Mini Review

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The DKTK EXLIQUID consortium – exploiting liquid biopsies to advance cancer precision medicine for molecular tumor board patients

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Abstract: Testing for genetic alterations in tumor tissue allows clinicians to identify patients who most likely will benefit from molecular targeted treatment. EXLIQUID – exploiting liquid biopsies to advance cancer precision medicine – investigates the potential of additional non-invasive tools for guiding therapy decisions and monitoring of advanced cancer patients. The term "liquid biopsy" (LB) refers to non-invasive analysis of tumor-derived

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circulating material such as cell-free DNA in blood samples from cancer patients. Although recent technological advances allow sensitive and specific detection of LB biomarkers, only few LB assays have entered clinical routine to date. EXLIQUID is a German Cancer Consortium (DKTK)-wide joint funding project that aims at establishing LBs as a minimally-invasive tool to analyze molecular changes in circulating tumor DNA (ctDNA). Here, we present the structure, clinical aim, and methodical approach of the new DKTK EXLIQUID consortium. Within EXLIQUID, we will set up a multicenter repository of high-quality LB samples from patients participating in DKTK MASTER and local molecular tumor boards, which use molecular profiles of tumor tissues to guide targeted therapies. We will develop LB assays for monitoring of therapy efficacy by the analysis of tumor mutant variants and tumor-specific DNA methylation patterns in ctDNA from these patients. By bringing together LB experts from all DKTK partner sites and exploiting the diversity of their particular expertise, complementary skills and technologies, the EXLIQUID consortium addresses the challenges of translating LBs into the clinic. The DKTK structure provides EXLIQUID a unique position for the identification of liquid biomarkers even in less common tumor types, thereby extending the group of patients benefitting from non-invasive LB testing. Besides its scientific aims, EXLIQUID is building a valuable precision oncology cohort and LB platform which will be available for future collaborative research studies within the DKTK and beyond.

Keywords: liquid profiling; molecular profiling; precision oncology.

Introduction

Diagnostic procedures and treatment options for malignant diseases are constantly evolving. Cross-institutional molecular tumor boards (MTBs) aim at providing patients with individualized treatment recommendations based on comprehensive molecular tumor profiling. Especially patients who failed to respond to standard treatment can benefit from discussion in such MTBs. There is increasing evidence that implementation of recommended treatments result in improved overall responses, improved disease control rates, and improved progression-free survival [1]. So far, MTB recommendations mainly rely on molecular profiles obtained from tumor tissue. Such an approach is affected by sampling bias, intratumoral heterogeneity, and the availability of tissue from surgery or from invasive biopsies.

Liquid biopsies (LBs) contain tumor derived material such as circulating tumor DNA (ctDNA) and circulating tumor cells [2–4]. LBs can provide reliable information about the complete tumor molecular composition and dynamics in a patient over time by means of analyzing cell-free DNA (cfDNA) from blood samples [5–7]. Few studies, however, have investigated the value of LBs for treatment recommendations within an MTB setting so far.

The TRACERx study (TRAcking Cancer Evolution through therapy (Rx)) in early stage lung cancer patients uses longitudinal sampling with LBs to track cancer evolutionary trajectories [8]. In one pilot study with 29 MTB patients, profiled both in tissue and LB, LB was more sensitive in mutation detection than tissue biopsy analysis (48 out of 53 mutations in LB vs. 16 out of 53 mutations in

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tissue) [9]. As a result, for 11 out of 29 patients, LB revealed clinically actionable mutations providing additional theoretical clinical benefit. A phase IV clinical trial in lung cancer patients demonstrated that plasma sample can be considered for analysis of EGFR mutations if tissue samples are unavailable [10]. Another study comparing LB and tissue based mutation profiling in lung cancer patients demonstrated that plasma-based sequencing can increase the detection of actionable findings while at the same time reducing the required time for obtaining results. In general, tissue and liquid profiling for clinically actionable mutations perform equally well [11]. The Japanese GOZILA study showed in patients with gastrointestinal cancer that somatic profiling based on LBs compared to tissue can significantly reduce sample acquisition duration (median, 4 vs. 14 days), test duration (median, 7 vs. 19 days), and reduce failure rate (0.1% vs. 10.6%), without compromising the efficacy of the recommended treatment [12]. The CANCER-ID consortium pursues LB-based approaches with an emphasis on circulating tumor cells, for example to predict the treatment response to chemotherapy and targeted therapy [13]. Discoveries of our partner site Dresden highlight the benefits of ctDNA analysis extracted from various body fluids for both prognosis prediction and early detection of recurrence [14–16]. Studies of our partner site Tübingen have demonstrated that ctDNA dynamics are suitable biomarkers during cancer therapy [17, 18]. Using a similar approach, recent results from the DYNAMIC clinical trial with 455 patients with stage II colorectal cancer showed ctDNA detection 4 or 7 weeks after surgery can guide chemotherapy decisions and reduce chemotherapy usage from 28% to 15% with virtually identical recurrence-free survival at 2 years [19]. Using a tumor-informed personalized approach for ctDNA detection relying on multiplex PCR assays designed for individual tumor-specific single nucleotide variants, we plan to detect relapse before it becomes evident by clinical imaging.

