2024 CNAPS
Circulating Nucleic Acids in Plasma and Serum

Med Uni Graz

13th CNAPS International Symposium on

Circulating Nucleic Acids in Plasma and Serum

Graz, Austria
March 4-6th, 2024

ABSTRACT BOOK

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ORAL PRESENTATIONS

Unraveling the genetics of cell-free DNA biology using GWAS

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Background: Plasma cell-free DNA (cfDNA) enables non-invasive screening applications, such as Non-invasive Prenatal Testing (NIPT) and Cancer Liquid Biopsies. The fragmentation and concentrations properties of cfDNA are heavily studied, but the (potentially confounding) effect of genetic variation on these properties remains insufficiently explored. Moreover, the biological mechanisms behind cfDNA have been linked to (auto)immunity, inflammation, and coagulation.

Methods: To explore the effect of common genetic variants on concentration and fragmentation properties of cfDNA, we performed multiple genome-wide association studies (GWAS) on imputed genotypes obtained from low-coverage whole genome sequencing data from over 100.000 Dutch NIPT screens.

Results: We found that all cfDNA properties studied have significant, partially distinct, heritable components. Besides associations with known nuclease genes DNASE1L3 and DFFB, we detected hundreds of novel genome-wide significant loci. A common missense variant in DNASE1L3 (p.Arg206Cys) has the strongest effect on most cfDNA traits. Especially in samples which are imputed to be homozygous for this variant, sequenced fragments were longer, less frequently ended in CC/GG and plasma cfDNA concentrations were lower. For the clinical application of NIPT, we show that the altered fragmentation patterns cause predictors to overestimate fetal DNA fractions. Despite this apparent increase in fetal DNA, the reduced cfDNA concentration causes a 14x increased odds of NIPT failure. In addition, we found significant genetic correlations between our cfDNA traits and GWAS of blood cell indices, systemic autoimmune diseases and thrombosis.

Conclusion: In conclusion, we find that a considerable fraction of the variation in cfDNA fragmentation and concentration is attributable to common genetic factors which should be addressed in future clinical applications of cfDNA.

Elucidating the origins of circulating DNA in diverse cancer types using Nanopore whole-genome sequencing

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Background: Cancer patients exhibit elevated circulating DNA levels, including tumor DNA that is amenable to sequencing for detection and monitoring Oncology. Questions remain about the abundance and origin of immune-derived cell-free DNA in cancer. We address this using Oxford Nanopore whole-genome plasma sequencing (cfNano), which maintains the DNA methylation and fragmentation patterns of individual molecules, including longer fragments previously inaccessible using traditional short-read sequencing.

Methods: We screened cancer plasma samples using H3.1 nucleosome levels, selecting several dozen cases that spanned a range of cfDNA levels and included diverse cancer types including Colorectal, Lung, Prostate, Breast, Non-Hodgkin's lymphoma, and Acute Myeloid Leukemia. We profiled these along with 20 healthy individuals using cfNano, estimating cell type composition using a DNA methylation reference atlas of normal human cell types, and cancer fraction based on copy number alterations (CNA) from cfNano sequences as well as matched Illumina whole-genome sequencing. Total DNA concentration and nucleosome concentration (using Volition's H3.1 Nu.Q® platform) were measured to estimate absolute cell type DNA quantities.

Results: cfNano accurately predicted the cancer type's cell of origin in many cases, even with shallow genomic coverage (<1x). In samples with low DNA and nucleosome levels, detection was challenging; however, Nanopore-based CNA detection outperformed Illumina-based detection. High circulating nucleosome levels facilitated more robust detection of the cancer's cell of origin, despite substantial immune-derived DNA. Ultra-long immune-derived fragments (>2kb) were present at low frequency across all samples, with reduced proportions in samples with higher cancer DNA. AML samples exhibited notably high levels of these longer fragments.

Conclusion: Nanopore whole-genome sequencing is a potent tool for detecting and characterizing immune and tumor cfDNA in cancer, extending beyond the capabilities of short read sequencing to offer new insights into the origins of ultra-long DNA fragments.

A novel approach for DNA footprinting using short double-stranded cell-free DNA from plasma

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Background: Size distribution of cell-free DNA fragments has gained attention as a relevant factor for cfDNA analysis and utility for diagnostic purposes. Accordingly, fragmentomics revealed short cfDNA as a promising biomarker class derived from interaction of DNA-binding proteins with regulatory DNA-binding motifs. As short cfDNA only represents a minor fraction of total double-stranded cfDNA we therefore aimed for enrichment of such short ds cfDNA fragments to facilitate genome-wide footprint analyses.

Methods: We established a size-selective preparative gel electrophoresis enrichment approach for short double-stranded cfDNA fragments showing a length distribution of 20 to 60 bps out of blood plasma for high-throughput DNA sequencing and subsequent bioinformatic analyses for footprint analyses.

Results: Short double-stranded cfDNA is enriched at gene promoters, binding sites of transcription factors as well as structural DNA-binding proteins thus facilitating a genome-wide DNA footprint in liquid biopsies. In short double-stranded cfDNA from healthy individuals, we found a significant enrichment of 203 different transcription factor consensus motifs. Moreover, our data suggest that short double-stranded cfDNA is not a degradation product of regular cfDNA but rather represents a biological entity of its own. Levels of short double-stranded cfDNA at specific genomic regions correlate with DNA-methylation and H3K4me3 histone modification levels, as well as gene transcription. When comparing pancreatic ductal adenocarcinoma with colorectal carcinoma or septic with post-operative control patient samples, we identified 731 and 1107 differentially occupied loci, respectively. These regions facilitated discrimination between colorectal and pancreatic cancers as well as between septic patients and clinical controls from patient plasma samples.

Conclusion: Taken together, analysis of short double-stranded cfDNA fragments might provide the most accurate picture of a genome-wide transcription factor footprint in liquid biopsies. With the ability to identify the occupancy of disease-specific transcription factor binding sites, there might be considerable potential for liquid biopsy applications.

Fragmentomics-based methylation analysis (FRAGMA) of urinary DNA enables detection and localization of cancer in urinary system

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Background: Our previous work demonstrated that cell-free DNA (cfDNA) molecules were preferentially cleaved at the methylated cytosines of cytosine-phosphate-guanine (CpG) sites rather than unmethylated ones in plasma. We therefore developed a method named FRAGmentomics-based Methylation Analysis (FRAGMA), which allows us to predict CpG methylation of plasma cfDNA without using bisulfite treatment. In this study, we attempt to extend FRAGMA technology into urinary cell-free DNA (ucfDNA).

Methods: Paired whole-genome and whole-genome bisulfite sequencing have been done for ucfDNA samples from 6 healthy individuals to investigate the relationship between ucfDNA methylation and fragmentation. To test the potential application of FRAGMA in tissues-of-origin analyses and cancer detection, 172 ucfDNA samples from kidney transplant, bone marrow transplant patients, pregnant women, non-cancer controls, and patients with various cancers present in urinary system have been sequenced.

Results: The relative ratios of CGN to NCG motifs (where N represented any nucleotide) at 5' ends of ucfDNA (i.e., CGN/NCG motif ratios) were well correlated with its methylation levels. Normalized CGN/NCG motif ratios derived from various tissue-specific differentially methylated CpGs closely reflected ucfDNA tissue DNA contributions (e.g., blood cells, kidney, and placenta) (Pearson's r: 0.91-1.00). Thus, ucfDNA cleavage patterns were informative for ucfDNA methylation and tissue-of-origin analyses. Leveraging the fragmentomic features related to FRAGMA, we achieved an area under a receiver operating characteristic curve (AUC) of 0.91-0.98 in detecting patients with cancers of urinary system (i.e., bladder, prostate and kidney cancers). Furthermore, 70% - 96% accuracy has been reached in terms of tracing the tumor origins in urinary system.

Conclusion: This study demonstrated the feasibility of extending the FRAGMA technology to analysis of ucfDNA, enabling the detection and origin localization of cancers present in the urinary system. We believe that there are broad utilities of FRAGMA in different clinical scenarios with the use of different types of liquid biopsy.

Circulating, cell-free methylated DNA indicates source of allograft injury post-liver transplant

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Background: Molecular biomarkers to monitor dynamic changes after liver transplant are essential to guide clinical decision-making and improve patient outcomes. Current approaches have limited utility and are unable to differentiate amongst causes of graft injury. Here, we apply circulating, cell-free methylated DNA released from dying cells to monitor cellular damages post-liver transplant impacting the graft tissue as well as other native host organs.

Methods: We expand existing DNA methylation atlases from whole-genome bisulfite sequencing (WGBS) reference data of healthy tissues to include regions of critical importance to liver cell-types relevant to injury progression and tissue repair. Identified liver cell-type-specific methylation blocks are validated through multi-omic data integration and found to be enriched at open chromatin and regulatory regions specific to the respective cell populations. Cell-free DNA fragments were captured from patient serum by hybridization to CpG-rich DNA panels and mapped to the expanded DNA methylation atlases to inform tissue origins.

Results: We profiled 130 serum samples collected from 45 liver transplant patients at serial timepoints before and after transplant. We found that the procedure of liver transplant results in multi-tissue cellular damage across all patients that recovers in patients with allograft acceptance during the first post-operative week. Further, we reveal that sustained elevation of hepatocyte and biliary epithelial cfDNA beyond the first week are indicative of graft injury. Notably, the cellular makeup of liver-derived cfDNA is significantly different at time of biopsy-proven phenotypes, comparing hepatocellular and biliary etiologies of allograft injury.

Conclusion: We show that cfDNA composition can differentiate amongst causes of graft injury at timepoints corresponding to biopsy-proven tissue diagnosis. Thus, cell-free methylated DNA has the potential to improve care of liver transplant patients by facilitating early intervention and targeted treatment strategies.

Production and origin of circulating DNA and its diagnostic potential in inflammatory diseases.

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Background: For many years, circulating DNA (cirDNA) in blood was thought to be a waste product of cell death. Here, we introduce a new paradigm of cirDNA production by neutrophil extracellular traps (NETs) and its diagnostic potential for inflammatory diseases such as cancer, lupus and COVID-19.

Methods: We studied in vitro degradation of chromatin, NETs production by ex vivo activated neutrophils, in vitro NETs degradation in serum and cirDNA fragmentation in knockout mice to understand the impact of NETs on cirDNA fragmentation. We compared NETs markers and cirDNA fragmentation profiles in patients with inflammatory diseases. We quantified NETs markers using ELISA, cirDNA using qPCR, and performed fragment size analysis with shallow WGS and capillary electrophoresis. In addition, we conducted two clinical studies involving 279 and 549 individuals to assess the diagnostic potential of NETs markers for COVID-19.

Results: We observed that the degradation of chromatin and NETs in serum leads to the production of mononucleosome-associated DNA. The results have also shown an impact of NETs enzymes on DNA degradation. Additionally, we found an association of cirDNA levels with NETs markers as well as an altered cirDNA fragmentation in patients with inflammatory diseases. In the clinical study, we observed a strong correlation between cirDNA and other NETs markers with COVID-19 severity. Elevated NETs markers are also observed in asymptomatic patients with COVID-19 and persist for six months or more after acute infection.

Conclusion: We present evidence of NETs' auto-catabolic activity leading to the production of mononucleosome-associated cirDNA, independently of cell death. The studies highlight the diagnostic potential of cirDNA and NETs markers in inflammatory diseases and its pivotal role in COVID-19 pathogenesis. The presence of a low-grade inflammation due to the persistent formation of NETs could offer an explanation for post-infection sequelae of long COVID syndrome and perhaps in cancer patients.

High-resolution maps of human nucleosomes from circulating cell-free DNA

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Background: Circulating cell-free DNA (cfDNA) has recently emerged as an informative non-invasive marker for cellular turnover in various conditions and cell types. Additionally, enrichment of cfDNA at promoters and regulatory regions are indicative of gene expression levels and cellular composition. Yet, a comprehensive high-resolution map of human nucleosomes in the blood and plasma is not available.

Methods: We collected deep whole-genome sequencing data of cell-free DNA fragments, including bisulfite-free (total coverage > 500X) and bisulfite-treated (> 2000X) samples. We then developed robust computational algorithms that use length-specific fragment enrichment scores and sequence-specific features to robustly call nucleosome position and occupancy at base-pair resolution.

Results: We identified over 12 million nucleosomes across the entire human genome, in health and disease. These nucleosome maps exhibit remarkably low variability across dozens of healthy donors, including samples subjected to shallow sequencing or bisulfite treatment. These maps revealed distinct patterns at transcription start sites, corresponding with gene expression levels in remote tissues. Additionally, the analysis of cfDNA from various cancer patients highlighted nucleosomal changes at tumor suppressor genes and oncogenes.

Conclusion: Here we provide comprehensive high-resolution maps of human nucleosomes in health and disease. We show that nucleosome positioning and occupancy are extremely conserved in healthy individuals, but vary in disease. This opens exciting prospects for medical diagnostics based on liquid biopsy, including nucleosomal changes associated with misregulation of gene expression in various diseases including cancer.

Universal cell-free DNA Breakpoints in Cancer Patients: Unravelling Chromatin State Nucleosome Dynamics for Advanced Liquid Biopsy Detection

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Background: Maximizing the utility of circulating cell-free (ccfDNA) fragments for liquid biopsies requires a deeper understanding of chromatin structure and mechanisms of nucleosome positioning.

Methods: Our study employs digital PCR to analyze nucleosome protection and its impact on ccfDNA fragments. We investigate the concentrations of amplifiable ccfDNA in samples, focusing on nucleosome protection peaks and adjacent linker DNA. We then conduct extensive genome-wide bioinformatic analyses using windowed protection scoring, genome-wide maps of ccfDNA-derived nucleosome positioning and ChromHMM chromatin state labels derived from ChIP-seq.

Results: We find that amplicons targeting the nucleosome protection peaks of ccfDNA pools consistently exhibit higher concentrations in individual samples compared to amplicons between these peaks. This pattern suggests the presence of conserved ccfDNA breakpoints across the human population. Notably, this conservation can diminish the sensitivity of liquid biopsies, which we demonstrate in common cancer mutations including the canonical IDH1 R132 mutation and NRAS Q61. Our bioinformatic analyses reveal that the in vivo nucleosome repeat lengths (NRL) of euchromatin and heterochromatin are 182 bp and 187 bp, respectively. These NRLs align with two distinct families of nucleosome fiber topoisomers previously identified by in silico and in vitro studies. Notably, we show this NRL difference is halved in female X chromosomes, supporting the idea that these NRLs dynamically associate with changes in chromatin state. We further demonstrate that nucleosome phasing signals are a universal property of chromatin, falsifying the claim that occupied transcription binding sites and landmarks of gene regulation are flanked by well-positioned nucleosomes.

Conclusion: Our study highlights the need for comprehensive nucleosome maps of diverse tissue types to improve ccfDNA-based assay design and enable precise targeting of nucleosome-protected regions within circulating tumor DNA. Our integrated analysis leads us to develop a model in which open chromatin arises from a shift in NRL induced by CTCF/cohesion-mediated looping.

Complementary analysis of epigenetic and genetic features in plasma und urinary cell-free DNA at first diagnosis of prostate cancer for early detection and risk stratification

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Background: Prostate cancer (PCa) is a heterogeneous disease and the second most frequent malignancy among men. There is a quest for novel biomarkers to enhance early detection and risk stratification. Precise molecular characterization and determination of tumor aggressiveness at initial diagnosis would support optimal risk-adapted therapy decision. Multiparametric analysis of cell-free DNA (cfDNA) in plasma or urine show potential for addressing these challenges.

Methods: Liquid biopsy samples were collected from newly diagnosed PCa patients and cancer-free controls to perform a comprehensive analysis of localized low- and high-risk, as well as metastatic PCa at initial diagnosis. Therefore, cell-free methylated DNA immunoprecipitation sequencing and low-coverage whole genome sequencing were employed on matched plasma and urine from 73 localized or advanced PCa patients and 36 controls to assess methylation, copy number alterations (CNA), and fragmentation characteristics. Additionally, same procedure was applied on fresh-frozen PCa tissue and matched buffy coat samples from eight patients.

Results: Methylation analysis in PCa tissue revealed 3,003 differential regions compared to matched buffy coats and control plasma cfDNA. Methylation signals in these regions were also increased for plasma samples with detectable CNAs. Known early CNA events in PCa were detectable in PCa tissue and either in plasma or urine of advanced PCa patients (N1M0, M1). Fragmentation analysis of cfDNA revealed a prominent plasma-like peak besides small fragments in several urine samples, sometimes concordant to CNA detection.

Conclusion: Complementary analysis of epigenetic and genomic features, as well as assessment of both plasma and urine, enhances the detection of ctDNA in advanced PCa. Urinary cfDNA represents an eligible source for this purpose. Comparative analysis of matched plasma and urine could reveal insights into the biology of urinary cfDNA shedding. Additionally, AI-prediction models could facilitate the distinguishment between controls and advanced or even localized PCa by utilizing interplay between different data modalities.

Circulating Tumor DNA Shedding and Risk of Recurrence in Asymptomatic and Symptomatic Colorectal Cancer Patients: A Comparative Study

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Background: To reduce cancer mortality, early diagnosis is imperative. Circulating tumor DNA (ctDNA) analysis is a promising tool for early colorectal cancer (CRC) detection. However, multiple concerns still need to be addressed: What is the sensitivity for asymptomatic CRCs? And do these tumors shed ctDNA to the circulation to the same extent as symptomatic CRCs? Is the specificity of ctDNA analysis affected by common comorbidities in the target age-group? The present study addresses these concerns.

Methods: ctDNA analysis was performed in plasma from 215 patients with asymptomatic CRC, 117 patients with symptomatic CRC, and 804 non-cancer individuals using tumor-agnostic methylation-based droplet digital PCR. As validation, plasma from 368 asymptomatic and 722 symptomatic CRC patients was analyzed by tumor-informed mutation-based droplet digital PCR.

Results: Methylation-based analysis detected ctDNA in a significantly lower fraction of asymptomatic (50%) than symptomatic (82%) CRC patients ($p<0.001$), which was also demonstrated by regression analysis adjusted for tumor stage and size (odds ratio (OR): 0.30, 95% confidence interval (CI): 0.14-0.62, $p=0.001$). These findings were further corroborated through mutation-based ctDNA analysis of 1,090 independent CRC patients (OR: 0.69, 95% CI: 0.50-0.94, $p=0.018$). Furthermore, the asymptomatic CRC patients had a lower risk of recurrence compared to symptomatic CRC patients (subdistribution HR: 0.58, 95% CI: 0.36-0.91, $p=0.018$) adjusted for tumor stage and size. ctDNA analysis of 804 non-cancer individuals (50-75 years) revealed a low false positive rate (13/804, 1.6%) with no discernable association with any comorbidities.

Conclusion: Our findings indicate asymptomatic CRCs may shed less ctDNA and have a less aggressive phenotype than symptomatic CRCs. The findings stress the importance of evaluating ctDNA-based early cancer detection methods in asymptomatic patients to unravel the clinical pertinence of using the methods in this setting, and prompt further investigations of the biological differences between asymptomatic and symptomatic CRC.

Association between tumor driver mutations and the presence of post-surgical circulating tumor DNA: Insights into tumor biology

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Background: While molecular residual disease (MRD) detection using circulating tumor DNA (ctDNA) has been shown to be a prognostic biomarker for cancer monitoring, detection rates vary across tumor types, histology, and treatment. We investigated the relationship between tumor genomic features and post-surgical MRD rates.

Methods: Patients (N=45,139) with solid tumors who underwent whole exome sequencing to design a personalized ctDNA assay (SignateraTM) through February 2023 were included. Associations between genomic alterations and anytime postsurgical ctDNA positivity were assessed. Driver information was further combined with other clinicopathologic features (e.g., mutational signatures, clonal architecture, mutation burden). The most notable findings are presented, with a more detailed analysis available.

Results: We found 52 significant gene-cancer type associations with ctDNA detection (23 increased; 29 decreased); TP53 accounted for most significant associations. ctDNA detection rates were significantly different based on individual residues. Notably, ctDNA positivity was higher in patients with PIK3CA E545K variants in bladder (p=0.02), breast (p=0.004), esophagogastric (p=0.009) and lung (p=0.000003) cancers. ctDNA positivity rates in pancreatic cancer patients with KRAS G12R (25%) were lower compared to KRAS G12V (37%, p=0.0003) and KRAS G12D (41%, p=0.0000005). Pathognomonic driver mutations were more strongly associated with elevated ctDNA positivity in early-stage vs late-stage disease, such as KRAS in pancreatic cancer (early-stage: MRD-positive 72% vs MRD-negative 59%, p=0.003; late-stage: MRD-positive 61% vs MRD-negative 61%, p=0.85) or TP53 in lung cancer (early-stage: MRD-positive 35% vs MRD-negative 26%, p<0.001; late-stage: 37% vs 36%, p=0.65). Additional tumor genomic features were also investigated to determine correlation with ctDNA positivity.

Conclusion: In one of the largest datasets to date, we report on the influence of genomic drivers and cancer genomic features with ctDNA detection rates. Observed stage-dependent differences may provide novel genomic biomarkers for enhanced clinical risk staging in addition to histopathological factors.

Whole genome sequencing with ppmSeq™ enables efficient and scalable rare variant detection in cell free DNA

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Background: Identifying rare somatic single nucleotide variants (SNVs) in cell free DNA (cfDNA) plays a crucial role in early cancer detection, and in monitoring treatment response and disease recurrence. Discriminating true somatic variants from sequencing errors is challenging due to the availability of only a single supporting read. While molecular barcoding and redundant sequencing have been utilized in bespoke panels [Wan et al, 2020] as well as in whole-genome assays [Cheng et al, 2023], these methods invariably require significant over-sequencing and cause loss of sensitivity.
We present ppmSeq™, a PCR-free library preparation technology that uniquely leverages Ultima Genomics' clonal amplification process. Here, DNA denaturation is not required prior to clonal amplification so both native strands are clonally amplified on the same sequencing bead, allowing for a linear increase in duplex recovery and scalable duplex coverage without unique molecular identifiers or redundant sequencing.

Methods: We applied standard and ppmSeq protocols on a cohort of eight cancer patients, sequenced both sets on a UG 100™ sequencer, then applied machine-learning guided single read mutation calling to create a robust framework for rare variant detection.
Results: We achieved 10-40x whole genome duplex coverage with inputs as low as 10ng. Thus, our method enables whole-genome duplex coverage that is ~20-fold higher than prior duplex Whole Genome Sequencing (WGS) methods and accurate detections of circulating tumor DNA (ctDNA) at concentrations below $1E^{-5}$. ppmSeq has demonstrated SNV error rates below $1E^{-6}$ (median), enabling ctDNA detection with high sensitivity.

Conclusion: Our study introduces a breakthrough in ctDNA analysis with a ppmSeq protocol for the UG 100 sequencer, achieving extremely low SNV error rates while preserving the breadth of whole-genome sequencing and without the need for bespoke panels or over-sequencing. This approach could pave the way for routine, high precision ctDNA monitoring in the clinic.

Conflict of Interests
Authors IR, EM, OB, ZS, DST, SG, RS, GK, AJ, DL are employees and shareholders of Ultima Genomics.
Author SS provides consulting services to Ultima Genomics.

HPV-ctDNA correlates with disease stage in multiple liquid biopsy analytes in cervical cancer and dysplasia patients

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Introduction: Cervical cancer (CC) is the 4th most diagnosed cancer among women, with ~528,000 deaths annually. Liquid biopsy has emerged as a minimally invasive approach to detect and monitor disease. However, little is known about the presence of circulating tumour (ct)DNA in different liquid biopsy analytes in CC. The aim of this study was to compare the presence of HPV-ctDNA isolated from different liquid biopsy analytes in patients with CC and cervical dysplasia (CD).

Methods: Blood, urine, vaginal swabs, and pap tests were collected from 91 patients: 24 CC, 43 High-grade squamous intraepithelial lesions (HSIL), and 24 low-grade squamous intraepithelial lesions (LSIL)/normal pathology patients. 25 patients were sampled longitudinally before and after treatment. DNA was extracted using a commercial kit and tested for ctDNA by custom digital droplet PCR assay for HPV16/18/31/33/35/45/52/58. DNA fragmentation was analyzed using the Agilent Bioanalyzer 2100.

Results: HPV-ctDNA was found to be detectable 23/24 (96%) CC samples and 30/43 (70%) HSIL and 7/24 (29%) LSIL or normal pathology patients in plasma, urine, and/or vaginal swab. Among cancer patients, plasma ctDNA presence and concentration were associated with greater disease severity (P=0.03 for Stage I/II vs. III/IV). Across all analytes, HPV ctDNA was higher with increasing disease stage. In the longitudinally sampled patients, significant reductions were seen in ctDNA concentrations following treatment (P=0.03 for plasma, P=0.007 for urine). DNA fragment size varied by analyte used, with larger fragments seen in urine and vaginal swabs.

Conclusions: HPV-DNA was detectable in all liquid biopsy analytes sampled in patients with CC and CD, with significantly higher levels in CC samples. Plasma HPV-ctDNA was an indicator of disease severity in CC patients. Treatment led to reductions in ctDNA across analytes. Globally, this data points to HPV ctDNA analysis as a promising method for detecting and monitoring HPV-related CC and CD.

Tumor-informed frequent ctDNA monitoring confirms the clinical validity in advanced cancer therapy

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BACKGROUND: The potential of tumor-informed ctDNA monitoring has been demonstrated as a new class of tumor markers. Ultimately, a tumor marker should reflect the real-time tumor burden with high accuracy. We have established a frequent ctDNA monitoring system that employs panel sequencing followed by individualized digital PCR (dPCR).

METHODS: A total of 522 advanced cancer (23 types) patients enrolled from 8 independent clinical studies conducted between 2015 and 2023 were analyzed. Three categories of the clinical validity defined as: (a) early relapse prediction; (b) treatment efficacy evaluation; and (c) no relapse corroboration, were assessed (Merker et al, J Clin Oncol, 2018). All patients first underwent panel sequencing to identify somatic mutations suited to ctDNA monitoring. The ctDNA was monitored using pre-optimized 1-6 digital PCR probes selected from the OTS-1000ex dPCR probe library designed for >1,000 somatic mutations (Quantdetect, Tokyo, Japan).

RESULTS: As of October 31, 2023, all enrolled patients have been sequenced. Using the OTS-1000ex library, tumor-informed ctDNA monitoring was performed on 45% patients (n=166), with an interval of approximately 3 months, with ~0.05% VAF detection limit. The fractions of cancer patients demonstrated at least one of the defined clinical validities are as follows: 89%(n=74), esophageal; 30%(n=10), stomach; 99%(n=77), colorectal; 100%(n=3), lung; 100%(n=2), breast; and others. Among esophageal cancer patients, the overall survival rate for patients whose ctDNA after one cycle of chemotherapy dropped by less than 10% from the pre-chemotherapy was significantly better than those of more than 10%. Among colorectal cancer patients, the ctDNA monitoring results indicate possible extension of periodical CT interval without compromising relapse detection. Hyperprogressive disease in patients received immune-checkpoint inhibitors may be predicted by the ctDNA monitoring a few weeks earlier than by CT scan alone.

CONCLUSION: Tumor-informed ctDNA monitoring facilitates the timely decision-making by tumor burden in the context of advanced cancer therapy.

Microbial Cell-free DNA Sequencing of Plasma with the Karius Test for Diagnosis of Pneumonia in Immunocompromised Patients

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BACKGROUND: Invasive diagnostic testing fails to identify the cause of pneumonia in a majority of cases, leading to substantial morbidity and mortality. Microbial cell-free DNA sequencing of plasma offers the potential to non-invasively diagnose infections throughout in the body. The effect of adding the Karius Test to Standard of Care (SoC) testing for pneumonia in immunocompromised patients was examined in the PICKUP study (Pneumonia in the Immunocompromised - Use of the Karius Test for the Detection of Undiagnosed Pathogens).

METHODS: PICKUP was a prospective multi-center observational study of 257 patients across 10 leading cancer institutions in the United States. Patients were enrolled at the time of diagnostic bronchoscopy, and results of rigorous Standard of Care testing were compared to Karius Test results. The primary endpoint was adjudicated diagnostic yield of the Karius Test in patients for which all other diagnostic testing failed to identify a cause of pneumonia at 7 days post-enrollment. An independent adjudication panel (4 pulmonologists, 4 infectious disease physicians) determined all etiological causes of infectious diseases in a two step review process of clinical records.

RESULTS: SoC testing, consisting of 15-40 diagnostic tests over 7 days, identified the cause of pneumonia in 30% of patients. Microbial cell-free DNA sequencing of plasma with the Karius Test at the time of enrollment identified approximately as many etiological causes of pneumonia as all SoC testing (n=48 vs 52), with an adjudicated diagnosis rate 2x to 50x higher than any individual SoC test. Overall, Karius Testing increased etiological diagnosis of pneumonia by 40%. In addition, 39% of enrolled patients were diagnosed with a Non-pneumonia infection upon adjudication of the Karius Test results.

CONCLUSIONS: The Karius Test increased the diagnosis of pneumonia and non-pneumonia infections in immunocompromised patients when added to standard of care.

Potential impact of microbial cfDNA-based metagenomic identification of pathogens on outcome for patients suffering from septic shock

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Background: Sepsis remains a major health threat with early optimization of the antimicrobial treatment regimen as a prerequisite for treatment success. Despite limited sensitivity and specificity, blood cultures (BCs) still represent the gold standard of diagnostic care. We aimed to overcome current diagnostic limitations by unbiased next-generation sequencing (NGS) of circulating microbial cell-free DNA (mcfDNA) in plasma samples of septic patients.

Methods: We performed a prospective, observational, non-interventional, multicenter study (ClinicalTrials.gov: NCT03356249/TIFOnet Critical Care Trials Group) to compare positivity rates for NGS-based identification of causative pathogens with standard-of-care BCs in patients suffering from sepsis or septic shock. An independent expert panel (n=3) retrospectively evaluated the plausibility of NGS-based findings and the potential for anti-infective treatment adaptations based on NGS results.

Results: The positivity rate of NGS-based diagnostics (NGS+) for plasma samples from 491 septic patients was 70.5% compared to positive BCs (BC+) with 19.4% within the first three days after sepsis onset. NGS+ results were evaluated as plausible in 98.6% of cases by the expert panel. Based on the experts´ recommendations, additional knowledge of NGS-based pathogen findings would have resulted in anti-infective treatment adaptations in 32.6% of all patients. Inadequately treated NGS+/blood culture negative (BC-) patients showed worse outcomes, including prolonged intensive care unit stay, mechanical ventilation and kidney replacement therapy.

Conclusion: The integration of NGS-based pathogen diagnostics in sepsis or septic shock has the potential to improve patients´ outcomes as compared to a treatment strategy based on standard-of-care microbiological diagnostics alone.

Comprehensive longitudinal tracking of lung cancer evolutionary clonal dynamics during therapy using circulating tumour DNA

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Background: Outgrowth of resistance cancer cell populations is a common mechanism of therapy failure in oncology. Effective personalised medicine relies on targeting of ubiquitous aberrations; however, tumours are highly heterogenous. Liquid biopsies have the potential to provide representative tumour sampling at regular intervals through disease course.

Methods: We analysed 1102 plasma samples from 202 lung cancer patients who underwent exome sequencing of tumour samples through the disease course. We designed personalised panels targeting from 200-600 mutations for each patient and sequenced cell-free DNA (cfDNA) around these variant sites to saturation. We used ECLIPSE to determine the clonal composition of low tumour fraction (<1%) ctDNA.

Results: Subclonal mutations which presented as clonal in a single sample could be seperated from truly clonal mutations using ctDNA, distinguishing appropriate therapeutic targets without multiregional sampling. We observed treatment associated clonal dynamics indicative of therapeutic resistance in several patients, detectable without known resistance associated mutations. Metastatic competent subclones were expanded to a greater extent in the primary tumour, measured using pre-operative plasma ($P < 0.001$, $OR = 4.5$). Application of a personalised neoantigen vaccine was associated with regression of clones harbouring a targeted EGFR mutation. However, copy number loss of the targeted EGFR mutation occurred in a liver lesion which grew over the treatment period on surveillance radiology alongside a novel ctDNA subclone. Upon vaccine treatment cessation the resistance-associated subclone and liver lesion underwent regression. EGFR mutant clones become dominant in the tumour thereafter suggesting a fitness cost associated with vaccine resistant EGFR mutation loss in this patient, measured using ctDNA.

Conclusions: Comprehensive tracking of tumour subpopulations in plasma can accurately profile longitudinal tumour cell dynamics during treatment and biological determinants of metastasis with the potential to guide personalised medicine.

Application of Nanopore sequencing for liquid biopsy analysis in children with cancer

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Background: Paediatric cancers are the leading cause of death in children post infancy in the Western world. Comprehensive molecular profiling is essential to elucidate the molecular basis of treatment resistant disease and to guide clinical decision making. Access to high-quality tumour material for genomic profiling is a challenge in children where tissue biopsies are small. The analysis of cell-free DNA (cfDNA) from liquid biopsies for the detection of circulating tumour DNA (ctDNA) offers a powerful, minimally invasive alternative to tumour profiling. However, ctDNA analysis is currently limited in sensitivity, scalability, turnaround time and cost, hindering its implementation into standard clinical care. Emerging Nanopore sequencing can report on native DNA, is rapid and scalable at low cost, making this technology highly attractive in the clinical setting.

Methods: Here, we exploit Nanopore sequencing for the multi-modal analysis of ~300 cfDNA samples from ~150 patients enrolled in the UK Stratified Medicine Paediatrics study. We develop a Nanopore-specific cfDNA copy number tool and perform analyses to detect cancer-specific aberrations and informative epigenetic alterations. Results are compared and validated using matched Illumina whole-genome sequencing and clinical reports.

Results: Our study demonstrates the utility of Nanopore sequencing to detect clinically relevant aberrations, such as ALK and MYCN amplifications from low volume blood draws. Copy number aberrations detected using Nanopore sequencing were highly concordant with those detected with Illumina sequencing. Using the ability of Nanopore sequencing to read out epigenetic modifications, we show methylation-based disease classifiers, and illustrate the integration of copy number, fragmentomics and methylation signal to enable monitoring of disease burden and detection of relapse in longitudinal plasma samples.

Conclusion: Together, our results suggest that Nanopore-based multi-modal cfDNA analysis may present a powerful platform to improve disease management in children with cancer by facilitating early detection, accurate diagnosis, and efficient serial monitoring of disease progression.

Characterization of pediatric Multi-System Inflammatory Syndrome in Children by liquid biopsies

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Background: COVID-19 was initially described as a mild disease in children, however these initial observations have been challenged by reports of severe cases and a rare hyper-inflammatory syndrome associated with COVID-19: multi-system inflammatory syndrome in children (MIS-C). MIS-C is a complex systemic disorder involving immune dysfunction and widespread tissue damage, making diagnosis and characterization challenging due to its non-specific symptoms.

Methods: To gain insights into the cellular death and immune activity associated with MIS-C, we conducted a comprehensive analysis of plasma cell-free RNA (n=129), plasma cell-free DNA (n=65), and whole blood RNA (n=217) from pediatric COVID-19 patients, patients with MIS-C, and healthy children (Loy et al., 2023).

Results: Our study, using cell-free RNA to characterize immune activity and cellular damage, revealed a notable enrichment of IL-6 and IL-8 pathways in MIS-C cases. Moreover, increased levels of cell-free RNA from endothelial and neuronal cell types pointed to damage from these particular cells. Using cell-free DNA to quantify cellular damage, we observed heightened levels in MIS-C patients, likely due to high levels of inflammation. Intriguingly, when comparing cell-free RNA and whole-blood RNA paired from the same blood draw, despite their different cell origin profiles and biomarkers, both analytes clustered the samples in a similar manner. This finding suggests overlapping information and highlights the potential of cell-free RNA as a diagnostic tool for pediatric inflammatory conditions.

Conclusion: Our work represents a comprehensive systems-level analysis of tissue damage and immune alterations during MIS-C and pediatric COVID-19, laying the foundation for future studies employing cell-free RNA in the diagnosis of pediatric inflammatory conditions.

