencing of methylation-tissue-specific markers to trace back the tissue of origin of cfDNA (pancreatic β-cell DNA, oligodendrocyte DNA, neuronal/glial DNA and exocrine pancreas DNA) in plasma and thus detect cell death in specific tissues from patients with type 1 diabetes and islet-graft recipients, relapsing multiple sclerosis, traumatic brain injury or cardiac arrest, pancreatic cancer or pancreatitis, respectively. These pioneering studies opened up the field for the study of cfDNA methylation patterns for early detection of cancer. Plasma cfDNA tissue of origin mapping was also confirmed by Sun et al.[114] while performing whole-genome-wide bisulphite sequencing (WGBS) on plasma DNA coupled with a deconvolution process to unravel the contributions of different tissue types to the plasma DNA pool, notably in the context of cancer disease.

Although promising, such studies are challenging to reproduce because of the high cost and the time-consuming nature of the genome-wide bisulphite sequencing (WGBS) technique. However, it is worth mentioning that only the relative contribution of cfDNA from different tissues is determined by methylation deconvolution based on a sequencing method and not the absolute concentration of cfDNA originating from each tissue. It would be particular interesting to ascertain the absolute concentration of cfDNA when more than one organ is suspected to release DNA, which is the case for metastatic tumours, for example. Consequently, and in order to overcome the high expenses and technical challenges that still present a hurdle in the methylation deconvolution process, digital PCR-based methods might be a solution due to their cost effectiveness and high turnaround time. Gai and co-workers developed a ddPCR assay for the detection and quantification of plasma DNA derived from the liver and the colon by targeting specific regions that are differentially methylated in the tumour-bearing tissue (liver and colon) when compared with other types of tissue.[115] In a broader approach, Shen et al. successfully used MeDIP coupled to sophisticated bioinformatics tools to distinguish multiple types of early stage cancers with high sensitivity;[101] this study also confirmed the consistent overlap of the epigenetic signature between the primary tumour and the plasma DNA as important prerequisite for future clinical applications of cfDNA methylation-based liquid biopsies.

VIRUS-SPECIFIC DNA ELEMENTS

The non-human origin of viral DNA makes it a highly interesting and specific marker for monitoring virus-associated cancers using liquid biopsy samples. We now know that several different cancer types are closely linked to specific viral infections. More than 99% of cases of cervical carcinoma are attributable to human papillomavirus (HPV) infection whereas around 30% of oropharyngeal head and neck squamous cell carcinoma (HNSCC) cases are considered to be caused by persistent HPV infection. HPV comprises a large group of double-stranded DNA viruses, of which around 15 are considered high risk types, causing different squamous epithelial cancers including cervical, vaginal, vulvar, penile, anal and oropharyngeal. The double-stranded DNA virus Epstein–Barr virus (EBV) as well as persistent infections (viral and bacterial) are associated with certain cancers such as nasopharyngeal carcinoma (NPC) and gastric cancer and non-Hodgkin lymphoma in children.[116,117]

Several studies have shown that circulating viral DNA is detectable the plasma of patients with HPV-and EBV-associated cancers, with plasma HPV DNA shown to be a highly sensitive and specific biomarker, especially when detected using digital PCR-based methods.[118] Table 2 shows studies in which the detection of circulating HPV DNA has been assessed in serum or plasma from patients with different HPV-associated cancers. Most studies on cervical cancer have involved rather small groups of patients, except for the larger 2019 study by Cheung et al., in which pretreatment blood from 138 patients with cervical cancer was analysed for the presence of HPV E7 and L1 sequences.[119] HPV DNA was detected in 61.6% of patients, and patients with a high viral load had an increased risk of disease recurrence and death at 5 years in univariate but not multivariate analysis. Furthermore, Cocuzza et al. showed that in 34.2% of women with low grade or precancerous cervical lesions, HPV cfDNA can be detected and quantified in plasma samples, an observation that paves the way for the potential use of blood as an additional prescreening tool in parallel with cervical smears.[120] For HNSCC, the results of larger studies have been published. In a 2018 meta-analysis of data from 600 HNSCC patients from five studies investigating circulating HPV DNA as a biomarker for disease progression, the pooled sensitivity in detecting recurrence was 54% (95% CI [confidence interval]: 32–74%) and the pooled specificity was 98% (CI: 93–99.4%), with a positive predictive value (PPV) of 93% and a negative predictive value of 94%.[121] The data clearly indicate that circulating HPV DNA is a promising tool for surveillance in patients with HPV-associated HNSCC. Interestingly, the combined use of HPV analysis in both saliva and plasma might increase the sensitivity and specificity of the assays. Ahn et al. showed that the posttreatment HPV16 DNA status was 90.7% specific and 69.5% sensitive in predicting recurrence within 3 years in HNSCC patients when plasma and saliva results were combined.[122] Wang et al. showed that the analysis of saliva seems to be especially sensitive in cancers of the oral cavity, whereas plasma is preferentially enriched for tumour DNA from other sites.[123] Additional papers on saliva-based liquid biopsies have also shown promising results, especially in oropharyngeal cancer.[19,124,125]

The role of circulating EBV in NPC has also been assessed in many studies.[126] The presence of plasma EBV-DNA has been shown to be of clinical value in prognostication,[127,128] monitoring of recurrence[129,130] and even in screening for NPC.[131] Leung et al. showed that EBV-DNA load at the midpoint of a radiotherapy course can predict outcome in NPC patients.[129] Of the 107 patients investigated, 35 patients failed therapy; EBV-DNA was detectable in 74% of these patients. EBV detection was more predictive of outcome than was tumour stage.[129] In another similar study of a cohort of 949 NPC patients, high EBV-DNA loads before treatment, at mid-treatment and at the end of treatment were all associated with significantly poorer overall survival, distant metastasis-free survival and progression-free survival.[132] Recently, Lv et al. quantified cfEBV copy numbers longitudinally in 673 locally advanced nasopharyngeal carcinoma patients. The inter-patient heterogeneity in viral copy number clearance was used to define prognostic phenotypes distinguishing early, intermediate, late and no responders to chemotherapy. These data suggest that real-time monitoring of cfEBV response adds prognostic information and might have potential utility for risk-adapted treatment in NPC.[133]

Table 2. Studies measuring circulating HPV DNA in different HPV-associated cancers.

Cancer entity	Number of patients	Detection method	Detection rate	Clinical association	Reference
Anal carcinoma	57	ddPCR (HPV16)	91.1% at baseline samples, 38.9% after 5 months of chemotherapy	Residual HPV cfDNA detected at completion of chemotherapy was associated with shorter PFS and 1-year OS	[148]
Anal carcinoma	33	ddPCR (HPV16 or 18)	87.9% of stage II–III patients at baseline. After chemoradiotherapy 17%	HPV cfDNA after chemoradiotherapy was significantly associated with shorter DSF	[149]
Cervical carcinoma	138	ddPCR (E7 and L1)	61.6% at baseline	High viral load (≥20 E7 or L1 copies in 20 µL reaction volume) had increased risk of recurrence and death at 5 years	[119]
Cervical carcinoma	21	junction-specific PCR	23.9% at preoperation	HPV cfDNA significantly associated with reduced PFS	[150]
Cervical carcinoma	19	ddPCR (HPV16 and 18)	100% at baseline, 0% in healthy controls	Persistent clearance of HPV cfDNA was only observed in patients with complete response	[151]
Cervical ($n = 47$), anal ($n = 15$) oro-pharynx ($n = 8$) carcinoma.	70	ddPCR (HPV16 and 18, E7)	87% at baseline	HPV cfDNA levels in cervical cancer were related to the clinical stage and tumour size	[152]
Cervical carcinoma and dysplasia	68	PCR + RFLP	11.8%		[153]
Cervical carcinoma	16	qPCR (HPV16 and 18, E7)	81.2%	HPV cfDNA concentration in patients serum was related to tumour dynamics.	[154]
Cervical dysplasia	120	qPCR (7 HPV variants)	34.2%		[120]
HNSCC	200	TaqMan-qPCR (HPV17 and18)	14%	Baseline HPV cfDNA was associated with higher N stage and stage IV	[155]
HNSCC	47	ddPCR (HPV16 or 18)	86% at baseline	The combined saliva and plasma analysis detected in 96% HPV cfDNA	[123]
HNSCC	70	qPCR (E7)	17%		[156]
Oropharyngeal carcinoma	262	qPCR (HPV16 E6/7)	87% at baseline among HPV-pos patients, 11.5% in HPV-neg patients	Baseline HPV cfDNA was associated with higher N stage and overall disease stage.	[157]
Oropharyngeal carcinoma	93	qPCR (HPV16 E6/7)	67.3% at baseline	The combined saliva and plasma posttreatment HPV cfDNA status was 90.7% specific and 69.5% sensitive in predicting recurrence within 3 years.	[122]
Oropharyngeal carcinoma	40	qPCR (E6/7)	65% at baseline	HPV cfDNA correlated significantly with the nodal metabolic tumour volume with persistent clearance in patients with complete response	[158]

DFS disease-free survival, *ddPCR* droplet digital PCR, *qPCR* quantitative PCR, *HNSCC* head and neck squamous cell carcinoma, *HPV* human papilloma virus, *OS* overall survival, *PFS* progression-free survival, *RFLP* restriction fragment length polymorphism.