In general, LBs can provide valuable information in addition to tissue-based analysis. First, they can capture the entire mutation spectrum of a potentially heterogeneous tumor that may not be detectable in a tissue biopsy. Second, they allow tracking of tumor evolution by following changes in the composition of molecular variants.

Here, we describe EXLIQUID — exploiting liquid biopsies to advance cancer precision medicine. The EXLIQUID project aims at harnessing the potential to analyze the molecular profile of a patient's advanced cancer under treatment from blood samples at several time points with the focus on rare solid cancers.

Aims of EXLIQUID

The establishment of a multicenter biobanking repository with high-quality LB samples from patients with advanced solid cancers who receive comprehensive tumor profiling in MTBs is the basis of the EXLIQUID project. We use these samples from a variety of tumor types to study ctDNA--based kinetics of tumor-specific variants revealing the molecular response, onset, depth, and duration as well as clonal evolution. Our findings will provide a basis for future improvement of molecularly stratified treatments. In addition, we will assess the potential of ctDNA-based epigenomic markers for therapy monitoring and, in combination with tumor mutant variants, for early LB-based prediction of therapy resistance.

Structure of the German cancer consortium for translational research (DKTK) and the EXLIQUID consortium

The DKTK

Promising novel findings in cancer research should be translated into clinical studies and clinical practice as fast as possible. The DKTK, one of six German Health Research Centers, was founded in 2012 with the goal to promote expertise in clinically oriented cancer research and consists of university hospitals and academic research institutes at eight sites across Germany. More than 20 academic research institutes and university hospitals at seven partner locations cooperate with the German Cancer Research Center (DKFZ), the consortium's core center. A robust joint funding program allows for multicenter projects and clinical trials involving multiple sites sharing expertise and best practice. In 2020 alone, DKTK researchers published their scientific work in 1,197 articles. In the same year, published scientific work affiliated with the DKTK was cited 49,689 times (www.dkfz.de/zbi/nolink/Publikationen-DKTK-2020.pdf).

The EXLIQUID consortium

In 2017, two of the authors (Sültmann and von Bubnoff) initiated the DKTK working group "Liquid Biopsy". This group encompasses DKTK scientists and clinicians with expertise in LB. The EXLIQUID initiative, which emerged

from this group, is an interdisciplinary alliance bringing together clinicians, natural scientists, biobankers, and medical data specialists working in the field of LB at all eight DKTK sites. It currently includes the associated partner sites with the university medical centers of Lübeck and Mannheim and is open for further extension (Figure 1). In 2021, the DKTK steering committee considered the project as promising for connecting LB research with clinical practice and provided funding. The EXLIQUID consortium commenced its work in late 2021.