Dynamic size and association profiles of tumour-derived DNA during pancreatic cancer progression

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Background: Although circulating tumour-derived DNA (ctDNA) is most often described as <200bp and associated only with histones, longer forms have also been found associated with extracellular vesicles (EV). Where ctDNA is most enriched has important implications for how blood should be processed to best detect and monitor malignant disease.

Methods: To better understand the distribution of ctDNA throughout blood, we used differential centrifugation to separate the cells, platelets, different classes of EVs (apoptotic bodies (AB), large (LEV) and small (SEV) EVs) and soluble proteins (SP) from fifty-four blood samples donated by twenty-one pancreatic cancer patients at various stages of disease. To investigate further, we selectively enriched for long or short DNA species in the vesicle and soluble protein fractions using tagmentation- or ligation-based DNA amplification methods, respectively. This allowed us to use automated electrophoresis, digital PCR and sequence copy number variation analysis to establish the abundance and enrichment of tumour-derived DNA in each fraction.

Results: We first assessed the abundance of long and short DNA fragments associated with each component and found patients had increased levels of long and short DNA with ABs, as well as short DNA with LEVs and SP. When samples collected earlier and later in disease were separated, we found the greatest enrichment of ctDNA in long DNA associated with SEVs early, and short DNA associated with ABs late. This division also revealed a disease progression-dependent increase in non-EV particles. These could be visualized in the appearance of novel, low molecular weight bands on agarose gels, which were enriched in ctDNA in late-stage samples.

Conclusion:These findings suggest that targeting ctDNA associated with different blood components at specific stages of disease would improve ctDNA enrichment and the sensitivity of clinical diagnostics.

POSTER PRESENTATIONS

P01.001

A Novel Six-plex Droplet Digital Methylation-Specific PCR Assay for Identification of ctDNA Biomarkers in Prostate Cancer

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Background: Liquid biopsy assays for detection of circulating tumor DNA (ctDNA) containing cancer-specific alterations is a promising diagnostic approach due to its non-invasive nature. The DNA methylation patterns are involved in tissue-specific gene regulation. Aberrant DNA methylation pattern occurs during cancer development and the ctDNA methylation landscape in a prostate cancer (PCa) patient is expected to contain a mixture of ctDNAs with prostate-specific and PCa-specific methylation patterns. The aim of this study was to develop and validate a novel multiplex assay for detection of prostate ctDNA biomarkers with increased sensitivity.

Methods: Candidate CpG markers were selected using methylation array data from normal prostate and PCa tissues. Methylation-specific ddPCR assays were designed and experimentally validated on samples from normal and cancerous prostate tissues as well as plasma. The three top candidate assays were incorporated into a multiplex ddPCR assay with two previously reported PCa methylation-specific assays and a reference assay. The multiplex assay was analysed on the six-color multiplexing QX600 ddPCR system.

Results: The multiplex showed 100% specificity towards biomarkers from normal prostate and PCa tissues. Furthermore, the limit of blank was determined with a 95% confidence level in plasma from control patients to be less than two positive droplets for four of the five included prostate assays. All individual assays displayed similar sensitivities when used in the six-plex as when used individually with the reference assay. Results from plasma samples from PCa-patients will be presented at the meeting.

Conclusion: The current results indicates that the assay will be able to detect ctDNA in PCa patients with high sensitivity and specificity. Studies on plasma samples from PCa patients will provide knowledge on potential advantages of using multiplex ddPCR analyses that simultaneously detect prostate- and PCa-specific biomarkers.

P01.002

Impact of cfDNA Reference Materials on Clinical Performance of Liquid Biopsy NGS Assays

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Introduction: Liquid biopsy enables the non-invasive analysis of genetic tumor variants in circulating free DNA (cfDNA) in plasma to support personalized treatment. The majority of cancer patients present with actionable tumor variants with variant allele frequencies (VAFs) <1%. Therefore, accurate analytical validation of LB assays at the lower end of the measurement scale is required. Selection of appropriate reference materials is the first and key prerequisite for this purpose.

Methods: We analyzed six types of commercially available reference materials and 42 patient samples using a duplex-sequencing-based liquid biopsy NGS assay. With this we aimed to evaluate the suitability of cfDNA reference materials to be used for liquid biopsy NGS assay validation.

Results: We comprehensively evaluated the similarity of commercial cfDNA reference materials to native cfDNA. We observed significant differences between the reference materials in terms of wet-lab and sequencing quality as well as background noise. No reference material resembled native cfDNA in all performance metrics investigated. While some reference materials more closely resembled native cfDNA in terms of fragment lngth distribution and wet-lab performance, others presented with higher similarity in background noise. Based on our results, we established guidelines for the selection of appropriate reference materials for the different steps in performance evaluation. The use of inappropriate materials and cutoffs could eventually lead to a lower sensitivity for variant detection.

Conclusion: Careful consideration of commercial reference materials is required for performance evaluation of liquid biopsy NGS assays. While the similarity to native cfDNA aids in the development of experimental protocols, reference materials with well-defined variants are preferable for determining sensitivity and precision, which are essential for accurate clinical interpretation.

P01.003

Characterising circulating tumour DNA release in esophageal adenocarcinoma

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Background: Esophageal adenocarcinoma (EAC) has a 5-year survival rate of <20%. Circulating tumour (ct)DNA from liquid biopsies offers a minimally invasive way to monitor this disease, but significant knowledge gaps exist in our understanding of ctDNA release. Our aim is to determine the effect of cancer treatment on ctDNA release using 1) in vitro models and 2) longitudinal plasma samples from EAC patients.

Methods: 1) We established a cisplatin resistant model of OE19 cells and assessed ctDNA release kinetics in the supernatant of chemosensitive vs. chemoresistant cells during treatment. ctDNA was analyzed by Qubit, digital (d)PCR, and Bioanalyzer. Annexin-V/PI flow cytometry was used to assess percentages of apoptotic and necrotic cells to correlate ctDNA to release mechanism. 2) 48 blood samples were collected from 6 EAC patients at diagnosis, surgery, and before each neoadjuvant/adjuvant chemotherapy dose. cfDNA was isolated from baseline and surgical samples for targeted deep sequencing via a 127-gene panel alongside white blood cell controls and from all other timepoints for dPCR quantification.

Results: In the in vitro model, ctDNA emission kinetics correlated to cytotoxicity (apoptosis and necrosis as shown through flow cytometry), with higher levels of ctDNA released by chemosensitive vs. resistant cells ($p<0.05$). Additionally, chemotherapy treatment caused a shift in average ctDNA fragment size, with larger fragments observed during chemotherapy treatment in all cells, corresponding to an increased proportion of necrotic cells. In the clinical study, blood was sampled from patients with tumours in the distal esophagus or gastroesophageal junction. Patient clinical staging and treatment response varied (cT2-T4 N0-N3 M0-1, Grade IA-Grade III). cfDNA concentrations of samples sequenced ranged from 0.280-0.924ng/uL.

Conclusion: This study reveals that in vitro models are a powerful tool to study ctDNA kinetics. ctDNA release correlated to cytotoxicity and cell death, providing valuable insight into the interpretation of clinical liquid biopsy data.

P01.004

Development of a large volume automated cfDNA extraction for 1ml to 10ml sample volume and its implication on sensitivity and reproducibility of cfDNA analysis

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Background: Cell-free DNA (cfDNA) is a key analyte for liquid biopsy samples. Due to the very low concentrations of cfDNA found in plasma, sensitivity of the downstream applications and its implication for the result reporting are of highest importance.
Here, we compare the cfDNA recovery of a manual and automated cfDNA extraction kit for IVD applications. In addition, we report the linearity in cfDNA extraction for sample input volumes ranging from 1ml to 10ml.

Methods: Plasma and urine samples were collected from an in-house donation. CfDNA was extracted using an automated bead-based method on the QIAsymphony SP instrument or alternatively a manual column-based method. The extracted cfDNA was analyzed by qPCR, Qubit dsDNA assay and Tape Station.

Results: Equivalence in cfDNA extraction was shown for an automated cfDNA extraction kit, the QS DSP Circulating DNA Kit compared to the gold standard in the market, the QIAamp DSP Circulating NA Kit. In parallel, the automated cfDNA extraction kit revealed a linear cfDNA recovery for different input volumes ranging from 1-10ml from plasma and urine samples.

Conclusion:
Standardization of pre-analytical workflows for downstream applications with a clinical implication shall be considered to ensure reliable and reproducible results as well as improved sensitivity for cfDNA analysis from liquid biopsies.

P01.005

Comparison of Tapestation (Cell-free DNA ScreenTape) with digital PCR (dPCR; Fluidigm Biomark) for assessment of cfDNA quality and quantity.

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Background: Circulating cell-free DNA (cfDNA) isolated from blood plasma is usually found as 120-180 base pair long fragments. After extracting cfDNA from plasma there may be contaminating high molecular weight DNA, greater than 1kb and likely derived from genomic DNA (gDNA), present at variable concentrations. For standardisation of downstream analyses, DNA quantification is needed and different methods can be applied for this including dPCR, Qubit and TapeStation. Unrivalled sensitivity (down to a few copies) is offered by dPCR, but specifically quantifying the molecules of various sizes by dPCR is cumbersome. Qubit quantifies all double stranded DNA fragments regardless of size, but has a lower sensitivity compared to dPCR (Qubit-HS stated quantitative range: 5pg/µL to 120ng/µL). Recently Agilent released the Cell-free DNA ScreenTape for their family of Tapestation Systems (stated quantitative range: 100pg/µL to 4ng/µL), which enables rapid quantitation of material, automatically providing the cfDNA size profile and an assessment of gDNA contamination. We sought to compare the utility of cfDNA Tapestation quantitation to our standard dPCR based method.

Methods: We have accumulated matched dPCR (using dual assays for the human RPP30 locus and an exogenous spike in, analysed using a qdPCR™ 37K integrated fluidic circuit run on Fluidigm Biomark) and cfDNA Tapestation readings, across hundreds of cfDNA samples.
Results: We will present the outcome of our ongoing comparisons on hundreds of samples from healthy individuals and patients with different cancer types.

Conclusions: dPCR is more sensitive than cfDNA Tapestation for quantifying total DNA and offers an assessment of extraction efficiency of the exogenous spike in, but Tapestation offers a simplified and faster way to visualise and quantify the cfDNA fragments. In addition, Tapestation is less expensive, more amenable than dPCR to analysis of small batches and gives data on the level of gDNA contamination and other anomalies in size profile.

P01.006

Optimizing cell-free DNA (cfDNA) extraction: a comparative study of automated, semi-automated, and manual techniques

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Background: Circulating tumor DNA (ctDNA) has emerged as a vital biomarker in cancer management, aiding in diagnosis, prognosis, and monitoring therapy response, including the detection of minimal residual disease and therapy resistance. A primary challenge in maximizing ctDNA assay sensitivity, which is essential for developing practical, routine assays, lies in the typically low abundance of ctDNA molecules amidst a background of non-tumor DNA (cfDNA). This scarcity, coupled with high sample input demands of most assay types, often impedes the reliable detection of ctDNA. Consequently, selecting efficient and standardized cfDNA extraction methods is imperative to enhance the effectiveness of liquid biopsies in clinical environments.

Methods: We compared various automated and semi-automated methods against the manual gold standard using plasma from 12 pancreatic cancer patients and 12 healthy individuals, plus cell culture supernatant from a bone cancer cell line (143B). The extraction was performed using three commercial instruments and compared with the QIAamp Circulating Nucleic Acids kit. Specifically, we employed the Qiagen EZ2 Connect extraction automat, the Promega Maxwell RSC 48 instrument, and the Roche MagNA Pure 24 system with corresponding cfDNA extraction kits. The quantity and quality of extracted ctDNA was assessed with the Qubit Fluorometer and Agilent Bioanalyzer.

Results: Our findings indicated that while all methods yielded ctDNA of similar quality, there were notable differences in processing time and yield. The automated methods provided a significant time advantage but not higher yields.

Conclusion: The study concludes that the quality and quantity of extracted ctDNA are crucial for the sensitivity of liquid biopsy assays. Selecting an appropriate extraction method is essential for accurate results. As liquid biopsies integrate into routine clinical use, standardization and automation of extraction processes are key. Our evaluation of different extraction methods highlighted significant variations in cfDNA yields, underscoring the importance of method selection in clinical practice.

P02.001

Targeted cfDNA fragmentomics for cancer detection

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Background: cfDNA fragmentation is non-random and reflects epigenetic features of parental cells. Evidence suggests that fragmentomic cfDNA markers can be used for cancer detection. However, most of the fragmentomic studies rely on genome-wide data. We studied a targeted approach to the analysis of cfDNA fragmentation using both targeted high-throughput sequencing and PCR.

Methods: The cohort of 267 participants included healthy donors and individuals with three types of cancer: colon adenocarcinoma, clear cell/papillary cell renal cell carcinoma, and lung adenocarcinoma. The cfDNA samples were analyzed using an approach based on a modification of anchored multiplex PCR and NGS, which makes it possible to determine the coordinates of the ends of cfDNA fragments in regions of interest. Based on the NGS results, a ddPCR system was developed to compare the lengths of cfDNA molecules at the selected loci.

Results: We developed a machine learning classifier that was able to detect cancer samples with an AUC of up to 0.94 from NGS data. The method demonstrated efficacy for the detection of early-stage cancers, the median cancer score for such samples was 0.83 and 0.28 for healthy donors.
Further, we developed a ddPCR assay to compare the number of copies of cfDNA fragments of two lengths (<80 and >150 bp) in the three loci with increased fragmentation in cfDNA from colon adenocarcinoma patients. We found that analyzing a limited number of loci using digital PCR was not sufficient to obtain reliable sample classification, although we confirmed the prevalence of shorter fragments in the cfDNA of cancer patients.

Conclusion: We developed a targeted NGS-based cfDNA fragmentomic assay and tested it on a cohort of patients with different types of cancer. Transitioning to a more cost-effective PCR-based fragmentomic approach, if possible, would require further extensive search of relevant targets.

P02.002

Nanopore sequencing of native cell-free DNA (cfDNA) enables direct detection of fetal aneuploidy

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Background: Non-invasive prenatal test (NIPT) is a widely adopted method to screen the risk of fetus with chromosomal abnormalities. Currently, the cfDNA samples are sequenced using the 2nd generation sequencing technology (Illumina). Recently, the 3rd generation sequencing technology, such as Pacbio and Nanopore, has been demonstrated to provide similar performance, albeit a PCR enrichment step is necessary to obtain the required amount of DNA. With this study, we directly sequenced native cfDNA samples on Nanopore PromethION without PCR enrichment to test their applicability on NIPT analysis.

Methods: 83 samples were selected in this pilot study. Among them, 13 are known trisomy cases, 5 with other pregnancy issues without trisomy, the remaining samples are healthy controls. Native cfDNA was extracted directly from mother's blood sample. Depending on the initial amount of cfDNA available from each sample, 20 to 24 samples were pooled and sequenced on Nanopore PromethION. Raw sequenced reads were trimmed, duplicates removed, and then analyzed by in-house NIPT pipeline.

Results: Direct sequencing of native cfDNA obtained between 700,000 up-to 14m reads per sample. Only 2 sample failed to generate enough data. This variation is possibly due to the different amount of initial cfDNA available per sample. Although limited number of reads (< 1m) were obtained for some samples, all but one trisomy cases were correctly detected, and no trisomy was detected in healthy controls and in the samples with other conditions.

Conclusion: Our pilot study demonstrated that NIPT analysis can be performed successfully on data generated through a direct sequencing of native cfDNA sample without PCR enrichment using Nanopore technology. Further study is required to rigorously define the limited of detection of such approach. Without PCR, methylation signal can readily be detected from Nanopore reads. Its potential will be explored in the next phase of the project.

P02.003

Fragmentomic features of blood plasma cfDNA for cancer diagnosis

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Background: In recent years, it has become clear that also analysis of cfDNA fragmentation patterns can be helpful for certain diagnostic questions. The non-random generated cfDNA fragments are biomarkers that can inform about the tissue of origin and/or cancer type. cfDNA fragmentomics is an emerging field in liquid biopsy research, and includes fragment length, end-motif, nucleosome footprint analysis.

We hypothesize that the cfDNA fragmentomic features i.e. end-motifs and nucleosome footprint profiles are biomarkers to differentiate cancer (sub)types and estimate cfDNA tumor fractions present in the blood plasma, and also to infer gene expression from nucleosome footprint profile. In this context, we evaluate different approaches to perform cfDNA fragmentomic analysis.

Method: We generated around 100-200M paired-end, whole genome sequencing reads of 10 neuroblastoma, 10 healthy, 5 DLBCL and 5 lung cancer patient cfDNA samples. After read alignment, the published Griffin pipeline was used to generate nucleosome occupancy profiles, more specifically at sites surrounding the binding sites of 1300 transcription factors. Next, the 4-mer end-motifs frequency was computed from the alignment files. The performance to differentiate between the cancer (sub)types was assessed for both the TF binding site profile and the 4-mer end-motifs.

Results: Preliminary results show that based on the nucleosome occupancy features samples from some cancer patients clustered apart from healthy sample. Notably, within neuroblastoma samples, we observed a sub-clustering related to MYCN amplification status. The end-motifs features also allows for the differentiation between cancer and healthy samples. Currently, we are evaluating the differentiation potential across various cancer types.

Conclusion: Preliminary results show that nucleosome occupancy and end-motif data allows to differentiate cancer entities. Further research will include a) inferring gene expression status based on nucleosome footprints, b) estimating tumor fraction using fragmentomic features and c) optimizing the pipeline to reproduce results at shallow sequencing depth by downsampling the reads.

P02.004

Impact of liquid biopsy preanalytics on cfDNA fragmentation analyses

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Background: Liquid biopsy for non-invasive disease monitoring of cancer patients is progressing towards clinical practice. So far, the main focus is on circulating tumor DNA (ctDNA) analysis, targeting actionable somatic hotspot variants. Recent studies indicate the potential of the untargeted detection of ctDNA based on fragmentation analysis of total circulating free DNA (cfDNA). Here we performed a pilot study to evaluate the impact of preanalytics on cfDNA fragmentomics.

Methods: Blood samples from 10 healthy individuals and 3 patients with metastatic colorectal cancer (CRC) were collected in EDTA tubes at the partner site in Granada. To investigate the impact of sample shipping and cfDNA isolation on cfDNA fragmentation, cfDNA was isolated from each plasma. The effects of different preanalytical workflows on sensitivity and specificity of fragmentation-based ctDNA detection were evaluated using the whole-genome sequencing (WGS)-based LIquid biopsy Fragmentation, Epigenetic signature, and Copy Number Alteration analysis (LIFE-CNA), previously developed by the partner site in Munich.

Results: Plasma cfDNA concentration in samples collected from healthy individuals in EDTA tubes was significantly higher than in 55 healthy controls with blood samples collected in Streck tubes (p-value < 0.05). The different isolation methods did not result in significantly different plasma cfDNA concentrations. With LIFE-CNA, no major differences regarding the analyzed parameters, such as cfDNA fragmentation and chromatin signatures, were observed to be dependent on the preanalytical workflow in selected healthy controls and CRC patients.

Conclusion: Within our pilot study we showed that neither differences in sample collection tubes nor differences in cfDNA isolation workflows had a significant impact on cfDNA fragmentation analysis. These preliminary results suggest that cfDNA samples shipped across Europe can be used for fragmentation-based ctDNA detection. Thus, enabling us to initiate a larger study on the performance of targeted and fragmentation-based ctDNA analysis for the early detection of CRC.

P02.005

Characterization of cell-free DNA fragmentation profiles in an osteosarcoma cell line

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Background: Cell-free DNA (cfDNA) contains valuable genetic and pathological information within its variable population sizes, necessitating further investigation. Size profiles of cfDNA have been historically linked to cellular events such as apoptosis, necrosis, cell lysis, and potentially active release.

Methods: We examined cfDNA fragmentation patterns in human osteosarcoma (143B) cell cultures using various electrophoresis assays. This included four automated microfluidic capillary electrophoresis assays from Agilent (DNA 1000, High Sensitivity DNA, dsDNA 915, dsDNA 930) and a refined manual agarose gel electrophoresis protocol. These assays vary in their sensitivity and resolution, allowing for a detailed comparison of cfDNA sizing techniques.

Results: Our assay comparison revealed that: (i) higher sensitivity and resolution methods detect more complex nucleosomal patterns, including hepta-nucleosomes; (ii) cfDNA laddering extends beyond the typical 1–3 nucleosomal multiples detected by standard techniques; (iii) the perceived dominant size of long cfDNA is inflated by the limited resolving ability of electrophoresis, which conceals the presence of various larger poly-nucleosomal structures. The most sensitive assay, Agilent dsDNA 930, indicated a decrease in frequency of longer cfDNA fragments, consistent with a power-law distribution.

Conclusion: Our results demonstrate the variability in cfDNA size profiles as determined by different analytical techniques, highlighting the need for multiple methods to achieve accurate cfDNA characterization. The findings suggest a non-uniform process of DNA cleavage between nucleosomes, leading to an accumulation of shorter cfDNA fragments from the degradation of larger ones. This may explain the consistent size distributions seen across different biological processes and suggests caution in attributing cfDNA size distributions to specific mechanisms when based on a single analytical approach.

P02.006

Twist Pan-Cancer Reference Standards V2: Enhanced Precision and Reduced Errors in ctDNA Analysis

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Background: Reference materials are crucial during standardization of cell-free DNA (cfDNA) assay development to enable the precision required for discerning between malignant and benign genetic markers and the sensitivity needed for disease monitoring.

We initially introduced the Twist Pan-Cancer Reference Standards, serving as a cfDNA analog reference. This material featured a broad range of circulating tumor DNA (ctDNA) variants, closely emulating the size and distribution of cfDNA fragments. The reference standard includes over 400 synthetically-printed variant sites across 84 genes, including literature curated, clinically relevant variant sites as single nucleotide variants (SNV), insertions-deletions (InDel), or structural variants (SV). Despite its comprehensive features, the first version had its drawbacks. The method used to derive the wild-type background from donors led to the introduction of artifacts, which limited the ability to test for variant allele frequency (VAF) <0.1% at certain sites.

Methods: We have developed an enhanced version of the Twist Pan-Cancer Reference Standards, which offers a stable background genotype and a reduced background error rate.

Results: When evaluating this new reference standard with Twist library preparation, target enrichment and custom capture panels targeting clinically-relevant variants, there is a clear separation between measured VAF of the lowest variant-positive standard and WT background. In addition, a side-by-side analysis showed that the error rate of this standard (V2) is markedly lower than both its predecessor and other market competitors. Importantly, its accuracy aligns closely with that of native cfDNA, and we demonstrate that the Twist Pan-Cancer Reference Standards V2 is an appropriate substrate for 'limit of blank' studies of a given variant detection assay. The updated reference materials were created at multiple VAF dilutions, and characterized using both droplet PCR and NGS.

Conclusion: In summary, the Twist Pan-Cancer Reference Standards V2 emerges as a valuable asset for those using NGS-based liquid biopsy assays.

P03.002

Investigating origins of post-surgery persistence of circulating DNA (cirDNA) in cancer patients, revealing possible post-operative sequelae.

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The peri- and the post-surgery dynamics of cirDNA have been largely overlooked to date, despite their importance in determining the optimal timing for assessment of minimal residual disease (MRD).
We conducted two clinical studies to explore the dynamics of cirDNA quantity and of neutrophil extracellular traps (NETs) markers: (1) a peri-surgery study for a duration of up to 72 hours, which involved stage I-III colon (n=10), prostate (n=10), and breast (n=9) cancer patients; and (2) a post-surgery study that extended for up to two years post-surgery, which involved 74 stage III colon cancer (CC) patients. We assessed plasma levels of cirDNA using qPCR, and assessed two NETs markers using ELISA, in both cancer patients and healthy individuals (HI) (N=114).
(i), NETs formation contributes to post-surgery conditions; (ii), peri- and post-surgery cirDNA levels were highly associated with NETs formation in CC; (iii), each tumor type showed a specific pattern of the peri-surgery dynamics of cirDNA and of NETs markers; (iv), a significant proportion of patients showed pre- (58.1%) and post-surgery (80.4%) values of both type of markers higher than in HI, even 2 years following tumor resection, (v) these markers were either equal to or greater (23.2%) than their pre-surgery counterparts; and (vi), elevated values of these markers did not derive from chemotherapy toxicity.
We provide evidence that, for cancer patients in the post-surgery period, cirDNA originates mainly from NETs. This finding calls into question the current method of assessing MRD according to the fraction of mutant cirDNA. The peri-surgery dynamics of NETs formation and cirDNA release vary according to the surgical procedure and cancer type. In a significant part of patients (78%) with CC, NETs continue to persist for more than a year after surgery, even in cases without disease progression, highlighting the previously unreported long-lasting “sequelae” effects of cancer.

P03.003

CfDNA reflects exercise mediated neutrophil activation in healthy persons and patients with solid tumors

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Introduction: Methylation specific analyses enables to decipher the origin of circulating cell-free DNA. Recent research indicates that neutrophils are the major source of cfDNA in healthy persons, as well as in cancer patients with highly increased cfDNA levels.

Methods: We used targeted methylation specific analyses to identify the origin of cfDNA during exercise in healthy subjects (n=10). To analyze the potential association of cfDNA to extracellular vesicles (EVs), we isolated EVs using immuno-affinity capture, with or without subsequent DNase digestion, and analyzed DNA content. To identify exercise mediated effects in cancer patients, subjects with solid tumors (n=8) and age matched controls performed step wise incremental exercise testing, with subsequent analyses of cfDNA, DNaseI activity and 25 inflammatory cytokines. To determine differences in cfDNA and neutrophil elastase (NE) kinetics, we studied the release after an exercise test until exhaustion on a treadmill or on a bicycle ergometer in the same subjects (n=14).

Results: Methylation specific analysis revealed that physical activity almost exclusively triggered DNA release from neutrophils, with minor release from monocytes, but not from lymphocytes or other studied cell types. The release mechanism is independent of EVs and only 0.12% of the total DNA is associated EVs, and 80% of EV-DNA is on the surface of the EVs. In patients with solid tumors exercise leads to the increase of cfDNA and cytokines, which are highly expressed in neutrophils. The effect disappears 90 min after rest. Different exercise modalities lead to different activation of neutrophils in the same subject, which is reflected by neutrophil elastase, and more pronounced, by cfDNA release.

Conclusion: The results shed new light on the relevance of neutrophils as mediators of the exercise effect, putting cfDNA in a center point for clarifying the role of neutrophile activation during exercise and its relevance for health and disease.

P03.004

Bladder Cancer Diagnosis: Novel Insights from Urinary cfDNA Methylation Patterns

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Background: Bladder cancer ranks as the 10th most prevalent cancer globally and the 6th most common among men. Traditionally, its diagnosis involves invasive cystoscopy. Hence, the urgent need for accurate urinary biomarkers, facilitating non-invasive and cost-effective detection of primary and recurrent bladder tumors, is a significant challenge in the field of bladder cancer diagnostics. Addressing this need, we developed a method utilizing urinary cfDNA methylation patterns as a potential approach for diagnosing bladder cancer.

Methods: We identified 37 genomic loci that are differentially methylated in tumor vs normal DNA across various cancer types. We employed PCR amplification and Next-Generation Sequencing to analyze these sites in DNA extracted from 61 urine samples. Samples were sourced from healthy controls, patients with active bladder cancer, individuals with a history of bladder cancer but no active disease (NED), and patients without any history or active disease.

Results: Our study revealed that the average percentage of cancer methylation markers was significantly higher in the active bladder cancer group compared to all non-cancer groups combined (p-value<0.0001), with an area under the ROC curve of 0.8648 (p-value<0.0001). Intriguingly, the NED group exhibited a higher average cancer marker percentage than the control and active cancer patients, although it remained lower than that of the group with active cancer.

Conclusions: Our method offers a non-invasive approach to bladder cancer diagnosis through comprehensive methylation analysis. The potential applications of cancer-specific methylation markers extend beyond bladder cancer, holding promise for the non-invasive diagnosis of various malignant diseases utilizing urinary cfDNA or plasma cfDNA.

P03.005

Deep whole-genome bisulfite sequencing of circulating cell-free DNA for monitoring patients with heart failure

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Background: Recent progress in sequencing and analysis of cell-free DNA methylation data at a genomic scale allows unbiased evaluation of the remote cell death events, opening a broad window into studying normal and pathologic human tissue turnover. Heart failure (HF) is a clinical syndrome in which the heart is unable to effectively pump blood, affecting >60 million people worldwide. The condition ranges from mild to severe disease and may require surgical intervention. Yet, there is an unmet need for accurate biomarkers that can detect progression and assess treatment response.

Methods: We use deep whole-genome bisulfite sequencing (WGBS) on the novel UG100 platform to explore the utility of cfDNA methylation analysis in prognosis of HF. We assess cfDNA methylation in plasma samples taken from healthy individuals (n=17), patients after acute myocardial infarction (MI, n=14), and patients with HF (n=19). We use a comprehensive human methylation atlas and fragment-level whole-genome computational algorithms to infer the relative abundance of cell-free DNA released from specific cell types.

Results: This atlas allows to discern the contribution of DNA from multiple sources including cardiomyocytes, cardiac fibroblasts, vascular endothelial cells, and multiple immune and inflammatory cell types. HF patients showed elevated levels of cfDNA from vascular endothelial cells, whereas MI patients showed additional elevation in cardiomyocyte levels. Remarkably, we portray the tradeoff between sequencing depth and the number of markers used: 25 cell-type-specific markers sequenced at 500X are roughly equivalent to 250 markers sequenced at 30X, whereas the optimal balance should be tailored per cell type.

Conclusions: As we show, affordable deep whole-genome bisulfite sequencing of cfDNA has a tremendous potential as an accurate, unbiased and non-invasive diagnostic and monitoring tool in multiple areas of human medicine including various cardiological diseases.

P03.006

Multiple Myeloma Diagnosis and Prognosis using Plasma cfChIP-seq

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Multiple myeloma (MM) is a cancer that originates from plasma cells, leading to abnormal production of monoclonal paraproteins, bone destruction, and suppression of normal hematopoiesis. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM) are pre-malignant conditions where clonal malignant plasma cells proliferate without organ damage. These conditions have a lifelong risk of progression to MM, requiring indefinite monitoring. Early identification and prevention of progression are crucial, given the potential for irreversible organ damage in MM patients in case of delayed or missed diagnosis.

Here, we use Chromatin immunoprecipitation of cell-free chromatin (cfChIP-seq), which provides a genome-wide map of active promoters in the cells contributing to the circulating DNA. We performed cfChIP-seq on more than 100 patients with MGUS, SMM, and MM at different stages, along with a healthy control group. Our analysis revealed an increased representation of specific regions in the MM samples compared to the healthy controls. These regions consist of promoters of genes that are expressed in B-cells, plasma cells, and erythroblasts. Based on these regions, we calculated an "MM score", which showed a distinction between disease stages (i.e., MGUS, SMM, and MM). Using statistical methods, we estimated the tissue composition of each sample and identified genes that deviated from the expectation based on their composition.

We analyzed samples from ten patients at two different time points and observed a difference in the MM score between patients who progressed and those who did not, where the former had an overall higher score.

Our study highlights the potential of cfChIP-seq for differentiating disease stages and progression monitoring based on blood tests instead of invasive procedures (i.e., bone marrow sampling) and X-ray techniques (i.e., CT-PET). These findings lay the groundwork for further research into early identification and treatment of MM and predicting progression and response to treatment.

P03.007

Comprehensive cell-free DNA profiling can identify poor responders to lutetium-177-PSMA in patients with metastatic castration-resistant prostate cancer

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Background: Recently lutetium-177-PSMA (177Lu-PSMA) became available for the treatment of metastatic castration-resistant prostate cancer (mCRPC). We evaluated the prognostic value of circulating tumour DNA (ctDNA) profiling in mCRPC patients with 68Ga-PSMA or 18F-PSMA uptake in metastases on PET-CT, starting a new treatment with 177Lu-PSMA I&T.

Methods: Detection of genomic alterations in AR, PI3K signalling, and TP53 were associated with progression-free survival (PFS) using Kaplan-Meier and multivariate Cox regression analyses.

Results: We enrolled mCRPC patients (n=57) during a monocentric cohort study at AZ Groeninge (Kortrijk, Belgium). A median PFS of 3.84 months was achieved, with 21/56 (37.5%) evaluable patients achieving a ≥50% PSA response during treatment. In total, 46/57 (80.7%) patients provided a blood sample for ctDNA profiling prior to 177Lu-PSMA treatment. Circulating tumour DNA was detected in 39/46 (84.8%) patients, with increasing amounts correlating with a decremental PFS. Stratification on ctDNA levels identified three prognostic groups (low/undetectable, intermediate and high ctDNA) with different KM PFS estimates (median, 7.3 vs 4.3 vs 2.4 months, $p = 0.0023$). Genomic alterations were most frequently detected in AR, PTEN, TP53, TMPRSS2-ERG, and BRCA2. AR amplifications (HR 3.75, $p = 0.01$) and AR genomic structural rearrangements (HR 26.49, $p < 0.001$), and alterations in the PI3K pathway (HR 3.58, $p = 0.007$) harboured independent prognostic value. TP53, a biomarker of poor prognosis in context of androgen receptor signalling inhibitors, was not associated.

Conclusion: AR and PI3K signalling alterations are associated with poor 177Lu-PSMA outcome and warrant the prospective evaluation for patient selection in context 177Lu-PSMA treatment.

P03.008

Cell-free nucleic acid profiling for identification and characterization of complications post hematopoietic cell transplantation

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Background: Hematopoietic cell transplantation is a powerful tool in the treatment of various blood disorders, including blood cancers. Each year, over 30,000 patients globally undergo allogeneic hematopoietic cell transplants (HCT) to address both malignant and nonmalignant hematologic diseases. This patient group often encounters complications, including graft-versus-host disease (GVHD) affecting up to half of patients, infections such as bacterial and viral infections, graft failure, and significant risk of cancer relapse. The ablation of bone-marrow derived stem cells implicates a large-scale and rapidly changing immune profile of both acute and chronic patients that is well-suited to cell-free nucleic acids (cfNAs) as an analyte.

Methods: We performed longitudinal cfNA profiling at timepoints of preconditioning, day of infusion, engraftment, and 1, 2, 3, and 6 months post-transplant for patients receiving HCT treatment. cfNA characterization allowed us to obtain a dynamic window into the development and resolution of immunogenic responses associated with treatment and complications (Cheng, et al 2022).

Results: Our study showed a significant correlation between the abundance of solid-organ-derived cell-free DNA (cfDNA) in the blood one month post HCT and the likelihood of acute GVHD. Analysis of the cfDNA metagenomic profile revealed frequent instances of viral reactivation. The proportion of donor-specific cfDNA was found to be a reliable indicator of relapse and remission, and the fraction of tumor-specific cfDNA was indicative of cancer relapse.

Conclusions: The nucleic acid profiles, cell types of origin, and recovery trajectories suggest the utility of cell-free nucleic acids to inform tissue injury and immune response after HCT. Our study shows that utilizing cell-free nucleic acids could enhance the treatment of allogeneic HCT recipients by facilitating early detection and more accurate prediction of the diverse range of complications following HCT.

