NEW FRONTIERS IN LIQUID BIOPSIES

Data, Technology and Translational Potential

MAY 13–14, 2024

NATIONAL INSTITUTES OF HEALTH
9000 Rockville Pike
Bethesda, Maryland 20892
Natcher Conference Center, Bldg 45
Ruth L. Kirschstein Auditorium

Agenda | Poster Guide | Abstract Book

Agenda

New Frontiers in Liquid Biopsies:
Data, Technology and Translational Potential

May 13–14, 2024

Monday, May 13, 2024

Registration
8:00am–3:00pm
Lower level lobby

Coffee and tea
Supported by Foundation Medicine, Inc.

Bag storage available in Room D

Session 1: Welcome and opening keynote lecture
8:30–9:15am
Ruth L. Kirschstein Auditorium
Session chairs: Sean Agbor-Enoh, Esta Sterneck and Adam Sowalsky

8:30 **Introductory remarks**
Adam G. Sowalsky, Ph.D., National Cancer Institute, NIH

8:35 **Welcoming remarks**
James Gulley, M.D., Ph.D., Clinical Director, National Cancer Institute, NIH; *Introduction by Esta Sterneck, Ph.D., National Cancer Institute, NIH*
Richard Childs, M.D., Scientific Director, National Heart, Lung and Blood Institute, NIH; *Introduction by Sean Agbor-Enoh, M.D., Ph.D., National Heart, Lung and Blood Institute, NIH*

8:45 **Keynote Lecture: Improving cancer liquid biopsies through fragmentomics and population studies**
Dennis Lo, Ph.D., Professor of Chemical Pathology, The Chinese University of Hong Kong, 2022 Recipient of the Lasker-DeBakey Clinical Medical Research Award; *Introduction by Sean Agbor-Enoh, M.D., Ph.D., National Heart, Lung and Blood Institute, NIH*
9:05 Discussion

Session 2: Education session – foundations in liquid biopsies
9:15–10:30am
Ruth L. Kirschstein Auditorium
Session chairs: Bruna Pellini and Sarven Sabunciyan

9:15 **Foundations in placental liquid biopsies**
Angie Jelin, M.D., Associate Professor of Gynecology and Obstetrics, Johns Hopkins

9:30 **Principles and applications of RNA liquid biopsy**
Iwijn De Vlaminck, Ph.D., Associate Professor of Biomedical Engineering, Cornell University

9:45 **Plasma-derived extracellular vesicle and particle proteins as biomarkers for cancer diagnostics and prognostics**
David Lyden, M.D., Ph.D., Professor of Pediatrics, Weill Cornell Medical College

10:00 **Tapping into the genome: Foundations of CSF circulating tumor DNA liquid biopsy in CNS tumors**
Alexandra Miller, M.D., Ph.D., Associate Professor of Neurology, NYU Langone

10:15 **Foundations in ctDNA testing after curative-intent treatment in NSCLC**
Bruna Pellini, M.D., Assistant Member of Thoracic Oncology, Moffitt Cancer Center

Break
10:30–11:00am
Lower level lobby

Coffee and tea break, with light refreshments
Supported by Foundation Medicine, Inc.

Session 3: Liquid biopsies of cells and extracellular vesicles in circulation
11:00–1:00pm
Ruth L. Kirschstein Auditorium
Session chairs: Ignatia Barbara Van den Veyver and Esta Sterneck

11:00 **Extracellular vesicles and CTCs in lung cancer, clinical application**
Christian Rolfo, M.D., Professor of Hematology and Oncology, Icahn School of Medicine at Mount Sinai
11:20 Discussion

11:30 **Intact fetal cell-based and cell-free DNA-based approaches for non-invasive prenatal screening and testing**
Ignatia Barbara Van den Veyver, M.D., Professor of Obstetrics and Gynecology and Molecular and Human Genetics, Baylor College of Medicine
11:50 Discussion

12:00 **The utility of liquid biopsy in immuno-oncology: an emphasis on circulating tumor cells**
Catherine Alix-Panabières, Ph.D., Professor of Medicine, University Medical Centre of Montpellier
12:20 Discussion

12:30 **An indocyanine green-based liquid biopsy test for circulating tumor cells for pediatric liver cancer**
Sarah Woodfield, Ph.D., Assistant Professor of Surgery, Baylor College of Medicine
12:40 Discussion

12:45 **Circulating tumor extracellular vesicles to monitor metastatic prostate cancer genomics and transcriptomic evolution**
Irene Casanova Salas, Ph.D., La Caixa Junior Leader Fellow, Vall d'Hebron Institute of Oncology
12:55 Discussion

Break
1:00–3:00pm

1:00–2:00 **Boxed lunch, supported by Foundation Medicine, Inc.**
Lower level lobby

1:00–3:00 **Poster session**
Upper level atrium

1:15–2:00 **Lunch-table career discussions (advance sign-up required)**

Physician scientist in prenatal genetics
Angie Jelin, M.D., Associate Professor of Gynecology and Obstetrics, Johns Hopkins
Room C1/C2 combined

Thoracic medical oncologist and clinical/translational investigator at a large academic cancer center (clinical trials development and ctDNA applications in lung cancer)

Bruna Pellini, M.D., Assistant Member of Thoracic Oncology, Moffitt Cancer Center

Room F1/F2 combined

Genomic scientist in clinical trial correlative research

Alexander Wyatt, Ph.D., Associate Professor in Urologic Sciences, University of British Columbia

Room G1/G2 combined

Session 4: Development of liquid biopsy technologies

3:00–5:00pm

Ruth L. Kirschstein Auditorium

Session chairs: Alexandra Miller and Aaron Newman

3:00 **TEMPO: Integrating liquid biopsy proteomics with gene expression data**

Vinit B. Mahajan M.D., Ph.D., Professor of Ophthalmology, Stanford University

3:20 Discussion

3:30 **Leveraging AI in Nanotechnology for Precision Liquid Biopsies and Diagnosis**

Tony Y. Hu, Ph.D., F-NAI, F-AIMBE, Distinguished Professor of Biochemistry and Molecular Biology, Biomedical Engineering, and Microbiology, Tulane University

3:50 Discussion

4:00 **Solid and liquid determinants of cancer immunotherapy response**

Aaron Newman, Ph.D., Assistant Professor of Biomedical Data Science, Stanford University

4:20 Discussion

4:30 **Genome-wide cfDNA fragmentation patterns in cerebrospinal fluid informs medulloblastoma subtypes**

Venkata Yellapantula, Ph.D., Assistant Professor of Clinical Pathology, Keck School of Medicine of USC

4:40 Discussion

Tuesday, May 14, 2024

Registration
8:00am–3:00pm
Lower level lobby

Coffee and tea
Supported by Foundation Medicine, Inc.

Bag storage available in Room D

Session 5: Clinical implementation of cell free DNA technologies
8:00–10:30am
Ruth L. Kirschstein Auditorium
Session chairs: Aadel Chaudhuri and Trevor Pugh

8:00 **International R&D for ctDNA guided therapies for solid tumors: A clinical investigator's perspective**
Bob Li, M.D., Associate Professor of Medicine, Memorial Sloan Kettering Cancer Center and Weill Cornell Medical College
8:20 Discussion

8:30 **Multi-analyte and multi-modal cell-free DNA analysis to detect cancer early**
Aadel Chaudhuri, M.D., Ph.D., Associate Professor of Radiation Oncology, Mayo Clinic
8:50 Discussion

9:00 **Hereditary cancer surveillance using multi-modal cell-free DNA sequencing**
Trevor Pugh, Ph.D., FACMG, Senior Investigator, Ontario Institute for Cancer Research
9:20 Discussion

9:30 **Applying genomics to address health disparities in organ transplants**
Hannah Valantine, M.D., Professor of Medicine, Stanford University
9:50 Discussion

10:00 **The NIH IDENTIFY study: Do unusual prenatal cfDNA sequencing results act as a liquid biopsy to detect maternal malignancies?**
Diana Bianchi, M.D., Director, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH
10:20 Discussion

Break
10:30–11:00am
Lower level lobby

Coffee and tea break, with light refreshments
Supported by Foundation Medicine, Inc.

Session 6: Liquid biopsies - beyond genomics
11:00am–12:30pm
Ruth L. Kirschstein Auditorium
Session chairs: Julia Burnier and Simon Heeke

11:00 **Epigenomic features in ctDNA from metastatic prostate cancer**
Alexander Wyatt, Ph.D., Associate Professor in Urologic Sciences, University of British Columbia
11:20 Discussion

11:30 **Cystatin SN as an innovative salivary biomarker for periodontitis: a controlled pilot study**
Francesco Franco, Ph.D. candidate in Experimental Medicine and Therapy, University of Turin
11:40 Discussion

11:45 **Dissecting the circulating proteome in NF1-associated peripheral nerve sheath tumors**
Taylor Sundby, M.D., Assistant Research Physician, National Cancer Institute
11:55 Discussion

12:00 **Everything everywhere all at once: evolving ocular liquid biopsies**
Liya Xu, Ph.D., Assistant Professor, Keck School of Medicine of USC
12:10 Discussion

12:15 **Enrichment of tumor-associated fragmentomic features detected in cell-free DNA with sonobiopsy in glioma patients**
Pradeep Chuahan, Ph.D., Staff Scientist, Washington University School of Medicine in St. Louis
12:25 Discussion

Break
12:30–2:00pm

12:30–1:30 **Boxed lunch, supported by AstraZeneca**
Lower level lobby

12:30–2:00 **Poster session**
Upper level atrium

12:45–1:30 **Lunch-table topical issue discussions (advance sign-up required)**

International multi-stakeholder collaboration on liquid biopsy
Bob Li, M.D. (Memorial Sloan Kettering)
Room F1/F2 combined

At the edge of innovation: Leveraging insights of molecular residual disease to propel precision medicine
David Fabrizio (Foundation Medicine) and Diana Vega, Ph.D. (AstraZeneca)
Balcony C

Circulating tumor cells interest lunch group (no sign-up required)
Esta Sterneck, Ph.D. (NCI) and Catherine Alix-Panabières, Ph.D. (University of Montpellier)
Room G1/G2 combined

Session 7: Late breaking topics in liquid biopsy research
2:00–3:00pm
Ruth L. Kirschstein Auditorium
Session chairs: David Takeda and Kevin Gerrish

2:00 **Circulating, cell-free methylated DNA reveals cellular sources of graft injury after liver transplant**
Megan McNamara, M.D./Ph.D. candidate, Georgetown University School of Medicine
2:10 Discussion

2:12 **COVID-19 proteins in extracellular vesicles are linked to immune, coagulation, and cardiovascular complications and poor outcomes in severe disease patients**
Diego de Miguel Perez, Ph.D., Postdoctoral Fellow, Icahn School of Medicine at Mount Sinai
2:22 Discussion

2:24 **Cell-free DNA methylation signatures: bridging the gap in ALS biomarker discovery**
Yulin Jin, Ph.D., Postdoctoral Fellow, Emory University School of Medicine
2:34 Discussion

2:36 **Combined donor- and recipient- cell-free DNA signatures provide accurate detection of cardiac allograft rejection**