Within EXLIQUID, we aim at implementing LB assays for therapy selection and monitoring, especially in less frequent tumor entities where the diagnostic potential of LBs is largely unknown. To this end, we are building a multicenter repository of high-quality LB samples from patients participating in local MTBs at DKTK sites as well as in the DKTK MASTER (Molecularly Aided Stratification for Tumor Eradication) program [1, 20]. Here, we are focusing on less common entities for which a single site would not be able to enroll a sufficient number of patients and samples. We will include only patients for whom the molecular profiling led to a treatment recommendation by the MTB. Blood samples of these patients are collected with the patient's inclusion in the MTB, at the beginning of the treatment, and at each subsequent visit. We subject these longitudinal samples to comprehensive profiling of cfDNA on two levels: First, we focus on ctDNA-based kinetics of tumor mutant variants. Here we will evaluate the predictability of early treatment failure, the median lead time between molecular and clinical progression, and the clonal evolution dynamics associated with therapy resistance. Secondly, we will explore the potential of epigenomic alterations in ctDNA as predictive marker. Aberrant DNA methylation is a hallmark of cancer, and tumor-specific hyper- or hypo-methylated genomic sites can be used to monitor molecular tumor dynamics irrespective of DNA mutation status. Thus, methylation information may contribute to early prediction of therapy resistance. Furthermore, the epigenomic analysis may reveal information on the tissue of origin in patients with cancer of unknown primary (CUP).

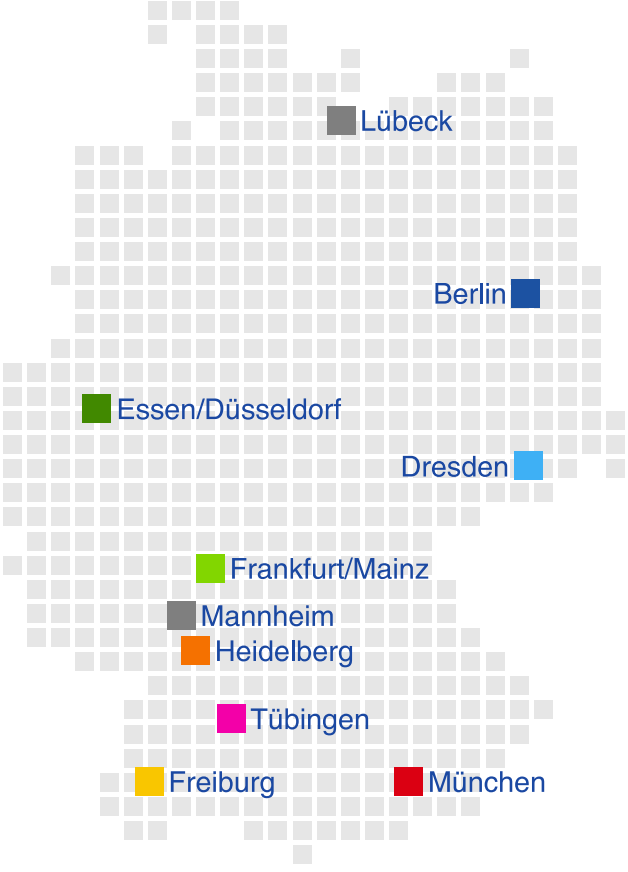

Figure 1: Map of the EXLIQUID sites.
Depicted are the eight DKTK sites contributing to EXLIQUID (colored squares) and the two associated sites (gray squares).

IT infrastructure

When storing and documenting biological samples along with high-quality clinical data under strict data protection requirements harmonized across multiple sites, logistics become a crucial factor. For this purpose, the Clinical Communication Platform (CCP) had been set up within the DKTK several years ago as a data hub and system for networked research. The CCP has established infrastructure and services that allow sites to integrate patient data (clinical care, biological samples, and research data in pseudonymized form) into a local data hub, the so-called bridgehead, to enable the network-wide linkage of this data for retrieval, sharing, and merging in compliance with all data protection requirements [21]. In addition, the CCP coordinates continuous harmonization processes with participating local cancer registries and biobanks from all sites covering datasets and sampling. In combination, CCP services and infrastructure allow the rapid identification of patient cohorts based on clinical criteria and availability of respective samples as well as data exchange.

EXLIQUID utilizes and contributes to the CCP in various ways. We tag participating patients in the CCP infrastructure via a dedicated pseudonymization service provided by the CCP [22]. On the one hand, we use a pseudonym generated for EXLIQUID to retrieve harmonized clinical data and sample information from routine care via the CCP bridgehead. On the other hand, this pseudonym links the research data generated in the project

to further data in the bridgehead, enhancing the potential use for subsequent research.

To allow the real-time querying of patient recruitment, sample availability and data collection across the participating EXLIQUID sites, the CCP provides a central EXLIQUID dashboard to track the progress of prospective patient recruitment and sample collection at participating sites. Based on pre-selected criteria, the dashboard reports key parameters for recruited patients and collected samples such as counts per site. The dashboard is an enhancement of the CCP's already established federated IT infrastructure, which consists of local and central IT components, taking into account the heterogeneity of local systems and data protection requirements [23]. The software developed for the EXLIQUID dashboard will be made available under an open source license, which allows sustainable maintenance and use in other projects.