Cheng, et al., Cell-free DNA profiling informs all major complications of hematopoietic cell transplantation. PNAS 119(4), (2022)

P03.009

Circular DNA as a potential biomarker for lung cancer

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Human plasma DNA originates from all tissues and organs, and has the potential to act as a versatile marker of disease. This is especially true for cancer, as plasma contains fragments of cancer-specific alleles. While linear DNA as a liquid biomarker has undergone intense study, the role of circulating circular DNA in cancer is less established, in part, due to a lack of comprehensive testing methods. In our laboratory, we have developed a method for the profiling of extrachromosomal circular DNA (eccDNA) in plasma, which integrates solid phase reversible immobilization (SPRI) bead purification, the removal of linear and mitochondrial DNA followed by sequencing. We initially examined the method's biological and technical variations through a dual laboratory analysis of plasma samples from four patients with lung adenocarcinoma and four healthy and physically fit individuals. We observed a fourfold eccDNA increase in plasma from cancer patients compared with the levels in plasma from healthy controls in both laboratories. We also noted a consistency in eccDNA sample counts (537 + 121 eccDNA/mL in controls) and sizes in sample from the same individual. Furthermore, our analysis found that eccDNA covered up to 0.4% of the genome/mL of plasma and only had a small sequence overlap in samples from the same person. Still, we observed that cancer samples contained a subset of fragments from the same genetic origin, which were absent in controls, suggesting a non-random eccDNA formation mechanism. Our method has since been used to compare eccDNA from 75 LC and 150 controls, which has been incorporated in our results. Our studies reveal that there is little eccDNA-sequence overlap between samples from the same individual and between individuals, why other eccDNA characteristic should be considered if eccDNA is to be used as a predictive cancer biomarker.

P03.010

Longitudinal tracking of drug resistance in metastatic prostate cancer

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Background: Liquid biopsies have emerged as valuable tools for monitoring cancer progression. In our effort to integrate them into clinical practice, we harness patient information collected during routine clinical visits along with data derived from ISO and CEN/TS conform collected cell-free DNA (cfDNA) and circulating tumor cells (CTCs).

Methods: This study focuses on patients with metastatic castration-resistant prostate cancer (mCRPC) undergoing a change in therapy due to disease progression. Over a twelve-week interval, liquid biopsy samples were collected. Plasma-derived cfDNA underwent shallow whole-genome sequencing to extract tumor DNA fractions and copy number alterations. Simultaneously, CTCs were isolated and stained using in situ padlock probe hybridization with a panel of mRNA markers. An automated trained classifier facilitated comprehensive analysis.

Results: The study enrolled 26 patients, generating a total of 145 longitudinal sample. On average, each patient contributed data from 6 time points, with some reaching up to 13 visits and monitoring extending up to 132 weeks. Sequencing data revealed detectable tumor DNA fractions in 66% of sample, ranging from 2% to 62% (mean tumor fraction = 13%). The comparison of tumor fraction burden with PSA levels demonstrated a concordant increase or decrease, providing insight into therapy response. The trained classifier identified treatment-relevant transcripts (PSMA, AR-V7, DLL3, SLFN11), with longitudinal monitoring unveiling treatment-associated changes in CTC profiles indicative of epithelial-mesenchymal plasticity.

Conclusion: Our findings underscore the importance of combining diverse analyses of liquid biopsies with clinical data to obtain a comprehensive view of a patient's disease progression. The parallel response patterns of PSA levels and tumor DNA fraction to therapy changes suggest their potential in early detection of drug resistance formation, preceding observable radiographic responses. This data is further reinforced by the dynamic changes observed in CTCs, providing a detailed understanding of mRNA and predictive marker alterations over time.

P03.011

Noninvasive prenatal genetic and epigenetic analyses using five-letter sequencing: applications to fetal de novo mutation detection and methylation-based maternal inheritance inference

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Background: The detection of fetal de novo mutations in maternal plasma has been limited by a relatively high error rate of current next-generation sequencing technologies. On the other hand, conventional methods for assessing DNA methylation that involve bisulfite or enzymatic treatment of DNA may fail to detect the common cytosine-to-thymine mutations. A recently developed five-letter sequencing technology enables simultaneous determination of genetic and epigenetic states of DNA. In this study, we assessed the feasibility of using the five-letter sequencing technology for a genome-wide analysis of fetal de novo mutations and maternal inheritance of the fetus.

Methods: We first obtained the germline de novo mutation profile and the placental somatic mutation profile by analyzing fetal and placental tissue samples with five-letter sequencing and amplicon sequencing. We then sequenced the cell-free DNA (cfDNA) in maternal plasma with five-letter sequencing.

Results: Using five-letter sequencing and a series of bioinformatics filters, we achieved a detection rate of 88.8% and a positive predictive value of 85.5% for the genome-wide detection of fetal germline de novo mutations from maternal plasma. Furthermore, we developed an approach called methylation-inferred haplotype imbalance (MIHI) for deducing the maternal inheritance of the fetus based on the imbalance in the methylation levels between the two maternal haplotypes in the maternal plasma. The combined use of MIHI and relative haplotype dosage (RHDO) analysis could significantly improve the accuracy of genome-wide deduction of maternal inheritance of the fetus (99.9 – 100.0% versus 97.7 – 98.7% by RHDO alone; proportion test, $p < 0.001$).

Conclusion: We demonstrated the feasibility of using five-letter sequencing to analyze cfDNA in maternal plasma for accurate genome-wide detection of fetal de novo mutations, including the cytosine-to-thymine mutations.Integrative analyses of cfDNA in maternal plasma using MIHI and RHDO further improved the accuracy of genome-wide deduction of the maternal inheritance of the fetus.

P03.012

Various degrees of hemolysis in blood samples did not significantly affect cfDNA analysis for predicting allograft rejection.

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Background: A large study for estimating allograft rejection in kidney and lung transplant patients, had an unusually high incidence of mild to severe hemolysis (50%). This was concerning because hemolysis may affect the amount of background DNA and concomitantly alter the measured fraction of cfDNA originating from the donor organ.

Methods: A subset of 427 hemolyzed samples were analyzed by the ProsperaTM test and compared to 509 non-hemolyzed samples. The ProsperaTM test is a transplant rejection assessment test to evaluate the risk of rejection of a transplanted organ, through the use of advanced cell-free DNA technology. Output measurements that were compared included Total cfDNA (genome copies per ml plasma) and Donor Fraction Estimate (DFE) which is the ratio between the Donor Derived cfDNA quantity (dd-cfDNA) and Total cfDNA.

Results: Results showed that Total cfDNA and DFE were not consistently affected by the degree of hemolysis in both Lung and Kidney transplant samples. However, there was a trend towards a reduced number of samples that surpassed the Prospera Risk threshold (1% DFE) when samples had severe hemolysis.

Conclusions: Although there is conflicting data in literature , hemolysis could potentially lead to lysis of nucleated cells and increase the amount of high-molecular weight (HMW) DNA in the sample. This may result in an underestimation of the DFE and lower the sensitivity of the assay. However, these effects were not observed in the results of the ProsperaTM test, which uses protocols that are insensitive to HMW DNA. Nevertheless, as a precautionary measure the degree of hemolysis should be recorded at the time of processing and severely hemolyzed samples could be flagged or removed from analysis.

P03.013

Epigenetic liquid biopsies reveal elevated vascular endothelial cell turnover and erythropoiesis in asymptomatic COVID-19 patients

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Background: The full spectrum of tissues affected by SARS-CoV-2 infection is crucial for deciphering the heterogenous clinical course of COVID-19 and long-covid.

Methods: We analyzed DNA methylation and histone modification patterns in circulating chromatin to assess cell type-specific turnover in severe and asymptomatic COVID-19 patients, in relation to clinical outcomes, and long-covid patients.

Results: Patients with severe COVID-19 had a massive elevation of circulating cell-free DNA (cfDNA) levels, which originated in lung epithelial cells, cardiomyocytes, vascular endothelial cells, and erythroblasts, suggesting increased cell death or turnover in these tissues. The immune response to infection was reflected by elevated B cell and monocyte/macrophage cfDNA levels, and by evidence of an interferon response in cells prior to cfDNA release. Strikingly, monocyte/macrophage cfDNA levels (but not monocyte counts), as well as lung epithelium cfDNA and vascular endothelial cfDNA, predicted clinical deterioration and duration of hospitalization. Asymptomatic patients had elevated levels of immune-derived cfDNA but did not show evidence of pulmonary or cardiac damage. Surprisingly, these patients showed elevated levels of vascular endothelial cell and erythroblast cfDNA, suggesting that sub-clinical vascular and erythrocyte turnover are universal features of COVID-19, independent of disease severity. Interestingly, long-covid patients had elevated levels of erythroblast and monocyte/macrophage cfDNA compared to individuals who fully recovered from COVID-19, suggesting the role of these cell types in the pathophysiology of the phenomena.

Conclusions: Epigenetic liquid biopsies provide non-invasive means of monitoring COVID-19 patients, and reveal sub-clinical vascular damage and red blood cell turnover.

P03.014

Megakaryocyte- and erythroblast-specific cell-free DNA patterns in plasma and platelets reflect thrombopoiesis and erythropoiesis levels

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Circulating cell-free DNA (cfDNA) fragments are a biological analyte with extensive utility in diagnostic medicine. Understanding the source of cfDNA and mechanisms of release is crucial for designing and interpreting cfDNA-based liquid biopsy assays. Using cell type-specific methylation markers as well as genome-wide methylation analysis, we determine that megakaryocytes, the precursors of anuclear platelets, are major contributors to cfDNA (~26%), while erythroblasts contribute 1-4% of cfDNA in healthy individuals. Surprisingly, we discover that platelets contain genomic DNA fragments originating in megakaryocytes, contrary to the general understanding that platelets lack genomic DNA. Megakaryocyte-derived cfDNA is increased in pathologies involving increased platelet production (Essential Thrombocythemia, Idiopathic Thrombocytopenic Purpura) and decreased upon reduced platelet production due to chemotherapy-induced bone marrow suppression. Similarly, erythroblast cfDNA is reflective of erythrocyte production and is elevated in patients with thalassemia. Megakaryocyte- and erythroblast-specific DNA methylation patterns can thus serve as biomarkers for pathologies involving increased or decreased thrombopoiesis and erythropoiesis, which can aid in determining the etiology of aberrant levels of erythrocytes and platelets.

P03.015

Unveiling complementary insights from the analysis of blood plasma and urine in prostate cancer

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The study of body fluids other than blood has gained increasing attention in the field of liquid biopsy. In this context, in urogenital cancer, cfDNA from urine (ucfDNA) could improve the sensitivity of liquid biopsy through proximal sampling, i.e. analysis of biofluids collected proximal to the tumor site. While evidence suggests that ctDNA in urine can be informative in renal and bladder cancer, less is known about ucfDNA in prostate cancer patients. Here, we aimed to determine the presence, levels, and potential clinical applications of ctDNA in plasma and urine of metastasized prostate cancer patients.
Blood was collected in PAXgene Blood ccfDNA Tubes (PreAnalytiX). Since in-house preanalytical studies have demonstrated that ucfDNA is rapidly degraded after donation, we used preservatives for urine collection. Matched urine and plasma samples from 77 metastatic prostate cancer patients were analyzed using a shallow whole genome sequencing approach. The tumor fraction and somatic copy number alterations (SCNA) were assessed using the ichorCNA algorithm.
Our data shows that even though the tumor fractions do not significantly differ in plasma and urine, there is a great variability at patient level. SCNAs were detected with a high concordance in plasma and urine in 20.8% of patients, while in 26% and 16.9% of patients SCNAs were detected in plasma or urine only, respectively.
Our data indicate that the presence of tumor-derived DNA in urine provides complementary information about the tumor that the sole analysis of plasma may miss. Since urine represents a desirable proposition given the quantities that can be collected at great ease, ucfDNA holds promise in the clinical management of prostate cancer.

P03.016

Enrichment of microbial DNA in plasma to improve diagnosis of sepsis

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Introduction: One of every 3-5 patients who develop sepsis in the intensive care unit (ICU) dies. Microbial cell-free DNA (mDNA) is a potential analyte for detection of causative pathogen(s) in sepsis. However, the very low abundance of mDNA (<0.01%) in plasma remains a technical challenge. mDNA is shorter and more fragmented as compared to human cell-free DNA. We hypothesized that mDNA enrichment using a combination of single-stranded DNA (ssDNA) sequencing library preparation and size selection (SS) can improve the sensitivity of pathogen detection.

Methods: Serial blood samples were collected from 48 trauma patients in the ICU on days 1-10 of admission. For this analysis, we identified a subset of patients with culture-proven infections. We extracted plasma DNA and prepared sequencing libraries using both, double-stranded DNA (dsDNA) and ssDNA approaches. We applied size selection to both libraries to exclude fragments larger than 115 bp. Following sequencing, we computationally classified microbial reads and calculated total and pathogen-specific mDNA fractions. Differences in mDNA fractions between conditions were evaluated using a Mann-Whitney U test.

Results: Five of the 48 ICU patients had culture-proven infections. We analyzed 46 samples from these patients, including 7 samples taken at timepoints with positive microbial cultures. Compared to dsDNA libraries, ssDNA-SS increased total mDNA fraction by an average of 202-fold across patient samples. Using dsDNA alone, pathogen-specific DNA was detected in 3/7 samples (43%). Using ssDNA-SS, sensitivity for pathogen-specific DNA was improved to 86% (6/7 samples). In 3/5 patients with infections, we detected pathogen-specific DNA before the clinical work-up for infection.

Conclusions: Microbial DNA is enriched 202-fold using ssDNA-SS as compared to standard dsDNA libraries. Our results demonstrate that sensitivity for detection of pathogen-specific DNA is improved by combining ssDNA library preparation with size selection. This may enable earlier detection of pathogen-specific DNA in patients with sepsis.

P03.018

Systematic analysis of liquid biopsy Next Generation Sequencing (NGS) assay thresholds on the achievable diagnostic yield.

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Background: Next Generation Sequencing (NGS)-based circulating free DNA (cfDNA) analysis from liquid biopsies enables non-invasive identification of genetic variants. Thereby, cfDNA analyses can e.g., guide personalized treatment of cancer patients or serve diagnostic purposes in patients with suspected mosaic diseases. As most patients present with causative and/or potentially actionable variants in cfDNA with variant allele frequencies (VAFs) <1%, stringent performance evaluation of validated NGS pipelines is mandatory to ensure highly sensitive and specific variant detection in NGS data and to maximize the diagnostic yield.

Methods: Following the recent revalidation of our liquid biopsy NGS assay, where we established an improved limit of blank (LOB) of 0.1% VAF for variant calling, we reanalyzed patient data with cancer and overgrowth syndromes. We systematically evaluate the impact of different specificity cutoffs on the diagnostic yield across three diagnostic laboratories in Germany.

Result: We show that a methodological revalidation of liquid biopsy NGS data using reference materials directly impacts on the diagnostic yield. We compared the detection rate of causative variants in patient samples based on three cutoffs for variant detection: 0.5%, 0.25% and 0.1%. We observed that a lower cutoff for variant detection correlated well with a higher diagnostic yield in all three laboratories. We further present the clinical and molecular data of two patients with suspected overgrowth syndrome, where likely causative variants were revealed by data re-analysis with an adjusted, highly specific cutoff.

Conclusion: The efficiency of diagnostic liquid biopsy NGS assays depends on the choice of appropriate reference materials for analytical validation. Our study provides real-world patient data on how the ideal choice of reference materials for analytical validation increases the diagnostic yield across indications.

P03.019

Variation in ctDNA detection in lung cancer patients according to their histological subtypes

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Background: Studies on next-generation sequencing have shown that circulating tumor DNA (ctDNA) detection can vary among patients with non-small cell lung cancer (NSCLC) according to their histological type, with higher detection observed in plasma from patients with squamous cell carcinoma (SCC) than adenocarcinoma (AC). Other methods can be more sensitive and increase detection rates for patients with AC. We investigated cell-free DNA (cfDNA) and ctDNA amounts in the two histological groups of NSCLC using a methylation-based tumor-agnostic approach.

Methods: Plasma was collected before treatment from two cohorts of NSCLC patients receiving first-line chemotherapy (N1 = 151) or checkpoint inhibitor immunotherapy (N2 = 69). Patients had stage III (N1 = 15.9%, N2 = 14.5%) or stage IV (N1 = 84.1%, N2 = 85.5%) cancer. The majority of cases were AC (N1 = 82.8%, N2 = 81.2%). Total cfDNA (albumin) and ctDNA (methylated HOXA9, met-HOXA9) were measured using droplet digital PCR. Samples with ≥ 5 met-HOXA9 containing droplets were considered ctDNA positive.

Results: For both cohorts, we observed a higher number of ctDNA-positive samples for patients with SCC (N1 = 96%, N2 = 92%) than AC (N1 = 73%, N2 = 79%). In N1, a higher average of met-HOXA9 copies/ml ($p = 0.005$) and met-HOXA9 % ($p = 0.002$) was found in samples from patients with SCC than those with AC. However, no significant difference in total cfDNA was observed between samples from patients with AC and SCC in both cohorts.

Conclusions: ctDNA detection in NSCLC patients with methylation-specific ddPCR has a higher sensitivity for SCC than AC. The total amount of cfDNA is similar in samples from patients with SCC and AC, so the biology of ctDNA release must differ between the two sub-groups of lung cancer. This highlights the importance of considering histological sub-grouping when conducting studies focusing on NSCLC.

P03.020

EQA on molecular tumor profiling using circulating tumor DNA-based methodologies routinely used in clinical pathology within the COIN consortium

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Background: Identification of tumor-derived variants in circulating tumor DNA (ctDNA) has potential as a sensitive and reliable surrogate for tumor tissue-based routine diagnostic testing. However, variations in pre(analytical) procedure affect the efficiency of ctDNA recovery. Here, an external quality assessment (EQA) was performed to determine the performance of ctDNA mutation detection workflows that are used in current diagnostic settings across laboratories within the Dutch COIN consortium.

Methods: Aliquots of three high-volume diagnostic leukapheresis (DLA) plasma samples and three artificial reference plasma samples with predetermined mutations were distributed among sixteen Dutch laboratories. Participating laboratories were requested to perform ctDNA analysis for BRAF exon15, EGFR exon18-21, and KRAS exon2-3 using their regular circulating cell-free DNA (ccfDNA) analysis workflow. Laboratories were assessed based on adherence to the study protocol, overall detection rate, and overall genotyping performance.

Results: A broad range of preanalytical conditions (e.g., plasma volume, elution volume, extraction methods) and analytical methodologies (e.g., ddPCR, small panel PCR assays, NGS) were used. Six laboratories (38%) had a performance score of >0.90; other laboratories scored between 0.26-0.80. Although thirteen laboratories (81%) reached a 100% overall detection rate, the therapeutically relevant EGFR p.(S752_I759del) (69%), EGFR p.(N771_H773dup) (50%), and KRAS p.(G12C) (48%) mutations were frequently not genotyped accurately.

Conclusions: Divergent (pre)analytical protocols could lead to discrepant clinical outcomes when using the same plasma samples. Standardization of (pre)analytical workflows can facilitate the implementation of reproducible liquid biopsy testing in the clinical routine.

P03.021

Exertional and exogenous heating modalities at equivalent core temperatures evoke different releases of cell-free DNA and hormones in young healthy men

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Background: Increased body core temperature (Tc) is a common symptom in clinical conditions and training or is a consequence of rising ambient temperatures. As a versatile biomarker, circulating cell-free DNA (cfDNA) is involved in immunomodulation, inflammation, and pathophysiological diseases, which can be accompanied by core temperature fluctuations, contributing to the increase in cfDNA levels. Here, we investigated the effect of identical thermal stress during exertional (exercise) (Exe-H) and exogenous (passive) heating (Exo-H) on cfDNA release and stress-related hormones.

Methods: 15 young male participants completed a physical exercise bout (cycling at 60% VO2max until Tc reached 39 °C) and hot water (43-44 °C) immersion (until Tc reached 39 °C). Blood samples for cfDNA and hormone analysis were taken each time the Tc rose by 0.5 °C or returned to baseline during or after cycling and hot water immersion. ELISA kits were performed to measure concentrations of catecholamines and prolactin.

Results: The time required to reach a certain Tc was not significantly different between Exe-H and Exo-H ($F_{7,145.9}=0.616$, $p=0.742$). Significant increases in cfDNA were already noticed in both interventions after the first measurement following the Pre time point (Tc 38 °C, with higher values during Exe-H ($p<0.01$), and steadily increasing until Tc reached 39 °C. Norepinephrine exhibited a strong temperature-dependent response throughout the trials, showing a stronger correlation with cfDNA in the Exe-H ($r=0.66$, $p<0.001$). Plasma levels of epinephrine were more elevated in Exe-H (heating modality effect: $p<0.05$). A tendency for higher serum prolactin values was observed at 38.5 °C in Exo-H vs. Exe-H ($p=0.005$) and prolactin concentration peaked at 39 °C in Exo-H.

Conclusion: Our results suggest comparable thermal stresses imposed on subjects by exertional and exogenous heating while cfDNA release is more pronounced following exercise. The correlation of cfDNA and norepinephrine suggests analogous release principles, which should be further investigated.

P03.022

Impact of COVID-19 on plasma levels of circulating cell-free DNA

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Background: Several pathologies increase the levels and composition of circulating cell-free DNA (cfDNA) in blood plasma. Here, we determined the quantity and fragmentation of cfDNA after acute COVID-19 infections, in patients with post-COVID-19 syndrome (PCS) and in young healthy subjects.

Methods: Absolute concentration and fragmentation index (FI) of cfDNA were measured with qPCR[1] in 35 patients with COVID-19 who visited a general practitioner (GP) due to infection (age = 38 ± 13 y), 45 hospitalised patients (68 ± 18 y), 27 intensive care unit (ICU) patients (age = 54 ± 19 y), 12 patients with PCS (54 ± 9 y), and 33 healthy young subjects without infection (age = 25 ± 6 y). The different cohorts were studied prospectively to minimise preanalytical bias for cfDNA measurement. Welch-ANOVA with Games-Howell post-hoc tests were calculated to identify differences.

Results: Severity of disease significantly elevated cfDNA levels, with concentrations of 14.8 ± 7.2 ng/ml in GP patients, 189.2 ± 165 ng/ml in hospitalised patients, 848 ± 1069 ng/ml in ICU patients, 29.2 ± 11.7 in PCS patients and 17.3 ± 9.9 ng/ml in healthy controls. Similarly, ICU patients showed the lowest FI = 0.15, FI in hospitalised patients was 0.22 and in general practice patients FI = 0.33. PCS patients and healthy subjects showed similar FI, 0.43 and 0.44, respectively.

Conclusion: Severe COVID-19 massively increases cfDNA, whereas we did not detect any concentration differences between the healthy subjects and GP patients ($p > .05$). FI is reduced in association with disease severity during acute infection. Interestingly, FI does not appear to be reduced in PCS patients after they have overcome the infection and does not differ from healthy subjects ($p > .05$). We will continue to increase the number of PCS patients and include more laboratory and clinical parameters to identify further associations.

P03.023

DNA integrity and preanalytical handling of endocervical samples for use in liquid biopsy-based diagnostics and monitoring of gynecological cancer

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Background: Liquid biopsies from the gynecological tract can be useful for analysis of cell free DNA (cfDNA) (1, 2, 3). Sampling procedure and optimal preanalytical handling are poorly investigated. In this study, we aimed to evaluate how the profile and integrity of DNA, sampled from the female genital tract, were affected by preanalytical handling.

Methods: Biobanked/archival (stored at -80°C after 48h at room temperature (RT)) and freshly obtained clinical samples were analyzed together with standardized in vitro samples. Archival and in vitro samples were preserved in methanol-based solution, imitating clinical handling of endocervical samples which are initially stored at RT for 48h before further handling. The fragmentation profile of archival and in vitro samples was analyzed. Time series of in vitro samples at RT and 4°C, with extraction at 6-hour intervals from 0 to 96h, were performed. The fresh clinical samples were collected from the endocervix in methanol-based solution and from the vagina in a solution designed to preserve DNA and placed at 4°C resp RT. Extraction was performed within 4h and at 48h.

Results: Accumulation of short DNA fragments were observed in the archival samples and in the in vitro samples, indicating that fragmentation was initiated prior to 48h. Time series showed that the quantity of short DNA fragments gradually increased with time at both RT and 4°C but at a slower rate in 4°C. At RT the percentage of fragments between 100-350bp increased 12-fold (0.39% vs 4.75%, p=0.002). The freshly obtained endocervical samples showed a similar significant increase in short fragments in samples stored at RT compared with samples stored at 4°C (p= 0.02 for 100-230bp; p= 0.007 for 230-350bp; p= 0.03 for 100-2500bp) at 4h–48h.

Conclusion: Pre-analytical handling of endocervical samples can be optimized. Lowering temperature from RT to 4°C immediately haltered DNA fragmentation.

P03.024

Direct Quantification of Circulating Cell Free Mitochondrial DNA (cf-mtDNA) in Human Blood Plasma without DNA Extraction

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Background: Mitochondria play a crucial role in energy production, autophagy, homeostasis, and act as a key mediator of immune activation and pro-inflammatory response. Many pathological conditions have been considered to contribute to the release of mitochondrial DNA (mtDNA) into the circulation, leading to the activation of inflammatory pathways. Monitoring inflammation is an essential aspect for severity of serval diseases, highlighting the relevance of cell free mitochondrial DNA (cf-mtDNA) as a potential biomarker in the field of liquid biopsy.

Methods: Here we established an assay for the direct quantification of cf-mtDNA in human blood plasma samples and quantified cf-mtDNA concentrations in intensive care unit patients with sepsis and COVID-19. Primers were designed only targeting the mitochondrial ND-1 gene, excluding to amplify nuclear embedded mitochondrial DNA insertions. Accuracy and linearity of the assay was tested on a custom-made fragment of the ND-1 gene. Standard curves were generated by spinking the ND-1 gene fragment into mouse plasma as background matrix. Plasma samples were diluted in water and measured by direct real-rime PCR.

Results: cf-mtDNA could be quantified in 83 % of the plasma samples without DNA extraction. The assay showed low limit of quantification (LOQ) with 50 copies/µL. In a melt curve analysis, we confirmed specificity of the assay. Reliability of the assay was validated by re-analyzing a subset of plasma samples, showing that the percentage difference between the first and the second measurement was less than 30 %.

Conclusion: Our results show that cf-mtDNA can be directly and reliably quantified in human blood plasma in patients with COVID-19 and sepsis, lowering cost and time of DNA extraction and sample preparation. Due to its role in inflammation and immune response cf-mtDNA can be used as a promising biomarker for monitoring infectious conditions such as COVID-19 or sepsis.

P03.026

Relation of total cfDNA levels during the first hours after liver transplantation with patient evolution and ischemic reperfusion injury

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Background: High quality of the donated organ is a key factor for transplantation success. Ischemic reperfusion injury after transplantation has been suggested as an important cause of liver dysfunction following surgery. Cell-free DNA (cfDNA) is a useful tool for transplanted patients monitoring. Thus, donor specific cfDNA might predict the organ damage or rejection. Increased levels of cfDNA during the first days of evolution may be an indicator of worse prognosis and has been associated with inferior survival.
We propose to analyze the relationship of total cfDNA values during the first 48 hours after transplantation with the ischemic reperfusion and the evolution of the patients after transplantation.

Methods: 110 liver transplanted patients were included in the study. Serum samples were collected at the moment of transplantation (TX) and during hospitalization. cfDNA was determined by the amplification of beta-globin gen by qPCR .

Results: We observed higher levels of cfDNA on 10 patients that die during the first two years, although did not reach statistical significance. cfDNA levels at TX and during the first 48 hours were significantly higher in patients with complications and in patients who suffered any kind of liver damage during the first month. We also observe a significant correlation between the number of days at intensive care unit (ICU) after transplantation and cfDNA levels at TX. Thus, mean cfDNA was significantly higher in patients with longer ICU stay.
High cfDNA levels were significantly associated with longer organ ischemia time (greater than 5h and 25 min; cold and warm ischemia time respectively). Patients were stratified according to TX cfDNA values and significant differences were observed among groups related to liver damage and warm ischemia time.

Conclusions: Total cfDNA levels during the first hours after transplantation may be an important tool for patient management during the first weeks of evolution.

P03.027

Differentiation Between Refractory Septic Shock Survivors and Non-survivors Through Cell-Free Blood DNA

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Background: Life-threatening sepsis resulting from an uncontrolled inflammatory response is the leading cause of mortality in the ICU. Despite advances in treatment, predicting outcome remains difficult. Personalized care is desirable to increase the chances of survival. Various factors such as patient characteristics, metabolomic profile, oxidative stress marker, and platelet count have been investigated to predict sepsis mortality in adults but the accuracy of clinical prognosis is limited. We aim to investigate differences in cell-free blood DNA (cfDNA) quantities, including bacterial DNA, in survivors of refractory septic shock (SS) and non-survivors (NSS).

Methods: 36 survivors (age 63 ± 15) and 29 non-survivors (age 66 ± 14) of septic refractory shock were enrolled in the study and clinical data collected upon admission. CfDNA from whole blood plasma was isolated with QIAamp Circulating Nucleic Acid Kit (Qiagen), dsDNA and ssDNA concentration were measured with Qubit respective kits. Bacterial DNA was detected with broad-range 16S rDNA qPCR and relative concentrations were calculated from standard curve.

Results: We have found only mild differences in dsDNA (higher in NSS) and ssDNA (higher in SS) values between the groups ($p=0.08$ and $p=0.38$, respectively). As for 16S rDNA, NSS group had significantly lower bacterial DNA concentrations (0.0214 ± 0.0496 ng/ml for SS and 0.1907 ± 0.4308 ng/ml for NSS) ($p<0.05$). Gender has significant effect - females in NSS group have lower bacterial DNA load (0.2606 ± 0.5782 ng/ml) than both males in NSS (0.0320 ± 0.0695 ng/ml) and females in SS groups (0.0377 ± 0.0843 ng/ml) ($p<0.05$ and $p=0.0679$, respectively). No correlation was found between bacterial DNA load and ssDNA or dsDNA concentrations in cfDNA from blood.

Conclusion: We show higher bacterial DNA concentrations in cfDNA from blood in non-survivors females, which can be taken into account in predicting the possible refractory septic shock outcome.

P03.028

Extracellular Vesicles (EVs) and EV-associated DNA in exercising systemic lupus erythematosus patients

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Introduction: Systemic lupus erythematosus (SLE) is an autoimmune disease showing systemic inflammation involving auto-antibodies against the nuclear components of immune cells. To date, SLE is incurable but next to immunosuppressive therapy, physical exercise programs have shown to improve quality of life of patients. The mechanisms responsible for this positive effect of exercise is not understood. Extracellular vesicles (EVs) are involved in multiple processes in innate and acquired immune response and exercise is known to trigger the release of EVs into the circulation. Here, we study the effect of acute and regular physical exercise on circulating EVs and EV-associated DNA in SLE patients.

Methods: We recently performed a randomized controlled study with SLE patients implementing 12-weeks of physical exercise training which significantly improved aerobic capacity and the SLE-Disease-Activity-Index of patients. Blood was sampled during acute bouts of physical exercise, before and after the intervention. Total EVs were separated from plasma using size exclusion chromatography and analyzed on the ExoView platform. EV-subpopulations were isolated by CD63-immuno affinity capture (genuine EVs) or AnxV-binding (platelet EVs and apoptotic bodies) and surface epitopes were assessed in multiplexed flow cytometry. cfDNA and EV-associated DNA were measured using ultra-sensitive LINE1-qPCR.

Results: Acute bouts of physical exercise led to the release of cfDNA (~2.3-fold) and EVs (~1.7-fold) into the blood of SLE patients. A major part of the cfDNA (~99 %) is not associated with genuine or platelet/apoptotic EVs. Neither acute bouts nor the intervention induced a phenotypic shift of the analyzed EV populations.

Discussion: We could show a similar EV- and EV-associated DNA response in SLE patients as described for healthy persons, indicating that EV-mediated signaling in SLE patients is not disturbed. A potential beneficial contribution of EVs to the overall positive effect of physical exercise on health could, thus, be also present under SLE disease conditions.

P04.001

Low-depth sequencing of cell-free DNA reveals disease-specific chromatin reorganization states.

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Circulating cell free DNA (cfDNA) is a composite mixture of fragmented genomic DNA arising from healthy, nucleated blood cells and tissues during normal cell-death. In cancer and other diseased conditions, changes in underlying nucleosome occupancy and epigenetics often result in altered chromatin state. Such chromatin reorganization can modify the genomic regions exposed to nucleases during abnormal cell-death, consequently giving rise to disease-specific cfDNA fragmentation profile. We have developed a unique approach to extrapolate underlying disease-state chromatin organization status by using our flagship a single-stranded next-generation sequencing library preparation method, SRSLY®, to generate low-depth cfDNA sequencing data. Single-stranded approach retains short fragments and nicked DNA and are known capture more accurate cfDNA fragmentomic signals. Here, using cfDNA sequencing data from a cohort of patients with myelodysplastic syndrome, a hematological disorder that progresses to AML, we determine changes in fragmentation along the 3D genome. By integrating the mapped fragment length ratio (within a 250 kB window across the genome) and Hi-C information from a lymphocytic cell-line as ground truth, we identify chromatin reorganization signals at sequencing depth as low as 5 million reads. We find that this signal is specific to cases that demonstrate ring-sideroblasts phenotype, a histopathological feature in the bone-marrow that is associated with nuclear reorganization. Further, we show that these differences characterize the sub-compartments of the 3D genome - the reorganized signal is depleted for B1 and B2 sub-compartments. These sub-compartments contain nucleolar associated domains that bear histone modifications that are associated with the highly condense, heterochromatin. Thus, the disease-specific, cfDNA-derived reorganization signals analyzed here are presumably arising from genomic fragmentation at regions that are loosely wound and more labile to nucleases during cell-death.

P04.002

Cell-free chromatin immunoprecipitation in human urine

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Urinary Cell-free DNA (cfDNA) holds valuable molecular information about processes occurring in the urinary and renal systems. Plasma circulating cf-nucleosomes have been shown to retain some of the cell of origin's chromatin modifications in humans, which is informative of cell identity and cell-state. Here, we extend the use of cell-free chromatin immunoprecipitation followed by sequencing (cfChIP-seq) to human urine. Cell-free nucleosomes captured in urine retain multiple histone post-translational modifications and utilizing a promoter mark we delineated the major tissues contributing to cfDNA in healthy donors. Using urine cfChIP-seq we identify a contribution of the kidney to the urine cell-free DNA pool in healthy donors which is undetected in urine exfoliated-cells and matching plasma samples. We show the assay's ability to identify pathologically driven changes in the urine of patients with bladder cancer, reflecting transcription programs in the tumor and the immune response. Our results demonstrate that urine cfChIP-seq is a noninvasive assay useful in both basic-research of the renal physiology and in monitoring urinary pathologies.

P04.003

Cell-free DNA nucleosome positioning patterns in breast cancer preclinical models for CDK4/6 inhibitor resistance liquid biopsy biomarker discovery

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Background: CDK4/6 inhibitors (CDK4/6i) are standard treatment for ER+/HER2- metastatic breast cancer, but resistance in patients necessitates novel biomarkers. Liquid biopsy, analyzing tumor-derived cell-free DNA (cfDNA), offers a minimally invasive method for identifying such biomarkers. Epigenetic features of cfDNA, like nucleosome positioning patterns, hold potential for predicting CDK4/6i resistance.

Methods: We analyzed cfDNA nucleosome features in preclinical tissue culture models, having optimized a nuclease treatment to mimic nucleosomal distributions found in blood plasma, thereby avoiding dilution by hematopoietic-derived cfDNA. Using paired CDK4/6i sensitive and resistant breast cancer cell lines (n=6), we generated a dataset of simulated cfDNA WGS (~85X), WGS, RNA-seq, and ATAC-seq.

Results: First, we demonstrated that simulated cfDNA nucleosome profiling reflected cell-specific gene expression and chromatin accessibility. Comparing these profiles with patient plasma cfDNA, we observed stronger nucleosome positioning in simulated cfDNA at chromatin-accessible regions derived from patient tissue. Next, analyzing chromatin accessibility in CDK4/6i sensitive and resistant models revealed over 50,000 chromatin variants, with 331 and 133 variants showing enriched accessibility across all sensitive and resistant models, respectively. We observed significant differences in nucleosome positioning (central coverage, paired Wilcoxon $p<0.001$) within simulated cfDNA at these common chromatin variants. Next, we assessed 133 previously published gene expression signatures encompassing biological features relevant to metastatic breast cancer, finding significant differences in simulated cfDNA nucleosome positioning features between sensitive and resistant models (paired Wilcoxon $p<0.001$). Particularly, the RB1 loss of heterozygosity signature (345 genes) showed altered simulated cfDNA nucleosome positioning, along with other key genes associated with CDK4/6i resistance, like RB1, CCNE1, CDKN1A, and FGFR1.