A paradigm-shifting paper on the use of circulating viral DNA for NPC screening was published by Chan et al. in 2015.[131] Of 20,000 screened asymptomatic individuals, 309 tested persistently positive for EBV, 34 of whom went on to have confirmed NPC. The sensitivity and specificity of the presence of EBV-DNA in plasma was found to be 97.1% and 98.6%, respectively. Importantly, these 34 patients were detected at earlier disease stages and thus had a better outcome than patients in historical cohorts.[131] In order to improve the PPV for NPC screening, the same group further analysed the molecular nature of EBV-DNA in the plasma of subjects with and without NPC by target-capture sequencing and identified differences in both the abundance and size profiles of plasma EBV-DNA molecules. NPC patients had significantly more plasma EBV than disease-free patients and exhibited a reduction in the 166-bp peak (mean size of cfDNA), but showed a more pronounced peak at around 150 bp. Furthermore, compared with non-NPC subjects, NPC patients had fewer EBV-DNA molecules that were shorter than 110 bp. By combining quantitative and size-based characteristics of plasma EBV-DNA, the authors achieved a false-positive rate of 0.7% and a PPV of 19.6% using single time-point testing without the need for a follow-up blood sample.[134] EBV infections are also associated with gastric cancer, accounting for 8–9% of all gastric cancer cases. In a 2019 large prospective study of 2760 gastric cancer patients, 52.1% (73/140) of EBV-associated gastric carcinomas had detectable EBV-DNA.[135] Furthermore, the plasma EBV-DNA load was found to be associated with treatment response, with the load decreasing in responders but increasing with disease progression.

Taken together, the detection of viral DNA in plasma and, in certain cases, saliva in virus-associated cancer has shown a high specificity and even potential for early screening. However, many studies still lack the statistical power to detect disease recurrence, especially among cancer patients with good prognosis. Thus, large prospective studies such as those on NPC from Lo and co-workers[131] need to be more widely performed to evaluate the clinical relevance of these liquid biomarkers in other, different tumour entities.

CONCLUSIONS AND PERSPECTIVES

Increasing amounts of data have shown that it is possible to gain information beyond mutations from cfDNA obtained from the

blood plasma of cancer patients, such as from the analysis of fragmentation patterns or methylation status, which are particularly informative regarding the regulation of gene expression. Human malignant tumour cells exhibit pervasive changes in DNA methylation patterns, which consequently lead to perturbations in gene expression or genomic instability. Deciphering these aberrant epigenetic modifications is of primary importance in light of the potential clinical perspectives in cancer management, ranging from early cancer detection to estimating prognosis and monitoring therapy response. Studies on cfDNA have also shown the emerging clinical potential for the early detection of virus-associated cancers, taking advantage of the lower complexity of different causative viral DNAs compared with the complex spectrum of somatic mutations in solid tumours. Nevertheless, a remaining challenge will be to distinguish transient viral infections from cancer-causing persistent infections. The detection of viral ctDNA sequences can also provide important basic information on the biology and kinetics of cfDNA in blood plasma. Serial monitoring of EBV load in plasma from NPC patients who have undergone nasopharyngectomy revealed that plasma EBV cfDNA was cleared at a rate that followed the first-order kinetics model of decay with a median half-life of only 139 min.[136] The data show that the elimination of EBV-DNA is very rapid and a blood draw after surgery might be therefore an even better predictor for disease recurrence than the baseline measurement.

An important prerequisite for the introduction of the analysis of cfDNA into cancer diagnostics is the standardisation of preanalytical and analytical variables of the existing cfDNA technologies. For this purpose, international consortia including partners from academia and industry, such as CANCER-ID or the ELBS, have been established and ring experiments (same samples or methods used in parallel at several sites)—have been performed.[137] In addition, a better understanding of the parameters that affect the release of DNA by tumour cells and host cells, as well as the effects of renal clearance, carrier proteins or extracellular vesicles in the blood plasma, thereby influencing the concentration of ctDNA and cfDNA in cancer patients,[138] would be of great importance. Increasing data suggest that other non-blood-based liquid biopsy approaches based on e.g. saliva, CSF or urine are reliable for inclusion in future clinical trials. Finally, it should be mentioned that other liquid biopsy analytes, such as circulating tumour cells, circulating microRNAs, tumour-educated platelets or tumour-associated proteins, might provide complementary information on tumour evolution and response to therapy in cancer patients.[28,55,139] Consequently, the development of a complex multi-analyte biomarker panel, which would require sophisticated bioinformatics tools such as machine-learning algorithms,[140] could contribute significantly to the noninvasive management of individual patients with cancer.

To sum up, the concept of liquid biopsy introduced 10 years ago[141] has opened new avenues in cancer diagnostics, and interventional clinical trials with established outcome measures are now needed to further demonstrate the clinical utility of ctDNA and other biomarkers.

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AUTHOR CONTRIBUTIONS

L.K. and Y.B. contributed equally to the work. L.K., Y.B. and H.W. researched the data for the review and wrote the manuscript. K.P. worked on the manuscript and supervised the project. All authors edited the manuscript before submission.

ADDITIONAL INFORMATION

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7. Publikation 4

Current and Future Clinical Applications of ctDNA in Immuno-Oncology

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ABSTRACT

Testing peripheral blood for circulating tumor DNA (ctDNA) offers a minimally invasive opportunity to diagnose, characterize, and monitor the disease in individual cancer patients. ctDNA can reflect the actual tumor burden and specific genomic state of disease and thus might serve as a prognostic and predictive biomarker for immune checkpoint inhibitor (ICI) therapy. Recent studies in various cancer entities (e.g., melanoma, non–small cell lung cancer, colon cancer, and urothelial cancer) have shown that sequential ctDNA analyses allow for the identification of responders to ICI therapy, with a significant lead time to imaging. ctDNA assessment may also help distinguish pseudoprogression under ICI therapy from real progression. Developing dynamic changes in ctDNA concentrations as a potential surrogate endpoint of clinical efficacy in patients undergoing adjuvant immunotherapy is ongoing. Besides overall ctDNA burden, further ctDNA characterization can help uncover tumor-specific determinants (e.g., tumor mutational burden and microsatellite instability) of responses or resistance to immunotherapy. In future studies, standardized ctDNA assessments need to be included in interventional clinical trials across cancer entities to demonstrate the clinical utility of ctDNA as a biomarker for personalized cancer immunotherapy.

Introduction

Over the past decade, the identification of the molecular mechanisms by which tumor cells hamper immunity marked the coming of a new era in the management of cancer patients. Since first immune checkpoint inhibitor (ICI) approval in unresectable malignant melanoma (1), up to 15 different clinical entities, comprising both solid and hematologic malignancies, currently benefit from an FDA-approved indication for ICI-based treatment (2) and the field of applications is rapidly evolving. Notably, the repertoire of immune-oncology (IO) therapeutic options is constantly expanding by targeting additional immune checkpoints or costimulatory molecules, combining ICI with other therapeutic strategies (3, 4) and introducing innovative approaches based on T-cell bioengineering (5).

Early identification of relapse and early therapeutic intervention are essential determinants for improved overall survival. However, an objective biomarker associated with the efficacy of IO drugs is an urgent but still unmet clinical need.

The past decade has also seen the advent of liquid biopsy (6, 7). Contrary to tumor tissue biopsy, liquid biopsy gives access to tumor material in a minimally invasive way, therefore offering the patient a more acceptable, safer, and easily repeatable option to monitor tumor response. Liquid biopsy applies to detecting tumor cells or tumor-derived products like tumor DNA (referred to as circulating tumor DNA, ctDNA) mainly shed in peripheral blood and other body fluids. The field of ctDNA clinical applications is mainly based on mutation detection and has greatly benefited from significant improvements of detection methods in terms of sensitivity and multiplexing. The utility of monitoring tumor genomics through plasma ctDNA analysis has been widely investigated over the past years in diverse clinical settings (8, 9).

This review will present the different clinical applications of ctDNA analysis in the specific context of IO. We will discuss the capability of ctDNA, quantified either before or during therapy, to identify patients who will benefit from the treatment. We will finally describe ctDNA as a privileged substrate to study and monitor the genetic determinants of immunotherapy response, such as tumor mutation burden or microsatellite instability and underline the value of ctDNA-based decision-making in cancer treatments.

