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Concept and feasibility of the Augsburg Longitudinal Plasma Study (ALPS) – a prospective trial for comprehensive liquid biopsy-based longitudinal monitoring of solid cancer patients

https://doi.org/10.1515/labmed-2023-0156
Received December 22, 2023; accepted March 29, 2024;
published online April 19, 2024

Abstract

Objectives: Liquid biopsy (LBx) provides diagnostic, prognostic and predictive insights for malignant diseases and offers promising applications regarding tumor burden, tumor heterogeneity and clonal evolution.

Methods: The Augsburg Longitudinal Plasma Study (ALPS) is a prospective trial for patients with metastatic cancer that comprises sequential collection of LBx samples, tumor tissue, radiological imaging data, clinical information and patient-reported outcomes. Peripheral blood plasma is collected based on the individual patient's staging intervals and LBx-derived ctDNA analyses are performed using CAncer Personalized Profiling sequencing (CAPP-seq).

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Results: From April 2021 to October 2023, 419 patients have been enrolled. A total of 1,293 LBx samples were collected, 419 samples (100 %) at the beginning of the study and an average of 3 (range 1–12) during the 30-month follow-up period of the current interim analysis. 380 tissue biopsy (TBx) samples (90.7 %) were available at baseline and 39.6 % had ≥1 TBx samples at follow-up. Lung cancer patients are most prevalent in ALPS (n=147), followed by colorectal (n=38), prostate (n=31) and gastroesophageal cancer (n=28). On average, 12.0 ng/mL plasma cell-free DNA (cfDNA) could be isolated. First CAPP-seq analyses in 60 patients comprised 110 samples and demonstrated a detection sensitivity of 0.1 %.

Conclusions: The first interim analysis of ALPS confirms feasibility for comprehensive longitudinal evaluation of LBx and demonstrates suitability for ctDNA evaluation.

Keywords: liquid biopsy; ctDNA; longitudinal profiling; clonal evolution; spatial heterogeneity; translational research

Introduction

Advances in molecular tumor diagnostics have dramatically improved the functional understanding of oncogenesis and enabled comprehensive molecular characterization of many different types of cancer [1–4]. As a result, new predictive biomarkers have been identified and targeted anticancer drugs have been developed. Today, more than 80 targeted cancer therapies have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of solid malignancies, approximately half of which are linked to specific predictive biomarkers, enabling personalized cancer treatment [5]. However, the molecular mechanisms underlying response and acquisition of resistance are still incompletely understood [6].

Predictive biomarkers rely almost exclusively on genomic profiles obtained from pre-therapeutic tissue biopsies (TBx) and therefore depend on sample availability and sufficient quality. Moreover, they are susceptible to sampling biases arising from intraindividual or even intralesional spatial heterogeneity [6–8]. Tumor cells have the potential of adapting to the novel environment provided by the treatment and its selective pressure. Consequently, individual cells may develop resistance mechanisms over time [9]. Limited access to paired patient samples before, during and after therapy hampers the systematic characterization of clonal evolution resulting from the adaption processes of tumor cells. Longitudinal observation of molecular changes enables not only to track cancer evolutionary trajectories but also to understand resistance mechanisms and to identify secondary genetic alterations driving relapse or progression which can potentially be targeted [10, 11].

In addition to molecular profiling of tumor tissue, complementary information can be gained from non-invasive liquid biopsies (LBx). LBx can be generated from various biological fluids, such as peripheral blood, urine, or cerebrospinal fluid, to uncover critical information about a patient's tumor characteristics [12]. LBx aims to analyze tumor-derived material such as circulating tumor DNA (ctDNA), a subfraction of cell-free DNA (cfDNA), circulating tumor cells (CTCs), extracellular vesicles, peptides, and many others. Profiling of ctDNA enables monitoring of tumor burden and tracking of driver alterations over time, which has the potential to detect, characterize and respond to molecular relapse prior to clinical relapse [13–16]. However, few conclusive data are available to date evaluating the clinical utility of molecular tumor profiling over time in routine clinical practice. Moreover, data on clonal evolution tracked by LBx across entities treated with non-targeted therapies, e.g., chemotherapy, is sparse [17, 18].

Although LBx appears to have relevant advantages over TBx, substantial limitations remain, including problems with sensitivity and specificity, dependence on tumor size and type, and lack of information on tissue architecture [6, 18]. Overcoming these limitations is critical to fully evaluate the potential of liquid biopsy to improve cancer treatment and outcomes but also its role in clinical routine, e.g., as a complement or substitute for radiographic response assessment or as a universal tumor marker. While liquid biopsy shows great promise, further research and validation are needed to establish its reliability across different cancer types and stages. Standardization of testing methods and protocols is crucial to ensure consistent results [19, 20].

The prospective Augsburg Longitudinal Plasma Study (ALPS) provides a research platform to systematically investigate the value of longitudinal LBx profiling in solid malignancies with a particular focus on precision oncology.

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The study protocol focuses on the sequential collection of LBx and TBx samples and the comprehensive collection of clinical, imaging and outcome data to molecularly track and characterize individual patients in their respective clinical course. This design enables the systematic investigation of spatial and temporal heterogeneity of tumors and the characterization of developmental patterns in response to treatment.

Materials and methods

Study protocol and patient recruitment

Patient enrollment and prospective biobanking is performed after patients have provided written informed consent for the ALPS trial and simultaneously for biobanking at the Augsburg Central Biobank (ACBB). The essential feature of the study is that it accompanies the patient in routine clinical practice, that all additional examinations are performed as part of the clinical routine examinations, and that ALPS thus does not alter the clinical course of the patient. Main inclusion and exclusion criteria are given in Supplementary Methods. Eligible patients are identified through reviewing appointment lists, recommendations from tumor conferences and direct contact with the treating physicians. This trial is conducted in accordance with the Declaration of Helsinki, approved by the Local Ethics Committee and registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT05245136).