Table 1: Cancer types of patients seen in molecular tumor boards at DKTK sites in 2019.

Tumor	Total
Lung	2,800
Breast	350
Pancreas	230
Gynecologic	200
Gastrointestinal	190
Colorectal	190
Brain	160
Sarcoma	150
Urogenital	110
Hepatobiliary	100
Endocrine	90
CUP	80
Skin	80
Head and neck	60
Neurologic	50
Stomach	50
Bone and soft tissue	50
Neuroendocrine	30
Hematologic	20
Kidney	10
Other	190
Sum	5,190

Workplan

In EXLIQUID, researchers with complementary expertise are working together in order to adequately address the ultimate goal to accelerate clinical translation for LBs. Biweekly consortium meetings ascertain exchange of information to speed-up the project's progress. EXLIQUID consists of three parts: (1) sampling and biobanking of plasma and peripheral blood mononuclear cells (PBMC), (2) mutation analysis, and (3) methylation analysis of cfDNA.

Liquid biopsy sampling and biobanking

Within EXLIQUID, we establish a large and well-annotated multicenter repository of high-quality LB samples. EXLIQUID uses a decentral biobanking strategy based on well-established local liquid biobanks (German Biobank Alliance) as well as clinical data collection via the CCP and the DKTK MTB Alliance. We collect blood samples from patients participating in DKTK MASTER and local tumor profiling programs at each DKTK partner site (Table 1) at predefined time points (Figure 2). The first sample ($t0_a$) is collected at patient enrollment for molecular profiling of tumor tissue (estimated accrual rate of 1,000 patients per year). Subsequent samples are collected only from patients for whom (i) molecular profiling led to a treatment recommendation by the local molecular tumor board, (ii) the recommended drug is available, and (iii) the treatment is intended, referred to in the following as intent-to-treat (ITT) population. Based on a recent analysis of DKTK MASTER [1], the population comprises ~20–30% of the total patient population. Predefined time points for sample collection in the ITT population are before the start of the recommended treatment ($t0_b$), at the first clinical follow-up within two weeks after start of molecular treatment, at each visit for computed tomography (CT) scan until progression and at tumor progression (Figure 2). After local sample processing according to EXLIQUID standardized operating procedures (SOPs) for plasma and PBMC separation (see Supplemental Material), sample aliquots are stored in local central liquid biobanks. Biobanks document quality parameters such as time to centrifugation and time to freeze, and operate using the same EXLIQUID SOP. The relevant sample information is then registered in the local CCP bridgehead (see section "IT infrastructure"). Detailed information on the individual clinical course including CT scans from follow-up visits are collected for the ITT population. Access to samples is achieved by a use and access committee with members from the local EXLIQUID sites who contributed with samples.

Numbers are rounded to tens and include DKTK MASTER patients as well as local molecular tumor board patients.