Pretreatment Levels of ctDNA as a Prognostic Biomarker in IO

Clinical value of pretreatment ctDNA levels in metastatic patients

Supplementary Table S1 recapitulates the studies investigating the correlation of ctDNA measured before the treatment with the primary clinical endpoints. Most of the studies were conducted on melanoma and NSCLC populations who received ICI either as a first or later-line therapy, according to the timing of drug approval. Recent pan-cancer studies and hematologic malignancies, implementing new IO strategies, highlight the advantage of ctDNA to be implemented agnostic to cancer types and independent from a specific ICI treatment, as long as one mutation can be detected. There is a high level of heterogeneity between the studies about the number of included subjects, the types of clinical cohorts, and the methodology adopted to measure ctDNA,

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including the detection of a single mutation—usually the driver—versus multiple with gene panels, different sensitivity thresholds, and quantification strategies. Notably, most of the studies so far have only demonstrated the clinical validity of ctDNA as a biomarker (10). The use of pretreatment ctDNA value as a biomarker in the clinic will therefore necessitate establishing precise pretreatment ctDNA cutoff points for each particular assay and for each particular tumor type. Moreover, interventional studies are needed to demonstrate the clinical utility of ctDNA measurements. Nevertheless, several investigations have identified a congruent association between undetectable ctDNA or low ctDNA levels [inferior to the cohort's median variant allele frequency (VAF)] and a longer progression-free survival (PFS) and overall survival (OS) in univariate analyses (11–18). Owing to the close relationship between ctDNA and tumor burden, well established in NSCLC (19–21) and melanoma (11, 12, 15, 22–26), the underlying influence of anatomic tumor disease burden in the duration of response to ICI therapy might partially explain the pretreatment ctDNA association to PFS or OS (27, 28). However, in the up-to-now most extensive study encompassing 16 different tumor types in 790 patients, ctDNA association with OS after adjustment for Eastern Cooperative Oncology Group (ECOG) performance status, baseline liver metastases, baseline lymph node metastases, smoking status, tumor burden, and tumor PD-L1 score suggests that ctDNA is not simply a surrogate marker for baseline tumor burden (29). In this line, the mutation selected to quantify ctDNA could also play a role in associating ctDNA levels to the clinical outcome since specific mutations identified in tumor tissues could have different prognostic values (refs. 30, 31; cf. chapter 4.1). As an additional confounding factor, the capability to equally detect all different mutations in ctDNA remains unclear, as reported in melanoma (17).

Contrary to PFS and OS, the association to objective response rate (ORR) is poorly reported. When mentioned, ORR and pretreatment ctDNA levels association was not significant (Supplementary Table S1). This observation rather confers to ctDNA pretreatment levels a prognostic value than a direct link to clinical efficacy. Of note, the pretreatment ctDNA levels discrimination of patients with durable or nondurable clinical benefit reported by Nabet and colleagues can be explained by a different evaluation model of clinical response from immune Response Evaluation Criteria In Solid Tumors (RECIST) criteria (32).

Pretreatment ctDNA levels associated with PFS and OS should also be differently examined between the first- or the second-line treatment setting. For instance in melanoma, pretreatment ctDNA levels are only associated with clinical outcome in patients receiving ICI therapy as a first line (13, 17). Brain metastasis development in patients who relapsed after first-line therapy might be one potential explanation for the limited discriminative capacity in the second-line setting due to an insufficient ctDNA detection. This observation merits further clinical investigations, notably in other tumor types where ICI therapy can be proposed in the second line.

Clinical value of pretreatment ctDNA levels in adjuvant immunotherapy

Adjuvant immunotherapy, by definition, is being applied to tumor-resected patients. Several reports in melanoma (33–36) or lung (20) and colorectal cancer (37) have shown that the prevalence of ctDNA-positive patients after resection is low despite the use of highly sensitive digital PCR techniques. To increase the sensitivity of ctDNA testing, one could particularly recommend the interrogation of multiple mutations with personalized gene panels based on the primary tumor sequencing, the analysis of higher volumes of plasma, and repeated sampling to increase the sensitivity of mutation detection (38). However, in the adjuvant setting, the low quantities of ctDNA and sequencing artifacts currently limit the usage of large sequencing panel assays. Error suppression strategies to reduce background error rate will be necessary to improve the analytical specificity of ctDNA assays (39). In this line, ctDNA detection via personalized profiling by cancer personalized profiling by deep sequencing (CAPP-seq) was associated with a better outcome in a cohort of 28 locally advanced NSCLC patients receiving ICI as consolidation therapy after adjuvant chemoradiotherapy (40). In a clinical trial comparing adjuvant administration of the anti-PDL1 antibody atezolizumab versus observation in operable urothelial cancer patients, ctDNA positivity (detected by patient-specific mutation) at the beginning of the treatment identified a high-risk population who will benefit from adjuvant ICI therapy (41). This study design paves the way for additional high level of evidence studies in other clinical entities aimed to achieve clinical utility of ctDNA testing in the adjuvant setting.

On-treatment ctDNA Measurement to Predict Clinical Outcome

ctDNA measurements can easily be repeated throughout therapy. On-treatment levels of ctDNA either were used to calculate ctDNA changes by comparison with ctDNA levels at baseline or were directly associated with clinical outcome. Supplementary Table S1 also details the corresponding studies.

Early ctDNA dynamics after the onset of systemic therapy

The terms ctDNA "dynamics," "kinetics," and "variations" denote changes in VAF or concentration measured between before the first and before subsequent treatment infusions. It is worth mentioning here that, besides tumor driver mutations, those encoding for neoantigens (42) or even chromosomal number aberrations (CNA; refs. 43, 44) were used to quantify ctDNA changes.

ctDNA decrease is associated with a higher ORR, PFS, and OS. However, studies significantly differ by the ctDNA change threshold (20%, 50%, nonspecified increase or decrease, complete clearance) and time point (after one infusion or more, between 4 and 8 weeks) to assess molecular response. In the future, it will be critical to harmonize the strategy to adopt by a precise definition of a cutoff and of the time point to compare with baseline. In addition, a better knowledge of ctDNA intraday variation (45, 46) and the reproducibility of the methods is necessary to identify actual biological ctDNA variations correctly. Again, the reported studies have provided evidence for the clinical validity of ctDNA monitoring while demonstration of its clinical utility is still pending (10).

In the metastatic setting, the superior association of early on-treatment ctDNA changes to clinical efficacy over baseline ctDNA values is noteworthy (32, 47). Indeed, as a direct reflection of tumor burden (11, 12, 22–26, 48–54), ctDNA changes would encompass all variables that contribute to overall tumor response.

CtDNA variations evaluated in the early course of therapy correlated to radiographic best response evaluated 5 to 12 weeks later, suggesting an exciting capacity to anticipate tumor response in NSCLC or metastatic melanoma (55–58). However, this conclusion can be inherently biased by the study's design (i.e., most studies report on radiologic evaluations performed in daily clinical routine later than ctDNA sampling). Anticipating tumor response presents several advantages for the clinician, notably in case of treatment interruption due to severe side effects or in patients presenting with stable disease at their first assessment, to identify those who will finally go in clinical

response (29, 32, 55). Nevertheless, the agreement between the first radiologic evaluation of tumor response and ctDNA evolution profile is not total (59, 60). For instance, 23% of the patients present discordant ctDNA kinetics from the first RECIST evaluation (59). Pseudoprogression, defined as a radiologic finding of disease progression before response caused by various immune cells infiltrating the tumor mass, thus contributing to increased tumor volume, can be one source of discordance (61). Although not frequent (incidence range, 0% to 9.7%), pseudoprogression is a specific challenge associated with ICI treatment. One study in 29 metastatic melanoma patients treated with PD-1 antibodies has demonstrated that decreasing ctDNA profiles can accurately differentiate pseudoprogression from a proper disease progression (62). First RECIST evaluation cannot be considered as an accurate predictor of clinical outcome for ICI (32, 55), mandating additional response assessment at later time points during tumor evolution. Therefore, it will be necessary to explore early ctDNA variations with clinical benefit determined several months later to better understand its potential to guide clinician decisions. In this line, Nabet and colleagues acknowledge that ctDNA early kinetics misclassified 25% of NSCLC patients for durable clinical benefit (32), highlighting the need for continuous monitoring of ctDNA throughout the therapy. The limited value of ctDNA as a biomarker of intracranial response suggests that ctDNA measurements and clinical imaging are not redundant but rather complementary. In metastatic melanoma, intracranial disease control did not associate with on-treatment ctDNA favorable profiles or undetectability (11, 12). Properly designed studies with simultaneous assessment of tumor response by both methods would provide interesting hints to understand this complementarity better and build more accurate models to predict clinical outcome (32, 55, 59). Exploring the cerebrospinal fluid as another compartment for liquid biopsy would also be a good alternative for clinicians (63).