Sample collection, preparation, and storage

Peripheral blood is obtained by collecting five and three 9 mL EDTA containers at baseline and at each follow-up visit in addition to regular blood tests, respectively. Blood samples are processed within 2 h of collection. Plasma is separated by centrifugation at 2,000 *g* for 10 min (Hettich Rotina 38R, 20 °C, no brake), transferred to a new tube, and centrifuged at 2,000 *g* for 10 min at room temperature to eliminate any residual cellular debris. Plasma samples are then stored at −80 °C in 1.9 mL aliquots in the Augsburg Central Biobank (ACBB) for the duration of the study. Patient material remaining after the completion of the study will be stored at the ACBB for future Ethics Committee-approved research based on the biobank consent.

cfDNA isolation and library preparation for CAPP-seq based analyses

cfDNA extraction and library preparation is performed using the Roche Avenio ctDNA technology (Roche Holding AG, Basel, Switzerland) with a regular input volume of 4 mL plasma. cfDNA concentration is determined using the Qubit 1x dsDNA HS Assay Kit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). cfDNA quality and size distribution is assessed with the Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Santa Clara, CA, USA).

The sequencing libraries are prepared according to the manufacturer's protocol (Roche Avenio ctDNA Surveillance Kit) from the maximum possible input DNA for each sample. DNA concentration of pre-enriched and final libraries are measured with the Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). The fragment size and distribution of the libraries are determined using the Bioanalyzer High Sensitivity DNA Analysis (Agilent Technologies, Santa Clara, CA, USA).

Libraries are sequenced using paired-end 100 bp sequencing aiming at a theoretical coverage of 20,000fold on an Illumina Next Seq550 or on an NovaSeq 6000 platform (both Illlumina, San Diego, CA, USA, sequencing performed by the NGS Competence Center Tübingen, NCCT) ensuring a minimum analytical sensitivity of <0.1 %. These analytical sensitivity requirements comply with the Rili-BAEK guidelines, which stipulate a minimum sensitivity of 0.5 % for DNA from cell free body liquids [21].

The Roche Avenio Surveillance Assay is a commercially available assay validated for research use only. In addition, for validation purposes, we performed several preliminary tests with defined mutations (e.g., in the *BRAF* and *EGFR* genes) in which different dilutions were systematically compared and checked for validity using digital PCR (QuantStudio™ 3D, ThermoFisher Scientific, Waltham, MA, USA) and the Roche Avenio ctDNA Surveillance Kit.

Collection of clinical data and patient reported outcome measures (PROM), and asservation of germline material, preparation of tissue biopsies, and whole exome sequencing (WES) of TBx are described in Supplementary Methods.

Results

ALPS conceptualization and structure

ALPS was designed as an observational longitudinal study that does not interfere with the patient's clinical path and monitors the course of therapy at various levels. In addition to obtaining sequential TBx and LBx, these levels also include the documentation of the clinical course with clinical and laboratory parameters, the recording of diagnostic imaging at study inclusion and during sequential staging, and the recording of patient-related outcome measures (PROMs) (Figure 1A). All biosamples and data are collected in a registry platform, which forms the infrastructure level of ALPS, where different study endpoints are addressed and different projects and exploratory objectives defined in the study protocol are set up (Figure 1B).

In addition to the primary and secondary endpoints defined in the study protocol (concordance/discordance between TBx and LBx, resolution of spatial tumor heterogeneity and monitoring of patients treated with precision medicine), these projects include a number of exploratory projects and endpoints enabled by the open and flexible structure of ALPS. In addition to cfDNA-based genomic analyses, ALPS enables the analysis of other tumor components or information from LBx, such as genome-wide DNA methylation patterns from cfDNA and the isolation and analysis of extracellular vesicles and various peptides