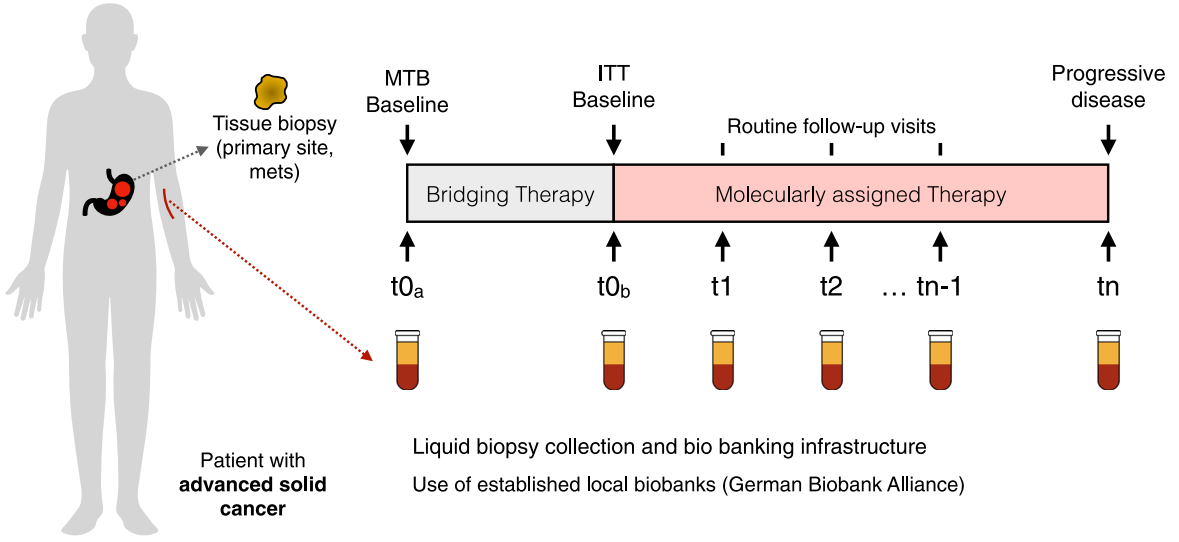

Figure 2: The EXLIQUID sampling scheme.
Sampling scheme of a cancer patient admitted to a molecular tumor board within the EXLIQUID consortium. The LB obtained and analyzed at $t0_a$ can be used to decide on a therapy matching to the molecular characteristics of the cancer. During routine follow-up visits liquid biopsies are used to monitor the therapeutic success until a progression of the disease can be detected.

Mutation analysis

We test the hypothesis whether assessment of the kinetics (onset, depth, duration) of the molecular response can improve the accuracy of outcome prognosis beyond standard CT-based imaging. In order to determine the spectrum of druggable alterations, which can be expected in prospective patient cohorts of DKTK MASTER and the local profiling programs, we performed a retrospective analysis of the first 100 patients included in the two molecular profiling programs (MASTER, TREAT20+) at the DKTK partner site Berlin [24]. This revealed that the genetic alterations resulting in MTB recommendations were mainly found in RAF/MEK/ERK, PI3K/AKT/mTOR signaling and DNA repair pathways [1]. Such an enrichment was also observed in the total DKTK MASTER cohort, where ~50% of recommendations proposed the use of drugs targeting these pathways. For these patients we use customized or commercially available panel NGS for tracking mutant variants in tumor suppressor genes and droplet digital PCR (ddPCR) for recurrent hotspot variants in tumor driver genes. We establish advanced protocols with adequate resolution for low-frequency mutant variants, necessary to monitor variants during follow-up in EXLIQUID. We achieve efficient recovery of ctDNA molecules down to allele frequencies of 0.004% [25–27] by a molecular barcoding strategy, ultra-high sequencing depths, and *in silico* elimination of highly stereotypical background artifacts – enabling sensitive ctDNA detection [26]. Moreover, by covering hundreds of genetic regions, our approach facilitates the identification of emerging genetic aberrations associated with resistance [28–30]. For cases displaying rare mutant variants which cannot be detected by the employed NGS panel, we pursue a complementary monitoring approach, which achieves its high sensitivity from the inclusion of multiple patient-individualized mutation assays [31]. We have established shallow whole genome sequencing (sWGS) of plasma cfDNA for longitudinal monitoring of lung cancer patients [29, 32–36]. The consortium plans to perform sWGS for the ITT population (see above) in order to determine the tumor load as the fraction of ctDNA compared to total plasma cfDNA. We compare the results with variant allele frequencies derived from panel seq and ddPCR analyses. In addition, we use sWGS to determine copy number variations in selected tumor entities for which high-level aneuploidy or large-scale structural rearrangements of chromosomes have been found based on the WGS/whole exome sequencing analysis of solid tumor biopsies.

Two strategies will be applied in the context of selecting NGS panels. The first is a tumor-informed approach, by which, based on the results of tumor tissue genotyping, 30 to 50 variants will be identified in order to design patient-individualized assays for ctDNA kinetics studies. The second is a tumor-naïve approach, whereby oncogenic drivers and/or gene variants associated with resistance will be used to construct the NGS panel. The precise content of the ctDNA assays thus remains to be

determined as it will be influenced by the tumor entity, as well as the assigned molecular treatments in the EXLIQUID patient cohort.