Conclusion: We evaluated chromatin accessibility and gene expression signatures in pure tumor-derived simulated cfDNA from CDK4/6i-sensitive and resistant breast cancer cells, finding altered nucleosome positioning profiles. Differences in cfDNA nucleosome positioning features could provide CDK4/6i resistance biomarkers for improved breast cancer treatment.

P04.005

Identification of an epigenetic profile of circulating nucleosomes in Non-Hodgkin Lymphoma as potential biomarkers of the disease.

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Background: Non-Hodgkin lymphoma (NHL) is a common type of hematological malignancy with lymph node biopsies the traditional diagnostic method. However, due to their highly invasive nature, tissue biopsies have many limitations. Conversely, liquid biopsy offers a promising diagnostic tool for cancer detection as it is noninvasive and easily repeatable over time compared to tissue biopsy. Dysregulation of histone post-translational modifications (hPTMs) has been associated with various solid cancers. Here, we proposed to use an enrichment method (Nu.Q® Capture) to characterize by Mass Spectrometry (MS) the epigenetic profile of nucleosomes in the blood of NHL patients.

Methods: K2EDTA plasma from NHL patients (n=9) and healthy donors (n=5) were subjected to Nu.Q®Capture-MS method and global nucleosome levels were investigated using Nu.Q®H3.1 immunoassays (Belgian Volition SRL). The identified hPTMs were then validated in an independent cohort (n = 24 NHL and n = 35 healthy donors) using Nu.Q® immunoassays targeting the specific hPTMs at diagnosis and during the treatment follow-up of two patients.

Results: The Nu.Q®Capture-MS analysis revealed a higher level of eight specific hPTMs peptides (H3K9Ac, H3K14Ac, H3K18Ac, H3K23Ac, H3K36Me1/2/3 and H3K27Me2) in NHL samples compared to healthy ($p<0.05$). Then, using Nu.Q® immunoassays, we validated the increase of H3.1-nucleosome levels in NHL vs Healthy (median 483.6ng/mL vs 12.11ng/mL, respectively) as well as 6 hPTMs-nucleosome (median NHL vs Healthy: H3K9Ac = 6.93ng/mL vs 3.83ng/mL ; H3K14Ac = 42.99ng/mL vs 18.67ng/mL; H3K18Ac = 12.40ng/mL vs 8.15 ng/mL, H3K9Me1 = 90.03ng/mL vs 13.97ng/mL; H3K27Me3 = 112.09ng/mL vs 8.79ng/mL; H3K36Me3 = 76.27ng/mL vs 10.14ng/mL, $p<0.05$). Those nucleosomes' concentrations and epigenetic profile are also altered with response to chemotherapy.

Conclusions: Our results indicate that levels of circulating H3.1-nucleosomes as well as nucleosomes containing specific histone modifications are elevated in NHL patient samples at diagnosis, and may be a useful tool to detect NHL and monitor treatment response.

P05.001

A blood based multi-omics multi-cancer early detection test: combined retrospective and prospective cohort studies

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Background: Recent studies have demonstrated that blood-based multi-cancer early detection (MCED) approaches may hold promise for identifying asymptomatic cancer patients from general population. However, most studies only exploit a single aspect of cancer hallmarks, which is challenging for the biological reasons as cancer is a heterogenous disease with a wide spectrum of pathological and clinical behaviors. Here we report a multi-omics MCED assay named SeekInCare, a CE-IVD Mark blood test, which incorporates genomic hallmarks: copy number aberrations, fragment size, end motifs and oncogenic viruses via shallow whole genome sequencing from cfDNA, and seven plasma protein tumor markers in 8 ml blood.

Methods: SeekInCare was developed using several retrospective cohorts and the method has been described in a publication (DOI: 10.1016/j.jmoldx.2021.06.003).

Results: We present the validation in a retrospective cohort consisting of 584 non-cancer individuals and 617 cancer patients covering 27 cancer types. SeekInCare achieved 65.5% sensitivity at 97.9% specificity, resulting in an AUC of 0.936. The sensitivities were 46.9%, 60.0%, 68.9%, 81.8% in stage I, II, III, IV patients. The sensitivities of 10 common cancer types are as the following: breast (46.2%), stomach (46.4%), colorectum (56.5%), gallbladder (60.0%), lung (62.8%), pancreas (64.7%), lymphoma (68.5%), esophagus (70.0%), liver (77.5%), and leukemia (86.7%). These cancer types account for 73.5% of cancer incidence and 81.8% of cancer-related mortality in China. We prospectively evaluated SeekInCare in a real-world cohort consisting of 1203 individuals who received the test as a laboratory developed test (median follow-up time: 753 days) in which it achieved 60.0% sensitivity, 96.1% specificity, 11.5% PPV and 99.7% NPV.

Conclusion: The performances of SeekInCare in both retrospective and prospective studies demonstrate that SeekInCare is an effective blood-based MCED test with similar performance as Grail's Galleri test, which paves the way for clinical utility as a cancer screening test in average-risk populations.

P05.003

Whole-genome sequencing of cell-free DNA reveals DNA of tumor origin in plasma from patients with colorectal adenomas

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BACKGROUND: Advancements within circulating tumor DNA (ctDNA) analysis have greatly improved the ability to detect ctDNA, even at low tumor burdens. Current efforts are now focused on utilizing ctDNA as a screening tool for early cancer detection and, if possible, precancerous lesions. However, the extent to which precancerous lesions, such as colorectal adenomas, release ctDNA into the bloodstream remains unclear. Here, we leverage the exceptional sensitivity of MRD-EDGEsnv, a whole-genome sequencing (WGS) approach for tumor-informed ctDNA detection, to evaluate ctDNA release from precancerous colorectal adenomas.

METHODS: The study included 40 healthy individuals and patients with stage III colorectal cancer (CRC; n=93), symptomatic adenomas (n=22), or asymptomatic adenomas (n=20). Tumor and normal WGS were used to establish a mutational compendium for each patient. MRD-EDGEsnv was then applied to plasma from healthy individuals and pre-operative (pre-OP) plasma samples from each patient. The threshold for defining a plasma sample as ctDNA positive was determined using plasma from healthy individuals and CRC patients. This threshold was subsequently used to determine the ctDNA status of plasma from patients with colorectal adenomas.

RESULTS: MRD-EDGEsnv showed excellent performance in distinguishing between CRC patients and healthy individuals, achieving an area under the curve of 0.98. At 95% specificity, the sensitivity for ctDNA detection was 96% in plasma samples from CRC patients. Notably, ctDNA was detected in 50% (11/22) of plasma samples from symptomatic adenomas and 25% (5/20) of plasma samples from asymptomatic adenomas. The observed plasma TFs in patients with adenomas were significantly lower compared to those in CRC patients ($p<0.0002$).

CONCLUSION: Using a tumor-informed approach, we demonstrate that a fraction of colorectal adenomas releases ctDNA into the circulation, albeit at very low levels. Thus, detection of colorectal adenomas through tumor-agnostic ctDNA analysis may be a viable goal, provided that the applied methods have substantial sensitivity.

P05.004

Epigenetic liquid biopsy for the differentiation of benign and malignant forms of plasma cell disorders.

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Plasma cell disorders (PCD) encompass a spectrum of monoclonal plasma cell proliferation disorders, including premalignant conditions- Monoclonal gammopathy of undetermined significance (MGUS) and Smoldering Multiple Myeloma (SMM) which may progress to the highly malignant multiple myeloma (MM). Predicting this transition is a significant clinical challenge.

We explored the utility of cell-free DNA (cfDNA) methylation patterns as a non-invasive biomarker for diagnosing and monitoring PCD, using plasma cell-specific methylation markers and cancer-specific, entropy-based methylation changes. We hypothesize that stable methylation markers of plasma cells and disordered methylation patterns typical of cancer can serve as a potent diagnostic and surveillance biomarkers in cfDNA of MM patients.

We isolated plasma cells from bone marrow of PCD patients and performed deep whole-genome bisulfite sequencing on samples of healthy individuals, and patients with MGUS, SMM and MM. We then developed a targeted assay for sensitive detection of plasma cell DNA and MM-specific methylation changes in cfDNA. We applied the assay to cfDNA samples from patients with MGUS (N=61), SMM (N=43) and MM(N=37), and compared findings to clinical progression.

MM patients have increased plasma cell cfDNA levels compared with patients with pre-malignant conditions. Patients with MGUS and SMM that clinically progressed showed elevated plasma-cell derived cfDNA compared with those that did not progress. Locally disordered methylation patterns were characteristic of the cfDNA of patients with MM, indicating that this is a biomarker of advanced disease.

We conclude that normal and cancer-specific cfDNA methylation patterns are a promising biomarker for diagnosis and surveillance of plasma cell disorders.

P05.005

Blood-based differentiation of malignant and benign pancreatic lesions using analysis of fragmentation patterns in cell-free DNA

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BACKGROUND: Pancreatic ductal adenocarcinoma (PDAC) remains lethal, with a five-year survival of <10%. For patients with pancreatic cystic lesions (PCL), analysis of DNA from cyst fluid has shown promise in determining the risk of malignant transformation. However, obtaining cyst fluid requires an invasive procedure. We recently developed an approach for blood-based cancer detection by analyzing fragmentation characteristics in plasma cell-free DNA (cfDNA). In this study, we evaluate the performance of this method in differentiating between patients with malignant and benign pancreatic lesions.

METHODS: We collected plasma samples from 81 patients at the time of endoscopic evaluation or surgical resection of a PCL. We simultaneously obtained plasma samples from 209 PDAC patients and 56 healthy individuals. Using plasma cfDNA whole genome sequencing data from patients with cancer and healthy individuals, we trained and cross-validated an ensemble machine learning model based on 10 genomic features capturing plasma cfDNA fragmentation patterns. This model was applied to sequencing data from patients with PCL for independent evaluation of diagnostic performance.

RESULTS: Patients with PCL included 51 women (63.0%), and the median age was 70 years (IQR, 62 to 77 years). The mean cfDNA concentration across the samples was 10.5 ng/mL (SD, 14.9 ng/mL). Ten patients (12.3%) were found to have a malignant PCL, with either high-grade dysplasia or invasive carcinoma. The trained model showed an area under the receiver operating characteristic curve of 0.78 for differentiating malignant from benign lesions, achieving 50% sensitivity at 95% specificity.

CONCLUSION: Our results are a novel demonstration that a peripheral blood test based on plasma cfDNA analysis can enable differentiation between malignant and benign pancreatic lesions. This approach may improve risk-stratification and clinical decision-making regarding the necessity for surgical resection. Larger studies and clinical trials to validate these results and evaluate their impact on outcomes are warranted.

P05.006

Methylated cirDNA in plasma for early ovarian cancer diagnosis - a biomarker screening pipeline.

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Background: Ovarian cancer is typically diagnosed late because there is no effective screening test and early stages of the disease are asymptomatic. A liquid biopsy that detects ovarian tumour-derived circulating DNA (cirDNA) in plasma has potential to improve patient outcomes through early screening. Previously, we identified 73 regions of differentially methylated DNA which are promising ovarian cancer biomarkers for liquid biopsy. We aimed to develop methylation-specific qPCR (MS-qPCR) assays for the 73 biomarkers and identify the best performing biomarkers based on their sensitivity and specificity in tissue and plasma.

Methods: Methylation-specific qPCR (MS-qPCR) assays were optimised using fully-methylated and fully-unmethylated DNA. Assays were sequentially tested on DNA and cirDNA from cancer-free blood (n=96) and ovarian cancer ascites and tumour (n=16, n=226) to eliminate false positives and false negatives and create a methylated cirDNA ovarian cancer diagnostic signature.

Results: MS-qPCR assays were designed for 52/73 differentially methylated DNA regions and 35 of these were successfully optimized to be methylation-specific. These were tested on cancer-free buffy coat, which eliminated 6 regions due to false positive signal. The remaining 29 regions were tested on ovarian cancer ascites and the best 8 regions were tested on cancer-free plasma cirDNA. Two of the regions were removed as they were methylated in 80% and 84% of the cancer-free plasma samples, reflecting contribution from non-leukocyte sources. The remaining 6 regions were tested on ovarian tumour DNA samples with the best 3 biomarkers methylated in 71-92% of samples.

Conclusion: We have identified 3 promising ovarian cancer biomarkers based on methylation patterns in cancer-free cirDNA and in ovarian cancer samples. These will be optimised for sensitivity and tested in samples from an expanded ovarian cancer cohort to develop an early detection test based on methylated cirDNA in plasma.

P05.007

Comprehensive analysis of differentially methylated regions in colorectal cancer (CRC)

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Background: Epigenetic alterations, such as CpG methylation, govern many disease states, including cancer, and can distinguish cancer status and type. Here, differentially patterns of co-methylated CpG sites were defined between patients with CRC and healthy individuals.

Methods: Deep whole genome bisulfite sequencing (WGBS) was conducted on DNA samples from 100 healthy individuals (median effective coverage, 352x±38x) and 75 patients with CRC (median effective coverage, 48x±18x). The two cohorts were age-balanced (CRC: 58±11 years; healthy: 57±8 years) and represented major ethnicities in the USA. The CRC cohort represented all stages, histologies, morphologies, and microsatellite instability (MSI, 23%).

A novel machine learning model was developed to discover differentially methylated regions between the healthy and CRC cohorts and between CRC subpopulations (age, sex, stage, histology, MSI/microsatellite stability (MSS)).

Results: We discovered 132,911 differentially methylated regions between the cohorts, spanning more than 1.14 million CpGs. 76% of all discovered regions were hypermethylated (70% CpG islands, 1.3% CpG shelves, 21% CpG shores); the remaining were hypomethylated (7.3% CpG islands, 8.6% CpG shelves, 25% CpG shores). We discovered 220 regions that were differentially methylated between CRC MSI samples and CRC MSS samples and healthy cfDNA samples. Subtype-specific differentially methylated regions were discovered, including 62 regions that were differentially methylated between mucinous adenocarcinoma samples and the rest of the cohort.

Conclusions: We identified many differentially methylated regions based on deep WGBS as strong biomarker candidates for the early detection of cancer in representative cohorts eligible for CRC screening. Compared to recurrently mutated driver genes in CRC, differential methylation profiles of CRC samples are generally much more homogenous and are therefore ideal biomarkers for targeted sequencing panels for early cancer detection. Further, analysis of these patterns based on clinicopathologic factors enabled subtype specific marker discovery, which can ensure equivalent performance for underrepresented subtypes or subpopulations.

P05.008

High-Grade Serous Ovarian Carcinoma detected with TP53 mutation panel and SiMSen-Seq

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Background: Ovarian carcinoma (OC) has the highest mortality among gynaecologic malignancies due to absent or unspecific symptoms. The high-grade serous ovarian carcinoma (HGSC) accounts for over 60% of the OC associated deaths. The most frequent genetic aberrations in HGSC are mutations in the TP53 gene, detected in approximately 96% of the tumours. Previous studies have proposed potential diagnostic utility of detecting mutations in circulating tumour DNA (ctDNA) from OC patients. However, the need for diagnostic methods capable of detecting asymptomatic HGSC is unmet.

Methods: In this study, we designed a highly sensitive and robust screening tool using the simple, multiplexed, PCR-based barcoding of DNA for sensitive mutation detection using sequencing (SiMSen-Seq) technique for identification of HGSC (n=11) by TP53 mutation profile in various clinical specimen for diagnostic applications. Specimens sampled included primary tumours, ascites, plasma, vaginal samples, and both liquid and solid samples from cyst-, endocervical-, and endometrial origin (n=94).

Results: The in-house designed panel amplified 17 regions, encompassing 619 nucleotide positions within the TP53 gene. Pathogenic variants were identified in 10/11 of the primary tumours, with a VAF ≥ 9% (range: 9-91). Preliminary data indicated that all (100%) paired samples obtained corresponding somatic mutations between two or more of the different compartments from the same patient.

In total 10/10 ascites-, 16/16 cyst-, 8/8 plasma-, 5/8 vaginal-, 17/22 endocervical-, and 14/19 endometrial samples showed somatic variants at a consensus read depth of 3 and a minimum VAF ≥ 0.1%.

Conclusion: The present study suggests that the TP53 mutation panel can identify somatic variants in a variety of non-invasive liquid biopsies. Additionally, the assessment of the TP53 mutation panel exhibits potential for forthcoming clinical utilization as a diagnostic tool.

P05.009

Construction of a gene panel for liquid biopsy-based diagnostics of gynecologic cancer

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Background: Ovarian carcinoma (OC) is the leading cause of death from gynecologic cancers mainly due to late detection, where most patients are diagnosed at advanced stages (51% in stage III; 29% in stage IV). Despite an overall more favorable prognosis, endometrial carcinoma (EC) is the most common gynecologic cancer, with an increasing incidence and where some subtypes are accompanied by a poor prognosis. Identification of DNA mutations from early-stage OC and EC would allow for earlier intervention. Previous studies have shown that tumor-derived mutations can be detected in samples collected from the gynecologic tract. Herein, we aimed to construct a diagnostic gene panel corresponding to common mutation profiles of OC and EC to detect early-stage malignancy through a non-invasive approach.

Methods: A gene panel targeting mutations in OC and EC-associated genes was constructed using the NGS-method, Simple multiplexed PCR-based barcoding of DNA for sensitive mutation detection using sequencing (SiMSen-Seq). Hotspots were identified using the Catalogue of Somatic Mutations in Cancer (COSMIC) database and assays were designed for amplification of short DNA fragments. Included assays were validated in multiple steps using qPCR and fragment analysis.

Results: The two constructed multiplexes, together composed of 125 single assays targeting genomic regions in 34 EC and OC-associated genes, displayed good performance at low DNA input (10 ng). Validation of individual assays showed high specificity and efficient amplification where >80% of assays displayed a sequencing coverage >500 UMI counts at a consensus depth of 3. Patient coverage calculations of COSMIC data suggested comparable coverage as similar published approaches.

Conclusion: The results suggest that the constructed gene panel has great potential to detect mutations in liquid biopsies, with low tumor DNA levels. Validation of the two multiplexes by fragment analysis and sequencing showed high performance, hence, prospective for the approaching sequencing of liquid biopsies.

P05.010

Multiparametric Investigation and Stratification of Indeterminate Lung Nodules (MISIL1)

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Background: The implementation of low-dose CT screening in Europe will increase the number of lung cancers detected early but also the number of indeterminate pulmonary nodules (IPN) identified. The MISIL1 study aims to use a multi-omics approach and machine learning to create a multiparametric biomarker “score” to distinguish between cancer and non-cancer IPNs.

Methods: MISIL1 is a multisite study which recruited 90 patients between May 2021 and May 2022. Three patient cohorts were recruited: 30 negative controls (normal CT), 30 positive controls (stage 1 NSCLC), and 30 patients with IPN. Inclusion criteria were aligned with published screening trials and participants consented to a clinical dataset and a 40mL blood draw. Biomarker evaluation includes cfDNA whole genome sequencing (WGS), cfDNA methylation targeted sequencing, circulating proteome, cytokine, and autoantibody quantification, and circulating tumour cell (CTC) detection. Data will be integrated with clinical parameters and analysed using a machine learning pipeline to generate a risk score for lung cancer.

Results: Univariate proteomic and autoantibody analysis have identified 11 and 17 unique discriminator candidates respectively. Evaluation of the EarlyCDT® Lung autoantibody assay detection found the sensitivity was 12.7% (95% CI: 4.8%, 25.7%) and specificity was 100% in our cohort (95% CI: 90.7%, 100%). Whole genome sequencing is underway for the cfDNA of all 90 patients and the tumour samples of 41 individuals diagnosed with a lung cancer during the course of the study. Methylation sequencing, CTC detection and cytokine analysis is in progress.

Conclusion: We have successfully identified a number of potential protein and autoantibody candidates to discriminate between malignant and benign nodules. The EarlyCDT®-Lung has low sensitivity in early lung cancer but is very specific and may have a role in a combined biomarker score. Further biomarker analysis is underway and we will present the results of their combination into a multiparametric test.

P05.011

Epigenomic profiling of active regulatory elements by enrichment of unmodified CpG dinucleotides

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Background: Current approaches for the study of DNA methylation and other modified bases focus on the modified fraction of the genome and are particularly well-suited to the detection of DNA hypermethylation. However, the study of hypomethylation (loss of DNA methylation), which is typically associated with markers of active chromatin, has been largely overlooked, in part, due to the lack of a suitable methodology.

Methods: Here I present an enrichment-based and bisulfite-free approach for epigenomic profiling named 'Active-Seq' (Azide Click Tagging for In Vitro Epigenomic sequencing) that achieves genome-wide profiling of DNA, by enriching for non-modified CpG sites using a mutated methyltransferase enzyme.

Results: I will show that the genomic regions enriched by Active-Seq overlap with promoters, enhancers and partially methylated domains, all of which have had their methylation status linked to the development and progression of diseases. Active-Seq is a fast epigenetic profiling platform with a simple and streamlined workflow, performed in tandem with sequencing library preparation. The enzymatic chemistry is non-damaging toward the DNA which is critical for working with low concentration DNA input and will facilitate the future development of multiomics assays.

Conclusion: The results demonstrate robust and reproducible performance of Active-Seq using low DNA input in cell lines, liquid biopsies (cell-free DNA, cfDNA) and formalin-fixed paraffin embedded (FFPE) tissue.

P05.013

Serial sample collections to evaluate ctDNA detection before clinical diagnosis in women at high risk of breast and ovarian cancer

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Background: Breast or ovarian cancers are diagnosed in 62,000 women each year in the UK. There is a need for improvements in earlier diagnosis given that there is no effective screening test for ovarian cancer and 30% of breast cancer patients are underdiagnosed in the current breast screening programme.

Methods: To, most effectively, achieve high power and positive predictive value in the asymptomatic population, we sought to collect samples annually from women with substantially elevated risk of breast and/or ovarian cancer. We have leveraged the EMBRACE study of individuals with a mutation in a known breast or ovarian cancer risk gene (e.g., BRCA1 or BRCA2) and a new study (EMBED) enrolling women with a strong family history. In addition, women recalled from breast screening with suspicious lesions were sampled to optimise laboratory methodologies and analytical pipelines.

Results: Since 2020 we have recruited over 5484 women to these studies and collected samples at over 8021 timepoints. As of October 2023, 34 incident breast cancers and 23 other cancers have been reported: given delays in reporting the true number of cancers will be significantly larger.
In the breast screening cohort of 88 women with 20 cancers analysed to date we have detected cancer signal by analysis of somatic copy number aberration (SCNA) and methylation.

Conclusion: Cancer detection is likely to be improved by using multiple features of cfDNA and other analytes. Where generic methods (methylation, fragmentomics, SCNA, panel of commonly mutated genes) fail to detect ctDNA, we plan tumour-guided sequencing with sensitivity approaching AF~0.0001% to define ctDNA levels prior to diagnosis. Determining the level of ctDNA present in plasma in the months and years before clinical diagnosis will pave the way for the development of additional methods, including combination of ctDNA with analytes such as protein markers and tumour educated platelets.

P05.014

Circulating differential methylation allele fraction (DMAF) strongly correlates with circulating tumor DNA (ctDNA) Variant Allele Fraction (VAF)

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Background: Measurement of ctDNA has been established as a prognostic biomarker of cancer disease burden. Emerging evidence has demonstrated that differential methylation patterns can serve as an accurate biomarker both for cancer detection and for predicting the tissue of origin. While it is important to distinguish cancer-positive from cancer-negative patients, clinical decision making increasingly depends on the exact level of the measured biomarker. To that end, we report on the correlation between ctDNA VAF and DMAF across patients with colorectal cancer (CRC).

Methods: Pre-surgical plasma samples from patients with CRC (N=70) were sourced from the prospective GALAXY, observational arm of ongoing CIRCULATE-Japan study trial (UMIN000039205). Samples classified as ctDNA-positive by a tumor-informed ctDNA assay (SignateraTM) were included in this analysis and ctDNA VAF (%) was calculated. We performed deep methylation sequencing (Illumina Novaseq) across multiple target regions. DMAF was computed by estimating the fraction of differentially methylated alleles for circulating cell-free DNA across target regions.

Results: Among 70 CRC patients, 68 had positive ctDNA results. Median age at testing was 64.0 years and male individuals represented 55.9% (n=38) of the cohort. Among them, 9 (13.2%) were stage I, 24 (35.3%) were stage II, 22 (32.3%) were stage III, 8 (11.7%) were stage IV, and 5 (7.3%) had unknown staging. The correlation of DMAF with ctDNA VAF was 0.894 (Spearman's Rank Correlation Coefficient) and was independent of disease stage, histology, age, and sex. Among MSS (micro-satellite stable; n=58) and MSI-H (micro-satellite instable n=10) patients, the DMAF correlation was 0.9 and 0.915, respectively.

Conclusion: These data demonstrate that abundance of differential methylation in CRC patients correlates with VAF regardless of clinicopathologic features of patients with CRC. Methylation-based assays are a promising tool for cancer detection and quantifying the disease burden.

P05.015

Second Primary cancer cohORT (SPORT) – a valuable resource for assessing novel tools for early detection of lung cancer

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Background: Early cancer detection is crucial to enhance patient survival rates, however, there is currently an urgent need to develop highly sensitive early detection methods and progression in the field necessitates validation of these in patient samples collected prior to diagnosis. The improved survival of lung cancer patients has led to increasing numbers of individuals at elevated risk of recurrence and second primary cancers (SPCs). This provides opportunities to collect samples prior to diagnosis.

Methods: The Second Primary lung cancer cohORT (SPORT) is an ongoing, multi-centre, observational cohort study, actively recruiting 850 lung cancer survivors who have reached their second anniversary post completion of curative treatment for a primary lung cancer. Blood samples are prospectively collected at six-month intervals for up to five years, alongside clinical data and records of new second cancer diagnoses and recurrences. Patient recruitment is expected to conclude in September 2024. Samples from lung cancer patients and non-cancer controls will be analysed using a combination of ctDNA detection methods such as cancer-specific DNA methylation, sequence changes and copy number aberrations and non-DNA alterations including protein biomarkers.

Results: The SPORT study is nearing patient recruitment completion, with 702 individuals currently enrolled. More than 32,000 sample vials have been collected across 12 sites in the UK and the first two participants have reached 8 return visits corresponding to 4 years in the study. Excluding 23 deaths, the study retention rate is 90%, and 38 participants have been diagnosed with a SPC (20 lung) and 28 with a recurrence (23 lung). Updated recruitment numbers and preliminary data on assay development will be presented at the meeting.

Conclusion: The SPORT study, featuring pre-diagnosis plasma, serum and tumour-educated platelets, serves as a unique resource for assessing the potential of ctDNA and other markers for detection of early-stage cancer and recurrence.

P05.016

Effective pre-operative diagnosis of ovarian cancer using minimally invasive Liquid Biopsies by combining HE4 and cfDNA in patients with an ovarian mass.

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Background: Pre-operative diagnosis of ovarian cancer in patients with an ovarian mass is challenging as sensitivity and specificity of current methods are not optimal. Besides, ultrasound-based methods require training and are prone to inter-observer variability. We aimed to explore new methodologies for pre-operative risk of malignancy assessment in patients with an ovarian mass based on scalable, objective and minimally invasive liquid biopsy-derived data types.

Material and Methods: In this case-control study, we included 100 patients with an ovarian mass clinically suspected of early-stage ovarian cancer. Of these 100 patients, 50 were confirmed to have a malignant mass (cases) and 50 had a benign mass (controls). Using WisecondorX, an algorithm used extensively in Non-Invasive Prenatal Testing (NIPT), we calculated the benign-calibrated Copy number Profile Abnormality (bCPA) score. This score represents how different a sample is from benign controls based on copy number profiles. We combined the bCPA score with human epididymis protein 4 (HE4) serum concentration to separate cases and controls.

Results: Combining the bCPA score with HE4, we obtained a model with a significantly higher sensitivity (42% vs 0% ($p < 0.002$)) at 99% specificity as compared to the Risk of Malignancy Index (RMI) which is currently employed in clinical practice. Investigating performance in subgroups, we observed especially large differences in the advanced stage and non-high grade serous ovarian cancer groups.

Conclusions: We show that cell-free DNA (cfDNA) can be successfully employed to perform pre-operative risk of malignancy assessment for ovarian masses. However, results warrant validation in a more extensive clinical study.

P06.001

Unraveling the potential clinical utility of circulating tumor DNA in colorectal cancer – evaluation in a nationwide Danish cohort

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Background: Increasingly, circulating tumor DNA (ctDNA) is proposed as a tool for minimal residual disease (MRD) assessment, with the potential to guide postoperative treatment decisions. Low ctDNA levels immediately after surgery necessitate extremely sensitive detection methods. Digital PCR (dPCR) offers low analysis costs and turnaround times of less than a day, making it ripe for clinical implementation. Our aim was to assess the potential clinical utility of monitoring MRD using a tumor-informed dPCR strategy for ctDNA detection in a large cohort of colorectal cancer patients.

Methods: Stage II-III colorectal cancer patients (n=851) treated with curative intent were recruited from 12 Danish surgical centers. Whole exome sequencing was conducted on matched tumor and buffy coat from all patients. After thorough clonality assessment, a single patient-specific variant was chosen for digital PCR analysis. Plasma samples (8mL) collected within 60 days after surgery, were investigated for ctDNA using digital PCR. Additionally, a subset of patients (n=246) had serial samples collected every three months analyzed for ctDNA.

Results: Both postoperative and serial ctDNA detection was prognostic of recurrence (HR=11.3, 95%CI 7.8-16.4, P<0.001; HR=30.7, 95%CI 20.2-46.7, P<0.001), with a cumulative ctDNA detection rate of 87% at the end of sample collection in recurrence patients. The ctDNA growth rate was prognostic of survival (HR=2.6, 95%CI 1.5-4.4, P=0.001). In recurrence patients, postoperative ctDNA detection was challenging for lung metastases (4/21 detected) and peritoneal metastases (2/10 detected).

Conclusion: These results from 851 stage II-III CRC patients demonstrate that our personalized digital PCR approach effectively detects MRD immediately after surgery. Additionally, our approach shows promise for serial ctDNA detection for recurrence surveillance applications. With digital PCR being a widespread and cost-effective method with short turnaround times, clinical implementation of ctDNA analysis may be more forthright using this method over cost-intensive sequencing-based methods.

P06.002

Correlation between variant allele frequency and mean tumor molecules with tumor burden in patients with solid tumors

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Background: Several studies have demonstrated the prognostic value of circulating tumor DNA (ctDNA), with mean tumor molecules (MTM)/ml and mean variant allele frequency (mVAF, %) as two validated metrics used to quantify tumor burden. However, the agreement between these two metrics with clinical parameters is yet to be understood. Here, we assessed the performance of the mVAF and MTM/mL metrics for predicting survival after definitive treatment.

Methods: In this study, we analyzed ctDNA data in a pan-cancer cohort of 23,543 patients (stages I-IV) with 91,562 blood samples who had ctDNA testing performed using a personalized, tumor-informed assay (SignateraTM, mPCR-NGS assay) as part of clinical care. For ctDNA-positive patients, correlation between MTM/ml and mVAF was examined. A subanalysis was performed to assess the correlation between ctDNA dynamics and patient outcomes.

Results: On a global cohort, a positive correlation between MTM/ml and mVAF was observed (linear regression: logmeanVAF = 0.87, logMTM/ml -7.59, R2=0.932; LOESS regression: R2=0.940). MTM/mL displayed a continued dynamic range at around 10,000 MTM/ml, while we observed mVAF values plateauing at 100%. Among 18,426 patients with longitudinal ctDNA measurements, 13.3% had discordant trajectories between MTM/ml and mVAF at subsequent timepoints. Changes in ctDNA levels among a smaller well-characterized subset of patients treated with immunotherapy (N=51), expressed both in MTM/ml and mVAF, showed a statistically significant association with progression-free survival, however, the correlation with MTM/mL was numerically stronger (ctDNA by MTM/ml: HR: 16, 95% CI: 3.72-69.5, p<0.0001; ctDNA by mVAF HR: 8.8, 95% CI: 2.9-26.7, p<0.0001).

Conclusion: Though MTM/ml and mVAF are both validated metrics for ctDNA measurement, our study suggests that MTM/ml could be more representative of molecular disease burden and clinical outcomes. ctDNA quantification and dynamics measured in MTM/ml may improve correlation with clinical parameters when used as the unit of measurement for ctDNA.

P06.003

Accurate genome-wide multimodal cell-free DNA profiling using nanopore sequencing

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Background: Shallow genome-wide cell-free DNA sequencing has emerged as a promising avenue for non- and semi-invasive cancer detection and monitoring. It has the advantage of simultaneously assessing copy number alterations and cfDNA fragmentation patterns. However, a major downside of shallow genome-wide sequencing is that it cannot effectively capture single nucleotide variants in samples with low tumor fractions. We developed a new technique based on the nanopore sequencing platform, CyclomicsSeq, which boosts accuracy to a level required for somatic SNV detection in cfDNA and substantially lowers turnaround times.

Methods: CyclomicsSeq utilizes a concatemerization strategy and consensus base calling, achieving low error rate sequencing. We applied CyclomicsSeq to healthy controls and 18 patient samples encompassing three cancer types (ovarian carcinoma, granulosa-cell tumor, and esophageal adenocarcinoma), including a time series of a granulosa-cell tumor patient. We also performed dilutions and simulations of various ctDNA fractions using known mutations of various cancer types in PCAWG to determine the limit of detection for CyclomicsSeq.

Results: CyclomicsSeq achieves a base accuracy more than two times higher than paired-end Illumina NovaSeq, and five times higher than Nanopore. The lowest tumor fraction we detected in the patient group is 0.5%. Our in silico dilutions clearly demonstrate that the SNV-signal is critical for detecting tumor fractions below 2%. Simulations showcased that with the method presented in combination with PromethION platform, we can confidently detect tumor fractions down to 0.25%.

Conclusion: We utilized the flexible ONT Nanopore sequencing platform for accurate genome-wide cfDNA sequencing capable of delivering next-day results. Our multimodal approach is capable of capturing tumor-derived somatic nucleotide variants, copy number alterations, and fragmentomics all at once, and derived integrated tumor fractions for each sample based on these three modalities. The results demonstrate that CyclomicsSeq is a promising method for minimal residual disease detection and treatment monitoring in cancer.

P06.004

Circulating Tumor DNA for Assessing Neoadjuvant Treatment Response and Recurrence Risk in Rectal Cancer Patients

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Background: Each year, more than 700,000 people worldwide are diagnosed with rectal cancer, which is a major cause of cancer-related mortality. Disease recurrence after curative-intent surgery is the main factor affecting survival in rectal cancer patients, hence identifying patients at risk is important to optimize postsurgical treatment. In recent years, multidisciplinary treatment approaches, including neoadjuvant therapy, have optimized the management of rectal cancer, leading to increased focus on non-operative “watch-and-wait” strategies. However, clinical modalities for determining complete response following neoadjuvant therapy are not sufficiently accurate for selecting patients suitable for non-operative management. Novel biomarkers such as circulating tumor DNA (ctDNA) offers great potential for predicting neoadjuvant treatment response and identifying patients at risk of recurrence.

Methods: We recruited 118 patients with localized rectal cancer treated with neoadjuvant therapy and surgery. Plasma samples (n=331) were collected before initiation of therapy, after neoadjuvant therapy, and postoperatively. Analysis of ctDNA was performed using a tumor-agnostic droplet digital PCR test targeting three methylation markers.