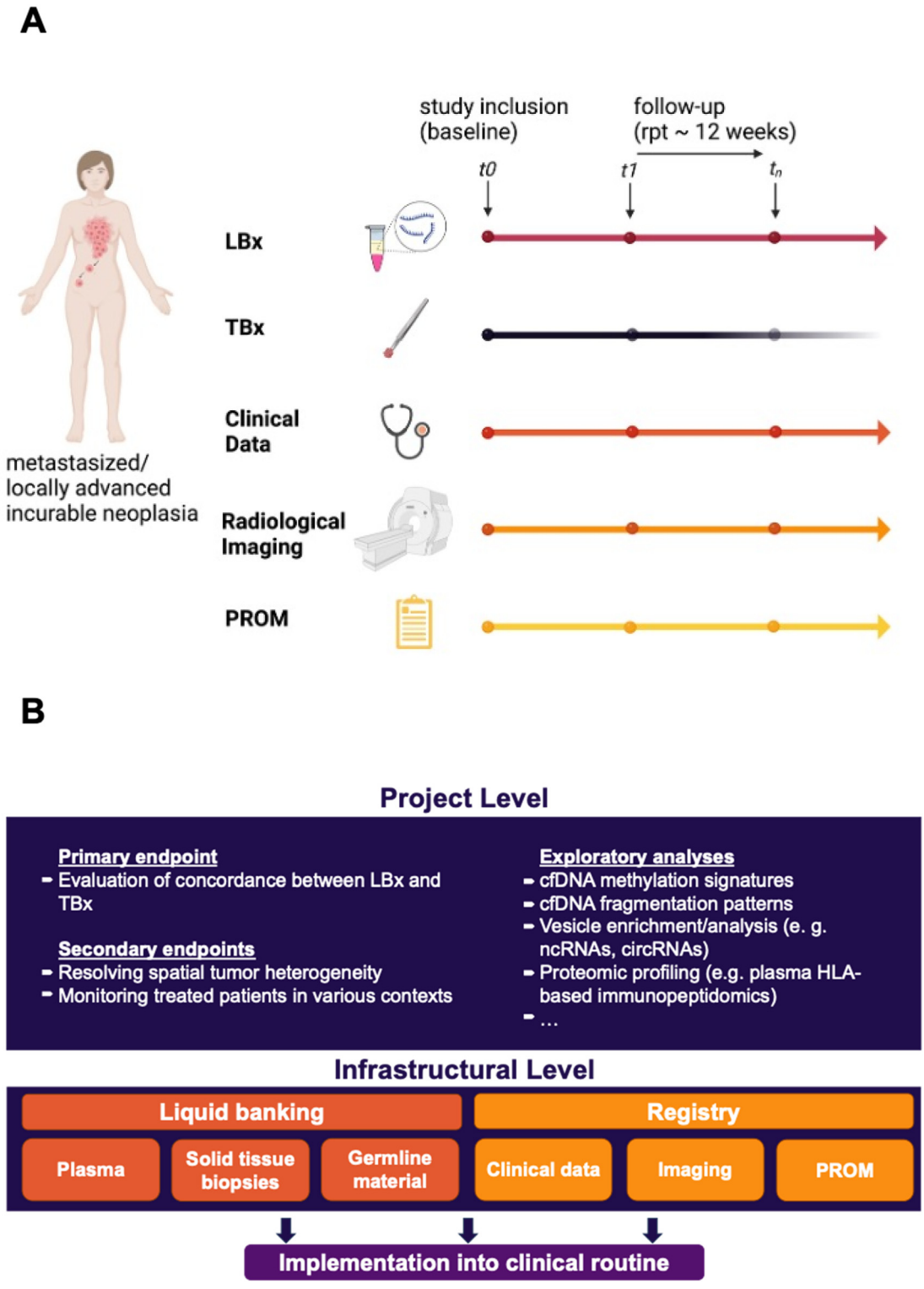

Figure 1: ALPS conceptualization, structure and data collection scheme. (A) Patients diagnosed with metastatic or locally advanced incurable tumor disease will be enrolled in the study at the time of diagnosis or change of treatment line and will be followed comprehensively throughout the course of the disease. As outlined in the study protocol, clinical data, radiologic imaging, liquid biopsy (LBx) and tissue biopsy (TBx) samples, and patient-reported outcome measures (PROMs) are systematically collected over time. The follow-up intervals (rpt) are determined by the physicians responsible for the treatment and are approx. 10–12 weeks for most patients. (B) Schematic representation of the structure of the ALPS trial. ALPS is based on an infrastructural level consisting of two pillars (liquid plus tissue biobanking and clinical registry) and on the project level, which is characterized by the primary endpoint, secondary study endpoints as well as a variety of exploratory analyses and respective satellite projects.

including immunopeptidomics (data not shown). Thus, biobanking in ALPS in combination with deep phenotyped data enables future translational research projects and further LBx analyses.

Patient enrollment and clinical characteristics

Recruitment for the ALPS cohort started on 04/01/2021 at the time of initial diagnosis or at the time of change to next treatment line for patients with metastatic or locally advanced, incurable malignant disease. Recruitment is currently open at Augsburg University Hospital for both inpatients and outpatients. Eligible patients are identified by screening appointment lists, clinical records, tumor board recommendations, and direct contact with treating physicians. Biomaterial for TBx is collected during routine clinical biopsy. LBx is collected as part of a routine blood draw. Clinical and patient-centered data are documented and LBx samples are collected synchronously with routine clinical presentations and staging examinations.

The current interim analysis with database cut-off on 10/18/2023 covers a recruitment period of approximately 30 months. During this period, 1,104 patients were screened and ultimately 419 patients who fulfilled the inclusion and exclusion criteria were consented and included in the study (Figure 2). This corresponds to a number needed to screen of 2.5. The screening rate was 28 patients per month from 04/2021 to 04/2022. Through optimizations in the screening procedure, the screening rate was increased to 39 patients per month from 05/2022 until the database cut-off 10/2023. The inclusion rate remained constant over the entire period at approximately 13.8 patients per month (Supplementary Figure 1). Of those screened, 668 patients could not be included in the study. Major reasons for screening errors were failure to meet the in- and exclusion criteria (n=416, 62.3 %), patient refusal (n=106, 15.9 %), loss of contact or loss to follow-up after screening and before inclusion (n=67, 10.0 %), organizational reasons related to the inpatient care of the patient (n=53, 7.9 %), and early death of the patient (n=26, 3.9 %). At time of interim analysis, 179 patients (42.7 %) were active patients in the study protocol. Of the 240 inactive patients, 179 (74.6 %) died and 57 (23.7 %) were lost to follow-up, two declined participation after inclusion and two further were removed a posteriori since criteria for inclusion/exclusion were wrongly assessed.

Patient characteristics are given in Table 1. Thirty-six percent of the included patients are female, median age is 67 years (range 25–91 years). The most common diagnosis in the ALPS cohort is lung cancer (35.1 %) followed by bowel cancer (9.1 %), prostate cancer (7.4 %), and cancer of unknown primary/CUP syndrome (6.2 %, Figure 3A, B). A total of 48 patients from the ALPS cohort (11.5 %) presented to the molecular tumor board (MTB) during their time on study and were thus subject to molecular follow-up via ALPS.

Longitudinal biobanking and follow-up

Biospecimens are collected at predefined time points based on sequential stagings of each patient. At patient inclusion, timely tissue samples were obtained from 380 patients (90.7 %) and LBx samples from all 419 patients (100 %). Similarly, germline material by means of peripheral blood mononuclear cells (PBMCs) was obtained from the same 419 patients. To reflect the longitudinal course of the patients, LBx samples and clinical data were collected synchronously with the re-staging examinations. For the first 419 patients (comprising a total of 1293 LBx samples), a total of 874 LBx follow-up samples in addition to the baseline LBx samples, are currently available. This corresponds to a median (mean) of 2 (3) (range 1–12) follow-up assessments (Figure 4). At follow-up visit 1, LBx samples were available from 65.2 % of patients, and at follow-up visits 2 and 3, 45.6 and 32.5 %, respectively. For 39.6 % of patients, at least one follow-up TBx (exceeding the re-biopsy rate of 15 % anticipated in the trial protocol) was obtained upon detection of tumor progression. The re-biopsy rate reflects routine procedures in a routine clinical setting. The main reasons for not performing re-biopsies were the risk of the procedure, the deterioration of the patient's condition and the difficulty of accessing the tumor lesion. The time between follow-up visits differed from patient to patient depending on their clinical follow-up and was 12 weeks in median (range 0–80, interquartile range (IQR): 9–16) at the time of interim analysis. These data represent a "snapshot" as the cohort continues to mature through the 181 patients in follow-up. Concurrent with the sequential preservation of LBx and TBx, we performed the longitudinal clinical documentation, storage of radiological image data, and PROM data.