Measuring ctDNA variations could also be applied to predict other immunotherapy regimes' efficacy. CtDNA clearance after the first cycles of treatment identified responders to adjuvant therapies in urothelial carcinoma or NSCLC (40, 41).

Recent advancements in immunotherapy have allowed treatment of relapsed or refractory diffuse large B-cell lymphoma thanks to CD19-targeted chimeric antigen receptor T cells (CAR T cells; ref. 64). In a pilot study of six patients, investigating specific clonotypic V(D)J rearrangements in ctDNA through the treatment could predict patient response to CAR-T cell therapy (65). In a subcohort of patients with metastatic cervical cancer treated with tumor-infiltrating lymphocyte therapy (TIL), a solid but transient HPV peak detected in cfDNA, immediately after the TIL therapy start was preferentially observed in patients with a complete and long-term response to TIL therapy (66). A similar post-TIL ctDNA "flair" was also observed in melanoma patients (67).

Association between on-treatment ctDNA concentrations and clinical outcome

Among the aforementioned studies, some have also directly correlated the ctDNA levels after the first cycles of ICI therapy to a clinical endpoint, with notable superiority to predict clinical efficacy over pretreatment levels of ctDNA (cf. Supplementary Table S1; refs. 11, 29, 47, 59, 68). Whether on-treatment levels or ctDNA variations is the most accurate way to predict the clinical outcome is still an open question. The disadvantage of ctDNA dynamics could be that, depending on its calculation mode, it can equalize patients presenting low pretreatment ctDNA and significant decrease with patients presenting high pretreatment ctDNA and smaller decrease. As such, an integrated metric defined as the ratio of on-treatment VAF to pretreatment VAF had a superior association with immunotherapy outcomes than on-treatment levels (29). On the other hand, ctDNA clearance was associated with the most favorable outcome profile (14, 29, 55, 59, 69), and conversely, detection of high levels of ctDNA is associated with future progression (14). Therefore, stratifying patients by both pretreatment and on-treatment levels and distinguishing ctDNA clearance should result in the most accurate evaluation of patient outcome, as initially suggested by Lee and colleagues in melanoma patients (12) or more recently by Zhang and colleagues (29). More studies comparing on-treatment ctDNA with on-treatment RECIST tumor evaluation would also be necessary to understand the complementarity between the two approaches better (70).

Even with favorable ctDNA kinetics, some of these patients will ultimately progress, and early ctDNA variations might not be able to discriminate long-term responders. Some studies have then evaluated ctDNA level at later time points of therapy. In NSCLC, 31 blood samples from patients achieving long-term benefit were collected at a median of 26.7 months after initiation of therapy (71). At this surveillance timepoint, 25/27 patients with undetectable ctDNA remained progression-free while all four patients with detectable ctDNA eventually progressed. A similar observation was reported in 38 melanoma patients evaluated after cessation of ICI therapy (72). Of the 28 patients with no progression, ctDNA was undetectable in 27 patients, and among the ten patients who progressed, four had detectable ctDNA at the time of treatment cessation. These independent observations corroborate the hypothesis raised by Bratman and colleagues in which ctDNA clearance at any time point during the therapy is associated with long-term survival (59). Concerning the lack of knowledge in the optimal treatment duration (and its consequences in terms of potentially severe side-effects exposure and financial costs), both studies pave the way for additional ctDNA evaluation later in therapy to better discriminate patient personal benefit. In this setting also, the usage of highly sensitive methods to detect ctDNA will be necessary to reduce the probability of false-negative results.

Genetic Determinants of Response to IO Therapies Assessed on ctDNA

In addition to a quantitative assessment, other genetic determinants of ICI therapy response can also be measured on ctDNA, such as the association of specific mutations to ICI therapy outcome, the assessment of tumor mutational burden and microinstability phenotype.

Status of specific cancer mutations relevant to therapy

As a surrogate of tumor tissue, plasma genotyping could also be used to directly evaluate the association of tumor-specific molecular alterations with response to ICI therapy or with the onset of immune-related adverse events. After excluding patients with no detectable ctDNA, Guibert and colleagues confirmed a better prognosis in patients harboring *TP53* or *KRAS* mutations and the detrimental effect of STK11 mutations and loss of *PTEN* compared with wild-type patients (30). Similarly, in 38 metastatic gastric cancer patients, the mutation status of *TGFBR2*, *RHOA*, and *PREX2* in ctDNA at baseline negatively influenced the PFS (31). In the same metastatic gastric cohort, patients with alterations in *CEBPA*, *FGFR4*, MET, or *KMT2B* detected in plasma at baseline had a greater likelihood of experiencing irAEs (31). In classic Hodgkin lymphoma (cHL), CHD8 mutation in ctDNA was only detected in patients with the longest PFS (73).

Repetitive sampling throughout therapy is the main advantage offered by ctDNA analysis. Large gene panels or whole-exome sequencing (WES) analysis on ctDNA depicting tumor clonal evolution can lead to identifying specific mutations implicated in resistance to immunotherapy. Mutation in *FOXL2* and *RHOA* genes and copy-number variation of *FGFR2* gene were identified as candidate resistant mechanisms after plasma analysis of 13 metastatic gastric cancer patients who had initially benefited from the treatment (31). Serial sequencing of ctDNA with a 329 pan-cancer--related gene panel and WES identified mutations in *PTCH1* and *B2M* genes in two out of four NSCLC patients with progressive disease (74). WES on ctDNA performed on eight different NSCLC patients reported alterations of Wnt-signaling pathway-related genes, an increase of copy-number aberrations in cancer-related genes, and loss of PTEN or B2M as molecular mechanisms associated with late progression (i.e., progression observed after six months of treatment) to ICI therapy (75). Considering the broader usage of comprehensive genome sequencing in the near future, one could strongly emphasize the need for additional studies across different clinical entities with regular plasma sampling to decipher the tumor molecular landscape at the onset of resistance to immunotherapy.

Tumor mutational burden

Following the hypothesis that the more nonsynonymous mutations are present in the tumor DNA, the more neoantigens will be presented at the surface of the tumor, tumor mutational burden (TMB; i.e., the number of somatic mutations per megabase of interrogated genomic sequence) has been extensively explored as an additional predictor of clinical benefit in ICI therapies. However, the correlation between a high TMB and better response to ICI therapy is still not completely established, varying between cancer entities (76–78). If WES would be the most accurate way to assess TMB in tumor tissue (named tTMB hereafter), panel sequencing-based estimates of TMB were mainly used in the clinic so far. Nevertheless, a lack of standardization in TMB score determination due to technical features (i.e., location and size of the sequenced regions, types of mutation detected, differences in the germline mutations filtering methods, and mode of calculation of TMB score) prevents TMB score comparison across platforms and tumor types (76, 77, 79–81) and has led to the recent initiative of establishing harmonization guidelines (82). Moreover, tTMB determination on a single biopsy can also be affected by intratumor heterogeneity and might evolve with treatment.

As an alternative to tissue determination, blood-based determination of TMB (bTMB) could overcome the double problem associated with repeated access to tumor material and tumor heterogeneity (83–86). However, bTMB assays face specific challenges, such as tumor-derived molecules' input varying upon cancer type and clonal hematopoiesis (87, 88). Importantly, standardization in bTMB assays is also currently lacking but should rapidly benefit from the harmonization efforts currently ongoing for tTMB determination.

Nevertheless, bTMB via ctDNA analysis with multiple gene panels was first evaluated as a surrogate for tTMB. Despite the use of different gene panels and independent cohorts of patients, a similar level of correlation (R around 0.6) between tissue and plasma was reported (89–91) The absence of a higher correlation between tTMB and bTMB could originate from the intratumor heterogeneity. However, a low VAF and an extended time interval between blood and tissue collection in some cases could also explain the reported level of correlation (90).

bTMB was then evaluated as a predictor of ICI therapy outcome. Like for tTMB, there is an association between a high bTMB score and a better ORR and improved PFS and OS in NSCLC patients (90, 92, 93). However, no association with OS was reported by several studies (94, 95), leading Wang and colleagues to question ctDNA-based TMB determination rationale. Patients with the highest amount of ctDNA have the highest number of mutations and the highest tumor burden, and both situations result in a contradictory effect on OS. Upon adjustment by VAF, bTMB-high eventually associated with improved ORR, PFS, and OS in uni- but also multivariate analysis (96). Still, prospective studies are needed to validate the predictive efficacy of low allele frequency bTMB. Interestingly, Nabet and colleagues recently addressed this issue by defining normalized bTMB as the ratio of bTMB and ctDNA level. Normalized bTMB was superior to both individual metrics (bTMB and ctDNA levels) for predicting durable clinical benefit (32).