The mutation analysis consists of three phases: First, after successful establishment of molecular tools and bioinformatics algorithms, we analyze the baseline samples collected at the time of patient recruitment for molecular tissue profiling ($t0_a$; Figure 2), before start of the recommended personalized treatment ($t0_b$). Comparative analysis of the results from tumor tissue versus LB profiling as well as the two different time points $t0_a$ and $t0_b$ reveals concordance rates, the influence of the bridging therapy on mutant allelic fractions, and any potential interference of tumor histology with these features. The second phase is dedicated to kinetics analysis of longitudinal samples ($t0_b$, t1, t2, … tn; Figure 2) for studying the onset, depth, and duration of the molecular response. Here, the comparative analysis of results from kinetic studies of mutant variants, tumor burden, and standard clinical follow-up procedures investigates the potential of LBs for early identification of responders/non-responders. The most promising liquid biomarkers of treatment efficacy and mutant variants significantly associated with both acquired and *de novo* (i.e., primary) resistance will be validated in the third phase, which is instrumental for identification of candidate markers for future clinical LB studies within the DKTK network.

Methylation analysis

Aberrant DNA methylation is a hallmark of cancer, and many hyper- or hypomethylated genomic sites can be used to monitor molecular tumor dynamics irrespective of DNA mutation status. In addition, methylation has successfully been used for the classification of tumor tissue samples [37]. In contrast, the analysis of methylation marks in cfDNA is less well established than mutation profiling, and novel technologies for their characterization are emerging [38, 39]. The focus of the EXLIQUID methylation analysis is to explore the potential of cancer-specific methylation alterations in cfDNA as markers of tumor progression and treatment response monitoring. To address this question, we restrict the methylation analysis to two time points where we expect a substantial amount of ctDNA: the baseline sample collected at the time of molecular tissue profiling ($t0_a$), and the sample collected at time of progression (tn, Figure 3). To this end, we leverage three different technologies towards generating proof-of-concept data for the identification of altered DNA methylation in ctDNA. On one hand, we apply global DNA methylation analysis of cfDNA using bisulfite treatment followed by WGS. On the other hand, we enrich for 5mC and 5hmC sites from cfDNA using low amounts (1–10 ng) of input samples, followed by NGS (MeDIP-Seq). Furthermore, we apply a DNA methylation assay based on methylation-sensitive restriction enzymes (MSRE), followed by ddPCR (Figure 3). By comparing the methylation profiles at progression versus baseline, we identify differentially methylated regions as potential markers of tumor progression and treatment resistance. In cases where genome-wide DNA methylation data are available as part of DKTK MASTER, we study the concordance of the methylation profiles of tissue and LB. We also plan to perform epigenomic analysis on patient samples which were also characterized for their mutational profile. This offers the opportunity for comparative analysis to assess the added predictive value of epigenomic alterations when combined with mutations.

In CUPs, the analysis of specific methylation patterns within ctDNA can be used to reveal the origin of the primary tumor, as specific tumors exhibit distinctive methylation patterns [40, 41]. To this end, we carry out bisulfite sequencing of cfDNA extracted from blood samples of patients suffering from cancers with a known primary origin. We use the obtained methylation data to match prospectively collected samples from CUP patients. For selected entities such as sarcoma, we investigate the feasibility to develop molecular classifiers for tumor sub-entities based on plasma DNA methylation patterns. Our efforts also utilize existing tissue-based methylation data from DKTK MASTER [1] and publicly available sources such as TCGA [8].

Outlook

Being in close contact with MTB clinicians, the EXLIQUID consortium plans to report back LB findings with clinically useful information for treatment decisions, thus pushing the progress of implementing LB into clinical routine. By establishing a high-quality LB biobank infrastructure for a national precision oncology cohort with a harmonized IT structure, EXLIQUID contributes to future collaborative research efforts within the DKTK and beyond. The EXLIQUID consortium is open for academic collaboration and additional university hospitals contributing with LB projects.