Median follow-up visits of the 48 patients who were also discussed in the molecular tumor board (MTB) at Augsburg University Hospital were 4 (range 1–12; IQR 2–6). Here, ALPS offers a unique opportunity to generate not only clinical and patient-centered outcome data but also molecular follow-up for MTB patients who were treated with molecular-guided treatment regimens.

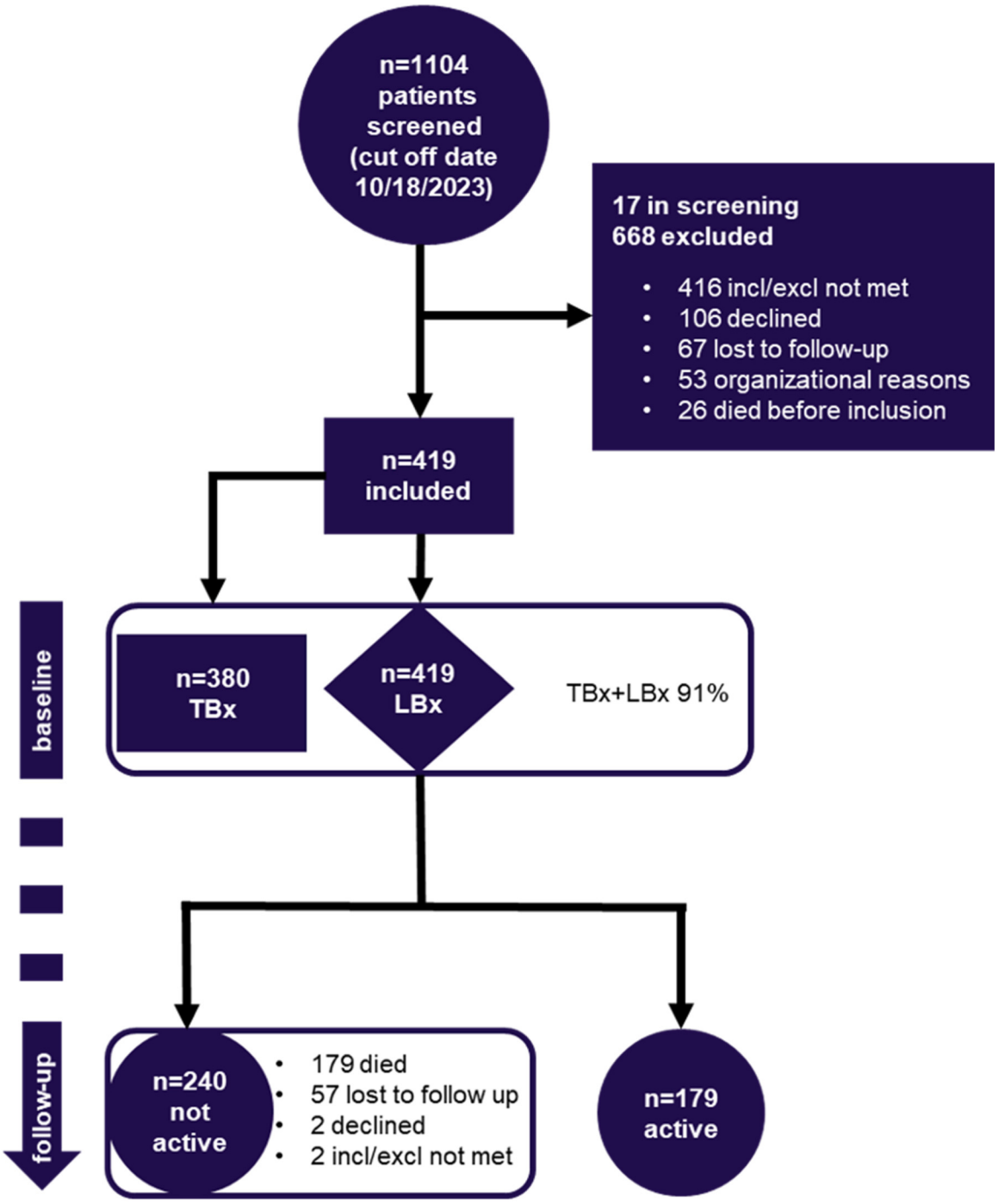

Figure 2: ALPS consort diagram showing screening, inclusion, liquid and tissue biopsy rates and patients currently under follow-up as of cut-off Oct 18, 2023.

Liquid biobanking, preanalytical quality and cfDNA preparation

The collection and storage of high-quality LBx samples is a central component of ALPS. In order to ensure the pre-analytical quality of the samples, tests with different blood container systems and latencies from sample collection to processing were carried out as part of the study preparation. The decay of hematopoietic cells from peripheral blood between sample collection and processing is a source of "contamination" of cfDNA with genomic DNA (gDNA). Since we minimized the latency time from collection to sample

Table 1: Basic patient demographics of the entire cohort, available biosamples and clinical data by cut-off Oct 18, 2023.

Total	n	419
Age, years	Median, range	67, 25–91
Sex	n (female:male)	151:268
TBx at baseline (t_0)	n (%)	373 (89.0 %)
≥1 TBx during follow-up (t_n)	n (%)	166 (39.6 %)
LBx at baseline (t_0)	n (%)	419 (100 %)
≥1 LBx during follow-up (t_n)	n (%)	273 (65.2 %)
PROM at baseline (t_0)	n (%)	348 (83.1 %)
≥1 PROM during follow-up (t_n)	n (%)	212 (50.1 %)
MTB patients in ALPS	n (%)	48 (11.5 %)

processing to <2 h in the current monocentric setting of ALPS, a cost-effective blood collection could be performed using conventional EDTA containers. A direct comparison with container systems containing cell-stabilizing agents showed similarly low levels of contaminating gDNA for all blood container systems in the period from 1 to about 8 h after collection (Supplementary Figure 2).

For the first 419 patients (comprising a total of 1293 LBx samples), the median time from blood collection to start of sample preparation was 30 min (IQR 21–47 min). Plasma was prepared from five 9 mL EDTA containers at baseline and from three 9 mL EDTA containers for follow-up visits. The median time from blood collection to cryoconservation of plasma samples was 90 min (IQR 72–111 min). Plasma samples were stored in aliquots of 1.9 mL at −80 °C.