Results: The pre-treatment ctDNA detection rate was 85.3%. Detectable ctDNA was associated with significantly worse recurrence-free survival, both after neoadjuvant therapy (HR 3.11, 95% CI 1.34-7.20, p=0.008) and after surgery (HR 5.45, 95% CI 2.29-12.97; p=0.0001). None of the nine patients with pathologic complete response (pCR) had ctDNA detected following neoadjuvant therapy. The sensitivity of ctDNA analysis for detecting residual disease following neoadjuvant therapy was 31.2%, and there was no significant association between pCR and ctDNA status following neoadjuvant therapy (p=0.58, Fisher’s exact).

Conclusion: In conclusion, we were able to demonstrate that ctDNA analysis is a valuable prognostic tool for recurrence prediction in neoadjuvant-treated rectal cancer patients. However, ctDNA analysis was unable to predict complete response to neoadjuvant therapy, and thus additional studies are needed to determine the role of ctDNA analysis in guiding non-operative management of rectal cancer patients.

P06.005

Signatera Research Use Only- a versatile oncology research tool

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Background: Several studies have utilized SignateraTM, a personalized, tumor-informed ctDNA testing tool for various clinical scenarios. Currently, Signatera is covered by medicare for colorectal, breast, muscle invasive bladder and immunotherapy-treated advanced solid tumors. Here we demonstrate the extended capabilities of a Signatera Research Use Only (RUO) for broader scientific applications, paving a path towards continued innovation, and improved outcomes for cancer patients.

Methods: The Signatera RUO assay allows flexibility of samples and data input for the exploration of ctDNA applications. Patient-specific ctDNA assays can be designed and developed from whole-exome and -genome sequencing data. The RUO modality allows investigating variable numbers and types of patient-specific variants, and development of defined custom panels depending on the cancer indication.

Results: To date, over 150 collaborative studies across a broad range of cancer types–including breast, gastrointestinal and genitourinary, liver, lung, ovarian, and melanoma–have been performed in collaboration with biotech/pharma and academic institutions. Signatera RUO has played a key role in the retrospective analysis of biobanked samples since these are often limited by non-standardized sample input. Data generated with Signatera RUO has successfully been used to inform prospective clinical trial design, evaluate the effectiveness of various experimental treatments, elucidate the optimal time window for molecular detection of recurrence or disease progression ahead of standard-of-care imaging modalities.

Conclusion: There is an ongoing need for continued innovation using ctDNA as a minimally-invasive biomarker in order to understand the full potential of ctDNA detection for cancer patients. The flexibility of the Signatera RUO workflow has powered ongoing innovation to drive the knowledge for best individualized patient care. Signatera RUO is a valuable research tool in the oncology space, allowing for investigation of the potential novel application of ctDNA detection technologies across a broad range of research and clinical questions.

P06.006

Cell-free DNA genomic and fragmentomic features for early outcome prediction in high grade B-cell lymphoma

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Background: High grade B-cell lymphoma (HGBL) patients need an accurate and early risk stratification strategy as prompt therapy escalation might improve survival for those not responding to the frontline treatment.

Methods: We evaluated cell free DNA (cfDNA) tumor fraction, fragment size, and a fragmentomic feature in 196 serial plasma samples collected from 94 patients enrolled in the HOVON-152 trial using Whole Genome Sequencing (median 3.7 coverage). We defined a new metric called ACT score (Aberrations, Contribution of short fragments, fragmenT) using machine learning by combining four cfDNA signals. ACT score was compared to the International Prognostic Index (IPI) and correlated to interim positron emission tomography/computed tomography (iPET-CT), end-of-treatment (EOT) response.

Results: Individual cfDNA features were successfully computed in all 92 patients included in analysis, and were altered in non-responders compared to responders after one cycle of immunochemotherapy (T1). Tumor fraction/Copy Number Aberrations by ichorCNA had the highest predictive performance The ACT score at T1 built with a balanced random forest in all patients outperformed all individual cfDNA features to predict EOT response (AUC = 0.74) in the validation set and had similar performance to the interim PET-CT (AUC = 0.77) carried out after three cycles.

Conclusion: ACT score consisting of four cfDNA features harnessed from T1 plasma samples can predict clinical outcomes earlier than the interim PET-CT. This low-cost and easy-to-interpret test does not need molecular information from tissue biopsies or a priori knowledge of mutations. Future studies will evaluate whether the ACT score, alone or in combination with radiomics, could assist the early selection of lymphoma patients who may benefit from risk-adapted treatment strategies.

P06.007

Impact of postoperative circulating cell-free DNA on ctDNA-based MRD detection and associated patient outcomes in patients with stage I-III colon cancer

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Background: Next generation sequencing with higher depth allows detection of circulating tumor DNA (ctDNA) in a background of non-tumor derived cell-free DNA (cfDNA). Specifically, after surgery or during systemic therapy, a cfDNA surge can potentially impact assay sensitivity. Limited studies have explored the ideal timing of ctDNA testing post-surgery.

Methods: In this retrospective analysis of real-world data obtained from patients with stage I-III colon cancer across 2,350 institutions, data from commercial tumor-informed ctDNA testing (Signatera™) collected from 06/2019 to 09/2023 were analyzed. Within the overall cohort (N=21,744, n=90,865 plasma samples), partial clinical information and follow-up data was available for 1,356 patients (n=8,695). Here we explore postoperative cfDNA kinetics and quantify ctDNA prior to surgery and postoperatively (MRD-window; 2-12 weeks post surgery, prior to therapy).

Results: In the majority (~60%) of cases, ctDNA sampling started within the first 12 weeks post-surgery, with a peak at 4 weeks. Median measured cfDNA concentration was 5.7 ng/mL (range: 1.1-229.9 ng/mL). cfDNA levels were higher within 0-2 weeks post-surgery, with a median of 8.3 ng/mL. Although cfDNA levels remained elevated during postoperative weeks 2-4 (median 6.6 ng/mL), ctDNA was consistently detected by week 2 post-surgery (ctDNA-positivity rates at week 2, 3, 4, 5 were 20.1%, 16.5%, 16.0%, 16.9%, respectively). ctDNA-positivity during the MRD window was associated with a higher recurrence rate of 62.1% (46/74), compared to 8.9% (31/349) in ctDNA-negative patients, yielding a marked reduction in disease-free survival (HR: 10.9, 95% CI: 6.9-17.3, p=0.001).

Conclusion: Our data suggests that standard MRD testing could potentially start as early as week 2, as postoperative elevated cfDNA concentration does not impact the ability to detect ctDNA.

P06.008

Investigating and defining significant changes in ctDNA dynamics and their correlation with clinical outcomes: An exploratory study

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Background: Limited research has explored the significance of change in circulating tumor (ct)DNA levels in relation to clinical outcomes. Here we examined the correlation between substantial ctDNA changes and patient outcomes in cancer patients.

Methods: Patients with advanced solid tumors (training set) with known response to immunotherapy and plasma samples available for tumor-informed ctDNA testing (SignateraTM) were evaluated to establish correlations between ctDNA dynamics and clinical outcomes. ctDNA dynamics were evaluated by; 1) comparing consecutive mean tumor molecules/mL (MTM/mL) confidence intervals (CIs), where non-overlapping CIs were considered statistically significant, 2) computing the monthly rate of change (MRC) in ctDNA. Results from the training set were tested in patients with resected stage I-IV colorectal cancer (CRC) and with ≥2 consecutive ctDNA-positive plasma samples between 8-12 months post-surgery (presumed to be off-treatment).

Results: In the training set (N=60), statistically significant changes in MTM/mL had 78.3% accuracy (82.1% PPV, 75% NPV) in determining progressive disease (PD). MRC and final ctDNA level thresholds were established, achieving 78.3% accuracy (95% PPV, 70% NPV) in determining PD. In the CRC test set (N=410), 318 exhibited statistically significant ctDNA changes (132 reduced; 186 increased). As expected, median MRC was higher in patients with significant ctDNA changes versus non-significant changes, regardless of whether ctDNA increased (significant: 243.14%, IQR 478.14%; non-significant: 26.7%, IQR 62.38%, $p<0.0001$) or decreased (significant: -72.55%, IQR -44.50%; non-significant: -22.65%, IQR -31.53%, $p<0.0001$). MRC thresholds established in the training set distinguished CRC patients with significant ctDNA increases (N=186), likely indicative of PD, from those with non-significant increases (N=52) with accuracy of 86%.

Conclusion: Findings from the training set indicate that integrating MRC with final ctDNA levels correlates with clinical outcomes. While the MRC threshold distinguished between statistically significant and non-significant changes in ctDNA in an independent cohort of CRC patients, correlation with clinical outcomes is ongoing.

P06.009

Ultra-Sensitive Minimal Residual Disease (MRD) Monitoring For Cancer Patients Using SuperRCA Mutation Assays With Flow Cytometer Readout

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Background: Rare tumor-specific mutations in patient samples serve as excellent markers to monitor the course of malignant disease and responses to therapy in clinical routine, and improved assay techniques are needed for broad adoption. superRCA assays provides rapid and highly specific detection of DNA sequence variants present at very low frequencies. Using a standard flow cytometer we demonstrate precise, ultra-sensitive detection of single-nucleotide mutant sequences from malignant cells against a 100,000-fold excess of DNA, to follow the course of patients treated for Colorectal Cancer patients.

Methods: Sequence of interest are enriched by targeted amplification and converted to DNA circles that are subjected to rolling-circle amplification (RCA). Genotyping probes interrogate repeated sequences of the RCA products with exquisite specificity, followed by RCA of the circularized probes. The large DNA clusters that result from each starting DNA circle are referred to as superRCA products.

Results and Conclusions: Plasma DNA collected at diagnosis and follow-up from 25 CRC patients was analyzed using a multiplex superRCA mutation detection assay together with genomic DNA (gDNA) from tumor and normal tissue from 20 patients. The lower limit of detection for the multiplex variants are in the range of 10-5, and when analyzing cfDNA from plasma with a typical input of 33 ng, the detection limit becomes ~0.01% mutant allele frequency (MAF). In 17 of 19 patients with identified hotspot mutations in tumor gDNA, at least one hotspot mutation could be detected in plasma DNA at the time of diagnosis. The MAF increased at subsequent time points in four of the patients who experienced a clinical relapse. Multiplex superRCA analysis of the remaining six patients failed to detect any hotspot mutations. In conclusion, superRCA assays proved suitable for detecting hotspot mutations in ctDNA, and dynamic changes in MAF were observed in patients with clinical relapse.

P06.010

Quantitative multiplexed analysis of tumor-derived structural variants in plasma DNA for minimal residual disease detection

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Background: Recent results have demonstrated the promise and the limitations of circulating tumor DNA (ctDNA) as a biomarker for minimal residual disease. Most current assays rely on interrogating multiple patient-specific point mutations to achieve exquisite limits of detection but struggle with polymerase errors, sequencing artifacts and limited conversion of input DNA. To overcome these challenges, we developed an approach called Structural Variant Enrichment and Normalization (SVEN) that targets patient-specific genomic rearrangements to achieve sensitive detection and quantification of ctDNA.

Methods: Consensus structural variants (SVs) were called from whole genome sequencing of two breast cancer cell lines, along with 21 early-stage breast cancer patients with FFPE tumor and buffy coat DNA. From these consensus SVs, multiplexed SV-specific primers were designed for each subject. We then conducted PCR amplification of cell-free DNA followed by sequencing, amplicon identification, and quantification. Analytical validation was conducted using 284 replicates of cell line dilutions from 1% to 0.003%, and 5 to 40 ng of input.

Results: We developed multiplexed assays targeting 25 and 32 SVs across two cell lines, and 3-43 SVs across breast cancer patients (median 23 per patient). 91% and 42% of PCR targets were successfully validated for cell lines and FFPE samples respectively. Cell line dilutions demonstrated a sensitivity of 89% at 0.003% tumor fraction with 10ng input or more. Tumor fractions quantified using SVEN agreed with known values ($R^2 = 0.989$). Application of validated panels to patient plasma samples and tumor/germline dilutions is ongoing.

Conclusion: Our results demonstrate a novel approach for tumor-guided ctDNA detection and quantification using multiplexed analysis of patient-specific structural variants. On-going studies are investigating the performance of this approach for ctDNA detection in patients with breast cancer treated with neoadjuvant therapy.

P06.011

Investigating the use of circulating tumor DNA-guided patient management in renal cancer

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Background: The incidence of renal cell carcinoma (RCC) is increasing worldwide. A quarter of cases are diagnosed at an advanced disease stage and ~25% of localized RCC patients, initially treated with curatively intended surgery, relapses. The high recurrence rates may be explained by presence of micrometastatic disease prior to surgery or minimal residual disease (MRD) after treatment that is undetectable with present imaging modalities. A few studies have proposed circulating tumor DNA (ctDNA) as a promising prognostic tool in RCC, but analyses have been challenged by the trace amounts of ctDNA shedded by RCC tumors.

Methods: In this study, ctDNA will be measured in pre- and post-operative plasma samples from 200 localized RCC patients by looking at genomic and epigenomic variants using the sensitive cfMeDIP-seq method. Furthermore, 100 metastatic RCC patients', all part of the randomized clinical NORDIC-SUN trial, will have plasma samples analyzed longitudinally during immunotherapy treatment. ctDNA will be assessed by both copy number- and methylation alterations, where differentially methylated regions between patients relapsing/not relapsing will be called and used to build a classifier for prediction of relapse risk. ctDNA levels/detection from both methods will be correlated to clinical outcome measures, to evaluate the prognostic potential of ctDNA in RCC.

Results: We hypothesize that ctDNA can be used to determine the risk of recurrence and identify patients with MRD after surgery who need further treatment. Furthermore, we believe that measures of tumor burden in metastatic RCC patients by analysis of ctDNA during treatment will be able to detect clinical relapses earlier than currently employed surveillance modalities. Preliminary results from the initial patients analyzed will be presented at CNAPS 2024.

Conclusion: Results will benefit both patients and the health care system as expensive oncological treatment can be administered only to patients that need it and will benefit from it.

P06.012

Ultra-sensitive molecular residual disease detection through whole genome sequencing with single-read error correction

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While whole genome sequencing (WGS) of cell-free DNA (cfDNA) holds enormous promise for molecular residual disease (MRD) detection, its performance is limited by WGS error rate. Here we introduce AccuScan, an efficient cfDNA WGS technology that enables genome-wide error suppression, achieving an error rate of 4.2x10^-7, which is about two orders of magnitude lower than a read-centric de-noising method. When applied to MRD detection, AccuScan demonstrated analytical sensitivity down to 10^-6 circulating tumor allele fraction (cTAF) at 99% sample level specificity. In colorectal cancer, AccuScan showed 90% landmark sensitivity for predicting relapse. Overall, AccuScan provides a highly accurate WGS solution for MRD, empowering circulating tumor DNA detection at parts per million range without high sample input nor personalized reagents.

P06.013

Improve Molecular Residual Disease monitoring by combining ctDNA molecular profiling and circulating H3K27Me3-nucleosome levels in NSCLC plasma samples

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Background: Patients diagnosed with stage IV NSCLC are treated by non-surgical therapies. The molecular profiling of circulating tumor DNA (ctDNA) is a helpful tool not only to define the most appropriate cancer treatment, but also to identify patients whose cfDNA includes residual tumor associated mutations. However, molecular residual disease (MRD) is missed in a significant number of patients leading to a delayed treatment or suboptimal treatment selections. We propose a novel molecular profiling paradigm by combining circulating nucleosome and molecular profiling analysis and investigate its potential use in disease monitoring.

Methods: K2-EDTA plasma samples from 319 patients with a NSCLC at diagnosis, and from 304 independent NSCLC patients under treatment were collected at Lyon University Hospital were analysed with a custom-validated NGS panel covered 78 genes involved in cancer. Samples from 201 healthy donors were also collected. Both NSCLC and healthy samples were analysed for four nucleosome structures (H3K27Me3-, H3K36Me3-, H3K9Me3-, and H3K4Me2-nucleosomes) using Nu.Q® immunoassays (Belgian Volition SRL).

Results: Significantly elevated concentrations of H3K27Me3-nucleosomes were found in plasmas at the diagnosis (median=24 ng/ml), and during the treatment follow-up of NSCLC patients (median=16.9 ng/ml), compared to healthy donors (median=8 ng/ml; p-value < 0.0001). During patient follow-up, somatic mutations was still detected in 43.1% of the samples. A high H3K27Me3-nucleosome level was found in 15.1% of the samples, despite no somatic mutations being detected, allowing the identification of a potential disease progression from 43.1% to 58.2% over molecular profiling alone.

Conclusion: Measuring H3K27Me3-nucleosome levels in combination with ctDNA molecular profiling performed better than ctDNA molecular profiling alone and may be a promising new method for MRD monitoring, during and/or after treatment in patient with late-stage NSCLC. Further clinical study would be required to prove that its use in the clinic may lead to a better patient management.

P06.014

Fully automated cell-free DNA extraction from up to 10 ml plasma enables sensitive mutation detection with digital PCR and next-generation sequencing

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Cell-free DNA (cfDNA) is found in blood, urine and other body fluids and is an important analyte for screening and treatment monitoring e.g. in cancer research. Since cfDNA is present in very low amounts, there is a need to process large input volumes leading to higher sensitivity of rare mutation detection and for high degree of automation to increase sample throughput. The EZ1&2 ccfDNA process uses magnetic beads to enable efficient purification of cfDNA from up to 24 samples without manual pre-enrichment from 1-10 ml human plasma or urine in 35-90 minutes processing time.
cfDNA from plasma or urine from healthy donors was extracted using the new EZ1&2 ccfDNA Kit.
cfDNA yield was determined using Qubit, qPCR and dPCR and quality assessed by fragment length analysis in comparison to other cfDNA extraction solutions. cfDNA material with defined mutations was spiked into healthy donor plasma and eluates were analyzed with dPCR for detection of variants with allele frequencies (VAF) below 0.5 %. Furthermore, the new QIAseq Targeted cfDNA Ultra Panel was evaluated for low VAF detection using SeraCare reference material extracted on the EZ2 Connect.
cfDNA can be isolated efficiently using the EZ1&2 ccfDNA Kit from 1-10 ml plasma in either 75 µl or 40 µl elution volumes with no discernible inhibitory effects in Qubit, qPCR, dPCR, fragment length analysis or NGS-based methods. This fully automated cfDNA extraction method proved to be equal or better than other cfDNA extraction methods and enabled the detection of VAFs as low as 0.1 % in dPCR and NGS.
The novel bench-top sized system provides robust and fast cfDNA extraction in as little as 35 minutes from up to 24 samples. Our data demonstrates equal or better performance in comparison to alternative methods including crucial detection of rare mutations.

P06.015

Simsen Personal – A personalized, tumor-guided sequencing platform for ultrasensitive ctDNA detection in clinical trials

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Detecting extremely rare variant alleles is becoming an increasingly recognized biomarker in clinical trials for treatment response monitoring, minimal residual disease detection, and patient stratification. Detection of rare circulating tumor DNA fragments (ctDNA) is hampered by low numbers of mutant DNA molecules and challenging sample types. Personalized ctDNA assays track dozens of mutations chosen based on each patient's unique tumor mutational profile.

Simsen Personal harnesses a highly optimized workflow for creating tumor-guided personalized ultrasensitive sequencing assays for ctDNA detection. This includes advanced in silico assay design based on the SiMSen-seq technology, carefully optimized reaction chemistry, and a machine-learning enhanced variant calling algorithm.

Our enhanced enhancements significantly increase the dynamic range and sensitivity to detect rare mutations and recover target molecules. We demonstrate the high sensitivity and specificity of Simsen Personal using both reference materials and human plasma samples. Furthermore, we show how Simsen Personal enables ultrasensitive ctDNA detection in clinical trials using real-world data.

Thus, Simsen Personal, a tumor-informed, personalized sequencing platform enables highly specific and sensitive detection of ctDNA, orders of magnitude greater than what can be achieved by standard sequencing approaches.

P06.016

Clinical validity of post-surgery circulating tumor DNA testing in stage III colon cancer patients treated with adjuvant chemotherapy: the PROVENC3 study

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Introduction: Surgery followed by adjuvant chemotherapy (ACT) is standard of care in stage III colon cancer. However, only 15-20% of patients benefit from ACT. Detection of circulating tumor DNA (ctDNA) after resection of the primary tumor is a strong prognostic biomarker in non-metastatic colon cancer and offers a promising approach to better stratify stage III colon cancer patients for ACT treatment decisions.

Aim: The PROVENC3 study aims to determine the clinical validity of whole genome sequencing (WGS)-based post-surgery ctDNA detection in stage III colon cancer patients treated with ACT.

Methods: Blood was collected pre-surgery, post-surgery and post-ACT. Tumor-informed ctDNA detection ctDNA was performed through integrated WGS analyses of formalin-fixed paraffin-embedded tumor tissue DNA (80x), germline DNA (40x), and plasma cell-free DNA (30x).

Results: Results from 209 patients (median follow-up: 40 months) show that ctDNA-positive patients post-surgery (n=28) had a higher risk of developing a recurrence within 3 years than ctDNA-negative patients (HR: 6.3, [95%CI: 3.5-11.3], P<10-8). Combination of post-surgery ctDNA status with established clinicopathological risk classification of low-risk (T1-3-N1) and high-risk (T4 and/or N2) resulted in a 3-year cumulative recurrence risk of 82% for high-risk ctDNA-positive patients compared to 7% for low-risk ctDNA-negative patients (HR 28.9 [95%CI: 10.6-78.2]; P<10-10). Results from 170 patients with post-ACT blood available showed that post-ACT ctDNA-positive patients (n=24) were at a higher risk of developing a recurrence than ctDNA-negative patients (HR 7.9 [95%CI: 3.5-15.9]; P<10-8). The patients relapsing had higher aggregate ctDNA variant allele frequencies than patients not experiencing a recurrence (post-surgery: P<0.001; post-ACT:P<0.001).
Conclusion: Post-surgery ctDNA detection by tumor-informed WGS is a strong prognostic biomarker in stage III colon cancer patients. Importantly, the post-surgery ctDNA-positive patients disease-free were likely cured by ACT. These data enable the design of clinical practice-changing ctDNA-guided interventional trials in stage III colon cancer to personalize adjuvant treatment decisions.

P07.001

Longitudinal profiling of circulating tumour DNA for tracking colorectal cancer evolution

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Background: Circulating tumour DNA (ctDNA) is a valuable resource for monitoring the evolving molecular landscape of cancers under the selective pressure of therapy. Here, we are evaluating ctDNA molecular dynamics in metastatic colorectal cancer patients (CRC) enrolled into the MAYA trial, whereby patients with microsatellite-stable and O6-methylguanine–DNA methyltransferase silenced tumours receive temozolomide (TMZ) as immune sensitizing strategy followed by immunotherapy (low-dose ipilimumab + nivolumab).

Methods: ctDNA was isolated from serially collected plasma samples at baseline, post-TMZ induction, during immunotherapy and, when available, at disease progression. Low-pass whole genome sequencing (WGS) and deep whole exome sequencing (WES) were carried out on Illumina platforms. Long-read ctDNA sequencing was performed on PromethION (Oxford Nanopore Technologies).

Results: A total of 90 plasma samples from 29 patients were used for ctDNA analysis. To assess the purity of ctDNA samples, low-pass WGS was initially carried out. Tumour fraction (assessed by ichorCNA) varied among the cohort, with non-responder patients having higher ctDNA levels compared to responders (Mann-Whitney test, p=0.005). Next, deep-WES data revealed that mutations in CRC driver genes like APC, KRAS and TP53 were detected in approximately 40% of the patients and they were under positive selection as confirmed by dN/dS analysis (dN/dS >1 in both non-sense and truncating mutations). Post-treatment ctDNA samples (post-TMZ and during immunotherapy) showed the emergence of unique mutations in each patient (not observed at baseline). In addition, mutational signature analysis detected an increased SBS11 signature (caused by alkylating agent) in post-TMZ ctDNA samples which was reflected in an increase of tumour mutational burden. Finally, we selected paired ctDNA samples (from two patients) for a pilot nanopore sequencing and found that we can accurately recover both copy number and DNA methylation profiles.

Conclusions: These results underscore the potential clinical utility of longitudinal ctDNA analysis for studying real-time colorectal cancer evolution.

P07.002

Variations in the utilisation and perceived importance of liquid biopsy for patients with metastatic breast cancer: A survey study

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Aim: Although recommendations for using Liquid Biopsy (LB) approaches for certain indications in metastatic breast cancer (mBC) have been embedded in guidelines from NCCN, AGO, ASCO and ESMO, uptake in clinical practice is rather slow. Here, we aimed to assess the utilization and key issues for implementation of LB across Europe.

Methods: Using the Redcap platform, we developed an online questionnaire including 19 questions investigating three main areas: respondent demographics, awareness, knowledge and access to LB approaches, as well as future perspectives of liquid biopsy. The survey was distributed to networks of surgical oncologists, via email, social media, and the ESSO-EYSAC website from March 2023 to May 2023.

Results: A total of 292 participants from 38 different countries replied. Only 20% (58/292) of participants reported implementation of guidelines regarding LB testing. Of those 23% (66/292) of participants reported the use of ctDNA testing, while 19% (56/292) of respondents reported that their centres performed CTC analyses. The top three indications for liquid biopsy utilization were clinical studies (107/292, 37%), the evaluation of therapeutic possibilities (109/292, 37%) and prognostication (59/292, 20%)
The major reasons for not using liquid biopsy were lack of accessibility (37%) and lack of reimbursement/high costs (39%). Importantly, most participants (93.4%) without access to liquid biopsy would use it if it was accessible.
CTC monitoring makes up for 2% of all biomarkers that are used for the guidance of duration of treatment in metastatic breast cancer patients.

Conclusion:Even though we discovered a low rate of LB utilization, there is extensive interest to implement this technique in clinical practice. Access to the technology and economical reimbursement were identified as key issues for the implementation of LB.

P07.003

Comparison of ctDNA profiles from HR+/HER2-low and HR+/HER2-0 advanced breast cancer patients

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Background: Despite the recent advances in the systemic treatment of hormone receptor-positive (HR+) breast cancer, a substantial number of patients still face poor prognosis. A meaningful proportion of these patients have HER2-low disease, and novel treatments with HER2-targeted antibody-drug conjugates have promising results in this subpopulation of patients. Yet, the differences in circulating tumor DNA (ctDNA) profiles between HER2-low and HER2-0 patients have not been comprehensively investigated.

Methods: We analyzed 92 plasma samples from 83 metastatic breast cancer patients (HR+/HER2-low, n=62; HR+/HER2-0, n=30) taken before starting 1st or 2nd line treatment. Tumor fractions were assessed using an untargeted aneuploidy screening (mFAST-SeqS) and expressed as z-scores. The mutational landscape of ctDNA was established using a 77-gene panel (AVENIO ctDNA Expanded). We compared tumor fractions, variant allele frequencies (VAF) and the number of somatic variants between HER2-low and HER2-0 patients.

Results: HER2-low patients had significantly higher z-scores compared to HER2-0 patients (median 3.04 vs. 1.45, p-value 0.005). The groups did not significantly differ in the highest or the average VAF. HER2-low patients had a median of 3 detected variants (range 1-20), with 2 clonal and 1 subclonal variants. HER2-0 patients had a median of 4 detected variants (range 1-12), including 3 clonal and 1 subclonal variants. Contrary to prior reports, PIK3CA variants were more common in HER2-0 (53.3%) than HER2-low patients (32.3%), and TP53 variants had similar prevalence in the two compared groups (23.3% in HER2-0 and 21% in HER2-low).

Conclusion: We found significant differences in plasma tumor fractions between HER2-0 and HER2-low patients. Our cohort's mutational landscape differed from previous studies, highlighting the need for further research into the unique features of HER2-low breast tumors.

P07.004

A cell-free DNA methylation biomarker outperforms irRECIST in predicting treatment outcome

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Background: Immune checkpoint inhibitors (ICI) are established as first-line treatment for inoperable esophageal adenocarcinomas (EAC), in combination with chemotherapy (CTX). Unfortunately, response to ICI is highly variable, despite most EAC cases featuring a high mutation burden. EAC response to ICI is assessed using radiological criteria such as irRECIST, but their ability to predict long-term survival is poor. Consequently, early recognition of a failing therapy is challenging. More accurate early biomarkers of response to ICI are therefore of critical importance.

Methods: Cell free DNA (cfDNA) samples were isolated from patients enrolled in the LUD2015-005 trial, in which ICI was administered for four weeks before adding standard-of-care chemotherapy. For each patient, we collected cell-free DNA (cfDNA), tumor and adjacent normal tissue at diagnosis, before ICI+CTX, during and at the end of treatment, in addition to radiological clinical imaging.

All samples were then sequenced using the TET-Assisted Pyridine-borane Sequencing (TAPS) method recently developed in Oxford, providing high-depth, base-resolution mutation and DNA methylation information even from low-input samples.

Results: We identified a tumor-cell-specific genome-wide hypomethylation signature from that is present in most EACs. Using this signature, we modelled the admixture of tumor and normal methylation patterns to quantify the amount of tumor methylation detected (TMD) in cell-free DNA (cfDNA).

Patients classified as TMD-high had strikingly worse overall survival (37 weeks) compared to TMD-low patients (112 weeks). In comparison, classification of patients into responders and non-responders via irRECIST, based on measurements taken at the same time-point, was less predictive of treatment outcome.

Conclusions: We report the discovery of a circulatory biomarker that measures molecular response to ICI in EAC and identifies poor prognosis patients that appear stable from radiological imaging. Furthermore, patients with favorable molecular response were found to have a median OS almost 3 times that of patients with unfavorable molecular response.

P07.005

Plasma cell free DNA hydroxymethylation profiling reveals anti-PD1 treatment response and resistance biology in non-small cell lung cancer

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Background: Treatment with immune checkpoint inhibitors (ICIs) targeting programmed death-1 (PD-1) can yield durable anti-tumor responses, yet not all patients respond to ICIs. Current approaches to select patients who may benefit from anti-PD-1 treatment are insufficient. 5-hydroxymethylation (5hmC) analysis of plasma-derived cell free DNA (cfDNA) presents a novel non-invasive approach for identification of therapy response biomarkers which can tackle challenges associated with tumor biopsies such as tumor heterogeneity and serial sample collection.

Methods: 151 blood samples were collected from 31 non-small cell lung cancer (NSCLC) patients before therapy start and at multiple timepoints whilst on therapy. Blood samples were processed to obtain plasma-derived cfDNA, followed by enrichment of 5hmC-containing cfDNA fragments through biotinylation via a two-step chemistry and binding to streptavidin coated beads. 5hmC-enriched cfDNA and whole genome libraries were prepared in parallel and sequenced to obtain whole hydroxymethylome and whole genome plasma profiles, respectively.

Results: Comparison of on-treatment timepoint to matched pre-treatment samples from same patients revealed that anti-PD-1 treatment induced distinct changes in plasma cfDNA 5hmC profiles of responders, as judged by RECIST, relative to non-responders. In responders, 5hmC accumulated over genes involved in immune activation such as IFN and IFN response, inflammatory response, and TNF signaling, whereas in non-responders 5hmC increased over epithelial to mesenchymal transition genes. Molecular response to anti-PD-1 treatment, as measured by 5hmC changes in plasma cfDNA profiles were observed early on, starting with the first cycle of treatment. Comparison of pre-treatment plasma samples revealed that anti-PD-1 treatment response- and resistance-associated genes can be captured by 5hmC profiling of plasma-derived cfDNA.

Conclusion: These results demonstrate that 5hmC profiling can identify response and resistance associated biological pathways in plasma samples, offering a novel method for non-invasive prediction and monitoring of immunotherapy response in NSCLC.

P07.006

Genome-wide methylome profiling of cell-free DNA enables prognostication of patients with advanced prostate cancer

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Background: Metastatic castration-resistant prostate cancer (mCRPC) is an incurable condition due to high rates of therapy resistance. Development of biomarkers to guide treatment decisions in mCRPC is needed. Analysis of cell-free DNA (cfDNA) offers a promising strategy for biomarker discovery, as detection of tumor-derived cfDNA (ctDNA) in plasma has been associated with poor prognosis across several cancers. Here, we aimed to establish a methylation-based approach for detecting ctDNA (methylated ctDNA; me-ctDNA) in mCRPC plasma.

Methods: We generated methylome profiles of plasma cfDNA collected from a training cohort of 48 mCRPC patients and 18 controls, using methylated cfDNA immunoprecipitation followed by sequencing. Based on a subset of the training cohort, we established a methylation signature (cfMeCaP) consisting of 48 genomic regions specifically hypermethylated in mCRPC. Using the cfMeCaP methylation score of controls as cutoff, plasma samples were classified as me-ctDNA positive or me-ctDNA negative. This cutoff resulted in 100% sensitivity and 100% specificity for detecting me-ctDNA in the full cohort of 48 mCRPC patients and 18 controls.

Results: In a validation cohort of 85 mCRPC patients, 95.3% patients classified as me-ctDNA positive by the cfMeCaP signature, using the fixed cutoff. Furthermore, in the training cohort (48 mCRPC patients), high cfMeCaP methylation levels were associated with significantly shorter progression-free and overall survival, which was also validated in the external cohort (85 mCRPC patients).
Finally, in plasma from patients with earlier stage metastatic hormone-sensitive PC, me-ctDNA was detected in 17/37 (45.9%) patients using cfMeCaP. Here, me-ctDNA positive patients also had significantly shorter time to CRPC progression as compared to me-ctDNA negative patients, suggesting that the prognostic potential of cfMeCaP is not limited to mCRPC patients.

Conclusion: These results highlight the promising potential of plasma me-ctDNA analyses across multiple stages of prostate cancer to identify high risk patients that may benefit from intensified treatment.

P07.007

Predicting Response to Treatment in Metastatic Breast Cancer using Longitudinal ctDNA Dynamics

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Background: Evaluation of response to treatment is essential in the management of metastatic disease. Monitoring of circulating tumour DNA (ctDNA) has been proposed as an attractive (inexpensive and minimally invasive) method to track response to treatment. Here we describe a probabilistic framework to predict response to treatment in metastatic breast cancer (mBC) using longitudinal ctDNA data.

Methods: A cohort of 188 mBC patients had DNA extracted from serial plasma samples (total 1098, median=4; median (range) follow-up 1.15 (0-12.4) years). Plasma DNA was assessed using sWGS and the ctDNA levels were estimated using ichorCNA. Response to treatment was determined through CT imaging. We then modelled both the evolution of the longitudinal ctDNA levels and the dynamics of the response to treatment.

Results: We show that longitudinal ctDNA monitoring predicts response to treatment in mBC. We found that (1) the incorporation of ctDNA to monitor response to treatment offers clinical utility, outperforming CA 15-3, and (2) the model allows for individualised dynamically updated predictions as additional ctDNA measurements become available.

Conclusion: Our results demonstrate the promise of ctDNA monitoring in predicting treatment response and its potential for personalised clinical decision-making. We provide quantitative estimates of response to treatment. Our proposal considers the probability of treatment response for each patient individually, by doing so, it can identify individual patients with extremely high-risk of non-response to treatment for which the treatment should be discontinued. We anticipate our modelling framework will be a starting point for more sophisticated models in additional cancer types.

P07.008

Monitoring treatment response and toxicity in immune-checkpoint-inhibitor treated metastatic melanoma from cell-free methylated DNA

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Background: Immune checkpoint inhibitor (ICI) combination treatment can achieve a 5-year overall survival rate greater than 50% in patients with metastatic melanoma, with response rates approaching 60%. Still, approximately 40% of patients do not respond, and existing biomarkers fail to distinguish this subset of patients who do not benefit from ICI therapy. Additionally, as many as 80% of patients may experience immune-related adverse events (irAEs), which range from mild dermatological symptoms to severe myocarditis. Here, we explore the use of circulating methylated cell-free DNA (cmeDNA) to measure response and toxicity in the context of ICI-treated metastatic melanoma.

Methods: Serial serum samples were collected from patients with BRAFV600-mutant metastatic melanoma treated with ipilimumab/nivolumab. Methylated cell-free DNA was isolated from pre-treatment and on-treatment samples and converted enzymatically to identify methylated cytosines from sequencing. Using an atlas of human cell type-specific methylomes, deconvolution was performed to determine the abundance of cell type-specific methylation patterns in cmeDNA in patient serum at different time points of treatment.