Microsatellite instability

In colorectal cancer, microsatellite instability (MSI) was associated with a high Th1/CTL infiltration and upregulation of immune-checkpoint proteins, suggesting a link between MSI and response to ICI (97). Like for TMB, minimally invasive determination of MSI is highly desirable in a context of a constantly expanding usage of ICI therapy.

Next-generation sequencing (NGS)-based approaches can nowadays determine MSI by measuring the length of altered microsatellites sequences (98–101). Several NGS-based assays were recently developed on cfDNA to determine tumor MSI status by overcoming the technical challenges associated with detecting low-level allele length polymorphisms in coexisting excessive amounts of wild-type DNA and PCR originating errors on long mononucleotides repeats (98, 102–104). Despite a lack of consensus on the selected loci number and nature, the different NGS assays had a sensitivity around 0.1%–1% tumor fraction and presented a high concordance with tissue MSI status (102–104). Landscape studies performed in large plasma samples sets from cancer patients reported an MSI-high prevalence among tumor types similar to the one observed with tissue-based analyses (102, 104). This approach paves the way for a pragmatic strategy to identify better the subset of patients who might benefit from ICI therapies, especially in tumor types where the benefit of the IO treatment is not yet fully established. In small cohorts of gastrointestinal cancers treated by ICI therapy, patients detected with an MSI phenotype had significantly prolonged PFS (98, 102, 104), demonstrating clinical validity of the developed assays.

NGS-based methodologies present the advantage to enable simultaneous determination of the MSI status of the tumor together with detection of other genomic determinants of response to ICI therapy like TMB. The European Society for Medical Oncology (ESMO) recommendations on MSI tissue testing for immunotherapy in cancer stated that the relationships between MSI and TMB are complex and differ according to tumor types (105). Studies exploring the complementarity between these two biomarkers are needed to predict the outcome of ICI more finely. In this line, Willis and colleagues observed a significantly superior number of SNV in MSI-high than in microsatellite stable (MSS) patients (102). Wang and colleagues, in a pan-cancer plasma analysis, questioned this putative complementarity by dichotomizing the bMSS patient's cohort into bTMB-high and bTMB-low subsets. bMSS-TMB-high and the bMSI-high groups collectively predicted significantly improved outcome, indicating that bMSI combined with bTMB may maximize the scope of ICB therapy (104).

General Conclusions

The last years witnessed a growing body of evidence supporting the use of ctDNA's multiple features (e.g., ctDNA levels, mutations,

Table 1. Clinical applications of ctDNA in IO.

Biomarker	Clinical entities	Biomarker study type	Clinical trial number and ICI therapy evaluated	Refs.
Quantitative analysis of ctDNA[a]	Melanoma—metastatic	Evaluated in IO clinical trial[b]	NCT02374242 + NCT02089685 (nivolumab, pembrolizumab)	(12)
		Standard-of-care cohorts	Pembrolizumab/ipilimumab/nivolumab	(11, 13–18, 22, 58, 121, 122)
	NSCLC—metastatic	Evaluated in IO clinical trial	NCT01693562 + NCT02087423 (durvalumab)	(123)
			NCT01903993 + NCT02008227 (atezolizumab)	(32)
			NCT02475382 (nivolumab)	(124)
		Standard-of-care cohorts	Nivolumab, pembrolizumab	(30, 42, 47, 55, 57, 125, 126)
	NSCLC—localized	Evaluated in IO clinical trial	Durvalumab + NCT02525757 (atezolizumab)	(40)
	Gastric cancer—metastatic	Standard-of-care cohorts	Nivolumab, pembrolizumab, toripalimab, sintilimab	(31)
		Evaluated in IO clinical trial	NCT02589496 (pembrolizumab)	(89)
	Biliary tract—metastatic	Evaluated in IO clinical trial	SHR1210-GEMOX-BTC-IIT03	(95)
	Classic Hodgkin lymphoma	Evaluated in IO clinical trial	NCT03114683 (sintilimumab)	(73)
	Urothelial cancer—metastatic	Evaluated in IO clinical trial	NCT01693562 + NCT02087423 (durvalumab)	(123)
	Urothelial cancer – localized	PRCT designed to address clinical utility	NCT02450331 (atezolizumab)	(41)
	Multiple clinical entities—metastatic	Evaluated in IO clinical trial	NCT02644369 (pembrolizumab)	(59)
			NCT01693562 (durvalumab), NCT02087423 (durvalumab), NCT02261220 (durvalumab + tremelimumab)	(29)
Tumor mutational burden estimation	NSCLC—metastatic	Evaluated in IO clinical trials	NCT01903993 + NCT02008227 (atezolizumab)	(90, 91, 96)
			NCT02453282	(93)
			NCT02478931	(92)
			NCT02848651	(89)
	Biliary tract—metastatic	Evaluated in IO clinical trial	SHR1210-GEMOX-BTC-IIT03	(95)
Microsatellite instability estimation	Gastric cancer—metastatic	Evaluated in IO clinical trials	NCT02589496 (pembrolizumab)	(102)
	Gastrointestinal cancer—metastatic	Standard-of-care cohorts		(104)
	Prostate cancer—metastatic	Standard-of-care cohorts		(103)
	Multiple clinical entities—metastatic	Standard-of-care cohorts	NCT01876511 (pembrolizumab)	(98)

Note: This table summarizes the main clinical applications of ctDNA sorted by disease severity (localized or metastatic) and tumor type. For further details on the cited studies, please refer to Supplementary Table S1.
Abbreviation: PRCT, prospective randomized controlled trial.
[a]Please note that ctDNA quantification refers to both quantification before the treatment and early on treatment.
[b]The clinical trial was initially designed to measure drug efficiency/safety and ctDNA was measured as an observational parameter.

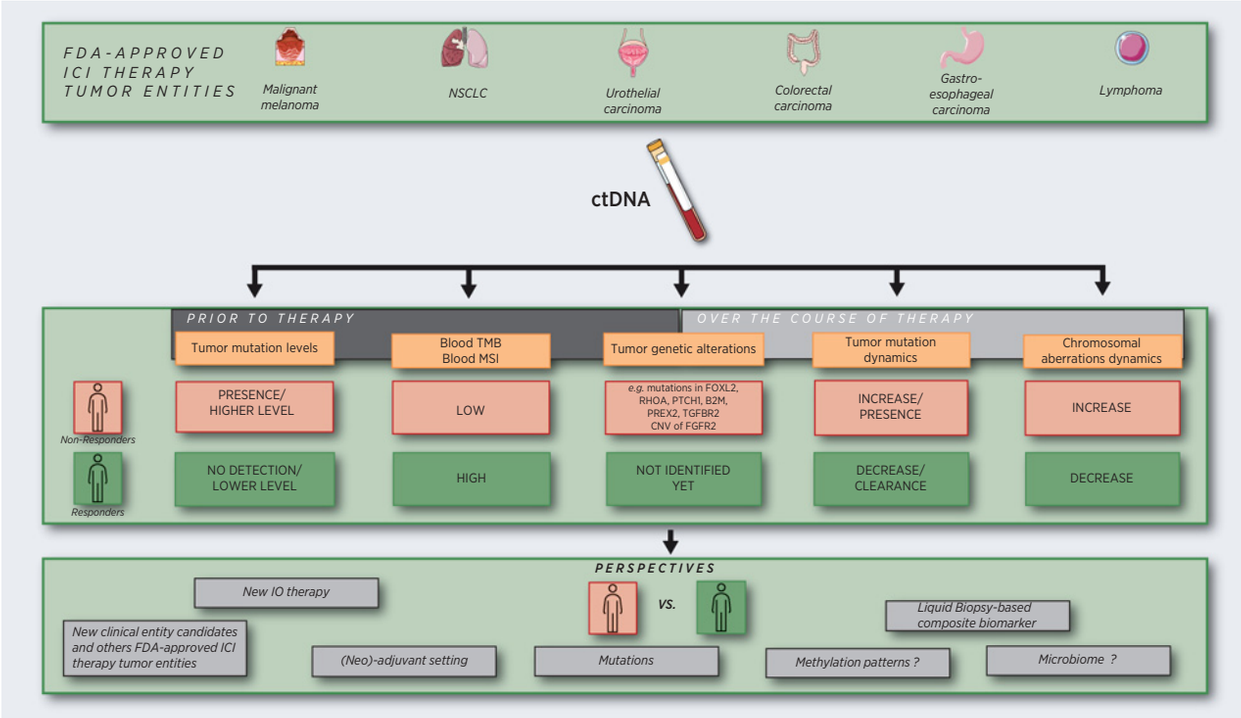

Figure 1.