So far, cfDNA was isolated from 330 LBx samples. The concentration of cfDNA varied significantly between entities, individual patients, samples, and time points. On average, 12.0 ng/mL plasma (range 1.2–2300.0 ng/mL plasma) could be isolated. Figure 5A shows the concentration at the time of study inclusion (baseline) differentiated by individual entities. During follow-up, cfDNA was also successfully detected in subsequent LBx samples, although the amount of cfDNA isolated here varied greatly between individuals and timepoints (Figure 5B).

CAPP-seq analysis was successfully performed on the cfDNA of the first 48 patients and demonstrated that the isolated material was suitable for the planned analyses and endpoints and could be processed accordingly. The sequencing parameters were chosen with 60x 10^6 reads per sample based on an on-target rate of 40–60 % and a duplicate rate of 20–30 %. The aim was to generate an average unique coverage of 6,000–7,000-fold to achieve a sensitivity of approximately 0.1 % for the detection of variants. CAPP-seq analyses was performed for the first 110 samples from 60 patients and yielded between 3.93 and 29.72 million reads with an on-target rate of 40–80 %. After first level processing and deduplication of the sequencing data, we achieved an average read depth of 5,218 reads (range 1,390–9,132) and a detection sensitivity for variants of <0.1 % per sample, demonstrating the applicability of CAPP-seq to samples of the ALPS cohort and the feasibility of our approach. In four cases, the median sequencing depth was below 3,000 (2,475). In these patients, it could not be guaranteed that the sample

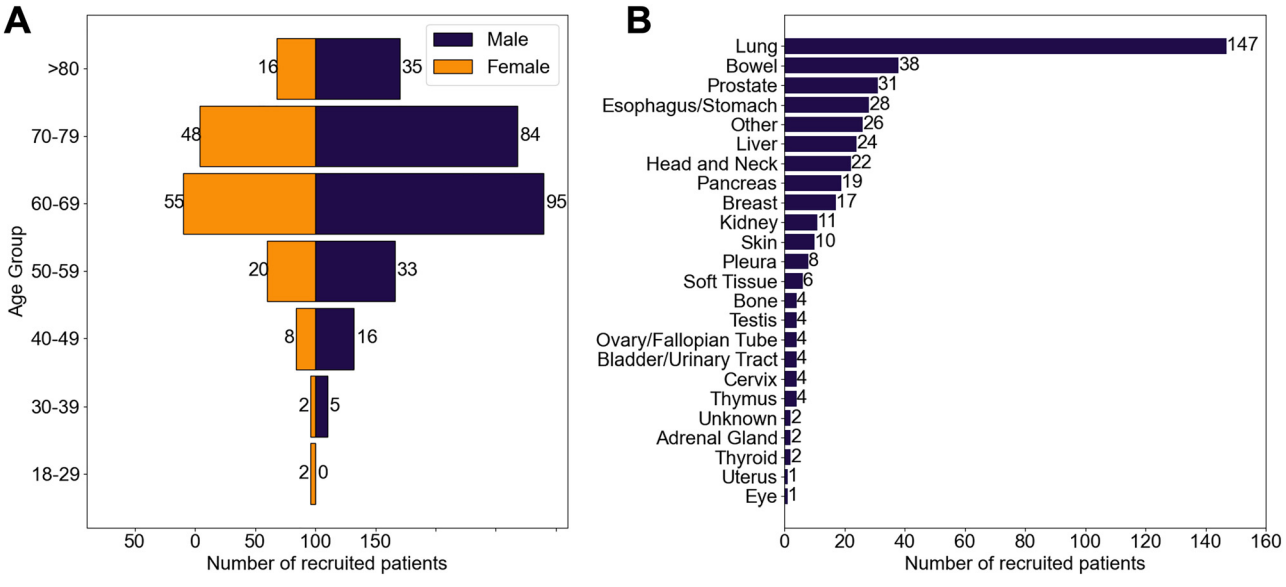

Figure 3: Age and entity distribution in the ALPS collective. (A) Age distribution in age groups of 10 years for female and male study participants and (B) distribution of cancer entities (according to the MSKCC OncoTree nomenclature) by cut-off Oct 18, 2023.