Temesgen Andargie, Ph.D., Postdoctoral Fellow, National Heart, Lung, and Blood Institute, NIH

2:46 Discussion

2:48 **Detecting small cell transformation in patients with EGFR mutant lung adenocarcinoma through epigenomic cfDNA profiling**

Jacob Berchuck, M.D., Assistant Professor of Medicine, Harvard Medical School

2:58 Discussion

Session 8: Panel discussion: The future of liquid biopsies

3:00–4:00pm

Ruth L. Kirschstein Auditorium

Session moderators: Dennis Lo and Sean Agbor-Enoh

3:00 **Panel discussion**

Alexander Wyatt, Ph.D., Associate Professor in Urologic Sciences, University of British Columbia

Diana Bianchi, M.D., Director, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, NIH

Ignatia Barbara Van den Veyver, M.D., Professor of Obstetrics and Gynecology and Molecular and Human Genetics, Baylor College of Medicine

Philip Castle, Ph.D., M.P.H., Director, Division of Cancer Therapeutics, National Cancer Institute, NIH

3:50 Audience Q&A

Conference co-chairs
Sean Agbor-Enoh, M.D., Ph.D.; National Heart, Lung, and Blood Institute
Aadel Chaudhuri, M.D., Ph.D.; Mayo Clinic
Alexandra Miller, M.D., Ph.D.; NYU Langone
Aaron Newman, Ph.D.; Stanford University
Bruna Pellini, M.D.; Moffitt Cancer Center
Antoinette Perry, Ph.D.; University College Dublin
Adam Sowalsky, Ph.D.; National Cancer Institute
Esta Sterneck, Ph.D.; National Cancer Institute
Ignatia Barbara Van den Veyver, M.D.; Baylor College of Medicine

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Hallie Gaitsch, B.S.: National Institute of Neurological Disorders and Stroke
Kevin Gerrish, Ph.D.; National Institute of Environmental Health Sciences
Marina Sharifi, M.D., Ph.D.; University of Wisconsin
Sarven Sabunciyan, Ph.D.; Johns Hopkins University

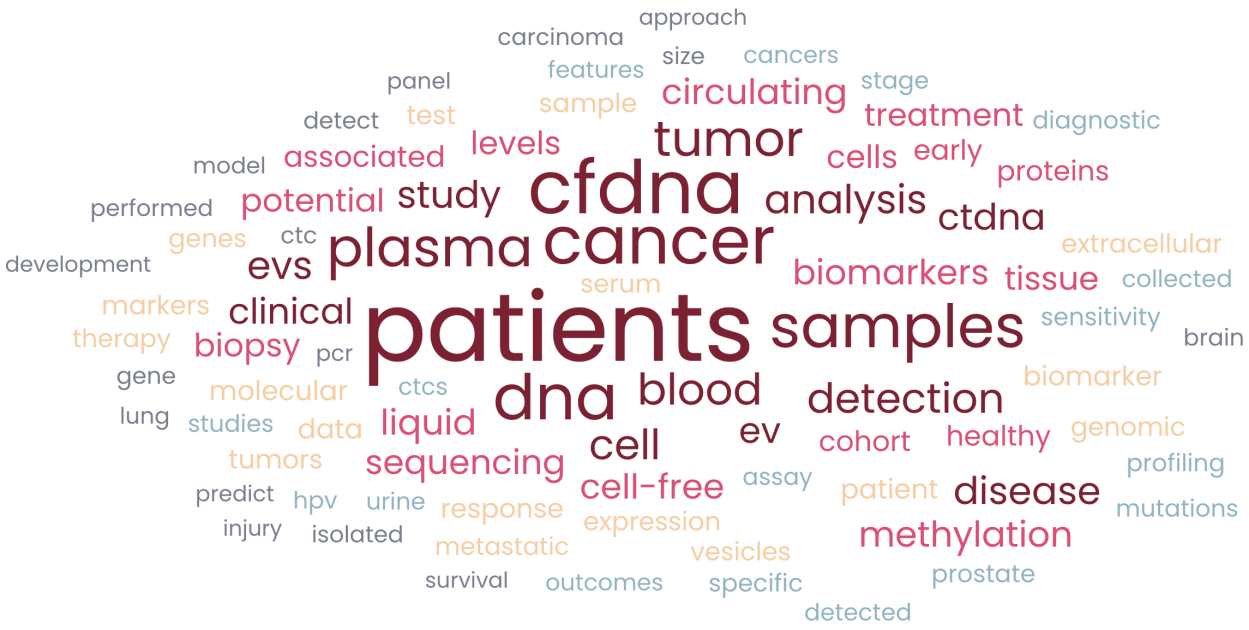
Keynote Lecture

Yuk Ming Dennis Lo, Ph.D.
2022 Recipient of the Lasker-DeBakey Clinical Medical Research Award
Professor of Chemical Pathology, The Chinese University of Hong Kong

"Improving cancer liquid biopsies through fragmentomics and population studies"

Cell-free DNA (cfDNA) in plasma offers a relatively noninvasive source of materials for the detection and monitoring of cancer. cfDNA consists largely of short fragments of DNA. Recently, it has been realized that the fragmentation process of cfDNA is non-random. The study of such a fragmentation process and the development of biomarkers associated with this process is called fragmentomics. Our laboratory has developed a number of fragmentomics cfDNA markers, including end motifs, jagged ends and fragmentomics-based methylation analysis (FRAGMA). The applications of cfDNA fragmentomics in cancer liquid biopsy will be discussed. We have also tested the feasibility of cfDNA-based cancer screening using population-screening of nasopharyngeal carcinoma (NPC) as a model system. We have conducted a large-scale study of over 20,000 subjects in Hong Kong, using plasma Epstein-Barr virus (EBV) DNA as a cfDNA marker. This study has demonstrated that earlier cancer detection and reduction in cancer-associated diagnoses are achievable through cfDNA-based screening.

Posters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	patients reveals novel mediators of early disease progression and therapeutic resistance		

#001

GENE-GENE FUSIONS AS BIOMARKERS FOR PERSONALIZED THERAPY IN GLIOBLASTOMA MULTIFORME BY MEANS OF LIQUID BIOPSY

GIWA OD[1], BAUM G[1], SIEGAL T[2], FRENKEL-MORGENSTERN F[1]
[1]Cancer Genomics and BioComputing of Complex Diseases Lab, The Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel
[2]Rabin Medical Center, Petah Tikvah, Israel

Fusions or chimeric transcripts are a result of the slippage of exons or introns from two different genes. These fusions can be generated by mechanisms such as cis-splicing, trans-splicing, and chromosomal aberrations. Over the years, the fusions were discovered to be biomarkers and therapeutic targets in cancers. Liquid biopsy is a non-invasive diagnostic technique that uses circulating cell-free DNA (cfDNA) with an exciting potential for a real-time molecular profiling of patients. This approach includes the analysis of circulating nucleic acids that are shed from primary tumors and their metastatic sites into peripheral blood. In this study, we intend to validate special fusions in glioblastoma using several methods such as targeted deep sequencing using hybridization panels, Polymerase Chain Reaction (PCR) assays and Sanger sequencing. It will help us to understand the sensitivity and specificity of the targeted sequencing method for fusion detection and biomarkers potential for glioblastoma diagnostics such as mutations in IDH, EGFR, FGFR3, RB1, PTEN etc. Furthermore, we will use the liquid biopsy technique to follow patients' response to therapy and investigate the effect of therapeutic intervention on cfDNA levels in Glioblastoma cell line medium. We developed a computational method for detecting unique human chimeras called Chimeric Transcripts in High-throughput sequencing data (ChiTaH). This was validated further using molecular biology techniques. We also performed a correlation analysis between cfDNA concentration and MRI parameters in glioblastoma tumor progression to follow patients' response to therapy. We discovered a low negative correlation between cfDNA concentration and MRI parameters that will be further investigated. Validated fusions such as, FGFR3-TACC3, EGFR-SEPT14 can be used for novel drug targets and Precision Medicine for Glioblastoma patients as drug targets to improve the patient's survival outcomes in the future.

#003

THE EMERGING ROLE OF CTRNA IN LIQUID BIOPSY – INSIGHTS FROM TWO CLINICAL TRIALS IN ONCOGENE-DRIVEN NON-SMALL CELL LUNG CANCER

HEEKE S[1], GANDHI S[2], TRAN HT[1], LE X[1], VOKES N[1], ANTONOFF M[3], LAM VK[4], SAAD MB[5], Pek M[6], NGEOW KC[7], WU J[1,5], ZHANG J[1], HEYMACH JV[1], ELAMIN YY[1]

[1]Department of Thoracic Head & Neck Medical Oncology, UT MD Anderson Cancer Center, Houston, USA
[2]Department of Radiation Oncology, UT MD Anderson Cancer Center, Houston, USA
[3]Department of Thoracic and Cardiovascular Surgery, UT MD Anderson Cancer Center, Houston, USA
[4]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins, Baltimore USA
[5]Department of Imaging Physics, UT MD Anderson Cancer Center, Houston, USA
[6]Lucence Health, Palo Alto, USA
[7]Lucence Diagnostics, Singapore

Background: Liquid biopsies have revolutionized clinical care in patient with non-small cell lung cancer (NSCLC) and circulating-tumor RNA (ctRNA) is increasingly used, especially for the detection of gene fusions while ctRNA expression is increasingly investigated. To understand how integration of ctRNA in liquid biopsies can improve clinical care, we analyzed longitudinal plasma samples from two clinical trials of patients with *ALK* fusions and *EGFR* mutations.
Methods: We retrospectively analysed 134 plasma samples from two clinical trials: BRIGHTSTAR (NCT03707938) enrolled 34 patients with confirmed *ALK* rearrangement. We used a targeted NGS assay that analyses ctDNA and ctRNA to detect gene rearrangements and mutations in 80 genes. CROSSOVER (NCT04479306) enrolled 38 patients with *EGFR* mutations with resistance to osimertinib and we used an updated version of the same assay that includes the analysis of ctRNA expression of 165 genes. Up to 5ml of plasma was utilized in both trials.
Results: In BRIGHTSTAR, *ALK* fusions were detected in 15/28 patients (54%) at baseline, of which eight were detected in both ctDNA and ctRNA, four were detected in ctDNA only, and three in ctRNA only. *ALK* fusions were detected exclusively in ctRNA in two patients before LCT, while *ALK* fusions were cleared after LCT in all patients. The detection of *ALK* fusion at baseline by either ctRNA or ctDNA was associated with significantly worse progression-free survival (P=0.033; HR (95%CI) ctDNA: 5.0 (1.0-24); ctRNA: 3.1 (0.6-16)).
In CROSSOVER, ctRNA expression demonstrated varying correlation to tissue expression, with 19/165 (12%) of genes with absolute spearman $\rho > 0.5$ and PECAM1 (ρ=0.82; P=0.0012) and CXCR3 (ρ=0.79; P=0.0024) demonstrating the highest correlation to tissue. We further performed differential ctRNA gene expression analysis for patients with and without brain metastasis. *CXCL9* was the most significant differentially expressed gene (P=0.0012) with higher expression in NSCLC patients without brain metastasis suggesting a role of ctRNA for differentiation of metastatic sites.
Conclusions: The addition of ctRNA to liquid biopsies increased the detection of *ALK* rearrangements, and the detection of *ALK* fusion transcripts at baseline was associated with significantly worse progression-free survival, highlighting the added value of ctRNA in detecting gene rearrangements. In addition, although we observed limited impact for the inclusion of ctRNA expression data in this cohort, our data suggest potential avenues for using these data to infer further information about cancer.