Results: DNA methylomes generated from normal adult melanocytes show previously unreported melanocyte-specific methylation patterns that distinguish melanocytes from other cell types and remain conserved in melanoma. We characterize the dynamics of melanocyte-specific cmeDNA over the course of treatment, and track cell-type-specific methylation markers indicative of possible irAE-mediated damage to normal tissue.

Conclusions: We establish melanocyte-lineage methylation markers and evaluate the use of cell-type specific DNA methylation to monitor treatment effects of immune checkpoint inhibitors in metastatic melanoma. The dynamics of cmeDNA derived from melanocytes and other cell types provide insights into treatment response and adverse effects.

P07.009

Monitoring of treatment efficacy using γδ T cell repertoires in peripheral blood of sarcoma patients

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Background. Non-invasive monitoring of tumor responses to therapeutics and whether resistance or recurrence are occurring in sarcoma patients are important for timely and effective clinical management. However, comprehensive liquid biopsy-based biomarkers are limited in sarcomas owing to their heterogeneity.

Methods. We here used a simple and ultrasensitive sequencing technique, SiMSen-Seq, adapted for analysis of immune repertoires. We describe a 32-plex γδ T-cell receptor (TCR) assay for analysis of non-enriched cells. The assay performance was evaluated using various amounts of commercially available genomic DNA derived from peripheral blood of healthy donors. We employed this approach to monitor the dynamics of γδ TCR repertoires in blood samples collected from 16 sarcoma patients at diagnosis, during treatment and follow-ups. The result was combined with circulating tumor DNA (ctDNA) data from the same patients to evaluate the possible added clinical value.

Results. We developed SiMSen-Seq into an ultrasensitive technology suitable for direct analysis of non-enriched immune repertoires. Using 100 – 1000ng of the DNA derived from peripheral blood of healthy donors, corresponding to < 0.1 – 10ng γδ T-cell DNA, our assay captured γδ TCR repertoires predominated by δV2 subtype (>75%), demonstrating consistency with previous findings. In the analysis of non-enriched γδ T-cells from 158 serially collected peripheral blood samples from the 16 sarcoma patients, we observed diversity and clonal size distribution of the TCR repertoires. Unlike in the healthy individuals, δV1 and δV3 were the dominant subtypes. None of the clonotypes were shared between patients. Importantly, dynamics of the dominant clonotypes during the follow-ups complemented ctDNA data of the same patients and correlated to clinical parameters.

Conclusion. Simple and ultrasensitive analysis of immune repertoires opens up new means in cancer management, by detailed deciphering of immune cells. However, further studies are needed to determine potential values of non-invasive γδ TCR analysis in sarcomas.

P07.010

The ALPINE study: the identification of acquired resistance mechanisms with cell-free DNA-based next-generation sequencing of non-small cell lung cancer patients treated with Immune Checkpoint Inhibitors

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Background: In the absence of targetable genomic alterations, advanced-stage non-small cell lung cancer (NSCLC) patients are typically treated with immune checkpoint inhibitors (ICIs) with proven long-lasting therapeutic responses in a subset of patients. Current predictive biomarkers do not adequately discriminate responders from non-responders. Moreover, knowledge of ICI-induced resistance mechanisms in NSCLC patients that can be detected via ctDNA analysis is limited. Therefore, in this study, molecular tumor profiling using ctDNA sequencing of longitudinally collected blood samples during ICI treatment was performed to identify acquired resistance-related variants.

Methods: Twenty patients with advanced NSCLC treated with ICIs were included. Four subsequent blood samples were collected: before treatment initiation (t0), 1-2 months after the start of treatment and with clinical response (t1), after three months of treatment or at least three months prior to confirmed clinical progression and without clinical progression (t2), and at the time of confirmed progression (t3). Circulating cell free DNA (ccfDNA) was analyzed using the AVENIO ctDNA Expanded kit. CtDNA dynamics of recurring and acquired mutations were evaluated following international guidelines applied in routine molecular diagnostics.

Results: Based on preliminary results, in total 203 ctDNA variants were identified across the cohort. Of 17 patients (85%), ctDNA dynamics correlated with changes in the sum of longest target lesion diameters (RECIST v1.1). Remarkably, the three patients in whom the ctDNA levels remained stable at disease progression, survived 37-70 weeks after RECIST progression. Twenty-four variants solely detected at disease progression were observed in 13 patients (65%). Evaluation of a possible relationship between ctDNA variants that were detected and acquired resistance to ICI treatment is in progress.

Conclusions: CcfDNA-based next-generation sequencing (NGS) enables the detection of tumor-derived variants and monitoring ctDNA dynamics to potentially identify mechanisms of therapy resistance. Our findings contribute to the biological understanding of ICI-treatment resistance mechanisms.

P07.011

Copy number variation analysis from plasma cell-free DNA as a predictive indicator in NSCLC patients under immune checkpoint blockade

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Background: Anti-PD-(L)1 therapy is routinely used in metastatic non-small cell lung cancers (NSCLC) and favorably changed patient prognosis. However, response rates vary and reliable biomarkers predicting treatment efficacy are lacking. Liquid biopsies allow minimally invasive monitoring of residual disease during therapy. Residual circulating tumor DNA (ctDNA) after therapy represents a predictive marker for adverse outcome. Here, we assessed genome-wide copy number variations (CNVs) in longitudinal plasma samples of NSCLC patients receiving PD-(L)1 blockade.

Methods: 119 serial plasma samples from 50 metastatic NSCLC patients were collected at therapy baseline (n=49), after four cycles of anti-PD-(L)1 therapy (n=46) and at disease progression (n=24). Duration of progression-free survival (PFS) and baseline PD-L1 expression was available for all patients. Plasma DNA was subjected to WGS (genome coverage; median=3.7x). Copy number profiles and copy number profile abnormality (CPA)-scores were determined by WisecondorX using WGS data of 50 healthy donors as reference.

Results: We found significantly increased CPA-scores in NSCLC patient samples compared to healthy donors (Mann-Whitney U, p=3.8e-7). In total, 29.4% of samples and 38.0% of patients were considered ctDNA-positive as they exceeded the maximum CPA-score of our healthy controls. Rising CPA-scores towards disease progression (Wilcoxon paired, p = 0.001) indicated the suitability of plasma CNVs for response assessment. CtDNA-positivity at therapy baseline in combination with PD-L1 expression ≤1% identified patients with short PFS (log-rank, p=2.0e-5; median 4.9 vs. 9.5 months). Residual ctDNA after four cycles of anti-PD-(L)1 therapy was also indicative of short therapy response (log-rank, p=4.2e-4; median 5.1 vs. 11.4 months) and improved the prediction of response duration when compared to baseline PD-L1 expression alone (PD-L1 <1%, p=0.053).

Conclusion: Our results highlight the suitability of plasma-based CNV analysis for disease monitoring and as a predictive marker for response duration in NSCLC patients under anti-PD-(L)1 therapy.

P07.012

Increasing digital PCR performance in liquid biopsy using reporter emission multiplexing

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High specificity, sensitivity and robustness have made digital PCR (dPCR) a key technology in liquid biopsy. However, there is still a high need to further increase its capabilities for improved cancer characterization before, during and after therapy. Cancer diagnostics and monitoring typically require screening of multiple mutations. However, splitting a sample in order to perform separate singleplex assays leads to a dilution effect and decreases assay sensitivity. In order to increase information output, there are several approaches for multiplexing dPCR reactions: Colorimetric, amplitude or combinatorial methods. These methods use fluorogenic DNA probes to detect DNA sequences or single nucleotide polymorphisms (SNPs). However, these assays suffer from effects such as non-specific probe cleavage or weak population separation, which again decrease assay performance.

As a solution, we introduce reporter emission multiplexing (REM), which is based on mediator probe PCR technology. Therein, DNA sequence detection is performed by label-free mediator probes and signal generation is achieved by fluorogenic reporter molecules. This separation leads to single nucleotide specific probe cleavage and is consequently highly sensitive towards SNP detection. Furthermore, it enables an efficient design and optimization process for population-specific reporter (PSR) oligonucleotide probes, capable of differentiating multiple target populations per detection channel based on fluorescence intensity variations between different fluorophore types.

We demonstrated that REM can be used to increase multiplexing degrees of currently available dPCR devices. In addition, our results indicate that REM assay components are interchangeable, allowing to use constant PSR sets in combination with different targets. SNPs KRAS G12A, KRAS G12D, KRAS G12V, BRAF V600E, NRAS Q61R and NRAS Q61K were quantified down to a concentration of 0.4 copies / µl in a background of respective wild-type DNA.

In conclusion, REM enhances dPCR multiplexing capabilities for highly sensitive testing of mutation panels in liquid biopsy without splitting samples.

P07.013

Infrastructure FOr Rare Cancer in the NEtherlands, towards a comprehensive platform for early detection and diagnosis of rare cancers (FORCE).

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Background: One in five patients with cancer has a rare type of tumor. For these patients, compared to patients with a more common tumor, their prognosis is poor due to late or incorrect diagnosis and fewer available treatment options. Moreover, most patients with rare cancer do not benefit yet from recent developments in ultrasensitive profiling of circulating cell-free DNA (USccfDNA). The information needed to apply USccfDNA in most rare cancers is lacking, which delays innovation of personalized treatments and deprives these patients of a chance for a better outcome. FORCE aims to improve treatment outcomes for patients with rare cancers by systematically collecting their clinical data, tumor tissue and peripheral blood components.

Methods: Retrospectively and prospectively collected data are used to build a data warehouse that provides comprehensive insight into the long-term biology of a rare cancer. The data warehouse is visualized as a train. The locomotive represents the infrastructure, which uses web-based tools for FAIR, multidisciplinary, data collection, and storage. Each train carriage represents the data concerning a coherent set of rare tumor types.

Results: Neuroendocrine neoplasms (NEN) is the first carriage joined to the FORCE infrastructure and serves as a use-case for the FORCE infrastructure. Currently, the first 54 patients are being included in this clinical biobank. A legal joint data registry agreement and governance structure were developed to enable a shared clinical biobank and facilitate sharing of data and samples among participating institutes. Both documents will serve as an adaptable template for other carriages.

Conclusion: We established a unique infrastructure that combines the collection of clinical data and blood components of patients with a rare tumor, starting with NEN. The FORCE infrastructure will enable studies to optimize early primary detection, detection of minimal residual disease (MRD), and early recurrence by USccfDNA assays and facilitate future clinical trials.

P07.014

Structural and copy number variant detection from cell-free DNA using Linked Target Capture (LTC) technology

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Background: The non-invasive analysis of cell free DNA (cfDNA) from blood is an emerging tool in the detection, monitoring, and treatment of cancer and other conditions. In particular, targeted Next Generation Sequencing (NGS) has enabled the detection of many rare cfDNA biomarkers, typically SNVs and indels, in a single assay. However, many conditions, such as sarcomas, harbor complex alterations (structural variants (SVs), copy number changes (CNVs)) that can be challenging to detect with existing methods. Additionally, current methods can require a priori knowledge of the alteration to enable detection.

Methods: Linked Target Capture (LTC) is a novel targeted sequencing technology, utilizing physically linked capture probes and PCR primers to enrich entire cfDNA fragments by amplifying targeted molecules from library adapter sequences. The LTC workflow can be completed in a single work day, requires minimal hands-on time, and because it uses a linked probe-pair capture approach, has high sensitivity and specificity for desired sequences. LTC also enables the detection of gene fusions/SVs with unknown partners, since the linked probe-primer pair can capture across untargeted sequences. Additionally, LTC preserves start/stop locations of the original template, produces high uniformity (required for CNV detection), and can deliver high on-target rate regardless of panel size.

Results: Here, we demonstrate the use of LTC for detecting SVs and CNVs in cfDNA applications. We evaluated commercially available reference materials, cell lines, and patient cfDNAs. LTC results were compared to reference material Certificate of Analysis. Cell lines and patient cfDNAs were evaluated with ddPCR as an orthogonal method. We report high sensitivity for both variant types, down to <1% tumor fraction, without prior knowledge of the alteration. Additionally, we report 99.9% specificity for detecting any variant across 29 genes.

Conclusion: LTC technology can be successfully implemented in cfDNA applications to detect SVs and CNVs at relevant tumor fractions.

P07.015

MicroRNA-7 agomiR targets circ_RNA in liquid biopsy to reverse chemotherapy resistance in breast cancer via modulation of the DNA damage response.

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Background. Epigenetic modulation of gene expression by non-coding RNA molecules creates a cellular profile for hyper-repair of DNA that overcomes chemotherapy-induced DNA damage. Micro-RNAs are small non-coding RNA molecules that bind and promote degradation of RNA template, supressing gene expression. Circular-RNA are stable degradation-resistant templates that can “mop-up” micro-RNAs preventing RNA template degradation.

Methods. We have defined a circ_RNA that binds miR-7 to prevent degradation of DNA repair gene RAD51 template and the template of the RAD51 protein activator, protein kinase cABL1. The feedback loop is modulated by RAD51 capability as a chaperone for the transcription factor of the circ_RNA.

Results. We validated circ_RNA binding of miR-7 and correlated its levels in a serum liquid biopsy retrospectively with poor patient outcome and distant metastases. We further validated prognostic capability in tumour response to chemotherapy, prospectively. Having validated a prognostic liquid biopsy, we set about to modulate the function of the circ_RNA as a theranostic. We evaluated the activity of a targeted agomiR that mimics miR-7 binding of circ_RNA.
Conclusion. The agomiR was able to co-opt the miR-7 binding site resulting in reduced levels of RAD51, DNA repair and restored sensitivity to chemotherapy. The agomir has prophylactic potential to eradicate chemoresistant potential and limit secondary cancers.

P07.016

Plasma-derived circulating tumor DNA in patients with advanced stage EGFR exon 20 mutation-positive NSCLC treated with osimertinib: a pilot study

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Background
Plasma-derived circulating tumor DNA (ctDNA) is promising in monitoring responses and detecting resistance mechanisms in non-small cell lung cancer (NSCLC). NSCLC patients with EGFR exon 20 mutations (EGFRex20+) treated with high-dose Osimertinib (160 mg) showed modest anti-tumor activity (28% confirmed response rate) and acceptable toxicity. In this pilot study we aim to identify molecular response and resistance mechanisms using ctDNA collected during treatment.

Methods
Twenty-five EGFRex20+, p.(T790M) negative, NSCLC patients with WHO PS 0-2, received high-dose Osimertinib QD until progression or unacceptable toxicity. Cell free DNA collected at baseline, six weeks (T6), and progression was analyzed with the next generation sequencing (NGS) based Guardant360 CDx (73 gene panel). Molecular profiles of samples at progression were compared with matched pretreatment (baseline) samples to find resistance mechanisms to Osimertinib. A secondary objective is whether changes in ctDNA levels of the EGFRex20+ variant, at T6 and progression, compared to baseline, are associated with progression.

Results
Baseline ctDNA was analyzed in twenty patients. EGFRex20+ variant concordance between tumor biopsy NGS and ctDNA was found in 13/20 patients (65%). Fourteen patients underwent ctDNA profiling at all timepoints. Nine showed an EGFRex20+ variant decrease six weeks post-therapy initiation, of whom eight showed an increase again at progression. Two patients lacked the EGFRex20+ variant in ctDNA at baseline, but this variant was observed upon disease progression. Variants potentially associated with treatment resistance were identified in several genes at progression in 11/18 patients (61%): SNV (n=14), insertion (n=2) and gene-fusion (n=1). We found three variants that were previously related with resistance: TP53 (n=2) and EGFR p(C797S) (n=1).

Conclusion
This pilot study using a unique cohort of EGFRex20+ NSCLC patients treated with high dose Osimertinib highlights the complexity of variant dynamics during treatment and disease progression, suggesting a potential link between changed levels of variants detected at progression.

P07.017

Monitoring tumor burden with low-coverage cell-free DNA sequencing in radiotherapy patients

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Background
Monitoring cancer progression and response to treatment is crucial for improving patient outcomes. However, current imaging technologies have limitations in providing frequent and systemic information about the tumor. Particularly, the discrimination of radiotherapy effect on the tumor versus healthy tissue is challenging, and imaging response may lag behind actual response on the tumor cell level. Whole-genome cell-free DNA (cfDNA) sequencing offers a tumor-agnostic approach to cancer monitoring which is highly relevant in clinical settings with a heterogenous set of tumors, such as in Radiation Oncology.

Methods
Plasma cfDNA samples were collected from seven oligometastatic cancer patients and seven head-and-neck cancer patients at six different time points before, during, and after radiotherapy, and at one time point from seven healthy and seven polymetastatic volunteers. Low-pass whole-genome sequencing was performed on all cfDNA samples, and cfDNA alterations including copy-number alterations, fragment lengths, nucleosome footprints were compared to clinical and imaging findings.

Results
We detected copy number alterations in the majority of polymetastatic patients, but only in one oligometastatic and one head-and-neck cancer patient. Notably, these patients showed progression following radiotherapy as evidenced by imaging. Copy number alterations were not detectable in the other cancer samples, nor in healthy samples. We used non-negative matrix factorization to identifiy cfDNA fragment length features showing strong correlation with copy number-based tumor fraction estimates. While head-and-neck cancer patients' cfDNA samples had very low tumor content, we detected viral DNA in all HPV-positive head-and-neck cancer samples.

Conclusions
Our results suggest that an elevated cfDNA tumor content is associated with tumor aggressiveness, consistent with recent research from other groups linking high cfDNA tumor fraction to an unfavorable prognosis. This underscores the potential utility of cfDNA sequencing in detecting relapse and informing treatment strategies.

P07.018

A liquid biopsy-based approach allows longitudinal tracking of cutaneous melanoma dynamics and early resistance to treatment

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Background: Melanoma heterogeneity is an obstacle to the management of metastatic disease. Although the advent of targeted therapy has significantly improved patient outcomes, the emergence of resistance makes it imperative to monitor the genetic landscape of the tumor. Liquid biopsy could be an important biomarker to track the evolution of the disease. Therefore, we aimed to correlate liquid biopsy dynamics with treatment response/progression by developing a customized analysis applied to longitudinal tumor monitoring.

Methods: We exploited droplet digital PCR and Next Generation Sequencing (NGS) to track circulating tumor DNA (ctDNA) trend, profile tumor genetic landscape, and assess copy number variation. The approach was applied to 50 samples from 17 stage IV melanoma patients treated with BRAF/MEK inhibitors, followed up to 28 months.

Results: BRAF mutations were detected in the cfDNA of 82% of patients. There was a significant difference in ctDNA amount at baseline in responders versus non-responders/early progressing patients (p=0.039). Moreover, a cut-off able to discriminate complete/partial responders from non-responders was identified. The cutoff was also able to discriminate early progressing patients independently from the type of response (p=0.035). Undetectable BRAF-mutant ctDNA at the first treatment observational point correlated with best overall survival (p=0.024), and lack of BRAF-mutant ctDNA clearance up to the first 6 months of treatment correlated with non-response or early progression (p=0.015). Single nucleotide variants (SNVs) known or suspected to confer resistance were identified in 70% of patients. The sensitivity and specificity of the custom NGS panel were 96% and 100%, respectively, for MAFs down to 1% (PPV:100%, NPV:89%). Moreover, the number of baseline cfDNA SNVs correlated with progression free survival (p=0.041).

Conclusions: This work provides proof-of-principle of the power of this approach and paves the way for a validation study aimed at evaluating early ctDNA-guided treatment decisions in stage IV melanoma.

P07.019

A robotic microfluidic approach for rapid sample-to-answer analysis of cell-free DNA

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Background: Clinical utility of cell-free DNA (cfDNA) is currently under investigation in many trials. However, complex laboratory protocols cause a long turnaround time from sampling (e.g., blood draw) to obtaining results (e.g., cfDNA analysis by NGS). Reducing the sample-to-answer time can be advantageous in multiple domains: (1.) Patients could benefit from the fast initiation of therapy, e.g., in highly aggressive malignancies such as pancreatic cancer. (2.) Healthcare systems could benefit from accelerated workflows, e.g., reducing hospital stays. (3.) Small laboratories could be empowered to provide liquid biopsy analysis as a new service.

Methods: We investigated microfluidic approaches for cfDNA analysis with the aim of accelerating analysis down to a few hours, including all pre-analytical and analytical steps. As pilot workflow, we analyzed circulating tumor DNA (ctDNA) in plasma of colorectal cancer patients. We developed and characterized microfluidic modules for blood-plasma-separation (centrifugation and microfiltration), cfDNA isolation (magnetic nanoparticles), and mutation analysis (multiplex digital droplet PCR, ddPCR).

Results: In proof-of-concept studies, we were able to demonstrate fast blood-plasma-separation (< 10 min), cfDNA isolation from plasma (≥ 95 % recovery) and 4-plex ddPCR quantification of CRC-associated point mutations (KRAS G12V, G12A, G12D and wild type). These results allow us to assemble all microfluidic modules into a fully integrated sample-to-answer system. For this purpose, we are currently developing a new laboratory platform that combines microfluidic technology with robotic automation.

Conclusion: We demonstrated feasibility of all steps required for sample-to-answer analysis of cfDNA. Such sample-to-answer systems could enable rapid cfDNA testing in a decentralized fashion, e.g. in clinics or small laboratories, with positive impact on patients as well as healthcare economy. In future, we aim to integrate the analysis of further analytes such as microRNA or extracellular vesicles in our platform, providing a modular toolbox for various applications in oncology, infectious disease or transplant medicine.

P07.020

Identifying actionable targets from plasma ctDNA from mCRPC patients

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Background: Recent progress in evaluating the molecular landscape identified a variety of treatment options for castration-resistant prostate cancer (CRPC) patients. Patients with homologous recombination deficiency (HRD) can be directed to poly(ADP-ribose) polymerase (PARP) inhibitors or platinum chemotherapy, while loss of CDK12 has been associated with responses to immunotherapy. Moreover, poor prognostic features, such as loss of RB1, PTEN or TP53, might help guide future therapeutic strategies. Given that circulating tumor DNA (ctDNA) can serve as a proxy for tumor characteristics, ctDNA holds promise as a viable alternative for identifying suitable biomarkers for clinical decision making.

Methods: We have collected a cohort of 131 metastatic CRPC patients of which the majority exhibited an elevated ctDNA fraction (median: 24.09%, min: 0%, max: 80.34%). We employed targeted sequencing of homologous recombination repair genes (HRR)-related genes as well as the cell cycle regulator genes RB1, PTEN or TP53. To assess genomic instability and gene deletions we performed shallow whole genome sequencing (sWGS).

Results: Non-benign alterations in a classical HRR gene could be identified in 26/131 (19.85%) patients, which was associated with a high degree of genetic instability. At least one cycle control gene (TP53, RB1, PTEN) was altered in 47 patients (35.88%). Most frequently altered genes included TP53 (28.2%) and BRCA2 (7.6%), of which the majority was of germline origin. Notably, increased co-occurrence was observed between BRCA2 and RB1/PTEN alterations, but not with TP53. Conversely, co-exclusivity was observed between TP53 and RB1 alterations. The most common copy number alterations included MYC amplifications (30%) and PPP2R2A (20%) and RB1 (15%) losses.

Conclusion: Our data demonstrate that even with a small and cost-effective gene panel, combined with sWGS, a variety of clinically relevant alterations can be detected in cell-free DNA in CRPC patients. Therefore, ctDNA holds great potential in improving the clinical management of CRPC patients.

P07.021

Development and optimization of the off-the-shelf digital PCR primer/probe library for ctDNA monitoring

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Background: The clinical validity of circulating tumor DNA (ctDNA) has been demonstrated in terms of: (a) early relapse prediction, (b) treatment efficacy evaluation; and (c) non-relapse corroboration. Generally, the variant allele frequency (VAF) of ctDNA is below 1% even before treatment. Digital- PCR (dPCR) is one of the best methods for quantifying ctDNA; however, only a limited number of primer/probe sets are commercially available. Thus, a validated dPCR primer/probe library is needed for timely ctDNA monitoring.

Methods: To cover as many somatic mutations as possible with a primer/probe library, the hotspot mutations were selected from the Catalogue Of Somatic Mutation In Cancer (COSMIC) database and literatures. The cover rate was evaluated with the databases of The Cancer Genome Atlas (TCGA), the Center for Cancer Genomics and Advanced Therapeutics (C-CAT), and our MORIOKA study. Where possible, each primer/probe set was validated against human DNA or synthesized DNA fragments to make the library ready to use.

Results: Over 1,000 mutations were included in the master primer/probe library and 27 smaller sub-libraries were also established, with specific foci on organs, genes, and drugs. The cover rate of the master library, called OTS-1000ex (meaning “off-the-shelf”), was estimated at 59%, 71%, and 75% against the TCGA, C-CAT, and MORIOKA study databases, respectively. Some potential cross reactions of dPCR, such as when using the probes for KRAS G12D and G12V, have been finely optimized. Overall, 93% of the tested probes in OTS-1000ex share the same PCR conditions, facilitating its simultaneous use for multiple ctDNA monitoring.

Conclusion: The high cover rate of the OTS-1000ex library facilitates the selection of mutations for tumor-informed ctDNA monitoring in the majority of the cancer patients without dPCR optimization by users. The use of dPCR will substantially improve the quality of individual ctDNA monitoring.

P07.022

Monitoring of molecular response during immunotherapy by circulating tumor DNA using patient-tailored assays in non-small cell lung cancer

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Background: Patients diagnosed with Non-Small Cell Lung Cancer (NSCLC) can be treated with antibodies against PD-1/PD-L1, called immune checkpoint blockade (ICB). Analysis of circulating tumor DNA (ct-DNA) in blood plasma is a non-invasive method that can be used to monitor treatment. Following ct-DNA levels has shown to predict clinical response earlier compared with standard radiological examination. The study aims to evaluate both the amounts and detection of somatic tumor specific DNA-variants in ct-DNA during early treatment cycles to identify patients most likely to gain long-term clinical benefit.

Methods: Twenty-three stage III-IV NSCLC patients treated with ICB were included in this prospective study. The amount of ct-DNA was analyzed longitudinally at up to six timepoints during treatment. Four to thirteen tumor patient-specific somatic DNA-variants were selected from the initial tumor sequencing and analyzed at each timepoint using ultrasensitive sequencing assays (Simsen Diagnostics). Patients were categorized as clinical responders or non-responders based on RECIST criteria at 9-month clinical assessments after initiation of ICB.

Results: Thirteen of twenty-three patients were clinical responders, of which seven patients had no detectable ct-DNA at any timepoint. In the remaining six responders, ct-DNA was detectable at baseline, in two patients at high levels that significantly decreased to the next timepoint or were non-detectable during follow-up timepoints. Ten patients were non-responders and generally had higher ct-DNA levels at baseline which increased over time.

Conclusion:Ct-DNA can be used to monitor treatment by detecting specific DNA-variants at multiple timepoints. The ct-DNA amount is correlated with response or non-response in patients. During the first treatment cycles a probable response can be determined, indicating clinical progress earlier than in current radiological settings. By measuring ct-DNA at several timepoints using tumor-specific somatic DNA-variants an early determination of treatment response can be found, which might indicate clinical benefit or not for the patients.

P07.023

Personalised ctDNA analysis for monitoring disease and treatment efficacy in patients with metastatic breast cancer

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Background: Treatment response for patients with metastatic breast cancer (MBC) is normally evaluated using radiographic imaging every 3-4 months. As a result, patients with long-term stable disease will undergo multiple unnecessary scans, whereas other non-responders will continue treatment for several months before disease progression can be confirmed. Therefore, there is a need for improved methods to monitor disease and treatment efficacy. The overall aim of this study is to determine the clinical value of blood-based biomarker analysis for disease monitoring in MBC patients.

Methods: The PDM-MBC trial (NCT04597580) includes patients with MBC, receiving first line therapy with an aromatase inhibitor and a CDK4/6 inhibitor (n=97). Patients undergo routine imaging and study samples are collected pre-treatment and then serially until disease progression. To detect and quantify circulating tumour-DNA (ctDNA) we used SiMSen-Seq and tailor-made ctDNA panels that detect patient-specific mutations selected from sequencing of tumour tissue. Biomarker analysis also includes the tumour marker cancer antigen 15-3 (CA15-3).

Results: To date, ctDNA has been analysed in 25 patients, of which 20 (80%) of the patients had detectable ctDNA pre-treatment. Increased ctDNA levels at or prior to disease progression was seen in 12/13 (92%) of the patients with a median lead-time of 87 days (0-531). CA15-3 was detected in 15/25 (65%) of the patients in pre-treatment samples. Elevated CA15-3 was observed for 8/13 (62%) at disease progression with a median lead-time of 0 days (0-169).

Conclusion: Our preliminary results suggest that ctDNA shows great promise as a potential prognostic biomarker in predicting disease progression in MBC patients. Ultrasensitive and personalised ctDNA analysis opens up new avenues to guide the need of radiological follow-up. However, larger cohorts are needed to determine the clinical value of ctDNA.

P07.024

Multi-cancer application of liquid biopsy comprehensive genomic profiling of circulating free DNA

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Background: Personalized medicine has proven to be a very effective treatment strategy in different solid tumors. The analysis of free circulating DNA (cfDNA) represents an optimal alternative strategy to characterize tumor, moreover it allows to monitor, by a non invasive manner, the tumor molecular profile during treatment. We studied the feasibility, sensibility and informativeness of a comprehensive genomic profiling (CGP) applied to cfDNA of cancer patients in comparison with tumor tissue and to conventional molecular diagnostics.

Methods: Tissue and plasma samples were analyzed using the Illumina TSO500-HT DNA/RNA and TS0500 ctDNA kits, respectively. Sequencing was performed on Novaseq6000 and DRAGEN software was used for analysis.

Results: Samples were collected from 54 cancer patients: 13 advanced non-small cell lung cancer (NSCLC) treated with immune checkpoints inhibitors, 18 advanced BRAF-V600E colorectal cancer (CRC) and 23 locally advanced gastric cancer (GC) patients candidate to neoadjuvant chemotherapy (NCT). Analysis of cfDNA was conducted on plasma collected at baseline and at progression or post-NCT. cfDNA and matched FFPE tissue were sequenced reaching a median sequencing depth of 1705X and 307X respectively. 85% of plasma samples met sequencing quality specifications. On average, plasma samples exhibited a TMB of 12.5 mut/Mb, with a mean of 210 nonsynonymous SNV/indels called per sample. Conversely, only few CNVs were detected, predominantly in samples with high-TF. For 8 cases, FFPE-baseline-progression matched analyses were available. A concordance rate of 82% (66/80) was achieved considering mutations found at baseline cfDNA (classified by Varsome as pathogenic, likely-pathogenic, or VUS) which were also found on FFPE. At progression, 2 additional molecular alterations were identified.

Conclusion: CGP of ctDNA is feasible and can be adopted in several types of cancers, providing a wide spectrum of genomic information, potentially useful for patients' stratification and for therapeutic choice

P07.025

ctDNA monitoring of tumor response to epidermal growth factor receptor tyrosine kinase inhibitor in metastatic non-small cell lung cancer patients

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Background: Plasma circulating tumor DNA (ctDNA) analysis is used for genotyping advanced non-small cell lung cancer (NSCLC) patients. Those harboring EGFR mutations can beneficiate from Osimertinib, an EGFR-TKi (Epidermal Growth Factor Receptor – Tyrosine Kinase Inhibitor) that has revolutionized the treatment landscape of NSCLC patients with important improvement on response rate and progression free survival. We evaluated if monitoring dynamic ctDNA changes may be used to predict treatment outcome to EGFR targeted therapy.

Methods: We are prospectively monitoring tumoral EGFR mutation in plasma by digital PCR at baseline, 1 month, 3 months and every 3 months until progression or complete follow up (24 months) in a cohort of 40 EGFR-mutated adenocarcinoma patients (Lung Resist NCT NCT04222335).

Results: Tumor EGFR mutation was detected in 28/40 (70%) samples at baseline (median concentration 79.68 [0-628875] copies/mL of plasma). At one month, tumor EGFR mutation was still detectable [0-20.2 copies/mL] in plasma in 10/28 patients (36%). At present, 22 patients have progressed of which 19 (86%) had detectable EGFR mutation at baseline. 8/10 patients (80%) with EGFR mutation detected at 1 month progressed. EGFR mutation increased only at clinical progression in 9/22 patients (41%) while preceded progression in 7/22 patients (32%). No increase in EGFR mutation before progression was observed in 6/22 (27%) patients, among which 3 were negative at baseline.

Conclusions: ctDNA monitoring might anticipate tumor response to EGFR TKi treatment. An updated correlation to clinical outcome will be presented at the symposium.

P07.026

Improvement of molecular tumor board decisions with a ctDNA-based deep scale cross-entity NGS panel

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Background: Next-generation-sequencing (NGS) of circulating tumor DNA (ctDNA) gains increasing importance for genomic profiling of solid and hematological cancers. The non-invasiveness, ease, reproducibility and repeatability of liquid biopsy collection and the full reflection of tumor heterogeneity together with novel technological developments are important advantages over tissue-based profiling. However, its implementation in clinical routine and molecular tumor board therapy decisions is still pending. The goal of the proposed NGS panel is the prevention and/or early detection of recurrence and resistance development and the improvement of the prognosis of cancer patients across a broad range of both solid and hematological cancer entities.

Methods: A targeted capture high-throughput deep scale NGS panel to detect cancer-specific mutational changes has been designed and is currently being validated on a pilot study cohort. From each gene, the complete coding sequence and the most prominent mutational hot spots are sequenced.

Results: This pilot study cohort includes tissue and singly or longitudinally collected plasma samples from cancer patients. All samples have already been analyzed by whole exome sequencing (WES) or digital PCR (ddPCR) to serve for validation. The NGS panel will allow in parallel 1) detection of carcinogenic mutations across a broad range of different entities, 2) surveillance of minimal residual disease (MRD), 3) surveillance of resistance development and 4) detection of fusion genes (NTRK, ALK, RET, ROS1 and FGFR) in liquid biopsy-based ctDNA diagnostics. It includes 109 genes and 500kb of covered coding sequences and will be used to analyze samples from the molecular tumor board and several clinical studies.

Conclusion: With this NGS panel we aim to pave the way for including liquid biopsy-based genomic analyses of ctDNA in clinical routine. Furthermore, we aim to improve patient outcome by offering a non-invasive and fast-paced surveillance tool to increase success of molecular tumor board-decided cancer therapies.

P07.027

Monitoring treatment response in patients with metastatic colorectal cancer using cfDNA fragmentomics testing: the DOLPHIN trial

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Background: Accurate monitoring of therapy response is important for treatment decision-making in patients with metastatic colorectal cancer (mCRC). Currently, clinical response is determined by imaging-based assessment of tumor size changes. However, this monitoring approach is constrained by limited sensitivity in detecting lymph node and peritoneal metastases as well as inter-reader variability. Detection of circulating tumor DNA (ctDNA) is indicative for viable neoplastic cells and may complement CT imaging. We recently developed a tumor agnostic ctDNA-test to analyze the ctDNA tumor fraction based on the plasma cell-free DNA (cfDNA) fragmentome: The DELFI tumor fraction (DELFI-TF) score. Aim: The DOLPHIN study aims to investigate whether the DELFI-TF ctDNA assay can provide a sensitive, affordable and broadly applicable ctDNA-test to monitor treatment response of mCRC patients.

Methods: The prospective, observational, multi-center DOLPHIN study, a substudy of the Prospective Dutch ColoRectal Cancer cohort (PLCRC), collects clinical data, images and blood samples from 400 patients receiving systemic therapy. Blood samples are drawn longitudinally in conjunction with CT imaging. Plasma cfDNA is analyzed for tumor-specific fragmentation patterns using the DELFI-TF score. Droplet digital PCR ctDNA-testing of RAS/RAF hotspot mutations serves as reference. Primary endpoint is the association between ctDNA changes and clinical response. Secondary endpoints are the association between ctDNA changes and radiological response according to RECIST and to serum carcinoembryonic antigen (CEA), lead time of ctDNA-testing compared to imaging to detect progressive disease, and the prognostic value of longitudinal ctDNA-testing.