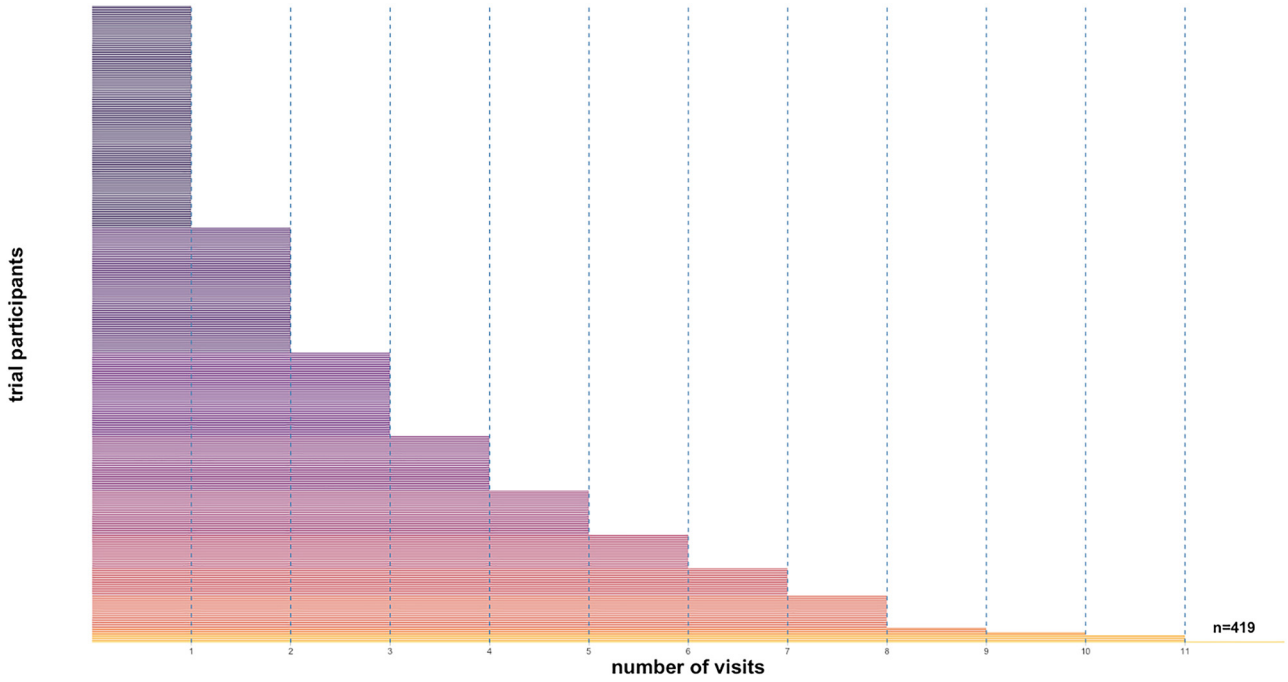

Figure 4: Swimmer's plot depicting the number of total visits (baseline plus follow-up visits) for all individual patients and by cut-off Oct 18, 2023. The Swimmer's plot shows the number of follow-up examinations for each individual patient by means of horizontal lines separated by vertical blue lines.

sensitivity reached the target detection sensitivity of 0.1 % as described above (Supplementary Figure 3A, B).

Using this approach, different numbers of cancer-related mutations and copy number variations were identified in all analyzed samples from five subcohorts defined by common entities (Figure 6A). These could be used to track tumor mass by estimating allelic burden and indicate clonal evolution under therapy. Figure 6B shows an example of the integration of LBx-derived data on the variant allele frequency of individual variants and the resulting tumor burden, radiological imaging data and the course of treatment.

Discussion and perspective

It has been shown that LBx can overcome the limitations of molecular characterization, such as spatial heterogeneity and molecular changes of the tumor over time, which significantly affect the validity of (repeated) TBx from single tumor lesions [6, 17, 22, 23]. A major advantage of obtaining tumor information from body fluids is that LBx rather than TBx can be employed to virtually continuosly monitor the molecular landscape and clonal evolution of malignant diseases and thereby generate real time updates on how the disease adapts to specific treatments [24–26]. Several exciting clinical trials such as the phase II CHRONOS trial have demonstrated that the real-time availability of this information can be successfully used to directly impact on treatment decisions [18]. Although a growing body of evidence supports the use of LBx as a disease monitoring and treatment management tool [27–29], its implementation in routine patient care still lags well behind its expected potential.

A major challenge for the evaluation and clinical application of longitudinal LBx data is to integrate it with all associated and relevant patient- and disease-related aspects as a multimodal longitudinal monitoring approach. For example, in addition to the clinical course, the collection of data from imaging (e.g., CT, MRI, etc.), other clinical and laboratory tests and PROM is relevant to placing LBx in a clinically applicable context. In short, this means providing a comprehensive overview of the "patient journey" while gaining deep insights into the dynamics of disease biology through LBx to better and more effectively shape patient care. This approach is particularly important for patients treated with unapproved drugs in precision oncology, e.g., in the context of molecular tumor boards [30].

The aim of ALPS is to systematically record longitudinal patient outcomes in a "real world" setting and complement them with structured assessment of LBx and TBx. By systematically performing LBx in patients with different malignancies in the context of their routine clinical course, ALPS aims to generate precise insights into disease biology, response to treatment and development of resistance.