#004

CIRCULATING TUMOR EXTRACELLULAR VESICLES TO MONITOR METASTATIC PROSTATE CANCER GENOMICS AND TRANSCRIPTOMIC EVOLUTION

CASANOVA-SALAS I[1], CÓRDOBA-TERREROS S[1], AGUILAR D[1], AGUNDEZ L[1], BRANDARIZ J[1], HERRANZ N[1], SIERRA A[1,2], SORIANO M[3], CRESTA P[1], SUANES M[1,2], HERNANDEZ-LOSA J[2], NONELL L[1], PEINADO H[4], CARLES J[1, 2], MATEO J[1, 2].

[1]Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain

[2]Vall d'Hebron University Hospital, Barcelona, Spain.

[3]Principe Felipe Research Institute (CIPF), Valencia, Spain

[4]Microenvironment and Metastasis Laboratory, Molecular Oncology Program, Spanish National Cancer Research Center (CNIO), Madrid, Spain.

Background: Liquid biopsies enable minimally-invasive molecular characterization of tumors, allowing the study of tumor evolution, and are valuable in supporting clinical decisions in metastatic prostate cancer. Extracellular vesicles (EVs) secreted by tumors are abundant in plasma, but their potential for interrogating the molecular features of tumors through multi-omic profiling remains widely unexplored.

Methods: We present a comprehensive analysis of plasma-derived tumor EV-DNA and EV-RNA by next-generation sequencing (NGS), in metastatic castration resistant prostate cancer (mCRPC). First, we used a range of in-vitro (PC3, LnCap, 22Rv1 and C4-2) and in-vivo models, including patient-derived tumor xenografts (PDXs), to optimize genomic and transcriptomic analysis of DNA and RNA contained in tumoral EVs. Next, we validated our findings in two cohorts of mCRPC patients (n=53) with longitudinal plasma samples collected upon systemic therapies with androgen receptor signaling inhibitors (ARSI) or taxane-based chemotherapy (n=132). Plasma from gender and age-matched healthy volunteers (n=11) and RNA from same-patient PBMCs (n=9) were analyzed as controls. Tumor copy number alterations (CNAs) obtained by low-pass whole genome sequencing (lpWGS) were used to infer tumor fraction (TF). In addition, transcriptomic signatures were obtained from EV-RNA sequencing.

Results: Genomic and transcriptomic profiling of circulating EV-DNA and EV-RNA isolated from *in-vitro* and *in-vivo* models of metastatic prostate cancer (mPC) revealed a high contribution of tumor material to EV-loaded DNA/RNA. These findings were corroborated in two patient cohorts comprising longitudinal plasma samples obtained during ARSI or taxane-based therapy. The genomic features of EV-DNA mirrored those of corresponding patient biopsies and demonstrated associations with clinical progression. Introducing a novel method for transcriptomic profiling of EV-RNA (RExCuE), we report how the circulating EV transcriptome is enriched with tumor-associated transcripts, providing insights into individual patient and tumor characteristics and reflecting on-therapy tumor adaptative changes.

Conclusions:

In sum, we show that EV profiling enables longitudinal transcriptomic and genomic profiling of mPC in liquid biopsy. Our work represents the first evidence for circulating EVs as a biomarker to monitor evolution of tumor genomics and transcriptomics in mCRPC on therapy, allowing the study of drug response and resistance mechanisms and with a unique potential to be developed as clinical biomarker.

#005

DETECTING SMALL CELL TRANSFORMATION IN PATIENTS WITH EGFR MUTANT LUNG ADENOCARCINOMA THROUGH EPIGENOMIC CFDNA PROFILING

BERCHUCK JE[1,2], ZARIF TE[1,2], MEADOR CB[3,4], QIU X[2], SEO J-H[,2], DAVIDSOHN MP[,2], SAVIGNANO H[,2], LAKSHMINARAYANAN G[1,2], MCCLURE HM[1,2], CANNIF J[1,2], FORTUNATO B[1,2], LI R[2], BANWAT MK[3.4], SEMAAN K[1,2,5], EID M[1,2], LONG H[2], HUNG YP[6], MAHADEVAN NR[1,7], BARBIE DA[1], OSER MG[1], PIOTROWSKA Z[3,4], CHOUEIRI TK[1], BACA SC[1,2,5], HATA AN[3,4] , FREEDMAN ML[1,2,5]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA
[2]Center for Functional Cancer Epigenetics, Dana-Farber Cancer Institute, Boston, MA
[3]Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA
[4]Massachusetts General Hospital Cancer Center, Boston, MA
[5]Eli and Edythe L. Broad Institute, Cambridge, MA
[6]Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA
[7]Department of Pathology, Brigham and Women's Hospital, Boston, MA

Histologic transformation to small cell lung cancer (SCLC) is a mechanism of therapeutic resistance in patients with advanced oncogene-driven lung adenocarcinoma (LUAD), which currently requires invasive biopsy for histologic review of tumor tissue for diagnosis. Prior studies suggest that epigenomic reprogramming is a molecular feature of small cell transformation. Herein, we sought to develop a liquid biopsy based on integrated epigenomic analysis of cell-free (cf)DNA methylation, histone modifications, and chromatin accessibility to non-invasively detect small cell transformation in patients with *EGFR* mutant LUAD. We first performed comprehensive epigenomic profiling of LUAD, *de novo* SCLC, and transformed (t)SCLC tumors, which revealed widespread epigenomic reprogramming between LUAD and tSCLC tumors, resulting in a large number of differential H3K27 acetylation (n=24,424), DNA methylation (n=3,298), and chromatin accessibility sites (n=16,352) between the histologic subtypes. We next leveraged these divergent epigenomic profiles to develop a cfDNA-based test to detect small cell transformation in patients with *EGFR* mutant LUAD. To do so, we utilized novel methods to perform genome-wide profiling of three distinct epigenomic features – the histone modification H3K27 acetylation, DNA methylation, and chromatin accessibility – from 1 ml of plasma collected from patients with *EGFR* mutant LUAD (n=20) or *EGFR* mutant tSCLC (n=12). We then developed a classifier to predict the likelihood of small cell transformation based on normalized signal at the SCLC-enriched versus LUAD-enriched sites derived from the differential tumor analysis above. Tumor-informed cfDNA analysis of each individual epigenomic feature resulted in accurate discrimination of patients with *EGFR* mutant LUAD versus tSCLC, with AUROCs of 0.87 for cfDNA H3K27ac acetylation, 0.85 for cfDNA methylation, and 0.82 for cfDNA chromatin accessibility. Comparative analysis of the LAUD- and SCLC-enriched genomic loci from the tumor analysis revealed that biologically informative sites for the three epigenomic features were largely non-overlapping. We therefore evaluated the performance of a multi- analyte classifier integrating cfDNA H3K27 acetylation, methylation, and chromatin accessibility data, which achieved an AUROC of 0.94 for accurate discrimination of patients with *EGFR* mutant LUAD versus tSCLC. In two patients with *EGFR* mutant LUAD with cfDNA samples collected before and aker small transformation, the integrated classifier accurately predicted the presence of tSCLC at the time of the biopsy-proven histologic diagnosis. These data support the immediate potential of epigenomic profiling to detect small cell transformation in patients with *EGFR* mutant LUAD and more broadly demonstrate the ability to non-invasively detect histologic transformation in patients with advanced cancer through multi-analyte epigenomic cfDNA profiling.

#006

MONITORING TREATMENT RESPONSE AND TOXICITY IN IMMUNE-CHECKPOINT-INHIBITOR TREATED METASTATIC MELANOMA FROM CELL-FREE METHYLATED DNA

JAIN SS[1], MCNAMARA ME[1], ALLEY A[1], MCDEED AP IV[1], GIBNEY G[1], ATKINS MB[1], WELLSTEIN A[1]
[1]Georgetown Lombardi Comprehensive Cancer Center, Washington, DC

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. 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. 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. 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.

#007

FRAGMENTOMIC ANALYSIS OF CELL-FREE DNA REVEALS CANCER ASSOCIATED SIGNATURES IN *TP53* MUTATION CARRIERS

WONG D[1], LUO P[1], TAGELDEIN M[1,2], ENSMINGER E[1,2], BRUCE J[1], OLDFIELD L[1], GONG H[3], FISCHER NW[3], LAVERTY B[2,3], SUBASRI V[2,3,4], SCHLIEN A[2,3,7], VILLANI A[2,3,7], KIM RH[1,3,5,6], MALKIN D[2,3,7], PUGH TJ[1,2,6]
[1]Princess Margaret Cancer Center, University Health Network, Toronto, Ontario, Canada
[2]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada
[3]The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada
[4]Vector Institute, Toronto, Ontario, Canada
[5]Toronto General Hospital Research Institute, Toronto, Ontario, Canada
[6]Ontario Institute for Cancer Research, Toronto, Ontario, Canada
[7]Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada

Introduction: Germline *TP53* mutations are the hallmark feature of Li-Fraumeni Syndrome (LFS), a hereditary cancer syndrome associated with a nearly 100% lifetime risk of cancer. Cell-free DNA (cfDNA) is an emerging technology shown to be clinically useful for early detection and disease monitoring. One rapidly developing "omic", fragmentomics, the study of cfDNA length, sequence, and positioning, is based upon the observation that the 3-dimentional chromatin architecture of a cell influences the representation of cfDNA fragments (size and location) released into circulation. While cfDNA has been extensively studied in sporadic cancer, few studies have been performed in the context of hereditary cancer syndromes.
Methods: 169 plasma samples were collected from 82 LFS individuals including cancer-free individuals with no history of cancer (n=58 samples), a previous history of cancer (n=74 samples), and individuals with active cancer (n=37 samples). Healthy *TP53*-wildtype control samples were also included (n=30). Samples underwent shallow whole genome sequencing (sWGS, ~1X coverage). Fragmentomic analyses included fragment size, end-motif, sequence context, fragment-end positioning, and nucleosome positioning.
Results: We find that LFS individuals exhibit an increased prevalence of A/T nucleotides at fragment ends, dysregulated nucleosome positioning at p53 binding sites, and loci-specific changes in chromatin accessibility at development-associated transcription factor binding sites and at cancer-associated open chromatin regions. These observations were independent of cancer status suggesting that germline *TP53* mutations result in an altered cfDNA fragmentomic landscape, likely due to differences in the epigenetic landscape of LFS patients (previously published). LFS patients with an active cancer exhibited further dysregulation cfDNA fragments consistent with those previously observed in sporadic cancers. Using machine learning classification, we were able to robustly differentiate between *TP53* mutant versus wildtype cfDNA samples (AUC-ROC = 0.710–1.000); and by using longitudinal analysis, we established patient-specific baselines that enabled early cancer detection in 8/8 patients, up to 10 months prior to clinical detection through current surveillance methods.
Conclusions: In this study, we show that germline events, such as *TP53* mutation in LFS, can influence cfDNA fragmentation which may have implications in other hereditary cancer syndromes. These fragmentomic signatures may be useful as a diagnostic tool in LFS patients and provides an important baseline for cancer early detection. Further, by using longitudinal samples and establishing patient-specific baselines, we show the utility of cfDNA fragmentomics as an effective tool for early cancer detection in LFS.