Current strategies and perspectives of clinical applications of ctDNA analysis in IO. Besides the detection and quantification of tumor mutations, other features like chromosomal aberrations can be quantified as a score, with the advantage of not requiring any prior knowledge of tumor mutation in an individual patient. Performing low-coverage genome-wide sequencing of cfDNA may also present interests in comparison with high-depth sequencing of targeted panels, in terms of cost and time. As a future perspective, the same kind of ctDNA analysis could be applied to new clinical entities where ICI therapy will be approved, or in new clinical therapeutic settings (neo/adjuvant). Other fascinating perspectives offered by studying ctDNA will be to identify other determinants of response to ICI therapy such as tumor-derived methylation patterns and the signatures of the host microbiome.

bTMB, bMSI) for discrimination of patient response to ICI therapy (**Table 1** and **Fig. 1**).

Most data have been obtained in metastatic patients with different types of solid tumors in the context of IO clinical trials, establishing the clinical validity of ctDNA quantification (before and early on-treatment) as prognosticator for response to therapy. The few reports on patients with localized disease in NSCLC and urothelial cancers suggest the capacity of ctDNA measurement to discriminate response from failure to therapy also in the adjuvant setting. The standardization of tests including the harmonization of cutoff points to discriminate ctDNA responders from nonresponders is now the priority task of international consortia like the European Liquid Biopsy Consortium (www.elbs.eu) or the International Alliance of Liquid Biopsy Standardization (ILSA; ref. 106). Indeed, most of the work so far reported was performed on patients included in standard-of-care cohorts or in the frame of a clinical trial initially designed to measure drug efficiency/safety. To introduce ctDNA measurements into clinical practice, interventional ctDNA-based clinical decision trials need to be designed to demonstrate the clinical utility of this biomarker. In this context, it is worth to highlight the pioneering clinical trial in localized urothelial cancers where ctDNA detection was used to personalize treatment selection for patients. In the same line, several clinical trials in early-stage NSCLC or triple-negative breast cancer are currently ongoing, in which adjuvant or neoadjuvant treatment choice is based on ctDNA positivity status after surgery (NCT04966663, NCT04849364, and NCT04585490). To better predict clinical benefit, ctDNA monitoring of tumor response could also open new avenues in the management of side effects and treatment costs. In the metastatic setting, such monitoring could also help to determine the best time point for switching from first- to second-line treatment. The CAcTUS trial in metastatic melanoma (NCT03808441) is a good example of this strategy; based on the determination of BRAF-mutant ctDNA levels patients receiving targeted therapy as first-line therapy are switched to immunotherapy as second-line therapy. In future studies, one could also imagine trials where increasing ctDNA kinetics will guide a switch from PD-1 monotherapy to a more aggressive PD-1 and CTLA-4 combination therapy while decreasing ctDNA will guide a deescalation from combination to the less aggressive monotherapy. Finally, medico-economic comparison with conventional radioimaging technologies is now also needed.

Despite the current technical challenges discussed above, ctDNA can also be used to estimate bTMB and bMSI, two genetic determinants of ICI therapy response. However, the overall response to immunotherapy is not solely dependent on tumor genomics. Tumor escape mechanisms driven at the transcriptional level and host immune system features have been highlighted as additional parameters involved in treatment efficacy (107–109). Therefore, it is very likely that multicomposite biomarkers capable of integrating several metrics will present the highest accuracy to predict tumor response to ICI. Thus, peripheral blood, including circulating tumor cells, circulating cytokines, peripheral T cells population profiles, and extracellular vesicles could be an ideal source to encompass simultaneously all

parameters involved in tumor immune response, and that have already been separately demonstrated as a candidate biomarker of clinical efficacy (110–114). Likewise, Nabet and colleagues have recently developed the DIREct-On score (Durable Immunotherapy Response Estimation by immune profiling and ctDNA) to predict the response of NSCLC patients receiving ICI-based therapies that incorporates three pretreatment biomarkers (ctDNA-normalized TMB, PDL1 tissue expression, circulating CD8 T-cell fraction) but also ctDNA levels after a single cycle of ICI therapy. This score outperformed each metric on the clinical classification accuracy and prognostic value and was the only feature independently associated with PFS in the multivariate Cox proportional model comprising age, ECOG, and line of therapy (32).

Besides mutations, other valuable information like methylation of specific loci or methylation patterns could be extracted from ctDNA analysis (115). Recently, the EPIMUNNE signature based on methylome analysis of the tumor tissue was successfully correlated to the clinical outcome of NSCLC patients treated by immunotherapy (116). Moreover, with thousand copies per cell, mitochondrial DNA in plasma represents an abundant source to exploit, potentially providing valuable information on both tumor and microenvironment (117, 118). Other exciting perspectives of exploiting plasma information could come from the emergent possibility to dissect the microbiome in peripheral blood that would make sense in this context owing to the putative role of intratumor bacteria in response to ICI therapy (119, 120). Thus, liquid biopsy analysis expands the offer to interrogate several features originating from both the host and tumor in a minimally invasive way, leading to the development of a personalized biomarker of response to ICI therapy.

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8. Zusammenfassung auf Deutsch und auf English

English: The avenue of blood-based biomarkers, namely liquid biopsy is considered as a breakthrough in translational research, owing to its minimally invasive character and the capacity of repeated sequential sampling to facilitate personalized monitoring of cancer patients. Though significant advances have been made in the field, many developed liquid biopsy detection approaches present with several limitations such as the high cost and standardization that still hampers their use in clinical routine. Nevertheless, targetable genomic aberrations present in non-small cell lung cancer (NSCLC) are relevant to cancer therapy and their detection using cell-free tumor DNA (ctDNA) have paved the way for ctDNA assays establishment in clinical practice. Moreover, the choice of immunotherapy as a prime treatment option for NSCLC patients makes PD-L1 assessment of primary clinical relevance. In the first part of this thesis, we validated the use of a novel sensitive assay to detect clinically relevant targetable genomic aberrations in NSCLC plasma derived ctDNA. The MassARRAY-based lung cancer panel targets 74 hot spot mutations in five genes commonly mutated in NSCLC in a clinically relevant time-frame and at acceptable cost. We detected mutations in 50% of our cohort with a variant allele frequency (VAF) ranging from 0.1 to 5%. We managed to detect mutations in ctDNA from 50% of the patients presenting with a limited oligo-brain metastatic disease. A comparison of the EGFR mutational status between matched plasma and tissue samples, showed a concordance of 78.6% with 6 discordant cases identified. We also proved in this study the clinical relevance of the MassARRAY for detecting minimal residual disease long before CT scan. With regards to CTCs, we conducted a prospective single-center study, where we compared PD-L1 expression on 3 different tumor materials: standard immunohistochemistry staining on tissue sections, cytological imprints and CTCs. We reported in our study agreement rates of PD-L1 positivity using different cut-off at 1% and 50% of PD-L1 tumor proportion score (TPS). Interestingly, addition of PD-L1 assessment on CTCs significantly improved the prediction capacity for PD-L1 positivity (1% cut-off); AUC= 91% [95% CI: 79-100%] and for high PD-L1 expression (50% cut-off); AUC= 84% [95% CI: 69-100%]. In conclusion, our data align with previous studies upholding the use of CTCs and ctDNA in clinical practice. While the combined analysis of cytological imprints and CTCs provided information on the tumoral PD-L1 and could represent an alternative source for standard tissue specimen, ctDNA analysis with the MassARRAY system allowed to track mutations and detect minimal residual disease, a step forward towards personalized lung cancer management.