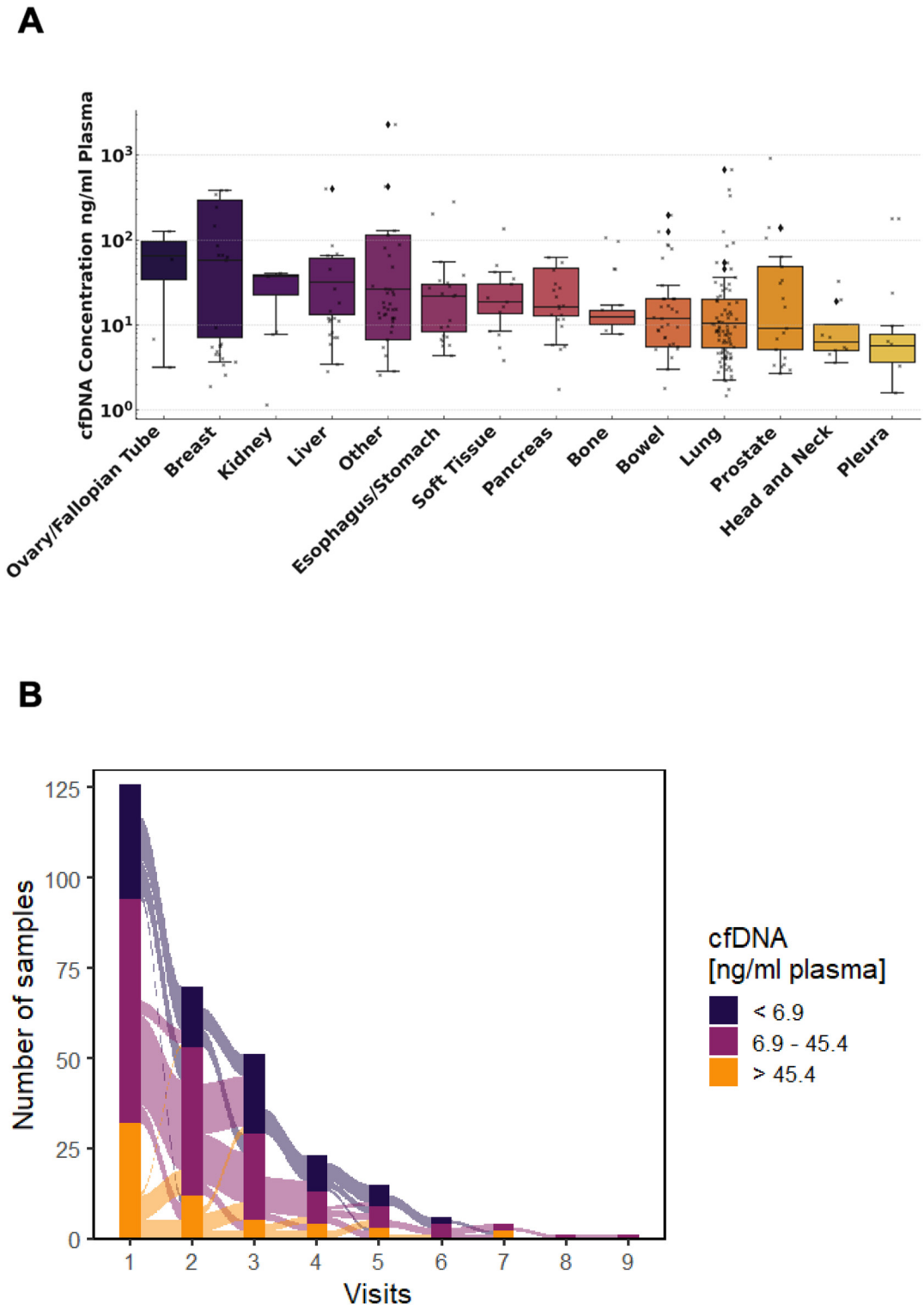

Figure 5: Overview of cfDNA quantity in ALPS. (A) Concentration of cell-free DNA (cfDNA) in ng cfDNA per 1 mL plasma from plasma isolations of the first 126 patients, grouped by cancer entity. (B) Changes in the cfDNA quantities isolated from individual patients over the course of the follow-up visits. The distribution of cfDNA concentrations of the first 126 patients was divided into quartiles at the time of study inclusion (with Q1of 6.9 ng and Q3 of 45.4 ng cfDNA/ml plasma). These quartile-related cut-off values were used for the distribution of the isolated cfDNA quantities at the follow-up visits in order to visualize cfDNA dynamics and relevant differences in cfDNA recovery.

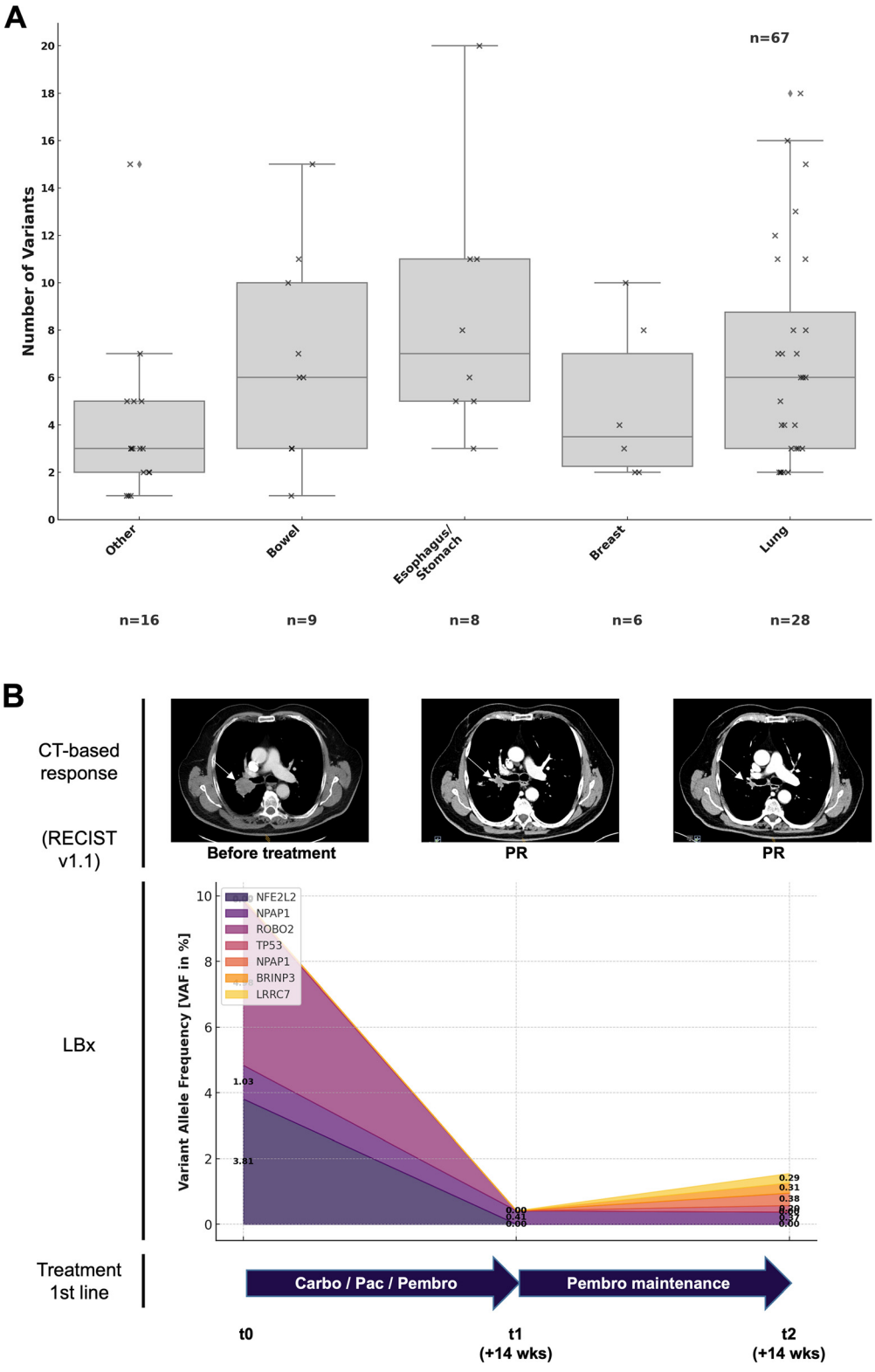

Figure 6: CAPP-seq based ctDNA analyses in ALPS. (A) Filtered variants by major entities. The entire spectrum of all entities is discussed in more detail in Figure 3B. In this illustration, CUP patients are listed in particular under "other". (B) Example of a patient with non-small cell lung cancer (NSCLC) who was monitored in ALPS from the start of first-line therapy. The upper panel shows CT-based imaging before initiation of therapy (t0), at time t1 after 14 weeks (wks) of immunochemotherapy and at time t2 after a further 14 weeks of immune maintenance therapy. Response (PR, partial response) is indicated according to RECIST criteria (v1.1). The white arrows mark the target lesion. The mean panel shows the variant allele frequency (VAF) in percent over timepoints t0-t2 in the genes/gene regions with detectable variants. Maximum detection sensitivity for variants is 0.1 %. A VAF of 0 is considered to be equivalent to not detected at a sensitivity level of 0.1 %. The lower panel depicts the course of therapy with the following substances: Carbo (carboplatin), Pac (paclitaxel), Pembro (pembrolizumab).