#008

CYSTATIN SN AS AN INNOVATIVE SALIVARY BIOMARKER FOR PERIODONTITIS: A CONTROLLED PILOT STUDY

FRANCO F[1], ROMANO F[2], DI SCIPIO F[2], ABBADESSA G[1], BAIMA G[2], AIMETTI M[2], BERTA GN[1]
[1]Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Italy
[2]Department of Surgical Sciences, C.I.R. Dental School, Section of Periodontology, University of Torino, 10126, Turin, Italy

Introduction: Periodontitis constitutes a significant health burden, impacting over one billion individuals worldwide. Unfortunately, this condition is characterized by irreversible damages to the tooth-supporting tissues, even from its initial manifestations. Consequently the necessity for early diagnosis, preceding the appearance of initial symptoms, becomes crucial. In this context, there is a growing recognition of saliva as a valuable tool for liquid biopsy, as it provides a non-invasive means to detect and monitor oral health conditions through the identification of biomarkers. In this regard, the present study aims at the identification of an innovative salivary biomarker whose expression correlates with the oral tissues' health status in the context of periodontitis.
Materials and Methods: In this study, a total of forty-five systemically healthy, non-smoker participants were consecutively recruited. The participants were categorized into three groups based on their oral health status: periodontally healthy conditions (H), severe periodontitis (P), and individuals with a reduction in periodontal tissue subsequent to periodontal treatment (T). Each group consisted of 15 participants. We applied two-dimensional gel electrophoresis followed by MALDI-TOF-MS to compare the salivary proteome of healthy individuals to that of patients with untreated and treated periodontitis. Subsequently, the obtained data were validated by Western Blot analysis.
Results: The salivary proteome of patients with untreated periodontitis showed a marked downregulation in the expression of Cystatin SN compared to the healthy ones; interestingly salivary expression of Cystatin SN is restored in patients who underwent the periodontal treatment. Western blot analysis confirmed the distinctive pattern of Cystatin SN expression observed through the proteomic approach.
Discussion: Cystatin SN is considered one of the most important salivary protease inhibitors therefore its reduction might trigger a higher protease activity in oral tissues leading to the canonical symptoms of the disease. Although our study is preliminary, we can speculate that Cystatin SN might be used as a potential biomarker of periodontitis. The discovery of an innovative salivary biomarker capable of monitoring the periodontal health status can address the unmet clinical needs associated with early diagnosis and therapy management of periodontitis, ultimately leading to improved patient outcomes. Consequently, further investigations should be conducted in order to clarify the molecular process that drives the changes in the salivary expression of Cystatin SN within periodontal diseases.

#009

CELL-FREE DNA TO UNVEIL POTENTIAL MECHANISMS OF RACIAL DISPARITIES IN LUNG TRANSPLANT

SANDERS A[1,3], ANDARGIE T[1,3], ALNABABTEH M[1,3,4], CHARYA A[1,2,3], JANG MK[1,3], KELLER M[1,3,4,8], MATTHEW J[8], ARYAL S[9], KONG H[1,3], PARK W[3], BERRY G[1,8], MARBOE C[1,10], ORENS J[1,8], SHAH P[1,8], NATHAN S*[1,9], VALANTINE H*[1,7], AGBOR-ENOH S*[1,3,8]

[1]Genomic Research Alliance for Transplantation (GRAfT), National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland
[2]Division of Pulmonary and Critical Care, University of Maryland Medical Center, Baltimore, Maryland
[3]Laboratory of Applied Precision Omics, Division of Intramural Research, National Heart, Lung and Blood Institute, Bethesda, Maryland
[4]Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland
[5]National Cancer Institute, Bethesda, Maryland
[6]Wayne State University School of Medicine, Detroit, Michigan
[7]Stanford University School of Medicine, Palo Alto, California
[8]Division of Pulmonary and Critical Care Medicine, The Johns Hopkins School of Medicine, Baltimore, Maryland
[9]Inova Fairfax Hospital, Fairfax, Virginia
[10]Department of Pathology, New York Presbyterian University Hospital of Cornell and Columbia, New York, New York

Background: The mechanisms contributing to the high rates of lung allograft failure in Black than White patients remain poorly defined. Here, we leverage the high sensitivity of cell-free DNA as a sensitive marker, to test if Black patients demonstrate high allograft and recipient tissue injury as a potential mechanism.
Methods: Design: The multicenter cohort of lung transplant recipients, the Genomic Research Alliance for Transplantation (GRAfT), collected serial sample and clinical data. Measure: Pre-transplant donor and recipient DNA was genotyped to identify SNPs. cfDNA was quantified by digital droplet PCR in copies per mL using primers directed at the SNPs for donor-derived (ddcfDNA) and recipient-derived (rdcfDNA) cfDNA or primers directed to mitochondrial DNA for mitochondrial cfDNA (cfmtDNA). Analysis: cfDNA was analyzed as a time-dependent covariate by race using a Linear Mixed Model to adjust for repeated measures within subjects. The average daily post-transplant cfDNA levels per subject was computed as the area under the cfDNA vs. time curve divided by time.
Results: The cohort included 152 White and 37 Black patients. Immediately after transplant surgery, Black patients demonstrated 3,052.776 copies/mL higher ddcfDNA ($p < 0.001$), 24,868 copies/mL higher rdcfDNA ($p=0.003$), and 565,176 copies/mL higher cfmtDNA ($p=0.001$) than White patients. Thereafter, cfDNA levels decayed for all patients. However, Black patients continued to show 2-fold higher average daily dd-cfDNA (Median (IQR)) (462 (185-1285) vs 242 (92-645), $p=0.039$) than White patients; daily rdcfDNA was similar in black and white patients (24,220 (9,163-61,645) vs 21,281 (4,415-76,064), $p=0.3249$). FEV1 was similar for White and Black patients after transplant surgery, and then rise to reach peak levels. Black patients displayed a 0.0059 units/day ($p=0.021$) slower increase in FEV1 reaching lower peak FEV1 than White patients.
Conclusion: Black patients demonstrate elevated allograft and recipient tissue injury than White patients correlating with lower peak FEV1. Studies to identify the mechanisms of the high tissue injury may uncover strategies to mitigate the high rates of allograft failure in Black patients.

#011

AQUEOUS HUMOR LIQUID BIOPSY: A FIVE-YEAR PROGRESS IN ESTABLISHING CLINICAL UTILITY FOR RETINOBLASTOMA DIAGNOSIS, PROGNOSIS, AND THERAPEUTIC OUTCOMES

LIYA XU[1,2,3,4] PATRICK PENG[1], PETER KUHN[4,5], JAMES HICKS[4,5], DAVID COBRINIK[1,2,3,4] JACLYN BIEGEL[3,6], XIAOWU GAI[3,6], VENKATA YELLAPANTULA[3,6] AND JESSE BERRY[1,2,3,4]

[1]The Vision Center at Children's Hospital Los Angeles, Los Angeles, CA

[2]USC Roski Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA,

[3]The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, CA

[4]Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA

[5]Convergent Science Institute in Cancer, University of Southern California, Los Angeles, CA

[6]Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California, USA

Purpose: Retinoblastoma (RB), a prevalent childhood cancer, presents distinct diagnostic challenges, impeding precision medicine due to inaccessible tumor biopsy. Our pioneering five-year research underscores the potential of aqueous humor (AH) as a safer liquid biopsy for RB-specific biomarkers. This prospective study aims to validate AH's clinical utility by establishing associations between cfDNA biomarkers at diagnosis and aggressive intraocular behavior. Routine detection of tumor-derived *RB1* mutations in AH, influencing genetic testing, and exploring AH as a distinct molecular biomarker correlating with treatment outcomes, seeks to revolutionize RB prognosis through personalized treatment decisions.

Methods: This prospective observational study involves AH liquid biopsy at RB diagnosis, with longitudinal sampling through therapy. Tumor-derived cell-free DNA undergoes targeted sequencing for single nucleotide variant (SNV) analysis of the *RB1* gene and shallow whole genome sequencing for detection of somatic copy number alterations (SCNAs). SCNAs, including 6p gain and MYCN amplification, are correlated prospectively with tumor response to therapy and globe salvage.

Results: In the diagnostic AH analysis of 26 eyes (21 patients), 73.1% underwent successful ocular salvage. Mutational analysis identified 23 pathogenic *RB1* variants and 2 deletions. SCNAs were discernible in 69.2% of diagnostic AH samples. Recurrent RB-SCNAs (gain of chr1q, chr2p, chr6p or loss of chr 13qm chr 16q) in 65.4% of eyes spanned diverse intraocular RB groups. Among Group D eyes, 66.7% exhibited 6p gain, significantly associated with intraocular recurrences/enucleation, in contrast to 25% salvaged with therapy. Prognostic markers, 6p gain and/or MYCN focal amplification correlated with overall ocular salvage outcomes.

Conclusions: AH liquid biopsy addresses inaccessible tumor tissue and the absence of prognostic molecular biomarkers in RB. This study underscores AH's promising potential as a companion diagnostic for RB prognostication, paving the way for advancements in personalized pediatric oncology.

#013

COMBINED DONOR- AND RECIPIENT- CELL-FREE DNA SIGNATURES PROVIDE ACCURATE DETECTION OF CARDIAC ALLOGRAFT REJECTION

ANDARGIE T[1], HILL T[2], FU S[3], REDEKAR N[2], PARK W[1], KONG H[1], JANG MK[1], YU. K[3], VALANTINE H[4], AGBOR-ENOH S[1,5]

[1]Genomic Research Alliance for Transplantation (GRAfT) and Laboratory of Applied Precision Omics, National Heart, Lung, and Blood Institute (NHLBI), NIH, Bethesda, MD.
[2]NIAID Collaborative Bioinformatics Resource, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD.
[3]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD.
[4]Department of Medicine, Stanford University, Stanford, CA.
[5]Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, MD.

Background: Donor-derived cell-free DNA (ddcfDNA) is highly sensitive for diagnosis of allograft injury and rejection, however its specificity is imperfect. The cfDNA released from recipient's immune cells that mediate allograft rejection (AR) and its potential for diagnosing AR have not been investigated. We hypothesize that combined donor and recipient immune cfDNA signatures could improve the performance to detect allograft rejection, while also differentiating acute cellular and antibody-mediated rejection phenotypes.
Methods: Plasma cfDNA was isolated and quantified from 76 heart transplant recipients (HTRs) enrolled in the multicenter prospective GRAfT study (NCT02423070) using ddPCR. Shotgun sequencing and whole-genome-bisulfite sequencing were performed to measure ddcfDNA and tissue-specific cfDNA, respectively. LASSO regression model was used to assess the performance of cfDNA features to distinguish AR (ACR grade > 1R and pAMR grade ≥ 1) from non-rejecting stable patients.
Results: A total of 94 AR episodes (45 ACR and 49 AMR) from 52 patients with confirmed endomyocardial biopsy, and 40 samples from 24 stable patients underwent for cfDNA analysis. HTRs with AR had significantly higher ddcfDNA levels as well as cfDNA from recipient immune cells, and vascular endothelium compared to stable controls. We then trained a LASSO regression model with 70% of the data and tested with 30% sets. The combination of donor and recipient cfDNA features had an AUC of 0. 96, compared to ddcfDNA alone (AUC = 0.88). The model achieved the highest performance in detecting AMR phenotypes (AUC: 0.98) compared to ACR (0.95).
Conclusion: These findings suggest that integrating the recipient signatures into ddcfDNA test improves the test performance for diagnosing AR, particularly for AMR. This approach advances the application of cfDNA as a reliable marker to monitor HTRs. Ongoing studies are acquiring samples from independent cohorts to validate our finding and identify cfDNA signatures that best distinguish antibody-mediated rejection from acute cellular rejection.