Results: Since the inclusion of the first patient in March 2023, 120 patients from seven hospital in the Netherlands have been included.

Conclusion : The DOLPHIN study will assess the added clinical value of longitudinal ctDNA-testing in treatment response monitoring of mCRC patients and whether ctDNA-testing can complement and/or partly replace imaging. This may lead to novel monitoring strategies and ctDNA-guided treatment decision-making in mCRC patients.

P08.001

Clinicial metagenomics from cell-free DNA: Overview and lessons learned from 4 years as a clinical metagenomics provider

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Background: Clinical metagenomics emerges more and more from academic research to clinical practice. Here, we share a few learning points regarding the implementation as a routine lab-service and CE-IVD marked software running in the cloud.

Methods: We use next-generation sequencing of cell-free DNA to digitally determine relevant pathogens from patients with systemic infections in blood and other bodily fluids using our CE-IVD marked DISQVER diagnostic platform. Until now we have collected microbial data from several thousand samples within four years of routine service.

Results: The set up of the DISQVER test is within the regulatory framework of CE-IVD marking of software as a service (SaaS) and ISO 13485 accreditation. This brings practical implications on database quality, software lock-in/development/testing, change and risk management, technical documentation, requiring substantial resources. Also the wetlab process runs within tightly defined and controlled QM boundaries, pre-set by extensive analytical validations.
We clinically validated our test for pathogen identification from blood within 8 clinical trials for intensive care medicine, hematology and co-infection in Covid-19, showing 2- to 3-fold higher identification rates for pathogens in comparison to standard of care blood culture. Furthermore, several case reports/ case series from different clinical backgrounds, frequently with difficult to diagnose pathogens, have been published. From the analysis of our routine processes, we could show that the turn-around time is very fast and can detect more species than blood culture. Especially the relatively high proportion of fungi and dsDNA viruses indicates superiority of the method compared to the standard diagnostic pathway.

Discussion: To bridge the gap between academic research and clinical implementation, many aspects of regulatory, technical and data protection requirements need to be addressed. Within the timeframe of commercial availability DISQVER from cell-free DNA found in the blood of infected patients is increasingly becoming a useful tool for infection diagnostics.

P08.002

Predicting progression free survival of advanced breast cancer patients in the 1st line of treatment by early circulating tumor DNA dynamics

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Background: CDK4/6 inhibitors (CDK4/6i) improve the outcome of patients with ER+/HER2- metastatic breast cancer (MBC). The recently reported SONIA trial shows no difference in progression free survival after two lines of treatment (PFS2) of CDK4/6i in first-line compared to second-line, while toxicity was higher when CDK4/6i is added in the first-line. Within this cohort, we investigated whether circulating tumor DNA (ctDNA) dynamics in the first two weeks enabled identification of patients with rapid disease progression on first line treatment.

Methods: Blood samples were obtained before start of first line treatment and after two weeks from 295 patients in both arms: the CDK4/6i + aromatase inhibitor (AI) combination arm and the AI monotherapy arm. Baseline cell free DNA (cfDNA) was sequenced with a focused NGS panel of 10 genes (OncomineTM Breast cfDNA panel V2). Dedicated digital PCR assays were used to subsequently track identified driver mutations over time. A ctDNA ratio (CDR) was calculated at two weeks relative to the baseline level to define ctDNA dynamics.

Results: At baseline, in 131 of the 295 (44.4%) patients one or more somatic mutations were detected. For 130 baseline ctDNA positive patients, ctDNA was followed after two weeks. Patients were divided in a limited ctDNA decline group (CDR > median level) and a strong ctDNA decline group (CDR < median level). Patients in the high ctDNA decline group had a substantially higher median PFS of 22.5 months compared to 16.8 months in patients in the limited ctDNA decline group (p = 0.048, log rank test).

Conclusion: A limited decline in ctDNA after 2 weeks of treatment is associated with a worse response on first line endocrine therapy, independent of CDK4/6i addition, in patients with MBC. Future studies are warranted to evaluate the clinical utility of our findings.

P08.003

Trial in progress: Optimization of treatment selection and follow-up in oligometastatic colorectal cancer – a ctDNA-guided phase II randomized approach - OPTIMISE

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Background: Patients with detectable ctDNA after radical intent treatment of metastatic spread from colorectal cancer (mCRC) have a high risk of recurrence, which might be prevented with intensified adjuvant chemotherapy (aCTh). In OPTIMISE, we investigate ctDNA-guided aCTh after radical intent treatment of mCRC. ClinicalTrials.gov identifier: NCT04680260.

Methods: Open-label phase II 1:1 randomized multicentre trial comparing ctDNA-guided aCTh against standard of care (SOC). Analyses of plasma samples for ctDNA by ddPCR, detecting CRC-specific mutations and NPY-gene hypermethylation. Key inclusion criteria: radical intent treatment for mCRC; clinically eligible for triple-agent chemotherapy. Patients randomized to the standard arm are treated according to SOC. In the ctDNA-guided arm, ctDNA-positive patients undergo a PET/CT scan and if clear, an escalation strategy with 4 months of FOLFOXIRI followed by 2 months of 5-FU. ctDNA negativity allows for a de-escalation strategy by shared-decision making. Primary endpoint; fraction of patients alive and free from recurrence at 2 years after inclusion. Secondary endpoints; toxicity, ctDNA negativity (6 months + 1 year), time to recurrence, rate of local and distant relapse, overall survival, QoL, cost-effectiveness. Feasibility measures for the pre-planned interim assessment; inclusion of 30 patients/12 months in two Danish hospitals, compliance with randomization>80%, PET-CT positive cases<20%, eligibility for triple-agent chemotherapy>80%.

Results: Results from the pre-planned interim assessment have been published (Callesen et al., Acta Onc, 2023) and complied with all feasibility measures. Furthermore, the median time to result (3 WD) and quality of analyses were adequate. Inclusion is ongoing, and currently, 49 patients are included. Sample size is 350 but will be revised based on findings in the run-in phase (n=100). Planned expansion with 5 sites.

Conclusion: To the best of our knowledge, OPTIMISE is the first randomized trial evaluating ctDNA-guided aCTh after radical-intent treatment for mCRC. Hopefully, ctDNA-guided aCTh and follow-up will prolong survival and reduce chemotherapy-related toxicity.

P08.004

CfDNA is an independent risk factor for all-cause mortality in patients with heart failure

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Background: The concentration of circulating cell-free DNA (cfDNA) has emerged as promising biomarker in acute cardiovascular pathologies and as predictor of mortality in myocardial infarction. First results indicate that cfDNA may be an independent risk factor for cardiovascular disease and overall mortality.

Methods: Here we analyzed cfDNA in 3109 EDTA plasma samples from a prospective cohort study on heart failure (NCT04064450). Two qPCR assays targeting a LINE1 element (cfDNA90bp and cfDNA222bp) were used to quantify cfDNA concentration and determine the fragmentation index (FI) of cfDNA (cfDNA222bp/ cfDNA90bp). Cox proportional hazards regression analyses were used to investigate the associations between cfDNA and cfDNA FI with all-cause mortality. The participants were classified as stage 0 (no heart failure) or stages A (at risk) to D (severe) according to the current Universal Definition of Heart Failure.

Results: The cohort included 3109 participants aged 34 to 85 years with 35.7% females. The concentration of cfDNA was lowest in the subjects at stage 0/A (n=571) with 10.99 (8.70/13.93) ng/ml (median (Q1/Q3)). Subjects with stage B (n=977) or symptomatic patients with stage C/D (n=1741) showed higher cfDNA concentrations with 13.37 (10.35/18.11) or 17.11 (12.56/22.80) ng/ml, respectively. Multivariable Cox regression indicated that the concentration of cfDNA90bp is a relevant prognostic marker for all-cause mortality after adjustment for age, sex, and cardiovascular risk factors [HR 1.428 (95% CI 1.315-1.551, p<.0001)]. After additional adjustment for medication intake, and NT-proBNP the effect estimates were lower, but still significant [HR 1.173 (95% CI 1.073-1.282, p=.0005)], while FI or cfDNA222bp lost their significance after final adjustment for NT-proBNP.

Conclusion: These results indicate that cfDNA is an independent risk factor for all-cause mortality, which contributes to the prediction of all-cause mortality in the study cohort independently of NT-proBNP. Further analyses will highlight the relevance of cfDNA levels for the prediction of cardiac death.

P09.001

ctDNA Detection in Colorectal Cancer Patient Plasma by Whole Genome Sequencing-Based Counting of Somatic Structural Variants

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Background: Circulating tumor DNA (ctDNA) is a cancer biomarker typically detected in plasma by analyses of tumor-specific mutations, DNA methylation, and fragmentation. The potential of somatic structural variants (sSVs) as ctDNA biomarkers remains underexplored, despite their high abundance and tumor specificity.

Methods: We present a tumor-informed whole genome sequencing (WGS) based pipeline for selecting and quantifying sSV for ctDNA detection in plasma. The pipeline estimates a sSV variant allele frequency (VAF) by counting sequencing reads mapping to sSV breakpoints in sequence-resolved sSV. The sSVs are identified in tumors by the SV callers Manta and DELLY, and the pipeline includes filtering steps to remove tumor-nonspecific sSVs. Our pipeline was applied to WGS-data from 144 stage III colorectal cancer (CRC) patients with serially collected plasma samples (n = 1291). CRC detection sensitivity was evaluated in pre-operative plasma samples (n = 136) and surveillance plasma samples from patients with recurrence (n = 182). Specificity was evaluated in surveillance plasma samples from non-recurrence patients (n = 731). Additionally, the performance was compared head-to-head with orthogonal point mutation-based ctDNA detection methods.

Results: Our pipeline excluded a median of 14%/9% (Manta/DELLY) sSVs predicted in fresh frozen tumors and 72%/36% (Manta/DELLY) sSV predicted in formalin-fixed paraffin-embedded tumors, leaving 12-390 sSVs per patient for ctDNA detection. Our approach exhibited 99,6% specificity assessed in surveillance samples from non-recurrence patients and a pre-operative sensitivity of 47%. We detected ctDNA in 67% (20/30) of recurrence patients with a median lead time of 6.5 months compared to radiological assessment. Head-to-head comparison of VAFs from our pipeline and point mutation-based ctDNA analyses revealed strong correlation (Pearson correlation, r2): 0.95 (droplet digital PCR), 0.93 (C2inform) and 0.95 (Signatera).

Conclusion: Our pipeline established a framework to use sSVs as powerful biomarkers for ctDNA detection with high specificity and strong concordance with point mutation-based ctDNA analyses.

P09.002

Utility of cell-free DNA fragment end and fragment positioning biological properties for cancer detection

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Background: Circulating tumour DNA (ctDNA) can be challenging to detect as it often constitutes only a minor fraction of cell-free DNA (cfDNA). Recent studies suggest that the biological properties of fragments differ between tumour and non-tumour derived cfDNA. Understanding and capturing these differences could help improve the detection of ctDNA in a tumour-naive way.

Methods: This study explores the cfDNA fragment ends sequence context and positioning throughout the genome using FRENDS (FRagment ENDs Sequence context) and MIDS (fragment distance to the MIDpoint of genomic elementS) methods. The FRENDS method is a collection of three methods which extract nucleotide frequency per position and motif frequency at the fragment start and end. The MIDS method assesses the distance of fragment midpoints to the region of interest's centre in 66 different genomic lists. These genomic lists represent the nucleosome positions, transcription start sites, regions with low coverage in cfDNA data from healthy donors (non-overlapping regions), open chromatin regions, repeat regions, genome segmentations and DNaseI hypersensitive regions.

Results: FRENDS and MIDS models, when applied to a multi-cancer cohort (n=462) of 18 cancer types showed an AUC of 0.98 and 0.94, respectively, in training (n=279) and 0.97 and 0.86 in test (n=183) samples, with AUC of >0.97 and > 0.84 in early stage (stages I and II) disease and AUC of > 0.99 and > 0.91 in late-stage disease. An ensemble model from FRENDS, MIDS and fragment length features showed even better detection (0.99 in train and 0.96 in test) with an AUC of 0.96 in early and 0.99 in late-stage disease.

Conclusions: Biological properties derived from cfDNA fragments, based on both fragment end and position, contain information that can be used to enhance cancer detection. The methods we have developed could help in extracting and utilising this information.

P09.003

A cfDNA methylation-based approach for the detection of tumour tissue-of-origin in Cancers of Unknown Primary

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Background: Cancers of Unknown Primary (CUP) comprise a heterogeneous group of metastatic cancers where the primary site remains undetectable. With limited access to targeted or immunotherapies without a primary tumour diagnosis, CUPs are the 6th leading cause of cancer deaths in the UK. Determining tissue-of-origin (TOO) by molecular profiling could improve treatment options, with tissue acquisition barriers mitigated via liquid biopsies. Here, we present the first reported application of a blood-based TOO assay in a CUP cohort.

Methods: An XGBoost machine learning classifier, termed CUPiD, has been developed using a data augmentation strategy to generate 'cfDNA-like' training data from publicly available DNA methylation array data mixed with non-cancer control (NCC) cfDNA data (276,108 mixtures, tumour fraction: 0.5–10%) to predict TOO across 29 tumour classes. Genome-wide cfDNA methylation profiles from 143 patients with known cancer types, 41 patients with CUP and 27 NCCs have been generated using T7-MBD-seq, a methylation enrichment approach, to validate the performance of CUPiD.

Results: CUPiD was first tested in cfDNA samples from patients with known tumour types, with overall multi-class sensitivity of 84.6% and accuracy of 96.8%. CUPiD predictions were made for 78.0% of CUP cases, of which 71.9% were clinically consistent with a subsequent or suspected primary tumour diagnosis. Furthermore, CUPiD predictions were made in 6/8 patients where no primary was ever suspected, exemplifying the potential of TOO molecular profiling in the most uncertain cases.
For 15/41 patients initially treated as CUP a subsequent primary tumour diagnosis was possible, although median time to this was 7.1 months (range 0.4-47.2 months) demonstrating the impact this approach could have if implemented at the time of initial suspected cancer diagnosis.

Conclusion: Methylation profiling of cfDNA provides accurate TOO predictions for patients with CUP, demonstrating the potential for diagnosis re-classification and/or treatment change in this hard-to-treat cancer group.

P09.004

Machine Learning to Detect the SINEs of Cancer

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Background: We previously described an approach called RealSeqS to evaluate aneuploidy in plasma cell-free DNA (cfDNA) through the amplification of ~350,000 repeated elements with a single primer. We hypothesized that an unbiased evaluation of the large amount of sequencing data obtained with RealSeqS might reveal other differences between plasma samples from patients with and without cancer.

Methods: This hypothesis was tested through the development of a machine-learning approach called Alu Profile Learning Using Sequencing (A-PLUS) and its application to 7615 samples from 5178 individuals, 2073 with cancer and the remainder without cancer. Samples from cancer patients and controls were pre-specified into four cohorts used for: 1) model training, 2) analyte integration and threshold determination, 3) validation, and 4) reproducibility.

Results: A-PLUS alone provided a sensitivity of 40.5% across 11 different cancer types in the Validation Cohort, at a specificity of 98.5%. Combining A-PLUS with aneuploidy and 8 common protein biomarkers detected 51% of the cancers at 98.9% specificity. We found that part of the power of A-PLUS could be ascribed to a single feature – the global reduction of AluS sub-family elements in the circulating DNA of solid cancer patients. We confirmed this reduction through the analysis of another independent dataset obtained with a very different approach (whole genome sequencing).

Conclusion: The evaluation of Alu elements therefore has the potential to enhance the performance of several methods designed for the earlier detection of cancer.

P09.005

Deep learning models of DNA methylation allows ultrasensitive age prediction from blood

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DNA methylation is associated with gene regulation and establishment of cellular identity throughout life. Yet, gradual methylation changes at specific sites pave the way for predicting age, poor health, and lifespan from blood DNA. While the biological basis for these changes is mostly unknown, various epigenetic clocks showed a prediction accuracy of ~3-4 years, typically using linear regression models of methylation array data.
Here we report an alternative approach based on Deep Neural Network analysis of targeted-PCR sequencing. We have collected and analyzed fragment-level sequencing data from 300 donors aged 18-78 years, across 41 genomic regions, each containing 3-17 successive CpGs. We then trained a deep fully connected neural network, to predict chronological age from (1) average methylation levels, (2) fragment-level information, and (3) combinatorial patterns of neighboring CpGs.
Linear regression analysis of these data allows estimation of chronological age with a median error of 3.2 years on held-out test donors. Strikingly, deep neural network analysis of a single locus (ELOVL2, 9 CpGs) achieves an accuracy of 1.8 years, with improved robustness when additional regions are added. Overall, we observed two potential processes of age-related methylation changes. Some loci show gradual stochastic changes occurring independently at each CpG. Conversely, other regions show a slow coordinated shift from fully methylated to fully unmethylated fragments, possibly due to changes in cellular state, in composition, or in sub-populations.
We present an ultrasensitive blood-derived clock of chronological age, based on PCR sequencing and deep learning. The simplicity and accuracy of our method, as well as its fast turnaround times, pave the way for studying the molecular mechanisms underlying aging in blood cells and in remote tissues and allow practical applications in forensics and aging research.

P09.006

Beyond basics: Key mutation selection features for successful tumor-informed ctDNA detection

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Background: Tumor-informed mutation-based approaches are frequently used for detection of circulating tumor DNA (ctDNA). Not all mutations are equally good candidates for ctDNA assay design. The objective of this study was to explore how mutational features, such as cancer cell fraction (CCF), multiplicity, and sequencing error rate impact the success of tumor-informed ctDNA analysis. Additionally, we report a practical and easily implementable analysis pipeline for identifying and prioritizing candidate mutations from whole exome sequencing data.

Materials and Methods: We used data from 390 plasma samples from three independent cohorts of bladder and colorectal cancer patients analyzed using three different tumor-informed sequencing-based ctDNA detection strategies.

Results: High CCF mutations were detected more frequently than low-CCF mutations (Fisher exact test, Cohort A: P=1.73e-12, Cohort I: P=1.73e-04, Cohort N: P=7.67e-03). The detection-likelihood was additionally improved by selecting mutations with multiplicity of two or above (Fisher exact test, Cohort A: P=3.85e-03, Cohort I: P=1.89e-03, Cohort N: P=1.73e-14). Furthermore, as the tumor fraction in the plasma cell-free DNA approached the lower limit, the mutations for which the ctDNA detection method had the lowest error rates, showed the highest detection rates (Wilcoxon signed rank test, P=1.6e-07).

Conclusion: Selecting mutational markers with high CCF, high multiplicity, and low error rate significantly improves the likelihood of ctDNA detection. Our easily implementable analysis pipeline enables qualified prioritization of mutations for tumor-informed ctDNA analysis.

P09.007

Cross-dataset pan-cancer detection: Correlating cell-free DNA fragment coverage with open chromatin sites across 487 cell and tissue types

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Background: The fragmentation patterns of low-coverage whole genome sequenced (lcWGS) cell-free DNA (cfDNA) have been used to detect and subtype cancers. Previously published methods were mostly validated within their original dataset or on a single external dataset. cfDNA lcWGS data is systematically biased, making broad generalization difficult and, in some cases, within-dataset classification easy. We propose that cross-dataset generalization should be one of the main evaluation criteria of lcWGS methods.

Methods: We present a novel cancer detection method that generalizes across 9 datasets and their 13 differing cancer types (996 non-cancer controls, 1526 cancer) obtained from different studies. Our method correlates bias-corrected fragment coverage across the genome with the presence of open chromatin sites for 487 cell and tissue types. These correlation coefficients indicate shifts in the cfDNA cell-type composition like the emergence of DNA from tumor cells. We used logistic regression on the correlation coefficients to distinguish cancer samples from non-cancer controls and tested the generalization via leave-one-dataset-out cross-validation. Two of the datasets were only used during training as they contained no or too few control samples.

Results: We tested the cross-dataset generalization for seven of the datasets and achieved ROC AUC scores of 0.93, 0.91, 0.82, 0.78, 0.77, 0.76, 0.68, and 0.59 (mean = 0.78). Bias correction generally improved generalization as datasets became more similar.

Conclusion: Our work demonstrates the feasibility of cross-dataset generalization of cancer detection models. We investigate the dynamics between the datasets and highlight challenges in generalizing cfDNA-based lcWGS methods. We argue that new cfDNA cancer detection methods should be evaluated on cross-dataset and cross-lab generalization to increase confidence that they rely on cancer-relevant signals.

P09.008

Correctly extracting biological features from cell-free DNA sequencing data

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Background: Circulating cell-free DNA (cfDNA) can be used for various clinical purposes such as cancer early detection, therapeutic decision-making and minimal residual disease detection. Fragmentomic features are emerging as important factors that can improve sensitivity, while using information in sequencing reads that don't contain sequence alterations. Their application depends on a fundamental process: extracting biological features accurately from sequencing reads. Tools that are customarily used have not taken this into account, and bespoke tools for cfDNA analysis are needed. We developed a customised workflow to improve robustness and accuracy of biological feature calculation.

Methods: To tackle these long-lasting challenges, we clarified existing problems in this research area and developed the Trimming and Aligning Pipeline (TAP) and cfDNAPro R package. They comprehensively consider the properties of cfDNA and establish standards for cfDNA data analysis in contrast to analysis of genomic DNA or tissue samples. The TAP pipeline can be used to trim adapters and align sequences, and cfDNAPro is tailored for robust downstream feature extraction and visualisation (i.e., Fragment length, motif, copy number, and mutation). We evaluated this on shallow-depth whole genome sequencing data generated from 10 different samples using 5 different library preparation kits.

Results: We built a Singularity image for the TAP pipeline and a user interface via the cfDNAPro package, that provides fast computation by parallelisation and serves as a unified platform for extracting and visualising features from cfDNA sequencing data. As of the date of writing, the cfDNAPro R package has accumulated more than 2,000 global downloads.

Conclusion: TAP pipeline and cfDNAPro R package enable cfDNA sequencing data analysis standardisation, and offer a reproducible and robust way to perform feature extraction.

P09.009

Cell-Type Decomposition with Variation in Program Signals in Liquid Biopsies using VarNMF

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In liquid biopsies, we can view samples as non-negative linear combinations of unknown programs.
Extracting these programs provides insights into the underlying processes of the system. Here we focus on this problem in the context of cell-free ChIP-seq (cfChIP-seq) data, which combines chromatin modification programs originating from various populations of cells across the body, that differ in type and state.

This decomposition task is typically approached using the Non-negative Matrix Factorization (NMF) model, resulting in estimations of mixture weights and constant programs that reconstruct the observed data. However, NMF only accounts for two types of variations between samples - disparities in the proportions of program contribution and observation noise. In many cases, and specifically in cfChIP-seq data, there is an additional source of variation between samples that is not explained entirely by these factors, but rather by a variation in the programs' signal itself. For example, the liver cells program can range among a spectrum of states including levels of inflammation, levels of metabolites, etc.

Here, we account for this variation by introducing a probabilistic model named VarNMF, wherein programs are modeled as distributions instead of constant vectors. These distributions are extracted from the mixed samples without observing any program directly.

Using simulated data, we show that in the presence of variation in program signals, VarNMF outperforms the generalization abilities of NMF. We demonstrate its real-world potential by applying it to cfChIP-seq samples from healthy donors and metastatic colorectal carcinoma (CRC) patients. VarNMF infers program distributions associated with cell types expected in this dataset. Specifically, it extracts cancer-associated program distributions that reflect inter-cancer variability directly from mixed samples and without prior knowledge.

Taken together, these results illustrate the potential of VarNMF as a tool for program decomposition in biological contexts.

P09.010

WGS INVAR: A novel software tool for the detection of circulating tumour DNA in liquid biopsy data

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Background: Analysis of circulating tumour DNA (ctDNA) for Minimal Residual Disease (MRD) aims to detect few molecules of tumour origin in liquid biopsy samples for the identification of disease presence or recurrence. The main technical challenge consists of identifying true somatic mutations from sequencing noise to high levels of confidence in low disease burden settings, such as early detection or low mutational burden cancers.

Method: We explore the use of a novel software tool for the detection of early stage cancers in low signal to noise conditions and across multiple sample types, such as whole blood and plasma. The software runs on whole genome sequencing (WGS) data and aggregates signals across tumour-informed loci. It uses neighbouring regions to gather a representation of the noise across the sample. By integrating multiple biological features into the classification, we show that the sensitivity of the model to real mutations can be increased.

Results: We present how our WGS based method is able to more accurately detect cancer in hard to detect samples, leading to the positive classification of signal in early stage cancers also prior to clinical diagnosis, and down to sensitivities of a few mutant molecules per million. It can also model background noise to levels several fold more accurately than previous methods.

Conclusions: We present a fully packaged detection tool with a non-technical user friendly interface, cross-platform compatibility and a high computational efficiency. Available on the Rosenfeld Lab GitHub(nrlab-CRUK), it is streamlined to accept external data formats and open source to allow for personalisation or further development. It is provided with comprehensive documentation, and we welcome input from external users. We benchmark the efficiency of this tool by applying it to previously described sequencing data, to assess the detection limits and rates of ctDNA in samples with low levels of ctDNA.

P09.011

Predicting the type of mutations in cell-free DNA by semi-supervised GAN models

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Background: Distinguishing between pathogenic cancer mutations and other somatic mutations found in cell-free DNA (cfDNA) is an important challenge in the field of liquid biopsy. Mutations in genes that give hematopoietic stem cells (HSCs) a selection advantage under certain conditions drive clonal hematopoiesis (CH). Although some CH driving variants have been identified experimentally or by epidemiological studies, the compendium of all CH driving mutations is far from complete. Misclassification of CH mutations as tumor-derived could lead to inappropriate diagnosis and therapeutic treatment in the field of liquid biopsy.

Methods: To overcome this problem, we developed a deep learning method by preparing an in-house reference collection of somatic variants including around 25000 SNVs with information of their origin in two different categories (tumor vs. CH). We trained our model based on the semi-supervised generative adversarial network (SSGAN) architecture.

Results: Our SSGAN model first learns the hidden features of cfDNA genomic variants (20000 labeled, 100000 unlabeled SNVs obtained from pan-cancer and prostate cancer respectively) in an unsupervised manner and then classifies raw variants based on the pre-trained GAN algorithm. We were able to achieve an AUC of 96% and 94% in the training and validation phases, respectively by integrating a nucleotide composition feature. Furthermore, variants labeled as CH or tumor by our model were compared with their matched tumor and whole-blood samples based on genomic and functional characteristics and showed a decent amount of concordance.

Conclusion: Overall, our proposed method based on semi-supervised learning resulted in faster performance and reasonable accuracy when compared with classic classification approaches. This work demonstrates that genomic feature prediction from cfDNA variant data is a promising and viable alternative to cost-prohibitive approaches that require multiple sequencing datasets from patients.

P09.012

Classifying disease subtypes from cell-free ChIP-seq profiles

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Chromatin immunoprecipitation of cell-free chromatin (cfChIP-seq) of active marks (e.g., H3K4me3) provides a genome-wide map of active promoters in the cells contributing to the circulating DNA. These chromatin marks are inherently related to gene expression in the cell of origin. Disease subtypes involve changes in gene expression and are often defined by these changes, suggesting that cfChIP-seq can allow the classification of disease subtypes. However, this task is hampered by the inherent property of cell-free DNA – the contribution of disease cells is mixed with contributions from other cells, and the proportion, or load, of the disease in the cfDNA pool varies between patients.

Here, we design statistical tests for differential genes and classification methods that take into account the confounding effect of variable disease loads. We start by learning the distribution of each gene's value in healthy cohorts. Based on deviations from the healthy baseline, we estimate both the disease load and distribution of genes in the disease. Next, we identify the differential genes between two or more subtypes, taking into account the load as a confounding factor. Using these genes, we can classify cell-free DNA samples of one disease into different subtypes using a naive Bayes classifier.

We use these aspects to classify subtypes with small disease loads (> 2%) and find differential genes. First, we tested the method using synthetic data, and later, we used different cohorts for different tasks: classify AIH versus other liver diseases, classify different stages of Multiple Myeloma, and classify different subtypes of small cell carcinoma. These findings hold promise for classifying and understanding non-trivial disease subtypes, even in cases with a minimal disease load.

P09.013

Using Better Base Quality(BBQ) to detect cancer mutations from cell-free DNA accurately

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Detection of circulating tumor DNA(ctDNA) in blood increasingly attracts attention as a promising cancer biomarker. Yet accurate detection of tumor mutations in whole-genome sequencing of cell-free DNA remains challenging, particularly when the tumor fraction is so low that the variant allele frequencies are close to the sequencing error rates. Therefore, it is important to estimate the error rate of a specific base in a specific read accurately.

In paired-end sequencing, when DNA fragments are shorter than twice the read length, the overlapping region emerges as an ideal resource for training models to discern specific base error rates, as the mismatches inside the overlaps must be an error generated either by sequencing procedures or alignment. In this context, we introduce, 'Better Base Quality'(BBQ), a method that leverages the mismatches within read overlaps to precisely estimate the error rate associated with different sequencing contexts. Specifically, we use the 7-mer encompassing the mismatch to predict error rates for all combinations of FASTQ base quality(BQ) and mutation type using kmerPaPa. In addition to the sequencing context, we hypothesized that multiple alternatives at a site within the read overlap suggest a higher likelihood of error; thus, we updated our model with the position-specific information. We applied a beta-binomial distribution to allow the posterior error rate to differ between sites based on the number of observed mismatches.

The model has been tested on cell-free DNA cancer plasma samples of 5% tumor fraction and can demonstrate significantly improved precision in calling tumor single nucleotide variations compared to Mutect2, Strelka2 and Lofreq as shown by the precision-recall curve. This work can improve the accuracy of detecting circulating tumor DNA mutations and ultimately benefit early cancer detection and timely relapse monitoring.

P09.014

A multi-omics model for non-invasive tumor load quantification from blood plasma in metastatic colorectal cancer

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BACKGROUND: The Variant Allele Frequency (VAF) of driver mutations detected in cell-free DNA (cfDNA) is a widely-used proxy for circulating tumor load, but this requires either a tumor-informed approach or large, costly panels to be applicable to all patients. Here, we compared deep mutational profiling to tumor-agnostic data modalities generated from blood-based liquid biopsies of patients with colorectal liver metastases.

METHODS: In 99 patients we obtained the following measurements: a) cfDNA quantification b) mutation profiling of 14 CRC driver genes (OncomineTM), c) genome-wide cfDNA methylation (cfDNAme) profiling, d) circulating tumor cells (CTCs) and tumor-derived extracellular vesicles (tdEVs) enumeration, and e) expression profiling of 78 CRC-specific genes in CTC-enriched fractions using qPCR (GE).
From cfDNAme and GE profiles, we estimated the tumor fraction via deconvolution algorithms using negative binomial regression and support vector regression, respectively. For 68 patients with detectable driver mutations, we used the VAF as ground-truth for the circulating tumor load and assessed how well the other features correlated with it. We also trained linear predictors of the VAF using a) cfDNAme, b) GE c) CTCs and tdEVs and d) all features.

RESULTS: cfDNAme-derived tumor fraction estimates showed the strongest correlation with VAF (ρ=0.80), followed by tdEV (ρ=0.41) and CTC (ρ=0.34) counts. Combining all features gave the most accurate VAF predictions (leave-one-out cross-validation), while all predictors outperformed a baseline with only age and sex. In 31 patients without a detectable mutation, tumor fraction estimates from the all-features model were significantly higher in patients that had progressive disease within one year compared to patients who did not (median of 7.98% vs 1.61%, p=0.009). This remained significant in a multivariate model including age and sex (p=0.035).

CONCLUSION: The integration of multiple tumor-agnostic modalities into one circulating tumor load estimate correlates with VAF and captures clinically relevant information related to disease progression.

P09.015

Inferring tumour-derived mutations from candidate variants in cfDNA whole-genome sequencing data

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Background: Whole-genome sequencing of cell-free DNA (cfDNA) is increasingly being used for a range of applications, including mutation analysis. The number of cancer-specific mutations found in WGS data depends upon the fraction of tumour-derived cfDNA and the tumour mutation burden.
Variant callers designed for tumour-normal pairs, such as MuTect2 and Strelka2 can identify cancer mutations if the tumour fraction in cfDNA is at least 5%-10%. However, the resultant cfDNA mutation lists may contain false positive calls due to clonal haematopoiesis and sequencing artefacts.
We developed a package for cfDNA that utilises cancer-associated features and mutational signatures to filter candidate variants and infer tumour-derived mutations.

Method: Input cfDNA variants are annotated using features, such as fragment length, count of overlapping mutant paired-end reads, and region-specific mutational density. Sequencing paired-end status and fragment-level information is extracted from the bam file while regional tumour features are derived from publicly available mutational datasets.
The cfDNA variants are utilised to generate a 96-trinucleotide substitution matrix designed for refitting cancer-specific mutational signatures. Relative cancer signature contributions are identified even at 3%-5% contribution, provided that the sample variant counts enable accurate deconvolution.
Variants are iteratively filtered while updating annotated feature cut-offs. The optimal filtering solution is selected based on attributed cancer signature weights and refitting accuracy.

Results: The efficacy of cfDNA mutation filtering was evaluated in a small set of cfDNA samples with matched tumour-normal data. The cfDNA mutation detection pipeline exhibited an enhanced concordance with the ground truth tumour variants, achieving a mean improvement of 5% in accuracy metrics, compared to non-filtered cfDNA variants. This functionality is being integrated into the cfDNAPro R Package.

Conclusion: This package leverages cancer-associated features and mutational signatures to infer the origin of candidate cfDNA mutations. It enables cfDNA-specific annotation and filtering which enhances point mutation analysis in liquid biopsy data.

P09.016

A pan-tissue human atlas of allele-specific DNA methylation patterns

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Background: Various methods for predicting disease involve cell type deconvolution of cell-free DNA methylation patterns. The efficacy of these depends on the ability to find high specificity markers for all cell types. In this work we compiled a whole-genome methylation atlas with over 200 samples purified to homogeneity in 39 cell types, to study bimodal methylation patterns, allele-specific methylation, methylation quantitative trait loci (meQTLs), and parent-of-origin derived methylation. While these regions are of great importance to understanding gene regulation mechanisms and genetic disease they have not been well characterized in humans outside of blood samples. Moreover, identifying regions where methylation depends on genetic sequence is critical for identifying SNP-dependent cell-free DNA markers, whereas bimodal methylation within purified samples could be indicative of cell type subpopulations.

Methods: Using a fragment-level analysis, we developed an efficient computational algorithm for joint analysis of epigenetic and genetic information, and identified bimodal and allele-specific methylation. The variety of individuals comprising our atlas allowed testing for associations between allele-specific methylation patterns and heterozygosity, thus identifying meQTLs or putative parent-or-origin derived methylation. We further test tissue-specific allele-specific expression using the GTEx dataset and associate these with regions exhibiting allele-specific methylation.

Results: Overall we created a cell-type-specific catalog with >300k bimodally methylated regions, 40k meQTL loci, 30k regions showing allele-specific methylation, and 460 regions with putative parent-of-origin derived methylation. Overl 100 of these "imprinted" methylation regions are novel. Moreover, these regions are near over 700 genes exhibiting allele-specific methylation in corresponding tissues.

Conclusion: The catalog sheds light on the molecular mechanisms underlying allele-specific expression, exposing regions of sequence-dependent methylation, which should be avoided as biomarkers, or alternatively used as genotype-dependent markers. Moreover, bimodally methylated regions with no apparent ASM suggest existence of cell type subpopulations which should be investigated as potential biomarkers for disease.

P09.017

Leveraging Deep Learning for the Early Detection of Multiple Cancers through Minimal cfDNA Quantities

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Background: Cell-free DNA (cfDNA) holds promise for early cancer detection, but distinguishing tumor-derived cfDNA in a normal background is challenging without complex sequencing or reference samples. Our hypothesis posits that advanced deep neural networks can surmount this obstacle by directly learning intricate cancer-specific patterns from raw sequencing data.