Metastatic cancer is characterized by spatial and temporal heterogeneity, which poses a challenge for clinicians. ALPS addresses this by systematically evaluating the complementary value of TBx and LBx and continuously monitoring LBx samples, enabling the identification of evolving molecular profiles. This dynamic approach ensures that future treatment decisions are based on the most up-to-date and relevant information. We are currently focusing our CAPP-seq-based analyses on the Roche Avenio Surveillance Assay because, despite its limited panel size of less than 200 kb, it covers 471 frequently mutated regions in 197 genes and yields robust results across all entity types included in our trial. Its high sensitivity, which enables reliable ctDNA analyses with an allele frequency of less than 0.5 %, facilitates sensitive longitudinal observations. Depending on specific sub-cohorts and specific questions, further tailored gene panels will also be used in the future.

In addition, initial pilot projects based on ALPS have already shown that the LBx samples not only serve as cfDNA resource, but also enable future analyses of e.g., extracellular vesicles, non-coding RNAs (e.g., lncRNAs and circRNAs) and proteomic changes (e.g., characterization of the soluble immunopeptidome).

Since 2021, a total of 419 patients with various metastatic malignant entities have been enrolled in ALPS. The lack of availability of TBx in routine clinical practice is one of the main reasons for non-inclusion resulting in a relatively high screening failure rate, indicating that repeated TBx are performed too rarely in the current clinical routine. In addition, a relevant proportion of patients refused to participate in ALPS, suggesting that participation in clinical trials may not be a high priority for patients in difficult and potentially life-threatening situations.

Recruitment and follow-up in ALPS is not time-limited, allowing for a constantly growing cohort that will continue to mature with increasing follow-up time. This will allow further valuable conclusions to be drawn in the future that go beyond the molecular genetic landscape, including the association with prognostic parameters and patient-relevant endpoints such as overall survival and quality of life.

To enable the feasibility of this project, which is based on a large LBx banking approach, appropriate infrastructures were implemented to reduce time and financial expenditure. Our data show that plasma collection with EDTA containers is feasible and provides high quality data by controlling the processing time. The data on the quality and quantity of cfDNA and CAPP seq-based molecular profiling confirm the procedure described for ALPS. Although we have standardized and controlled the pre-analytical parameters, such as the time from blood collection to processing, in the best feasible way, the quality of the samples remains a critical key factor for the success of ALPS and must therefore be constantly monitored and re-evaluated.

We have set up our analyses with the aim of achieving an analytical sensitivity of at least 0.1 % and thus clearly fulfilling the criteria of the Rili-BAEK guidelines for the measurement of DNA from cell-free body fluids. With this first series of sequencing results from 110 samples, we show that this analytical sensitivity for ctDNA can be achieved in almost all ALPS samples analyzed so far.

Despite its promise, the ALPS platform does have further potential limitations that need to be acknowledged. The availability of FFPE tissue instead of fresh frozen tissue poses a technical challenge, especially when comparing the molecular genetic changes between TBx and LBx. Additionally, there may be limitations and biases in the representation of entities in the study, which may not fully reflect the prevalence of certain cancer subtypes.

ALPS is currently designed as a monocentric study, which could contribute to a bias in the representation of patients represented in ALPS and affect the generalizability of the results. A multicenter roll-out is planned to adequately map entity prevalences, record rare entities and thus generate greater significance for the planned analyses. Furthermore, it is planned to expand the existing study concept and to transfer the comprehensive multimodal longitudinal LBx-based monitoring to the perioperative or periinterventional context and to include patients who are treated neoadjuvantly or adjuvantly. As the platform evolves and expands its scope and reach, the potential for improving patient outcomes and advancing our understanding of metastatic cancer remains significant.

However, in order to further introduce LBx into routine diagnostics and create financial reimbursement opportunities, not only the scientific evidence but also the legal requirements and regulatory conditions must be created. In Europe in particular, this includes the need to establish conformity with the *In Vitro* Diagnostic Regulations (IVDR) for diagnostic assays.

In summary, the ALPS cohort presented here is a successful attempt to implement a structure for a comprehensive LBx-based longitudinal profile for patients with solid malignancies. The overall goal is to consolidate the clinical value of LBx, provide evidence for LBx-based monitoring, establish structural requirements, and identify harmonization needs for the clinical use of LBx, thus helping to translate LBx into routine clinical practice.

Acknowledgments: We thank all patients and their relatives for their willingness to participate in the Augsburg Longitudinal Plasma Study (ALPS) while being confronted with the

challenging situation of fighting a serious disease. This work was partially funded by the intramural funding program of the Faculty of Medicine, University of Augsburg. We would like to thank the Medical Faculty of the University of Augsburg and all departments of the University Hospital Augsburg for their continuous support. We would also like to thank the staff of the Augsburg Central Biobank (ACBB) for their help in processing, storing, and documenting the samples. NGS methods were performed with the support of the DFG-funded NGS Competence Center Tübingen (INST 37/1049-1).

Research ethics: This trial is conducted in accordance with the Declaration of Helsinki, approved by the Local Ethics Committee and registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT05245136).

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission. Conception and Design: SS, MS, MT, RC. Provision of study materials or patients: LU, AAN, RB, CL, MK, AL, BM, BK, MT, RC. Collection and assembly of data: SS, MS, AS, SB, ME, TM, AS, LR, MC, MW, MM, NC, MP, SH. Data analysis and interpretation: SS, MS, KH, CL, MK, KT, TW, TK, MM, NC, MP, IS, MS, BK, MT, RC. Manuscript writing: SS, MS, KH, AS, MT, RC. Visualization: MS, KH, AS, RC. Final approval of manuscript: all authors.

Competing interests: The authors state no conflict of interest.

Research funding: Intramural funding program of the Faculty of Medicine, University of Augsburg. S.S. was supported by the Clinician Scientist program of the Faculty of Medicine of the University of Augsburg.

Data availability: The raw data can be obtained on request from the corresponding author.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/labmed-2023-0156).