#014

TUMOR-DERIVED EXTRACELLULAR VESICLES AS A LIQUID BIOMARKER OF TUMOR BURDEN IN PROSTATE CANCER

LUCIEN F[1*], ANDREWS J[2], KIM Y[1], HORJETI E[1], ARAFA A[3,4], GUNN H[5], SUTERA P[6], DE BRUYCKER A[7,8], PHILLIPS R[9], SONG D[6], KIESS A[6], CHILDS D[10], SARTOR O[10], ORME J[10], TRAN P[13*], MERCIER C[11,12*], OST P[7,11*], PARK S[11*]
[1]Department of Urology, Mayo Clinic, Rochester MN, USA
[2]Department of Urology, Mayo Clinic, Scottsdale, AZ, USA
[3]Department of Pharmacology, University of Minnesota, Minneapolis MN, USA
[4]Masonic Cancer, University of Minnesota, MN, USA
[5]Department of Quantitative Health Sciences, Mayo Clinic, Rochester MN, USA
[6]Department of Radiation Oncology and Molecular Radiation Sciences, John Hopkins University, Baltimore, MD, USA
[7]Department of Human Structure and Repair at Ghent University, Ghent, Belgium
[8]Department of Radiation Oncology, AZ Groeninge, Kortrijk, Belgium
[9]Department of Radiation Oncology, Mayo Clinic, Rochester, MN
[10]Department of Medical Oncology, Mayo Clinic, Rochester, MN, USA
[11]Department of Radiation Oncology, Iridium Network, Wilrijk (Antwerp), Belgium
[12]Translational Cancer Research Unit (TCRU), Center for Oncological Research (CORE), University of Antwerp, Edegem (Antwerp), Belgium
[13]Department of Radiation Oncology, University of Maryland Medical System, Baltimore, MD, USA,
[14]Department of Immunology, Mayo Clinic, Rochester MN, USA

Liquid biopsy is emerging as an attractive tool because their accessibility has the potential to reduce health inequities in cancer care by making screening, therapy selection and post-treatment monitoring less burdensome and invasive for patients and health professionals. Extracellular vesicles (EVs) are microscopic vesicles released by all types of cells including tumor cells. They carry cargo (DNA, RNA, proteins) from donor cells which make them attractive biomarkers for non-invasive detection of human diseases. Our multi-disciplinary research team has pioneered the study of tumor-derived EVs as a novel type of cancer liquid biopsy in prostate cancer. Notably, we have developed a standardized methodology based on high resolution flow cytometry to quantify the circulating levels of EVs from few microliters of blood or urine and within 1 hour. By analyzing hundreds of blood and urine specimens, we established the proof-of-concept that blood levels of tumor-derived EVs correlate with tumor burden and predict patient outcome in prostate cancer. In oligometastatic castration-refractory prostate cancer (N=79 patients), we demonstrated that pre-radiotherapy low levels of tumor-derived EVs predict a lower risk of disease progression (median RFS 6.6 months versus 3.5 months, p=0.0087; HR=1.35 (1.03-1.76) p=0.03). In this study, we tested the prognostic and predictive value of tumor-derived EVs in oligorecurrent castration-sensitive prostate cancer treated with stereotactic ablative radiotherapy (SABR). In patients diagnosed with both conventional (bone scan, CT/MRI, N=30) or advanced PET imaging (C11-Choline and Ga68-PSMA, N=127), low concentrations of tumor-derived EVs were found in patients with durable disease-free survival when treated with SABR alone. In contrast, patients with elevated levels of tumor-derived EVs do not benefit from SABR and develop new metastatic lesions shortly after treatment. These findings suggests the presence of micrometastatic disease undetected by imaging at diagnosis and these patients may benefit from treatment intensification with systemic therapy. Tumor-derived EVs is a novel prognostic biomarker of tumor burden and a predictive biomarker of response to SABR in oligometastatic prostate cancer. While the clinical value of tumor-derived EVs remains to be validated in prospective randomized clinical trials, our data supports the potential of EVs as next-generation liquid biomarkers for advanced cancers.

#015

STANDARDIZED REPORTING OF PRE-ANALYTICAL VARIABLES TO ENHANCE RIGOR AND REPRODUCIBILITY IN LIQUID BIOPSY RESEARCH: EXPERIENCE FROM THE ISEV BLOOD TASK FORCE

LUCIEN F[1,2*], GUSTAFSON D[3,4,5*], LENASSI M[6], LI B[7,8], TESKE JJ[1], BOILARD E[9], VON HOHENBERG KC[10], FALCON-PEREZ JM[11], GUALERZI A[12], REALE A[13], JONES JC[14], LÄSSER C[15], LAWSON C[16], NARAZENKO I[17], O'DRISCOLL L[18], PINK R[19], SILJANDER PR[20], SOEKMADJI C[21], HENDRIX A[22], WELSH JA[14], WITWER KW[23], NIEUWLAND R[8]

[1]Department of Urology, Mayo Clinic, Rochester, Minnesota, USA

[2]Department of Immunology, Mayo Clinic, Rochester, Minnesota, USA

[3]Department of Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, Canada; [4]Toronto General Hospital Research Institute, Toronto, Ontario, Canada

[5]Department of Public Health Sciences, Queen's University, Kingston, Ontario, Canada

[6]Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana; [7]Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China; [8]Laboratory of Experimental Clinical Chemistry, and Amsterdam Vesicle Center, Amsterdam UMC, location AMC, Amsterdam, Netherlands

[9]Centre de Recherche du CHU de Québec – Université Laval, Département de microbiologie et immunologie, Faculté de Médecine de l'Université Laval, Québec, Qc, Canada

[10]Translational Lymphoma Research Group, German Cancer Research Center DKFZ, Heidelberg, Germany;

[11]Exosomes laboratory and Metabolomics Platform, CIC bioGUNE, CIBERehd, Bizkaia Technology Park, Derio, Bizkaia, Spain. IKERBASQUE, Basque Foundation for Science, Bilbao, Bizkaia, Spain

[12]IRCCS Fondazione Don Carlo Gnocchi, via Capecelatro 66, 20148, Milan, Italy

[13]Division of Blood Cancers, Monash University - Alfred Health, Melbourne, Victoria, Australia; [14]Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

[15]Krefting Research Centre, Institute of Medicine at the Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

[16]Comparative Biomedical Sciences, Royal Veterinary College, UK

[17]Institute for Infection Prevention and Control, Faculty of Medicine, University of Freiburg, Freiburg, Germany; German Cancer Consortium, Partner Site Freiburg and German Cancer Research Center, Heidelberg, Germany

[18]School of Pharmacy and Pharmaceutical Sciences & Trinity Biomedical Sciences Institute & Trinity St. James's Cancer Institute, Trinity College Dublin, Ireland

[19]Faculty Health and Life Sciences, Oxford Brookes University, Oxford, UK

[20]EV-group, Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, and Drug Research Program, Faculty of Pharmacy, Division of Pharmaceutical Biosciences, University of Helsinki, Helsinki, Finland

[21]School of Biomedical Sciences, Faculty of Medicine, University of Queensland, Brisbane, Australia; [22]Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium

[23]The Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, and Department of Neurology, Baltimore, Maryland, USA.

Blood is the most used body fluid for extracellular vesicle (EV) research. The composition of a blood sample and its derivatives (i.e., plasma and serum) are not only donor-dependent but also influenced by collection and preparation protocols. Since there are hundreds of pre-analytical protocols and over forty variables, the development of standard operating procedures for EV research is very challenging. To improve the reproducibility of blood EV research, the International Society for Extracellular Vesicles (ISEV) Blood EV Task Force proposes standardized reporting of (i) the applied blood collection and preparation protocol and (ii) the quality of the prepared plasma and serum samples. Gathering detailed information will provide insight into the performance of the protocols and in the presence of confounders in the prepared plasma and serum samples. To collect this information, the ISEV Blood EV Task Force created the Minimal Information for Blood EV research

(MIBlood-EV), a tool to record and report information about pre-analytical protocols used for plasma and serum preparation as well as assays used to assess the quality of these preparations. This tool can be easily implemented in existing databases allowing for future meta-analysis and followed by evidence-based optimization of pre-analytical protocols. Taken together, insight into the quality of prepared plasma and serum samples will (i) improve the quality of biobanks for EV research, (ii) allow the exchange of plasma and serum samples between biobanks and laboratories, (iii) facilitate inter-laboratory comparative EV studies, (iv) improve the peer review process, and, finally (v) does not require modifications of established local pre-analytical protocols. The implementation of MIBlood-EV will be driven by the commitment of EV research groups to promote reproducibility by ensuring transparent reporting of blood sample quality and derivatives used in EV studies. To achieve widespread adoption, we recommend the endorsement of the MIBlood-EV by other international societies such as the International Society for Liquid Biopsy. Furthermore, we encourage support from the International Society for Biological and Environmental Repositories, the global biobanking organization in charge of disseminating best practices for biorepositories. By promoting the adoption of the MIBlood-EV, we can improve experimental rigor and reproducibility in EV research, accelerating the translation of discoveries into clinical practice. Finally, this initiative can serve as framework for the future development of standardization and reporting tools specific to other types of liquid biopsy such as circulating tumor DNA and circulating tumor cells.