Although the initial idea of EXLIQUID was to harness, harmonize, and unify the liquid biopsy expertise in the DKTK network within clinical studies, we are well aware of the urgent need to implement liquid biopsies into clinical

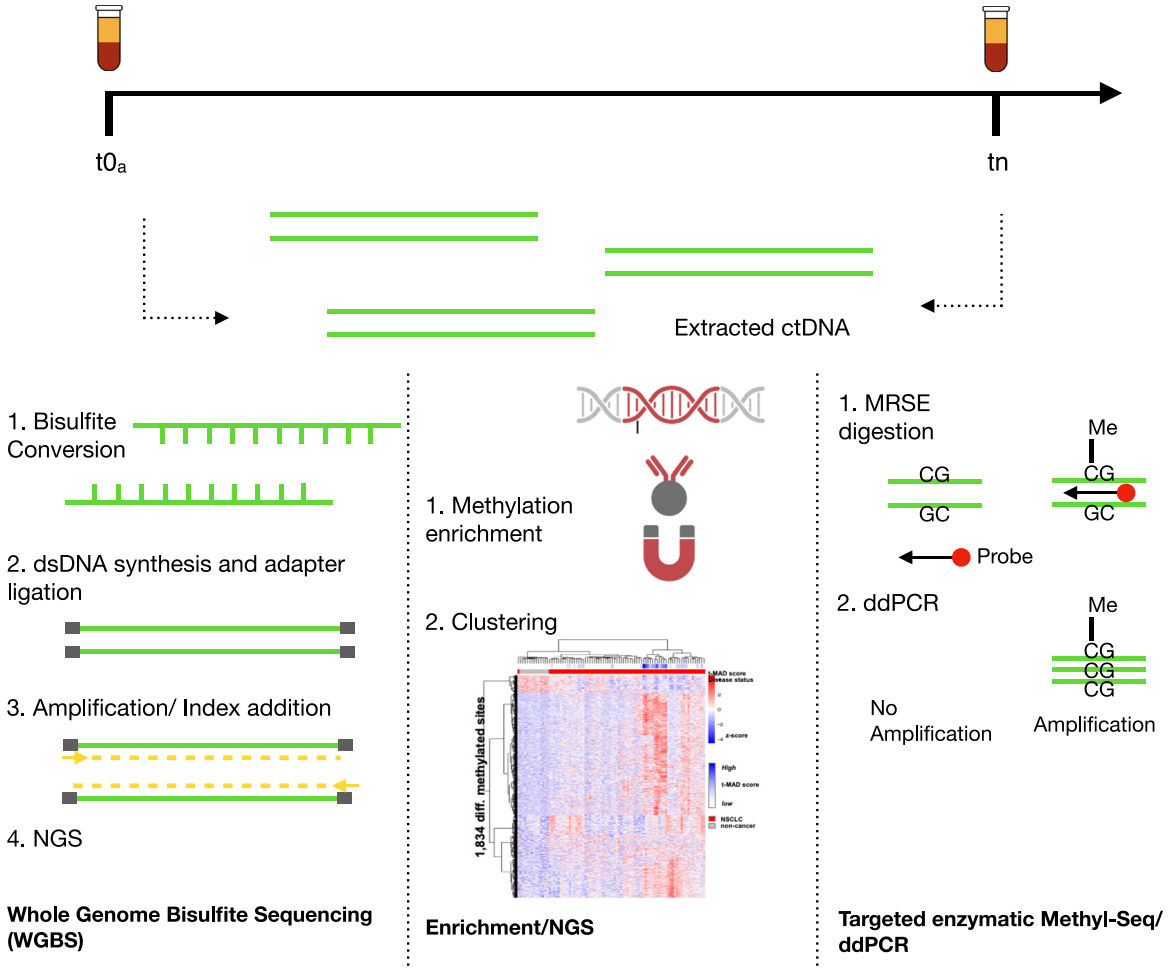

Figure 3: Illustration of the methylation techniques applied within the consortium.
Samples from $t0_a$ and tn are processed and analyzed with Whole Genome Bisulfite Sequencing (left panel), antibody enrichment/NGS Met Seq (middle panel), or targeted enzymatic Methyl-Seq/ddPCR (right panel).

routine diagnostics with respect to regulations (such as the European Union *In Vitro* Diagnostics Regulation), reimbursement, and clinical oncological guidelines. Hence, our plan is to actively pursue these implementation issues by public outreach activities and active participation in medical societies through members of the EXLIQUID consortium.

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and helped shape the research, the planned experiments, and the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Informed consent: Broad informed consent is obtained from all individuals included in this study.

Ethical approval: Sampling and biobanking is conducted under a broad consent established at each of the participating university hospitals after approval of the local Institutional Review Boards. Further analyses using samples or patient data will be performed after approval by the local Institutional Review Boards.

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