Deutsch (DeepL): Der Weg zu blutbasierten Biomarkern, und zwar mithilfe der Flüssigbiopsie, gilt aufgrund ihrer minimalinvasiven Eigenschaft und der Möglichkeit wiederholter sequenzieller Probenentnahmen als Durchbruch in der translationalen Forschung, wodurch schlussendlich eine personalisierte Überwachung erleichtert wird. Obwohl auf diesem Gebiet enorme Fortschritte erzielt wurden, weisen viele der entwickelten Nachweismethoden für Flüssigbiopsien einige Einschränkungen auf, wie z. B. die hohen Kosten und die Standardisierung, die ihre Anwendung in der klinischen Routine noch erschweren. Nichtsdestotrotz sind einerseits zielgerichtete genomische Aberrationen beim nicht-kleinzelligen Lungenkrebs (NSCLC) für die Krebstherapie relevant, und ihr Nachweis mittels zellfreier Tumor-DNA (ctDNA) hat den Weg für die Etablierung von ctDNA-Assays in der klinischen Praxis geebnet. Die Auswahl der Immuntherapie als wichtigste Behandlungsoption für NSCLC-Patienten zeigt andererseits die Relevanz der PD-L1-Bestimmung in der klinischen Analyse auf. In einem ersten Teil dieser Arbeit validierten wir in einer retrospektiven Studie die Verwendung eines neuartigen, sensitiven Assays zum Nachweis klinisch relevanter zielgerichteter genomischer Aberrationen in ctDNA, welches aus NSCLC-Plasma gewonnenen wird. Das auf dem MassARRAY basierende Lungenkrebs-Panel erfasst 74 Hot-Spot-Mutationen in fünf bei NSCLC häufig mutierten Genen in einem klinisch relevanten Zeitraum und zu akzeptablen Kosten. Wir haben in 50% unserer Kohorte Mutationen mit einer Varianten-Allel-Frequenz (VAF) von 0,1 bis 5% nachgewiesen. Bei 50% der Patienten, die begrenzte Oligohirnmetastasierung aufwiesen, konnten wir Mutationen in der ctDNA nachweisen. Ein Vergleich des EGFR-Mutationsstatus zwischen Plasma und Gewebe zeigte eine Übereinstimmung von 78,6%, wobei 6 Fälle identifiziert wurden, die nicht übereinstimmten. In dieser Studie konnten wir ebenfalls die klinische Relevanz des MassARRAY nachweisen, welches eine minimale Resterkrankung noch vor Durchführung eines CT-Scans erkennt. Im Hinblick auf CTCs führten wir eine prospektive Studie an einem einzigen Zentrum durch, in der wir die PD-L1-Expression anhand drei verschiedener Tumormaterialien verglichen: Standard-Immunhistochemie-Färbung auf Gewebeschnitten, zytologische Abdrücke und CTCs. Wir zeigten in unserer Studie Übereinstimmungsraten der PD-L1-Positivität unter Verwendung verschiedener Cut-off-Werte; bei 1% und 50% des PD-L1 Tumor Proportion Score (TPS). Interessanterweise verbesserte die zusätzliche PD-L1-Bewertung von CTCs die Vorhersagekapazität für PD-L1-Positivität (1% Cut-off) signifikant; AUC= 91% [95% CI: 79-100%] und für hohe PD-L1-Expression (50%

Cut-off); AUC= 84% [95% CI: 69-100%]. Zusammenfassend lässt sich sagen, dass unsere Daten im Einklang mit früheren Studien stehen, die die Verwendung von CTCs und ctDNA in der klinischen Praxis unterstützen. Während die kombinierte Analyse von zytologischen Abdrücken und CTCs Informationen über das tumorale PD-L1 lieferte und eine alternative Quelle für Standard-Gewebeproben darstellen könnte, ermöglichte die ctDNA-Analyse mit dem MassARRAY-System die Überwachung von Mutationen und die Erkennung der minimalen Resterkrankung, ein Schritt in Richtung personalisierte Lungenkrebsbehandlung.

9. Erklärung des Eigenanteils an den Publikationen

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Declaration of Contribution

The publications listed below are arranged according to time point release. The current impact factors (IF) of each journal are included in brackets.

Discovery of targetable genetic alterations in NSCLC patients with different metastatic patterns using a MassARRAY-based circulating tumor DNA assay - *Cells* [IF: 7.666]

This project was initiated by Prof. Dr. Klaus Pantel and Prof. Dr. Harriet Wikman in collaboration with Agena Biosciences. The aim of this retrospective study was to provide a first proof of principle for a validated ctDNA assay that can detect clinically relevant mutations based on a mass-spectrometry approach in advanced NSCLC patients, including patients with lower tumor burden such as oligo–brain metastatic disease. My role in this project was to isolate cell-free DNA from some of the samples for consequent MassARRAY-based analysis. Measurement of cfDNA quantity and quality control using TapeStation (Agilent) were performed and assessed by myself. All mutation analyses using the MassARRAY analysis were run by me or our technician Jolanthe Kropidlowski. All generated data were analyzed with Typer software version 4.0.26.74 (Agena Bioscience). Introduction to the software for data analysis and generation of mutations reports was performed by Alex Sartorini from Agena Bioscience. Data interpretation for final mutation call was performed in consultation with Prof. Wikman. As shared first author, I collaborated with Melanie Janning in consultation with Prof.Dr. Wikman, Prof Loges and Prof. Janning and Prof. Dr. Pantel in designing the manuscript concept and significantly contributed to writing the first manuscript draft and the consequent submission process. I was furthermore involved in rewriting the manuscript to its final version and aiding in the revision process prior to final publication.

YassB

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Prof. Dr. Med. Klaus Pantel

Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond – *British journal of cancer* [IF: 9.082]

The review was an invitation to participation by Prof. Dr. med. Klaus Pantel. The aim of this review was at first provide an overview of the main methods used to detect mutations in ctDNA and their cognate challenges. Focusing on additional features of cfDNA beyond the detection of point mutations that can be assessed using liquid biopsy samples from patients with solid tumors was the main scope of this work. My role in this review was to thoroughly and deeply report studies with regards to intra-tumoral heterogeneity, the methylation aspects related to ctDNA's clinical relevance but also fragmentation patterns driving ctDNA in the blood, regardless of tumor entity. I was responsible of designing both figures. Figure 1 was performed in consultation with Dr. Laura Keller. Once the literature overview was performed, a thorough and systematic synthesis and merge of findings of single, independents studies was performed. Additionally, I was substantially involved in writing the entire first manuscript draft in consultation with Dr. Keller, Prof. Wikman and Prof. Dr. Pantel. I was also involved in fine-tuning the manuscript to its final version with Dr. Laura Keller and substantially contributed to the revision process prior to final publication.

Yassine Belloum

Prof. Dr. Med. Klaus Pantel

Current and Future Clinical Applications of ctDNA in Immuno-Oncology – *Cancer Research* [IF: 13.312]

The review was an invitation to participation by Dr. Laura Keller and Prof. Dr. Med. Klaus Pantel. The aim of this review was to present review application of ctDNA analysis in the context of immune-oncology. My role in this project was to thoroughly and deeply report data from all studies present in PubMed investigating the role of ctDNA and tumor mutational burden analysis in different clinical setting with several tumor entities, with a major focus on lung cancer patients. I also took care of designing and reporting results from studies in table 1 in consultation with Dr. Laura Keller. Once the literature overview was performed, a thorough and systematic synthesis and merge of findings of single, independents studies was performed. Additionally, I was substantially involved in writing the entire first manuscript draft in consultation with Dr. Laura Keller and Dr. med. Julia Stadler. I was also involved in fine-tuning the manuscript to its final version with Dr. Laura Keller and substantially contributed to the revision process prior to final publication.

Yassine Belloum

Prof. Dr. Med. Klaus Pantel

Comparative evaluation of PD-L1 expression in cytology imprints, circulating tumor cells and tumor tissue in non-small cell lung cancer patients – *Molecular Oncology* [IF: 7.449]

The initial idea for this project was developed by Professors Wikman, Pantel and Reck. The aim of this prospective study was to investigate the relationship between PD-L1 expression on tumor tissue from standard immunohistochemistry with the PD-L1 expression of site-matched cytology imprints of primary tumor lesions and the detection rate of CTCs and their PD-L1 expression in patients with NSCLC. In my PhD, I took over the planning of necessary experiments in consultation with professor Wikman and professor Pantel and their implementation independently, with regard to liquid biopsy part. From culturing of the cell lines, determining the average cell size, performing spike-in experiments with healthy blood donors, staining the generated slides with a multicolor staining cocktail, to manual screening under the microscope of single cells. Once a clear protocol was established, I started processing the cancer patient samples for circulating tumor cell (CTC) isolation with the parsortix^{TM} system. I conducted the experiments myself. 137 patients were processed with the parsortix system and all the generated patients' slides were stained in batches (10 slides). Each patient slide was manually screened under the microscope. Introduction to the Zeiss software analysis was performed by Dr. Leonie Ott and the evaluation of the slides was performed by myself. Interpretation of CTC presence were reviewed together with professor Wikman. Statistical analysis was performed by Dr. Abdo. Cytological and histological analysis were performed at the "Lungenclinik Großhansdorf". Overall data interpretation of PD-L1 expression between the 3 different tumor material and the generated data were done in consultation with Prof. Pantel, Prof. Wikman, Prof. Reck and Dr. Abdo, all of whom provided valuable guidance and support. Additionally, I wrote the manuscript with Dr. Abdo. I was also responsible for rewriting the final manuscript to its final version and was tasked with its submission as well as conducting the revision process prior to final publication.

Yassine Belloum

Prof. Dr. Med. Klaus Pantel

10.Danksagung

Supervisors, colleagues, friends, family, to all of those who contributed to the finalization of this PhD thesis to smaller or greater extent, I want to express my deep gratitude and thanks.

To Professor Pantel, I want to thank you for giving me this opportunity to be within ELBA and conduct my thesis at the Institute of Tumor Biology (ITB). Thank you for your unconditional support throughout these years, for your valuable scientific input and mostly, for believing in me. Thank you for coming to Montpellier and have me discover the liquid biopsy field. It has been a pleasant journey, and for this, I want to express my sincere gratitude. Thank you!