#016

COMPARATIVE DNA METHYLATION PROFILING OF CIRRHOSIS WITH AND WITHOUT HEPATOCELLULAR CARCINOMA

LIM H[1], AMOS CI[1,2], EL-SERAG H[3], THRIFT AP[1,2]
[1]Section of Epidemiology and Population Science, Department of Medicine, Baylor College of Medicine, Houston, Texas
[2]Dan L Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, Texas
[3]Section of Gastroenterology and Hepatology, Department of Medicine, Baylor College of Medicine, Houston, Texas

Background: Hepatocellular carcinoma (HCC) is a leading cause of death worldwide. In the United States, HCC incidence and mortality have increased rapidly over the past two decades with a <20% 5-year survival rate. Most cases of HCC arise in the background of cirrhosis. DNA methylation has been shown to be associated with gene expression modulation without altering DNA sequence and plays an important role in both physiological and pathological processes. Aberrant DNA methylation occurs in an early phase of carcinogenesis and can be detected in peripheral blood mononuclear cells (PBMCs), which are thought to detect the epigenetic reprogramming of the host immune system during the development of malignant tumors. However, the difference of DNA methylation patterns in PBMC between cirrhosis and HCC is still unclear.
Methods: Twenty-two patients with cirrhosis who later developed HCC (cases) were gender/age-matched with 22 patients with cirrhosis who remained cancer-free during an average 4-year follow-up (controls). PBMCs were isolated from whole-blood samples collected at pre-diagnostic baseline from all cases and controls. All samples were analyzed by Infinium MethylationEPIC microarrays covering ~ 850K CpG sites. We used the Champ (Chip Analysis Methylation Pipeline) package to analyze the methylation comparing PBMC from cases and controls to identify differentially methylated CpGs sites and genes and their representation in specific biological pathways.
Results: In total, 508 differentially methylated probes (DMPs) were identified by comparing HCC patients and cirrhosis patients, with 161 hypomethylated and 347 hypermethylated (p-value < 0.05). Gene set enrichment analysis (GSEA) analysis revealed that the hypermethylated probes were mainly associated with functions such as regulation of nucleobase-containing compound metabolic process and heterocycle biosynthetic process. In contrast, the hypomethylated probes were mainly enriched in the organ system process. Protein-protein interaction analysis identified *RB1* and *HIST1HIE* with key pathways like the p53 pathway (PPI score=0.99, 0.99, respectively). Twenty-six CpG sites were located on promoter regions with p-value < 0.05 and $|\Delta \text{Beta value}| > 0.1$. The top 3 significantly hypomethylated CpG sites on promoter regions were cg04531182, cg10596483, and cg19263494 and corresponded to the *KLRC4-KLRK1*, *JRK,* and *PMF1* genes, respectively. The top 3 significantly hypermethylated CpG sites on promoter regions were cg25460273, cg08425810, and cg22706186 and corresponded to the *PTPRE*, *AGAP2*, and *FAM19A1* genes, respectively.
Conclusions: We identified the methylation landscape of cirrhosis and HCC and provided potentially new methylation markers of progression risk. Comprehensive epidemiologic and epigenetics studies are necessary to validate our findings in larger sample sizes.

#017

EVERYTHING EVERYWHERE ALL AT ONCE: EVOLVING OCULAR LIQUID BIOPSIES

LIYA XU[1,2,3]
[1]The Vision Center at Children's Hospital Los Angeles, Los Angeles, CA
[2]USC Roski Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA,
[3]The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, CA

Purpose: Ocular cancers, including Retinoblastoma (RB) and Uveal Melanoma (UM), demand precise yet unavailable precision medicine. The blood-brain barrier (BBB) limits liquid biopsy efficacy, emphasizing the need for alternative platforms. Aqueous humor (AH) offers a rich molecular composition, enabling comprehensive multi-omics analysis. This transformative research leverages AH liquid biopsy for personalized ocular cancer diagnostics and treatment.
Methods: This multi-center study employs AH to conduct a comprehensive multi-omics analysis in ocular cancers, with Institutional Review Board (IRB) approvals available through USC and CHLA for ethical oversight. Qubit quantifications of DNAs, RNAs, miRNAs, proteins and enumerations of extracellular vesicles (EVs) were correlated with clinical features, carrying implications for downstream clinical applications. Tumor mutations were identified with targeted NGS and somatic copy number alterations (SCNAs) were profiling with shallow WGS. Illumina Infinium BeadArray was employed for methylation profiling of DNAs from both AH and paired tumors. Single Particle Interferometric Reflectance Imaging Sensor (SP-IRIS) was employed for single vesicle resolution tetraspanin profiling. Macsplex, a multiplex bead-based flow cytometry assay, employed 39 capture beads coated with monoclonal antibodies for 37 different EV surface antigens. Single Extracellular Vesicle Nanoscopy (SEVEN) provided super-resolution imaging analysis of EVs from AH. The Proximity Extension Assay (PEA) for comprehensive proteomics, targeting over 3000 proteins, was used for AH proteomics analysis.
Results: dsDNA, ssDNA, miRNA, and proteins were quantifiable in up to 98% of samples. Median dsDNA concentration in RB diagnostic samples was significantly higher than in TX and END samples. UM diagnostic analyte levels correlated with advanced stages. Eyes with tumors <2 mm tall exhibited >70% quantifiable analytes. Post-brachytherapy samples had significantly elevated concentrations. The first and only CLIA-LDT genomic test for RB AH which was launched one year ago offers diagnosis, prognosis, and real-time monitoring of RB clinical management. Methylation profiling identified 245 genes directly regulated by methylation, influencing key pathways such as RB1, p53, p21, p16, and E2F. Single-vesicle resolution tetraspanin profiling revealed a tumor-associated extracellular vesicle (EV) subpopulation with CD63/81 positivity. CD133 emerged as an RB-specific biomarker, while MCSP was identified as a UM-specific biomarker detectable in Aqueous Humor (AH). Proximity Extension Assay (PEA) identified 45 differentially expressed proteins (DEPs) in AH, enabling classification of Gene Expression Profile (GEP) class 1 and 2, highlighting the potential for non-invasive stratification of metastatic risk.
Conclusions: This multi-faceted, multi-center approach establishes a robust foundation for precise diagnostics and personalized treatment strategies in ocular cancers.

#018

META-ANALYSIS OF PUBLICLY AVAILABLE DATASETS FOR THE IDENTIFICATION OF LIQUID BIOMARKERS FOR HIGH-GRADE SEROUS OVARIAN CANCER

JORDAN A[1,2], RODRIGUEZ A[3], MCCABE A[4], DEAN K[4], DAS S[3], PERRY AS[1,2]
[1]School of Biology and Environmental Science, O'Brien Science Centre, University College Dublin, Ireland.
[2]Cancer Biology and Therapeutics laboratory, UCD Conway Institute of Biomolecular and Biomedical Science
[3]School of Pharmacy and Biomedical Sciences, Royal College of Surgeons, Dublin, Ireland.
[4]School of Biochemistry and Cell Biology, Western Gateway 3.91, University College Cork, Ireland.

Background: Globally there are approximately 320,000 cases of ovarian cancer recorded annually. The disease has a relatively low 5-year survival rate at only 35-45% There is neither a screening test for ovarian cancer, nor a test that can detect the disease effectively at an early stage. This results in most cases (75%) being diagnosed at a late stage. When detected early, the 5-year survival rates increase to the 90th percentile. High-grade serous ovarian cancer (HGSOC) is the most common and aggressive form of the disease. The aim of this project is to develop an epigenetic blood and/or urine test which can detect HGSOC at an early stage.
Methods: We identified 22 datasets (140 HGSOC, 23 histologically normal fallopian tube (hnFT), 11 precursor lesions) with methylome data on HGSOC patients. Data were curated, normalised, and filtered to remove probes which hybridised, performed poorly and covered SNPs or non-CpG sites using the *rnbeads* package. Differentially methylated probes (DMPs) were classified as significant if they had a false discovery rate (FDR) p-value <0.05 and minimum mean β-value difference of 0.2. Differentially methylated regions (DMRs)were identified in HGSOC and precursor lesions compared to hnFT using the *DMRcate* package. DMRs were classed significant based on a harmonic mean FDR p- value <0.05, fishers multiple comparison p-value <0.05 and Stouffers score <0.05 and minimum mean β-value difference of 0.1.
Results: Preliminary analysis indicates a trend towards widespread hypomethylation in HGSOC. Comparing HGSOC to hnFT controls yielded 101,997 significant DMPs. Precursor lesions compared to unmatched and matched hnFT controls yielding 100,136 and 29,159 significant DMPs respectively. Of these significant DMPs 11,660 were shown to be consistently differentially methylated between the HGSOC vs hnFT and STIC vs hnFT comparisons. From these 11,660 probes, 447 DMRs were identified, 105 of which were hypermethylated and 342 of which were hypomethylated. Upon annotation within genetic and epigenetic domains, it was observed that the preponderance of hypermethylated regions was situated in 5' regulatory regions and CpG islands, while hypomethylated regions were predominantly associated with intergenic and open sea regions.
Next steps: The methylation of DMPs/DMRs will be analysed *in silico* in urine and blood datasets and other non-ovarian cancer cohorts, to ensure specificity of the DMPs/DMRs in liquid biopsy and ovarian cancer respectively. This will provide us with a panel of potential biomarkers which will then be analysed for their specificity and sensitivity in patient samples.

#019

AN INDOCYANINE GREEN-BASED LIQUID BIOPSY TEST FOR CIRCULATING TUMOR CELLS FOR PEDIATRIC LIVER CANCER

ESPINOZA AF[1], KURETI P[1], PATEL RH[1], DO SL[1], GOVINDU SR[1], ARMBRUSTER BW[1], URBICAIN M[2], PATEL KR[2], LOPEZ-TERRADA D[2], VASUDEVAN SA[1], and WOODFIELD SE[1].

[1]Pediatric Surgical Oncology Laboratory, Divisions of Pediatric Surgery and Surgical Research, Michael E. DeBakey Department of Surgery, Texas Children's Surgical Oncology Program, Texas Children's Liver Tumor Program, Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA.

[2]Department of Pathology and Immunology, Baylor College of Medicine, Texas Children's Department of Pathology, Houston, TX 77030, USA.

Background: Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are the most common malignant hepatocellular tumors seen in children. The aim of this study was to develop a liquid biopsy test for circulating tumor cells (CTCs) for these tumors that would be less invasive and provide real-time information about tumor response to therapy.

Methods: For this test, we utilized indocyanine green (ICG), a far-red fluorescent dye used clinically to identify malignant liver cells during surgery. We assessed ICG accumulation in cell lines (malignant liver: HepG2, Huh-6, Huh-7, Hep3B; malignant non-liver: A549 (lung), SH-SY5Y (neuroblastoma); non-malignant liver: HepRG; non-malignant non-liver: 293T), including one patient-derived cell line (HB17), using fluorescence microscopy and flow cytometry. For our CTC test, we developed a panel of liver tumor-specific markers, including ICG, Glypican-3 (GPC3), and DAPI and tested it with standard and imaging flow cytometry experiments with cell lines and non-cancer control blood samples. We then used this panel to analyze whole blood samples for CTC burden with standard and imaging flow cytometry with a cohort of 15 HB and HCC patients and correlated with patient characteristics and outcomes.

Results: We showed that ICG accumulation is specific to liver cancer cells, compared to non-malignant liver cells, non-liver solid tumor cells, and other non-malignant cells and can be used to identify liver tumor cells in a mixed population of cells. Experiments with the ICG/GPC3/DAPI panel showed that it specifically tagged malignant liver cells. The panel identified 1 cell or fewer per ml of blood with non-cancer control blood samples. Using patient samples, we found that CTC burden from sequential blood samples from the same patients mirrored the patients' responses to therapy.

Conclusions: Our novel ICG-based liquid biopsy test for CTCs can be used to specifically detect and quantify CTCs in the blood of pediatric liver cancer patients.