Methods: We developed a deep learning platform and applied it to multiple clinical datasets containing raw genomic sequencing data from cancer patients and healthy individuals, including sequencing data generated by our team. We assessed the performance of our deep learning models in classifying multiple types of cancer simultaneously and discretely. The models were evaluated through leave-one-out cross-validation method and were further tested on down-sampled sequencing samples, with the lowest sampling of 75,000 reads per sample (coverage < 0.01x). We compared our cancer detection performance to traditional cfDNA cancer detection methodologies.

Results: Our deep learning model achieved 80% sensitivity at 85% specificity in multi-cancer classification, which improved from sensitivity of 68% with multi-cancer model to 86% for cancer-specific model (breast cancer). Interestingly, our classification predictions per-sample were significantly correlated with the prediction scores of traditional cancer detection methods (Pearson correlation, r=0.53; P<1.1x10-8). Notably, while traditional cancer detection methods show a dramatic drop in performance with lower sequencing coverage, our method shows similar cancer detection ability across all tested depths (50Mil, 5Mil, 75K).

Conclusions: Deep learning shows potential for direct analysis of raw genomic data. With sufficient training data, optimized models may approach accuracies needed for clinical use. Our results demonstrate the feasibility of early cancer detection in cfDNA, specifically with an extremely low amount of sequencing data which enables employing this technique for a wide-scale screening. Ongoing work includes expanding the training data to nanopore sequencing, adding biologically relevant annotations and applying advanced natural language processing techniques (such as transformers and BERT architectures).

P09.018

GCparagon: Evaluating and Correcting GC biases in cell-free DNA at the fragment level

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Analyses of cell-free DNA (cfDNA) are increasingly being employed for various diagnostic and research applications. Many technologies aim to increase resolution, e.g., for detecting early-stage cancer or minimal residual disease.
However, these efforts can be confounded by inherent base composition biases of cfDNA analyses, specifically the over- and under-representation of Guanine (G) and Cytosine (C) rich sequences.
For sequencing data there is currently no universally applicable tool available to correct these effects for individual cfDNA fragments.

We present GCparagon, a two-stage algorithm for computing and correcting GC biases in sequencing data of cfDNA samples.
In the initial step, length and GC base count parameters are determined. Here, our algorithm minimizes the inclusion of known problematic genomic regions, such as low-mappability regions, in its calculations. Expected fragment attribute distributions are simulated for each processed genomic interval. GCparagon uses observed and expected values to compute weights for cfDNA fragments to counterbalance the observed distortion of cfDNA attributes. Correction weights are output as a correction matrix in compressed text format.
In an optional second step, these weights can be added as alignment tags to each sequence read alignment of the input binary alignment map (BAM file). Both the GC bias matrix and the tagged BAM file can be used for downstream analyses. Parallel computing allows for a GC bias computation in less than a minute and bias correction in less than 30 minutes for 0.1-60x whole genome sequenced samples.

We show that GCparagon greatly improves the analysis of regulatory regions, which often have specific GC compositional patterns. GCparagon is therefore suitable to contribute to the establishment of standardized cfDNA applications.

P09.019

Fragment-level multimodal analysis of cell-free DNA reveals complementary signals for cancer detection

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Background: Some recent work on cell-free DNA analysis has been directed toward the multimodal integration of genetic (e.g., copy number aberrations, variants) and epigenetic (e.g., methylation) profiles with application to cancer detection. To our knowledge, none of these methods is extensively and jointly modelling chromosomal instability, methylation and fragmentomic patterns. Moreover, the complementary of these different properties of cell-free DNA has not yet been properly discussed.

Methods: We performed enzymatic conversion and whole-genome methylation sequencing of blood samples from breast, ovarian and colon cancer cases (with a majority of stage I), as well as healthy controls with various sequencing depths (1-10x). Enzymatic conversion is indeed expected to have lower GC and fragmentation biases, therefore enabling joint methylome and fragmentome analyses. Accordingly, we devised a new analytical pipeline for extracting fragment-level information from these samples, and performing patient-to-patient comparisons at fragment-level in the form of kernel matrices. The latter were adjusted with respect to technical confounders using a novel correction algorithm, and finally used for downstream analysis using Machine Learning approaches for early cancer detection.

Results: We report 85%, 79%, 82%, 80% and 74% AUC after leave-one-batch-out cross-validation of cancers (regardless of the cancer type) using nucleosome positioning, fragment length, methylation, coverage and mutational information, respectively. AUC increased up to 88% when combining these approaches for multi-class cancer detection. When focusing on breast cancer cases only, which are the most prominent in our data set, we identified nucleosome positioning as the main driver for performance (92% AUC).

Conclusion: Performance gains obtained when combining cell-free DNA properties is indicative of their synergy and complementarity. Our method is highly interpretable, as kernel matrices are computed in an end-to-end fashion (from the raw data), and has potential for application to low-coverage whole-genome methylation sequencing data.

P09.021

A reference-free strategy for detecting circulating tumor DNA

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Background: Circulating tumor DNA (ctDNA) is emerging as a promising biomarker for postoperative monitoring of cancer patients. Precise cell-free DNA (cfDNA) tumor fraction estimation is crucial for evaluating treatment effects and timely detection of disease recurrence during the follow-up period. All current ctDNA detection methods that utilize whole-genome sequencing (WGS) data rely on the reference genome alignment of sequencing reads and often apply separate frameworks for detection of different variant types. However, confounded short-read alignment by heuristic approaches and combination of different alignment and variant calling tools can produce discordant results. The aim of this project was to develop a reference-free strategy that identifies tumor-specific somatic variation from cfDNA to detect ctDNA. The approach is not limited to specific genomic regions and can simultaneously capture different somatic variation types from raw sequencing reads.

Methods: The developed approach creates a set of k-mers (DNA sequences of length k) which are expected to be unique to the tumor genome from primary tumor WGS data. Unique tumor k-mers are identified by filtering out germline k-mers and setting constraints on the k-mer counts and sequence composition. Subsequently, unique tumor k-mers are searched for in the cfDNA samples to identify candidate ctDNA k-mers. Finally, tumor fraction is estimated by probabilistic modeling of candidate ctDNA k-mers' origin and count.

Results: We tested the method on postoperative plasma cfDNA from 31 stage III colorectal cancer patients with three years of follow-up. The tumor k-mer counting approach detected ctDNA in 76% (13/17) patients. For nine of these patients, ctDNA was detected earlier than clinical recurrence (mean 11 months), as detected by standard-of-care radiological follow-up.

Conclusion: In conclusion, the developed method is a novel reference-free approach to detect and quantify ctDNA based on tumor-specific variation presence in cfDNA and monitor cancer patients noninvasively for postoperative minimal residual disease.

P09.022

A comparison of statistical ctDNA detection methods on deep targeted panel-sequencing data

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Background: Circulating tumor DNA (ctDNA) can serve as an indicator of tumor burden in cancer patients, but its low abundance at minimal tumor loads makes detection difficult.
This study aims to evaluate ctDNA detection strategies using deep-targeted panel sequencing data, focusing on the DREAMS and Shearwater methods. Both methods identify cancer mutations by comparing mismatches against an error model. DREAMS employs a neural network for mismatch prediction using a rich set of features, while Shearwater uses error frequencies from a panel of normals.
Shearwater was originally designed for low-frequency cancer variant detection. We use it for ctDNA detection by integrating evidence across multiple variants and adapting it to include Insertions and Deletions (INDELs). We are currently extending DREAMS to also handle INDELs.

Methods: The performance was assessed using synthetic and deep-targeted sequencing data derived from Pre-Operative (pre-OP) plasma samples of CRC patients. The training included stage I-IV CRC patients (n=126) and controls (n=37), while the validation included stage I-III CRC patients (n=255) and controls (n=24).

Results: Initial results on synthetic data at six different allele frequency ranges show that DREAMS and Shearwater both achieve an Area Under the Curve (AUC) exceeding 0.95 in the Receiver Operator Characteristics (ROC) curve down to an allele frequency of 0.05%. DREAMS obtains higher AUC values for the ROC curve at all allele frequency ranges than Shearwater.
In the training cohort, Shearwater currently outperforms DREAMS in ctDNA detection, with a higher AUC (0.90 vs 0.87) and sensitivity (80% vs 76%) at 95% specificity. However, incorporating INDELS and other changes to DREAMS will likely improve its performance.

Conclusion: Both methods demonstrated exemplary performance in separating cancer from controls, with current results favoring Shearwater, though DREAMS' performance is expected to improve with the incorporation of INDELS.

P09.023

Enhancing Cancer Detection: Machine Learning Insights from Fragmentation Patterns of Plasma Cell-free DNA

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Cell-free DNA (cfDNA) offers a non-invasive avenue for cancer detection. cfDNA fragmentation patterns serve as a valuable resource that can be harnessed through machine learning methods. However, despite the promise for early cancer detection and intervention, the sensitivity and flexibility of existing methods remain limited. We aim to enhance cancer detection sensitivity through machine learning classifiers by gaining insights into the characteristics of cfDNA molecules.

Our study focuses on developing supervised machine learning classifiers for cancer diagnosis using cfDNA fragment-end patterns. These patterns, derived from the orientation of cfDNA fragment-ends in relation to the reference genome, serve as distinctive genomic signatures distinguishing cancer from control samples. We specifically extract these patterns from open-chromatin regions, labeling cancer sample patterns as 1 and control sample patterns as 0. Combining these fragment-end patterns and their corresponding labels within identical regions across both cancer and control samples, we construct a unified dataset. This dataset enables us to train classification models to predict the cancer status of specific genomic regions based on observed fragment-end patterns. The trained model then evaluates an unknown sample by analyzing its fragment-end patterns, generating probabilities for each pattern's likelihood of indicating cancer. Ultimately, sample-level predictions are obtained by applying a threshold to the mean probabilities.

In our initial analysis, we examined a cohort of 28 B-cell lymphoma and seven healthy control samples employing logistic regression, achieving 100% specificity and 85.7% sensitivity. To broaden the scope of our research, we intend to increase both the scale and diversity of our cohort, incorporating a broader range of cancer types. Additionally, we will explore the utilization of alternative machine learning methods, including deep learning, to expedite and improve cancer classification.

Our preliminary results underscore the potential of our method for enhancing cancer diagnosis and uncovering cancer-specific DNA regions, which may indicate tumor-derived signals.

P09.024

FinaleMe: Predicting DNA methylation by the fragmentation patterns of plasma cell-free DNA

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Analysis of DNA methylation in cell-free DNA (cfDNA) reveals clinically relevant biomarkers but requires specialized protocols and sufficient input material that limits its applicability. Millions of cfDNA samples have been profiled by genomic sequencing. To maximize the gene regulation information from existing dataset, we developed FinaleMe, a non-homogeneous Hidden Markov Model (HMM), to predict DNA methylation of cfDNA and, therefore, tissues-of-origin directly from plasma whole-genome sequencing (WGS). We validated the performance with 80 pairs of deep and shallow-coverage WGS and whole-genome bisulfite sequencing (WGBS) data.

P09.025

Quantification of cirDNA using standard qPCR, a novel approach

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Background: Circulating cell free DNA (cirDNA) is an important tool for diagnosis and monitoring of tumours (liquid biopsy). Its quantification using qPCR, however, can be challenging as the amount of target is often very small. Different methods (e.g., Cq, PCR-Miner, pyQPCR) yield different values thus reducing the confidence in the results and limiting the quantitative comparison of data from different laboratories.

Methods: In this work, a comparative analysis of several different quantification methods [1] is presented, with the aim of identifying their advantages and disadvantages. A novel metric that is particularly suitable for the evaluation of low input reactions is also presented. It relies on the variation from the baseline within each single trace and thus does not require a standard curve or other reference measures. Distance metrics such as the Bhattacharyya distance or the Kullback-Leibler divergence will be used to compare the results from different methods. Data for this analysis was acquired by extracting cirDNA from healthy plasma and conducting qPCR detecting endothelial-derived cirDNA.

Results: An analysis of the approaches available for the quantification of cirDNA through qPCR is presented. It includes a quantitative comparison of their results, together with a study of their advantages and limitations. These results are expected to improve robustness and reliability of cirDNA quantification.
A new metric specifically developed for low input reactions is also presented and valiated. It does not require a standard curve or other reference measures, thus overcoming some of the limitations of standard approaches.

Conclusions: This work improves the quantification of cirDNA using qPCR, by supporting researchers in choosing the best method for their data. Free availability of the platform-independent software developed for this work is expected to further encourage the widespread adoption of these methods and increase their benefit to the community.

[1] Pabinger et al (2014) doi: 10.1016/j.bdq.2014.08.002

P09.026

Scalable AWS Cloud Infrastructure for Bioinformatics R&D on High-Throughput Targeted Sequencing: A Focus on Non-Invasive Prenatal Testing (NIPT)

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Bioinformatics research and development (R&D) pipelines are essential for developing novel products that require high-throughput targeted sequencing, such as non-invasive prenatal testing (NIPT) from cell-free DNA (cfDNA). However, bioinformatics R&D pipelines often face challenges such as scalability, reproducibility, and cost-effectiveness. Here, we present a scalable Amazon Web Services (AWS) cloud infrastructure that supports bioinformatics R&D pipelines in the Workflow Description Language (WDL) framework. WDL is a domain-specific language that enables the specification of complex workflows in a human-readable and portable way. Our cloud infrastructure leverages AWS services such as Amazon EC2, Amazon S3, Amazon ECR, AWS Batch, AWS Fargate, and AWS EFS to provide a scalable, reliable, and cost-effective solution for running WDL workflows on the cloud. We demonstrate the performance and benefits of our cloud infrastructure using a case study of an NIPT analysis workflow that requires high-throughput targeted sequencing to informatically separate fetal DNA from maternal background. We show how our cloud infrastructure can handle large-scale data processing, automate workflow execution and monitoring, and optimize resource utilization and cost savings. We also discuss the challenges and future directions of our cloud infrastructure for bioinformatics R&D pipelines in the WDL framework.

P10.001

Genomic origin, fragmentomics and transcriptional correlation of long cell-free DNA molecules in human plasma

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BACKGROUND: Recent studies have revealed an unexplored population of long cell-free DNA (cfDNA) molecules in human plasma using long-read sequencing technologies. However, the biological properties of long cfDNA molecules (> 500 bp) remain largely unknown.

METHODS: We aggregated cfDNA data generated by Pacific Biosciences single-molecule real-time (SMRT) or Oxford Nanopore Technologies sequencing to profile the genomic representations and studied the correlation between long cfDNA molecules and gene transcriptional activity. We examined end frequencies of long molecules derived from regulatory regions. Moreover, we analyzed cleavage profiles surrounding cytosine-phosphate-guanine (CpG) sites to investigate long cfDNA fragmentomics in the context of cancer detection using 87 samples by SMRT sequencing. Additionally, cfDNA data from various nuclease-knockout mice were generated by SMRT sequencing to gain biological insights regarding the role of nucleases in the generation of long cfDNA molecules.

RESULTS: Analysis of plasma cfDNA using long-read sequencing revealed an uneven genomic origin of long molecules. Long cfDNA molecules showed overrepresentation in euchromatic regions of the genome, in sharp contrast to short DNA molecules. We observed a stronger relationship between the abundance of long molecules and mRNA gene expression levels, compared with short molecules (Pearson's r = 0.71 versus -0.14). Moreover, long and short molecules demonstrated distinct fragmentation patterns surrounding CpG sites. Leveraging the cleavage preferences surrounding CpG sites, the combined cleavage ratios of long and short molecules could differentiate patients with hepatocellular carcinoma (HCC) from non-HCC subjects (AUC = 0.874). We further investigated knockout mice in which selected nuclease genes had been inactivated, in comparison with wild-type mice. The proportion of long molecules originating from transcription start sites were lower in Dffb-deficient mice but higher in Dnase1l3-deficient mice, compared to that of wild-type mice.

CONCLUSION: This work provides new insights into the biological properties and potential clinical applications of long cfDNA molecules.

P10.002

Cell-free RNA signatures of myalgic encephalomyelitis/chronic fatigue syndrome

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Background: Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) affects approximately 3 million individuals in the United States alone, subjecting patients to debilitating symptoms of extreme exhaustion and widespread pain that can persist for years. Genetic and environmental factors can trigger the onset of ME/CFS; however, the underlying biological causes remain poorly understood. This knowledge gap leads to significant challenges when diagnosing ME/CFS. Current methods rely on symptom monitoring as well as excluding several other similarly presenting diseases, which frequently results in missed or incorrect diagnoses. Circulating cell-free RNA (cfRNA) offers a potential avenue for biomarker discovery and new diagnostic tools. Recent advancements in liquid biopsies of cfRNA from blood plasma demonstrate potential to diagnose diseases, including cancer and preeclampsia, as well as distinguish between infectious diseases, including COVID-19 and multisystem inflammatory syndrome in children.

Methods: In collaboration with the Center for Enervating NeuroImmune Disease at Cornell, we sequenced plasma cfRNA from 30 ME/CFS patients and 30 age and BMI-matched control participants. The plasma samples were collected before and 24 hours after a cardiopulmonary exercise test.

Results: Initial results identified numerous differentially abundant genes in ME/CFS patients compared to controls. In addition, we will present a novel cfRNA library preparation method which enhances our understanding of the biogenesis of the cell-free transcriptome. This method captures cfRNA in solution while retaining full-length molecules as well as all RNA isoforms.

Conclusion: Our research highlights the utility of cfRNA as an innovative tool for exploring the intricate and dynamic biology underlying diseases such as ME/CFS. We plan to demonstrate that machine learning and modeling techniques applied to this cfRNA data can identify potential disease-specific biomarkers and lead to advancements in ME/CFS diagnostics.

P10.003

Circulating Cell-Free RNA in Blood as a Host Response Biomarker for the Detection of Tuberculosis

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Background: Tuberculosis (TB) remains a leading cause of death from an infectious disease worldwide. This is due in part to a lack of tools to effectively screen and triage individuals with potential TB. Whole blood RNA signatures have been studied as potential biomarkers for TB, but they have fallen short of key diagnostic thresholds. Cell-free RNA (cfRNA) provides a new path into classifying disease since it represents a measure of RNA expression directly tied to cell death from circulating cell-types.

Methods: In this work, we investigated the utility of plasma cfRNA as a host response biomarker for TB. We sequenced cfRNA extracted from the plasma samples of 258 individuals with a cough lasting at least two weeks, who were seen at outpatient clinics in Uganda, Vietnam, and the Philippines. Of these individuals, 145 were diagnosed with microbiologically-confirmed TB. Our analysis of the plasma cfRNA transcriptome revealed hundreds of differentially abundant genes, which were then used to train 15 predictive machine learning models.

Results: The highest performing model led to the identification of a set of genes that had powerful diagnostic accuracy and ROC-AUC values greater than 0.9 across training, test, and a separate validation cohort. This gene signature performed extremely well when compared to the performance of the best-in-class whole blood RNA biosignatures. This gene-signature was also robust to differences in sample collection and geographic location.

Conclusion: Overall, our results demonstrate the utility of plasma cfRNA for the detection of TB and suggest the exciting potential for a point-of-care, gene expression-based assay to aid in early detection of TB.

P10.005

Exploring the Uncharted: Nanopore Sequencing Sheds Light on Long cfDNA and circRNA Isoforms in Cancer

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Background: The cell-free DNA (cfDNA) fragmentome is altered in cancer. Circular RNA (circRNA), a degradation-resistant RNA subtype involved in oncogenesis, exhibits biomarker potential. Due to the limitations of short-read sequencing (SRS), long cfDNA fragments and full-length circRNA isoforms are underexplored.
We aim to use long-read nanopore sequencing (LRS) to explore and characterize long cfDNA and circRNA isoforms in liquid biopsy.

Methods: DNA from plasma of lung cancer patients, controls and xenografted mice, and urine from bladder cancer patients and controls was extracted and processed by LRS and SRS. cfDNA data was processed using our ITSFASTR pipeline: preprocessed reads were mapped to the human reference genome and to the murine reference for xenografts; Somatic copy number aberrations (SCNA) and tumor fraction (TF) were estimated using ichorCNA; Fragmentation analysis was performed using FrEIA, fragmentation-based nucleosome positioning (FNP) analysis with Griffin.
circRNA was analyzed using our Ouro-seq protocol: total-RNA was extracted from urine samples of cervical cancer patients. circRNA was enriched, and cDNA libraries prepared for LRS. circRNA isoforms were called from filtered reads using CIRI-long.

Results: SCNA and TF were detected in 22/30 cancer patients and 0/5 controls, showing high correlation with SRS (SCNA: 0.81, TF: 0.98, $p<0.001$). Using LRS 54.1% of plasma cfDNA fragments were >300bp compared to 5.3% in SRS. 50% of the tumor-derived signal was >300bp, as confirmed by xenografts, with an increasing shift towards shorter sizes in the di- and trinucleosomal peaks. The FNP profile correlated between LRS and SRS.
Using Ouro-seq, we were able to explore full-length circRNA isoforms from the urine of cervical cancer patients.

Conclusion: Our tools allowed ultra-fast (<24h) cfDNA and circRNA analysis from liquid biopsies. Long cfDNA fragments were detected in plasma and urine, with a high proportion of tumor-derived DNA. circRNA isoforms analysis was possible from urine.

P10.006

Detection of Human CytomegaloVirus (hCMV) infection in pregnant women: Towards large-scale first trimester screening using data generated with whole-genome NIPT

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Background: We evaluated the possibility to use sequencing data generated by whole genome noninvasive prenatal testing (NIPT) for the detection of Human CytoMegaloVirus (hCMV) in the maternal blood, to identify pregnancies at risk for maternal-fetal transmission, possibly eligible for treatment to prevent transmission.

Methods: Raw NIPT data from 204,818 unselected pregnant women were coded reanalyzed for the presence of hCMV-cfDNA fragments. Diagnostic microbiologically tests were performed on a subset of both hCMV-cfDNA-positive (n=112) and -negative samples (n=127). Of four additional pregnant women, who presented in the 2nd/3rd trimester with fetuses with severe hCMV-related sequelae, the first trimester NIPT sequencing data were retrospectively studied for the presence of hCMV-cfDNA.

Results: In 0.94% of the samples hCMV-cfDNA fragments were detected, mostly with low viral loads. Of the subset of 112 microbiologically validated samples, 22 showed a relatively high hCMV-cfDNA load. Serology testing suggested a recent primary infection (PI) in 16/22, a past infection in 5/22 and 1/22 was seronegative, presumably a very recent PI without immune response yet. In 16/90 samples with low viral loads, serology data indicated recent PIs too (73/90 past infections and 1/90 seronegative). Recent PIs were not seen in the 127 hCMV-cfDNA-negative samples. In the NIPT data of the four additionally studied women, low viral loads were detected.

Conclusion: hCMV-cfDNA fragments were detected in 0.94% of the NIPT samples. High hCMV-cfDNA loads correlated well with recent PIs, but these were also seen in samples with low hCMV-cfDNA loads. More studies are needed to determine the clinical relevance of the different hCMV-cfDNA loads at this stage of pregnancy, especially with regard to potential treatment to prevent maternal-fetal transmission, but the data of the four women with hCMV-affected fetuses indicate that women with low hCMV-cfDNA loads should be regarded as at risk for maternal-fetal transmission and should receive follow-up testing.

P10.008

Dried blood spot sampling as a minimally invasive and accessible method to analyse ctDNA in cancer in challenging settings

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Background: Dried blood spots (DBS) are emerging as a promising tool in the field of circulating tumour DNA (ctDNA) analysis for cancer detection and monitoring. This minimally invasive sampling method offers several advantages, such as ease, frequency and low costs of collection, simplified shipping and long-term storage. DBS-based ctDNA analyses holds great promise in oncology, especially for applications such as treatment response monitoring and cancer monitoring. In this study, we explore the expanding relevance of DBS in the context of ctDNA, shedding light on its potential to transform cancer diagnostics and management.

Methods: Here we used a multifaceted approach to investigate the utility of DBS in cancer detection and monitoring. Our methods encompassed four key objectives: 1) Profiling multiple cancer types to ascertain optimal ctDNA signals for accurate detection. 2) Conducting DBS collection in resource-constrained, remote locations, such as sub-Saharan Africa, and evaluating their stability under room-temperature shipping conditions. 3) Exploring the feasibility of at-home DBS collection for longitudinal cancer monitoring. 4) Assessing the reliability and robustness of DBS-based cfDNA analysis through diurnal sampling.

Results: Our comprehensive investigation into the applications of DBS for cancer detection and monitoring yielded compelling results. Firstly, we successfully identified distinct ctDNA signals from multiple cancer types, enhancing the potential for accurate cancer detection. Secondly, DBS collected from resource-limited settings demonstrated notable stability during room-temperature shipping, emphasising the feasibility of utilising DBS in remote regions.

Conclusions: This research highlights the promising role of DBS in advancing non-invasive cancer diagnostics and monitoring. The versatility of DBS in capturing ctDNA from various cancer types, coupled with its accessibility in challenging environments, signifies a significant step forward in improving cancer care. These findings open avenues for cost-effective and patient-friendly at-home DBS collection, transforming cancer management strategies while ensuring timely and accurate monitoring.

P10.009

Enriching the Methylome using XR-methylSeq to Classify and Deconvolute Cerebrospinal Fluid and Plasma

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Background: Cerebrospinal Fluid (CSF) derived from the brain provides minimally invasive sampling during a diagnostic evaluation of primary or metastatic brain tumors. Our research revealed the presence of cell-free (cf) circulating tumor DNA in CSF of patients whose cytologic testing and/or flow cytometry yielded inconclusive results. However, our past approaches could detect but not classify the malignancies. Additionally, cfDNA in plasma is highly promising for tumor diagnosis. To allow classification, even with low tumor fraction and fragmented DNA, we created a methylation sequencing platform (XR-methylSeq).

Methods: We benchmarked XR-methylSeq with the K562 cell line (leukemia) and compared the correlation of methylation values between different approaches. Methylation classifiers were applied for 16 cytology-positive CSF samples, incorporating data from public references. We used t-distributed stochastic neighbor embedding (t-SNE) analysis for visualization in R. Additionally, we deconvoluted cell type fractions for CSF and plasma using wgbstools.

Results:
Benchmarks: XR-methylSeq has a 5-fold enrichment of the cell type-specific markers compared with whole genome bisulfite sequencing (WGBS). There is high concordance between XR-methylSeq and WGBS (Pearson's r = 0.97).
CSF: Thirteen cytology-positive CSF samples had copy number aberrations [IQR of estimated tumor fraction, 0.43 (0.41, 0.49)], and 85% of them had concordance between classifications and clinical diagnoses. By deconvolution, 9 cases (100%) of lung malignancies had the correct cancer cell-of-origin based on increased cell type contributions.
Plasma: CfDNA from plasma of a patient with acute liver injury had higher fraction of hepatocyte (22%) than the healthy control (8%).

Conclusions: This research highlights the potential of XR-methylSeq as an enriched methylation profiling method that is useful for cells-of-origin, tumor classification, deconvolution, and liquid biopsy applications.

P10.010

SaferSeqS: adaptation of a high-sensitivity method of mutation detection for cell-free DNA applications in cancer diagnostics

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Background: Circulating tumor DNA (ctDNA), comprising small fragments of DNA, is released into the bloodstream by cancer cells.Prior research has established a correlation between tumour volume and estimated variant allele frequency (VAF), tracking ctDNA VAF levels over time offers valuable insights into the dynamic landscape of cancer. Although common next generation sequencing (NGS) methods can detect somatic genomic tumour alterations at a limit of detection (LoD) of 0.01% VAF, their susceptibility to high PCR error rates and mutations from DNA damage often fail to detect low-frequency driver mutations, which are crucial for early diagnosis and recurrence detection in cancer.

Methods: To overcome this limitation, we employed SaferSeqS, a method previously developed by Cohen et al. (2021), that enhances sensitivity by incorporating identical molecular barcodes in both Watson and Crick strands of template molecules, along with target sequence enrichment through strand-specific PCR using gene-specific amplicons. This approach allowed us to compare mutations from both strands to identify true mutations. We developed custom pipelines and utilized the NGS-PrimerPlex pipeline for the SaferSeqS primer design workflow, expanding the pre-designed gene panel. Simultaneously, we generated whole-genome sequencing libraries from the same DNA molecules for copy number analysis.

Results: We have successfully established SaferSeqS targeted sequencing and whole-genome sequencing libraries using cell lines and plasma DNA.

Conclusion: Our results demonstrate the potential of SaferSeqS for detecting mutations across a larger gene panel while simultaneously acquiring copy number profiles from the same DNA molecules. We plan to apply this method in various clinical studies to improve sensitivity in combined mutation and copy number profiling, thus facilitating early cancer diagnosis and early detection of recurrence.

P10.011

Utilizing Non-Invasive Prenatal Test Sequencing Data Resource for Human Genetic Investigation

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Background: Non-invasive prenatal testing (NIPT) is a molecular diagnostic test that employs ultra-low-pass sequencing of maternal plasma cell-free DNA to detect fetal trisomy. NIPT has been rapidly adopted worldwide due to its high sensitivity, specificity, and safety. With over ten million tests conducted, it has become one of the largest human genetic resources for understanding population genetic variation and its associations with phenotypes.

Methods: We present methods to address the use of very large, very low-depth NIPT genetic resources, including detailed evaluations of analysis protocols. These methods comprise algorithms and software adapted for genetic variation detection, genotype imputation, and genome-wide association analysis.

Results: Our findings demonstrate that, by combining proper probabilistic models and population-specific haplotype reference panels, genotype imputation accuracy can range from 0.7 to 0.9 for genetic variants with alternative allele frequency between 0.01-0.05. This accuracy is achieved at sequencing depths ranging from 0.1x to 0.25x. Furthermore, we can achieve an R-square of greater than 0.9 for the estimation of genetic effect size using data from different sequencing platforms.

Conclusion: These results provide a theoretical and practical basis for using NIPT data resources to advance medical genetic studies, particularly in traits related to maternal and child health.

P10.012

Monitoring circulating tumour DNA levels in dried blood spots from patients with metastatic cancer

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Background: Proof-of-principle studies have shown that circulating tumour DNA (ctDNA) can be detected in minute volumes of blood, even dried blood spots (DBS). Such findings have raised attention to exploring DBS for the purpose of frequent monitoring of ctDNA. Adopting DBS for ctDNA collection and analysis may support novel clinical trial designs and increase the real-world utility of liquid biopsies, including studies involving time-points at which traditional phlebotomy is not feasible.

Objective: We investigate the translational potential of DBS analysing ctDNA from patients with metastatic cancer using tumour-naïve and tumour-informed approaches.

Methods: We collected serial finger-prick DBS, matching double-spun plasma, baseline tumour tissue and matched buffy coat from 10 patients with metastatic oesophageal cancer for DNA extraction. Isolated fpDBS DNA was size selected using cumulative cycles of bead-based purification to remove high-molecular-weight genomic DNA. Subsequently, libraries generated from fpDBS, plasma, and tumour DNA were paired-end whole-genome-sequenced at ~1X, 10X, and 50X, respectively. Using ichorCNA, t-MAD, cfDNAPro, and INVAR2, we analysed the WGS data for somatic copy number aberrations (SCNAs), fragment length, end motifs, tumour fraction, and single nucleotide variants.

Results: We identified SCNAs reflective of the primary tumour in plasma and fpDBS from advanced-stage oesophageal cancer patients and observed changes to levels of those SCNAs after treatment in 6/10 patients. Fragmentomics analyses revealed shorter fragment length of fpDBS compared to matched plasma. Each sample was classified using INVAR2, where tumour-derived mutations were detected and aggregated before being compared across the cohort.

Conclusion: Our data demonstrates that analysis of ctDNA from fpDBS is feasible for measuring tumour burden in this set of metastatic cancer patients. Changes to ctDNA levels measured by fpDBS can reflect disease progression or treatment response. These preliminary results suggest potential that DBS-based ctDNA assays may facilitate novel clinical trials to monitor treatment response and predict disease outcome.

P10.013

Toward characterization of cell-free RNA in human plasma

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Cell-free RNA (cfRNA) in human plasma, originating from both living and dying cells, offers real-time insights into diverse cellular processes throughout the body. While cfRNA shows promise as a non-invasive biomarker to many pathological conditions, its high fragmentation and low concentration in plasma pose analytical challenges. Most studies rely on targeted cfRNA capture, potentially missing crucial biomarkers. We aim to overcome these challenges by developing cheap and simple untargeted cfRNA sequencing pipeline compatible with automation to multisample processing, with complementary computational analysis.

Blood from healthy donors was collected into EDTA tubes and immediately centrifuged to remove cells and debris. cfRNA was extracted from 1 ml of fresh plasma using a standard commercial kit. Our RNA-seq protocol, inspired by scRNA-seq protocols, involves RT with template switch followed by second-strand synthesis with klenow, PCR and ribo-depletion. Both unique molecular identifiers (UMI) and sample specific barcodes are added during the RT reaction, allowing early pooling and accurate counting of molecules. This protocol is significantly cheaper than a standard commercial kit and is easy to perform on a large number of samples. In parallel, we prepared cDNA libraries using a commercial kit to compare between the methods.

Early results show that the home-in protocol yields close to comparable libraries. As shown in previous studies, the most abundant genes in cfRNA are of red blood cells (HBA1, HBA2, HBB). However, we identified several tissue-specific genes not expressed by blood cells.

While challenges persist, cfRNA holds substantial promise as a multi-dimensional biomarker that can shed light on ongoing processes within the body and provide valuable information for a range of conditions. Our high-throughput cfRNA sequencing pipeline is crucial for systematic exploration of this promise. Our preliminary findings highlight tissue-specific gene expression in cfRNA from healthy donors, underscoring its potential significance.

P10.014

Detecting circulating tumour DNA in archived whole blood samples

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Background: The detection of circulating tumour DNA (ctDNA) through liquid biopsy has primarily focused on plasma derived from the blood of cancer patients. However, collecting plasma requires trained personnel, specific equipment, and several millilitres of whole blood for plasma isolation and subsequent storage at -80°C. In this study, we aimed to explore the possibility of detecting ctDNA in archived whole blood samples from cancer patients.

Methods: We analysed ten samples collected more than ten years ago from melanoma patients, extracting DNA from whole blood ranging from 1mL to 50uL (representing a few drops of blood). Additionally, we investigated the potential for detecting tumour DNA prior to clinical diagnosis by analysing archival samples collected from high risk women who later developed breast cancer. Libraries from extracted DNA were submitted for paired-end, shallow whole genome sequencing (sWGS). We analysed the data for somatic copy number aberrations (SCNAs) and single nucleotide variants (SNVs) using in-house developed bioinformatics tools.

Results: In DNA extracted from archived whole blood samples, we detected SCNAs that reflected the profile observed in matched plasma DNA and tumour tissue samples collected from the same individuals. We will further report on evaluating SNVs analysis in archival blood samples collected before diagnosis, using a tumour-informed sequencing approach.

Conclusion: Our data proves that ctDNA can be detected from whole blood collected without plasma isolation and demonstrates the potential to analyse ctDNA in archived blood collection. Archival collections can offer opportunities to evaluate the potential to detect ctDNA before clinical diagnosis, which has implications for liquid biopsy applications. Compared to conventional protocols based on plasma cfDNA, the proposed process is novel, simple, and requires minimal volume of blood.

P10.015

Multiomics characterization of cell type repertoires for urine RNA liquid biopsies

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Urine is assayed alongside blood in medicine, yet current clinical diagnostic tests utilize only a small fraction of its total biomolecular repertoire, potentially foregoing high-resolution insights into human health and disease. In this work, we characterized the joint landscapes of transcriptomic and metabolomic signals in human urine. We also compared the urine transcriptome to plasma cell-free RNA, identifying a distinct cell type repertoire and enrichment for metabolic signal. Untargeted metabolomic measurements identified a complementary set of pathways to the transcriptomic analysis. Our findings suggest that urine is a promising biofluid yielding prognostic and detailed insights for hard-to-biopsy tissues with low representation in the blood, offering promise for a new generation of RNA liquid biopsies.