I would like to extend my sincere thanks to my PhD co-supervisor, Prof. Dr. Harriet Wikman. Dear Haiju, I am deeply grateful for the guidance and daily support you've provided me with. Thank you for helping me expand my knowledge and my critical perspectives on the different exciting projects we had. Thank you for always pushing me and I know it's for my own good and I am sincerely grateful for that. Thank you!

To the European commission, without whom this project would have never existed if not for their financial support, to my fellow ELBA partners, supervisors, coordinators, thank you all for your great work to make such a brilliant training network happen!

I would like to offer my special thanks to my thesis committee board members, Prof. Dr. med Klaus Pantel, Prof. Dr. med. Christoffer Gebhardt and Priv.-Doz. Dr. med. Faik G. Uzunoglu. Thank you for your willingness to review this manuscript and agreeing to be part of my examination committee.

To my fellow colleagues and friends at ITB, I want to express my sincere thanks and gratitude for giving me the chance to evolve in such a friendly atmosphere. Thank you Jolanthe for your daily work on the bench and for your willingness to always help us whenever needed. Thank you for the daily german conversations, even though sometimes we end up in sign language. Ich habe jeden Moment geliebt! Vielen dank! Svenja, Thais, thank you girls for supporting me this whole time. I am so grateful for being in the same office with you, to share our laughs but also to cope with troubling times. I also enjoyed our spontaneous scientific conversations. See! I am still putting up with the Wikman girls ☺ Thank you Lucija, for being my close friend and my colleague. Thank you! Antje, for always being in a nice mood, Cony, Leonie, Désirée, Katharina and all the technicians, thank you ladies for allowing me to be part of such a cheerful work-environment. To Laura as well, who's been helping me throughout my thesis. I enjoyed every moment of our delightful and mostly funny conversations.

A special thanks goes to all our partners and collaborators from the ITB and outside ITB: our nurses and medical doctors here at UKE, Agena Biosciences members and Lungen clinic Großhansdrof nurses and medical doctors who contributed to make these publications possible but also the ones collaborating on upcoming publications. Thank you all for your great contribution and valuable feedbacks and advices.

To my friends and to all the amazing people I met in Hamburg, and you will recognize yourself: you are the reason I am easily open to moving from one city to another. It's not about where we go, where we are but rather about who are we surrounded with, and you my dears have been the reason I enjoyed living in Hamburg. I am deeply grateful for your friendship! Thank you for always cheering me up and for all the moment of joy we shared together.

An meine Lauri, I guess I owe you loads of trips to Berlin! thank you for supporting me and cheering me up this whole time with your unexpectedly funny jokes. I know it has not been easy for you. Thank you! I look forward to our travelling plans to happen.

And finally, to my parents, to my little sis who's older than me, without whom none of this would have been possible. As we grow older in life, we learn more about what matters the most. And YOU do. My only regret is spending so little time with you since the day I left. I miss you every single day. Your sacrifice, your devotement for your children's future, your belief in education as the cornerstone for a successful life, your unconditional love and understanding, for this and more, thank you! I am deeply grateful! I know what this means to you and I hope I made you proud.

Love y'all !

11.Lebenslauf

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RESEARCH AREA

My principal research interests lie in the field of liquid biopsy. More precisely, I work on cfDNA (1-3) and rare single cells' detection and molecular characterization. My present research activity focuses on circulating tumor cells (CTCs) and their clinical relevance in non-small cell lung cancer (NSCLC) patients. In parallel, I work on the skeletal muscle, more precisely, I want to understand the molecular shield against CTCs' metastatic colonization and disseminated tumor cells' (DTC) incapacity to grow to overt metastasis in skeletal muscle.

INTERNSCHIPS/ EXPERIENCE

2016 – 2017 UMR-CNRS 9002 «institute of human genetics» Montpellier, **FRANCE**
Implication of Oct4 as a key player in limiting the pluripotency and tumorigenicity of Muscle-Derived stem cell (MDSC)
Supervision: Prof. Dr. Ned lamb & Prof. Dr. Anne Fernandez
(6 months internships).

2015 – 2016 UMR 866 INRA «muscle dynamics and metabolism» Montpellier, **FRANCE**
Implication of mitochondrial protein Redd1 in skeletal muscle metabolism (4)
Supervision: Prof. Dr. François Favier & Prof. Dr. Vincent Ollendorf
(6 months internships).

2014 – 2015 EA 1274 «movement, sport, health» Rennes, **FRANCE**
Cancer induced cardiac-cachexia: pathogenesis and impact of physical activity (5)
Supervision: Prof. Dr. Françoise Rannou-Bekonno
(4 months internships).

2013 – 2014 « medecine sport service », Rennes hospital Center (CHU), **FRANCE**
In collaboration with the Rennes University Hospital center and a handball club, this internship took place in the context of achieving a

discovery research work: “repeated sprints: physiological adaptations to exercise and training"
(2 months internships).

EDUCATION

2018 – 2023 PhD “Circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA) as blood-based biomarkers for managing non-small cell lung cancer (NSCLC) patients” (1-3, 5-8).
University medical center Hamburg-Eppendorf**, GERMANY**
Department of tumor biology (Institut für Tumorbiologie)
Funded under the European Commission’s Marie Sklodowska-Curie Programme.

Thesis director: Prof. Dr. med. Klaus Pantel
Thesis co-director: Prof. Dr. Harriet Wikman

2016 – 2017 International Master of Sciences (MSc) “Bac+5” in cancer biology

University of Montpellier, **FRANCE**

2015 – 2016 Master of Science (MSc) “Bac+5” in muscle pathology and physiology

University of Claude Bernard Lyon1, **FRANCE**

2015 – 2016 Master of Science (MSc) “Bac+5” in exercise physiology and muscle biology
University of Montpellier, **FRANCE**
Course: exercise physiology; cellular and molecular adaptation

2014 – 2015 Bachelor of Honor “Bac+4” in movement, sport and health

University of Rennes 2, **FRANCE**
Course: depth knowledge at the molecular level (cachexia, sarcopenia, myopathy, cardiomyopathy...), metabolic adaptations to exercise...

2013 – 2014 Bachelor “Bac+3” in sciences and techniques of physical activity and sports (“STAPS”)

University of Rennes 2, **FRANCE**

LANGUAGES

- Arabic (Tunisian) – Native
- French – Native
- English – Fluent

- Spanish (Intermediate) – B1
- German (Intermediate) – B1

PUBLICATIONS

1. **Belloum Y***, Janning M*, Mohme M, Simon R, Kropidlowski J, Sartori A, et al. Discovery of Targetable Genetic Alterations in NSCLC Patients with Different Metastatic Patterns Using a MassARRAY-Based Circulating Tumor DNA Assay. Cells. 2020;9(11).
2. Keller L*, **Belloum Y***, Wikman H, Pantel K. Clinical relevance of blood-based ctDNA analysis: mutation detection and beyond. British journal of cancer. 2021;124(2):345-58.
3. Stadler JC*, **Belloum Y***, Deitert B, Sementsov M, Heidrich I, Gebhardt C, et al. Current and Future Clinical Applications of ctDNA in Immuno-Oncology. Cancer Res. 2022;82(3):349-58.
4. Britto FA, Cortade F, **Belloum Y**, Blaquiere M, Gallot YS, Docquier A, et al. Glucocorticoid-dependent REDD1 expression reduces muscle metabolism to enable adaptation under energetic stress. BMC Biol. 2018;16(1):65.
5. **Belloum Y**, Rannou-Bekono F, Favier FB. Cancer-induced cardiac cachexia: Pathogenesis and impact of physical activity (Review). Oncol Rep. 2017;37(5):2543-52.
6. Nitschke C, Markmann B, Walter P, Badbaran A, Tolle M, Kropidlowski J, et al. Peripheral and Portal Venous KRAS ctDNA Detection as Independent Prognostic Markers of Early Tumor Recurrence in Pancreatic Ductal Adenocarcinoma. Clinical chemistry. 2023;69(3):295-307.
7. Nitschke C, Markmann B, Tolle M, Kropidlowski J, **Belloum Y**, Goetz MR, et al. Characterization of RARRES1 Expression on Circulating Tumor Cells as Unfavorable Prognostic Marker in Resected Pancreatic Ductal Adenocarcinoma Patients. Cancers. 2022;14(18).
8. Abdo* M, **Belloum* Y**, Heigener D, Welker L, Weihe Sv, Schmidt M, et al. Comparative Evaluation of PD-L1 Expression in Cytology Imprints, Circulating Tumor Cells and Tumor Tissue in Non-Small Cell Lung Cancer Patients. Molecular oncology. Molecular oncology. 2023 (In Press).

12.Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: Hamburg, 09 March 2023