#020

ISOLATION AND CHARACTERIZATION OF CELL-FREE DNA FROM CEREBRAL ORGANOIDS

SILVER BB[1,2], BROOKS A[3], GERRISH K[2], and TOKAR EJ[1]
[1]Mechanistic Toxicology Branch, DTT, NIEHS, Research Triangle Park, NC
[2]Molecular Genomics Core, DIR, NIEHS, Research Triangle Park, NC
[3]Biostatistics and Computational Biology Branch, DIR, NIEHS, Research Triangle Park, NC

Early detection of neurological conditions is critical for timely diagnosis and effective treatment. Identifying changes at the cellular level is essential for implementing therapeutic interventions prior to symptomatic onset of disease. However, monitoring brain tissue directly through biopsies is invasive and high-risk. Liquid biopsies, consisting of bodily fluids such as blood or cerebrospinal fluid, contain plentiful information in many forms, including proteins and nucleic acids. One such component, cell-free DNA (cfDNA), has gained rapid attention for its potential as a versatile biomarker. CfDNA can be released passively by dying cells within tissues or extruded actively in the form of extracellular vesicles. Prior studies suggest that cfDNA has promising use as a biomarker of neurological disease, but we still lack a complete profile of cfDNA released by brain tissue, and what changes in cfDNA occur in response to deleterious changes within the brain. Mapping changes in cfDNA to specific cellular events is difficult *in vivo*, where many tissues contribute to released cfDNA. Organoids are tractable systems of examining tissue-level changes consistently in a human background. However, few studies have investigated the release of cfDNA from organoids. Here, we examined cfDNA isolated from cerebral organoids. We found that cerebral organoids release quantities of cfDNA sufficient for downstream analysis with whole-genome sequencing. Further, gene ontology analysis of genes aligning with sequenced cfDNA fragments revealed association with terms related to neurodevelopment and autism spectrum disorder. Using droplet-digital PCR, we identified specific cfDNA sequences corresponding to regions within both mitochondrial and nuclear genes. From these data we conclude that cerebral organoids hold promise as tools for the discovery of cfDNA biomarkers related to neurodevelopment and neurological disorders.

#021

CELL-FREE DNA EXPOSES TISSUE INJURY PROFILES LEADING TO PRIMARY GRAFT DYSFUNCTION BEFORE SYMPTOMS DEVELOP

KONG H[1,2], ACASILLAN AJ[8], SUN J[3], HILL T[3], REDEKAR N[3], KONG MK[1,2], ANDARGIE T[1,2], PARK W[1,2], ALNABABTEH M[1,2,4], SHAH P[2,9], ARYAL S[5], ORENS J[2,9], NATHAN SD[2,5], KELLER M[1,2,4,9], DIAMOND J[6], CANTU E[7], BUSH E[8], AGBOR-ENOH S[1,2,9]

[1]Laboratory of Applied Precision Omics, Division of Intramural Research, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, MD.

[2]Genomic Research Alliance for Transplantation (GRAfT), Bethesda, MD.

[3]Integrated Data Sciences Section, Research Technologies Branch, National Institute of Allergy and Infectious Diseases, Bethesda, MD.

[4]Critical Care Medicine Department, Clinical Center, NIH, Bethesda, MD.

[5]Inova Heart and Vascular Institute, Falls Church, VA.

[6]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

[7]Division of Cardiovascular Surgery, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

[8]Division of Thoracic Surgery, Department of Surgery, Johns Hopkins Hospital, Baltimore, MD.

[9]Division of Pulmonary and Critical Care Medicine, The Johns Hopkins School of Medicine, Baltimore, MD.

Purpose: The mechanism of biomarker to predict of post-transplant outcomes and the time of treatment remain unclear. Our aim is to determine the relationship between peri-transplant cfDNA levels in the recipient and the development of primary graft dysfunction(PGD).

Methods: We used multicenter prospective cohort study. We included 47 lung transplant patients from the GRAfT cohort. At the 72 hours follow-up, PGD development was determined using ISHLT guidelines. Blood samples at 24 hours after lung-transplant surgery were used and total nuclear cfDNA(ncfDNA) level and mitochondrial cfDNA(mtcfDNA) in plasma were analyzed using multiplex digital droplet PCR. The origin of organ injury was determined by using tissue-specific cfDNA levels. Whole-genome-bisulfite sequencing and DNA methylation were measured.

Results: There were 21 no-PGD episodes and 26 PGD episodes. ncfDNA were significantly higher in PGD (median = 47364 copies/mL; IQR = 26302-95918) outcomes compared to no-PGD (median = 21106 copies/mL; IQR = 13522-44825), p=0.012. mtcfDNA were also higher in PGD (median =377468 copies/mL; IQR = 208240-551686) outcomes compared to no-PGD (median = 272100 copies/mL; IQR = 212300-497345), p=0.375. Importantly, pre-transplant cfDNA levels demonstrated higher in PGD group (median = 9865 copies/mL; IQR = 3531-33267) than no-PGD group (median = 4145 copies/mL; IQR = 3654-7436), p=0.078. Innate immune cells, erythrocyte progenitor cells, vascular endothelial cells, and lung cells were higher with PGD.

Conclusions: PGD outcomes in 72 hours of post-transplant demonstrates higher ncfDNA and mtcfDNA with systemic tissue injury from pre-transplant and 24 hours. The utility of cfDNA level from pre-transplant and peri-transplant will be benefit to predict post-transplant outcomes.

#022

GENOME-WIDE CFDNA FRAGMENTATION PATTERNS IN CEREBROSPINAL FLUID INFORMS MEDULLOBLASTOMA SUBTYPES

MARKOWITZ AL[1], MUNUGULA C[1], CHRISTODOULOU E[1], JI J[1], XU L[2] and YELLAPANTULA V[1]
[1]Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California, USA
[2]The Vision Center, Children's Hospital Los Angeles, Los Angeles, California, USA

Background: Fragmentation patterns of plasma cell-free DNA (cfDNA) has the potential to discern tissue of origin and tumor subtypes in select cancers, which is of diagnostic and prognostic relevance. Medulloblastoma is a brain cancer characterized by four distinct subtypes— WNT, SHH, G3, and G4—where cfDNA profiling of cerebrospinal fluid (CSF) was shown to have clinical indications, such as assessment of residual disease. In this study, we aim to distinguish medulloblastoma subtypes by applying machine learning approaches on genome-wide fragmentation patterns and copy number aberrations (CNA) through low-pass whole genome sequencing (LPWGS) of cfDNA derived from CSF.
Methods: We acquired LPWGS data from 122 medulloblastoma patients generated from clinical trial, NCT00085202 (Liu et al., 2021), and processed them uniformly by aligning reads to GRCh38 reference and calling CNA using ichorCNA. We subsequently generated a feature-set comprising of 1) genome-wide logR values of CNA (5 Mb bins), 2) short-to-long fragment ratios corrected for GC and mappability across the genome (5 Mb bins), 3) 4bp sequence motifs on the 5'-end of cfDNA fragments, 4) contribution of F-profiles by sequence motifs, and 5) tumor fraction values. Here, cumulative motif contributions from all samples were decomposed into seven distinct F-profiles using non-negative matrix factorization. Samples were then limited to those having at least 8% tumor fraction and feature selection was performed using recursive feature elimination.
Results: CNA defined by monosomy 6, 9q loss, focal amplification in 8q involving MYC and i(17q) enriched in WNT, SHH, G3 and G3/G4 subtypes, respectively were readily identified as key features. Interestingly in G3, F-profile 3 and ACAC-motif contributions were significantly enriched, while F-profile 6 and GAAA-motif frequency were lower in comparison with all other subtypes (Wilcoxon p-value<0.05). These features are particularly valuable to differentiate G3 and G4 subtypes, which are histologically challenging to distinguish and are typically discerned through methylation profiling of the tumor tissue. Building on these promising initial findings, samples were subsequently divided to train and test the validity of one-vs-rest (OvR) logistic regression models to distinguish MB subtypes. Overall, the model showed strong performance on a hold-out test set of 20% of total samples (macro-average AUC = 0.93) suggesting cfDNA profiles are distinctive among medulloblastoma subtypes.
Conclusion: This study shows that tumor subtyping in medulloblastoma can be performed with good accuracy using CSF, a finding that has important clinical implications. Large and diverse cohorts are necessary to improve generalizability and robustness of this model.

#023

SMALL EXTRACELLULAR VESICLES AS BIOMARKERS OF RESPONSE IN RECURRENT/METASTATIC HNSCC PATIENTS TREATED WITH IMMUNOTHERAPY

ZANDBERG DP[1], HONG CS[1,2], FERRIS RL[1], DIERGAARDE B[1,3], WHITESIDE TL[1,4]
[1]UPMC Hillman Cancer Center, Pittsburgh, PA, USA
[2]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[3]Department of Human Genetics, School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA
[4]Departments of Pathology Immunology and Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Background: The development of biomarkers that predict response to anti-PD-1 mAb therapy in cancer patients is an unmet need. Small extracellular vesicles (sEV), aka exosomes, are “molecular mimics” of parent cells and could serve as potential non-invasive cancer biomarkers. Here, we evaluate the utility of sEV as biomarkers of response to immunotherapy in recurrent/metastatic (R/M) head and neck squamous cell carcinoma (HNSCC) patients.
Methods: Plasma sEV were isolated by ultrafiltration/size exclusion chromatography from 24 R/M HNSCC patients prior to immunotherapy initiation. sEV were separated by immune capture with anti-CD3 mAb into T cell-derived CD3(+) and CD3(-) subsets. The CD3(-) sEV enriched in CD44v3(+) protein are largely tumor-derived (TEX). Stimulatory (stim) and suppressive (supp) protein profiles and ratios in CD3(-) sEV were determined by on-bead flow cytometry. Differences between groups were assessed using Wilcoxon-Mann-Whitney tests. Multivariate Cox proportional hazards regression was used to evaluate the relationship between pre-treatment CD3(-) sEV characteristics and overall (OS) and progression free survival (PFS).
Results: Immune capture yielded a small CD3(+) fraction, and CD3(-)CD44v3(+) sEV represented the majority of plasma sEV. Total CD3(+) sEV level did not correlate with lymphocyte count or neutrophil/lymphocyte ratio. High CD3(+) sEV level was associated with better OS (P= 0.007) and PFS (P=0.005) but not with best response. Total CD3(-)CD44v3(+) sEV level was not associated with outcome (best response, OS, PFS) in our study population. However, high supp score and stim score were both associated with better OS (P=0.02 and P=0.04, respectively), and the supp/stim ratio was associated with best response (P=0.02). Exploration of individual proteins showed that high PD-L1 was associated with better OS and PFS (P=0.005 and P=0.04, respectively). High CTLA-4 was, independent from PDL-1, also associated with better OS (P=0.03).
Conclusions: Evaluation of the sEV subsets, T cell-derived-CD3(+) sEV and TEX-enriched CD3(-) sEV, indicated their potential utility as predictive biomarkers of response to immunotherapy. High levels of T cell-derived sEV, and high supp and stim scores, supp/stim ratio, and PD-L1 and CTLA-4 expression levels in TEX-enriched CD3(-) sEV were associated with better outcomes.

#024

EARLY POST-TRANSPLANT GENOMIC STORM PREDICT ALLOGRAFT FUNCTION AND SURVIVAL IN LUNG TRANSPLANT RECIPIENTS

ALNABABTEH M[1], KELLER MB[1], LUCIA P[2], SHAH PM[3], MATHEW J[4], KONG H[5], ANDARGIE T[5], PARK W[5], CHARYA AV[6], LUIKART H[7], ARYAL S[8], NATHAN SD[9], ORENS JB[10], KHUSH K[11], JANG M[5], AGBOR-ENOH S[12]

[1]Critical Care Medicine, National Institutes of Health, Bethesda, MD, United States
[2]Division of Hospital Medicine, Johns Hopkins Bayview Medical Center, Baltimore, MD, United States
[3]Pulmonary and Critical Care, Johns Hopkins Univ, Baltimore, MD, United States
[4]Johns Hopkins University, Baltimore, MD, United States
[5]National Institutes of Health, Bethesda, MD, United States
[6]Pulmonary and Critical Care, University of Maryland Medical Center, Baltimore, MD, United States
[7]Stanford University, Stanford, CA, United States
[8]Advanced Lung Disease and Lung Transplant, Inova Fairfax Hospital, Falls Church, VA, United States
[9]Inova Fairfax Hosp, Falls Church, VA, United States
[0]Johns Hopkins Hosp, Baltimore, MD, United States
[11]Stanford, Stanford, CA, United States
[12]Laboratory of Transplantation Genomics, National Heart, Lung and Blood Institute, Bethesda, MD, United States

Introduction: Lung transplant recipients (LTRs) are closely monitored to identify high-risk cases for enhanced surveillance. Despite this, there's a significant early mortality rate post-transplantation. We hypothesize that early post-transplant systemic tissue injury, measured by recipient-derived (rd-cfDNA), correlate with allograft function and mortality risk.
Methods: *Design:* The study included 189 LTRs from the multicenter prospective study, GRAfT. Serial plasma samples were collected with bronchoscopy. Endpoints included: % predicted forced expiratory volume in the first second(%FEV1), primary graft dysfunction(PGD), chronic lung allograft dysfunction(CLAD), and death. *Measures:* We measured rd-cfDNA using digital droplet PCR targeting recipient single nucleotide polymorphisms. *Analysis:* We computed daily average rd-cfDNA for the first 90 days, using area under the rd-cfDNA-time curve, and stratified the cohort into tertiles. Cox regression assessed whether rdcfDNA tertiles predict mortality or CLAD.
Results: Median (IQR) rdcfDNA for low, middle, high tertiles were 2715 (1905-3516), 8682 (6790-12551), and 46737 (28097-79566) copies/ml, respectively. Patients in the high tertile, compared to low, had reduced %FEV1 at day 90 [53% vs 72%, $p < 0.001$] and day 180 [61% vs 75%, $p < 0.01$], higher mortality risk [HR: 2.278; 95% CI: 1.15, 4.52, *p =0.02*], and similar CLAD risk [HR: 0.98; 95% CI: 0.42, 2.30, *p =0.95*]. PGD patients had higher risk of being in the highest tertile [OR: 6.39; 95% CI: 2.49, 16.36].
Conclusion: High early post-transplant rd-cfDNA levels correlate with allograft injury and mortality. Validating rd-cfDNA as a prognostic marker, identifying tissue origins, and exploring its role in allograft complications is imperative for future research.

#025

SONOBIOPSY ENRICHES CIRCULATING TUMOR-SPECIFIC BIOMARKERS IN A MOUSE MODEL OF DIFFUSE INTRINSIC PONTINE GLIOMA

ZHANG D[1], YUE Y[2], GONG Y[2], YANG L[2], XU K[2], YUAN J[2], CHEN H[2,3,4]
[1]Department of Imaging Science, Washington University in St. Louis, St. Louis, MO 63112, USA
[2]Department of Biomedical Engineering, Washington University in St. Louis, Saint Louis, MO 63130, USA
[3]Department of Neurosurgery, Washington University School of Medicine, St. Louis, MO, 63110, USA
[4]Division of Neurotechnology, Department of Neurosurgery, Washington University School of Medicine, Saint Louis, MO, 63110, USA

Introduction: Diffuse intrinsic pontine glioma (DIPG) is a devastating pediatric brain cancer for which curative treatment remains elusive. The eloquent location of the brainstem and the infiltrative characteristics of the tumor prevent surgical resection. While cerebrospinal fluid (CSF) may contain reliable molecular biomarkers for DIPG, its collection entails invasive procedures. Blood-based liquid biopsies are non-invasive and repeatable alternatives. However, DIPG is usually characterized by an intact blood-brain barrier (BBB), which impedes the release of tumor-derived biomarkers into blood circulation for sensitive molecular diagnosis. Focused ultrasound (FUS) in combination with microbubbles has been shown to induce precise, transient, reversible, and noninvasive BBB opening (FUS-BBBO). Our group firstly leveraged the FUS-BBBO to promote the release of brain tumor-derived biomarkers to the blood for non-invasive and sensitive molecular diagnosis of brain tumor by blood based liquid biopsy (sonobiospy). We have demonstrated the successful enrichment of glioblastoma-specific circulating tumor DNA (ctDNA) using sonobiopsy in both preclinical and clinical studies. This study aims to assess the feasibility and safety of sonobiopsy in a mouse model of DIPG.
Methods: Mice were orthotopically injected with a murine DIPG cell line. Magnetic resonance imaging (MRI) was used to guide FUS-BBBO (frequency: 1.5 MHz, pressure: 0.55 MPa, pulse repetition frequency: 5 Hz, duty cycle: 3.35%, pulse length: 6.7 ms, and treatment duration: 3 minutes) at the tumor target. Contrast-enhanced T1-weighted (CET1w) MRIs were acquired pre- and post-sonication to assess changes in BBB permeability. Droplet digital PCR (ddPCR) was used to quantify tumor-specific biomarkers (EGFP and Fluc) in the plasma and CSF respectively. Histological staining was performed to assess potential tissue damage caused by FUS sonication
Results: MRI scans validated the significant enhancement of DIPG tumor permeability following FUS treatment. Sonobiopsy notably elevated the plasma levels of EGFP circulating tumor RNA (ctRNA) (5.5-fold, p=0.0112) and EGFP circulating tumor nucleic acid (ctNA) (4.3-fold, p=0.0173). In CSF, sonobiopsy markedly increased levels of EGFP circulating tumor DNA (ctDNA) (2.5-fold, p=0.0253), EGFP ctNA (2.5-fold, p=0.0087), Fluc ctDNA (2.7-fold, p=0.0253), and Fluc ctNA (2.9-fold, p=0.0207). No hemorrhage or tissue damage was observed attributed to FUS sonication.
Conclusions: This study demonstrates the feasibility and safety of sonobiopsy for enriching tumor-specific circulating biomarkers in both plasma and CSF within a DIPG mouse model. These findings lay groundwork for further investigations into sonobiopsy as a promising non-invasive molecular diagnostic tool for DIPG.

#027

ULTRA-SHORT CTDNA: A NEW PARADIGM OF CTDNA LIQUID BIOPSY

WONG D[1], FANG W[1], CHIEN-CHUNG L[2], WEI-LUN H[2], TANG, CY[3], HE H[4], KIM, Y[1], and FENG L[1]
[1]University of California Los Angeles, Los Angeles, California, USA
[2]National Cheng Kung University Hospital, Tainan, Taiwan
[3]AIONco LLC, Menlo Park, California, USA
[4]National Institute of Standards and Technology, Gaithersburg, Maryland, USA

Mononucleosomal ctDNA (mnctDNA, 157-bp, double-stranded) spearheads liquid biopsy (LB) for cancer detection, treatment, and clinical care. The bottlenecks of mnctDNA LB are low copy number of ctDNA and limit of detection of platform technologies. Recent discovery of ultra-short ctDNA (usctDNA, 52-54 nt, single-stranded) that harbors somatic mutations prompted us to explore the quantification and stoichiometry of usctDNA to mnctDNA in plasma and saliva of NSCLC patients. Paired plasma and saliva from 40 advanced NSCLC patients with tissue genotyped positive for L858R were processed for cfDNA extraction and quantification with Qubit and Tape Station. Digital PCR assays targeting L858R somatic mutation to generate amplicons of 57-bp (usctDNA) and 100-bp (mnctDNA) were performed by UCLA and independently by NIST. Results in Table 1 revealed that three findings of usctDNA that impact ctDNA liquid biopsy. First, the somatic mutation (L858R) is present in usctDNA. Second, the stoichiometric ratio of usctDNA to mnctDNA is similar in plasma and saliva at 1.6X. For the wild-type counterpart, the ratio is 1.1X for plasma and 0.6X for saliva. Third, the copy number of usctDNA and mnctDNA in saliva to plasma is 7.8X and 7.7X.
Conclusion: Detection of tumor-associated/specific *EGFR* L858R somatic mutations in uscfDNA is translationally impactful, adding 1.6X of the ctDNA targets for molecular detection, effectively cumulating 2.6X of total ctDNA targets, permitting earlier cancer detection, treatment monitoring, and therapy prognostication. The finding that saliva harbors 7X of the us- and mnctDNA further raises the prospect of advancing saliva ctDNA liquid biopsy for NSCLC detection as it effectively allow 15.5X (7.8X+7.7X) ability to detect malignancy-associated ctDNA.

		ctDNA (L858R)				cfDNA (WT)	
	usctDNA	mnctDNA	U/M		usctDNA	mnctDNA	U/M
Plasma	**1555** ± 326	**964** ± 209	1.6X		**15201** ± 1509	**13261** ± 1424	1.1X
Saliva	**12158** ± 1731	**7442** ± 1020	1.6X		**340896** ± 27170	**601733** ± 715730	0.6X
Saliva/ Plasma (folds X)	7.8	7.7	1		22.4	45.3	0.6

Table 1: L858R *EGFR* ctDNA, ultra-short and mononucleosomal, in plasma and saliva of advanced NSCLC patients.

#028

QUANTIFICATION OF SPINAL CORD-DERIVED CELL FREE DNA IN PLASMA OF PATIENTS WITH METASTATIC EPIDURAL SPINAL CORD COMPRESSION

RAN KR[1], AZAD TD[1], AL-KHINDI T[1], MATERI JD[1], RAJ D[1], LI M[1], JUDGE K[1], BLAIR C[1], DOUVILLE CB[2], YEGNASUBRAMANIAN S[2], THEODORE N[1], BETTEGOWDA C[1]
[1]Department of Neurosurgery, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
[2]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Metastatic epidural spinal cord compression (ESCC) can result in significant disability and lasting neurologic compromise if not promptly diagnosed and managed. In patients with acute neurologic deficit, surgical decompression may be required. While circulating tumor DNA (ctDNA) has been utilized to determine oncologic disease burden in patients with ESCC, there is no molecular correlate of neurologic function. Leveraging advances in methylation profiling, we developed an off-the-shelf approach for detecting spinal cord-derived cell free DNA (cfDNA) in the peripheral blood of patients with ESCC. We recruited a prospective cohort of pre-operative blood samples from 7 patients (57.1% female, 57.1% White, 3.3 ± 2.4 days with acute neurologic worsening, 42.9% primary colon cancer). We also obtained blood samples from 11 patients without cancer. Spinal cord-derived cfDNA was detected in 85.7% of ESCC patients (71.7 hGE/mL plasma) and 0% of control patients. Percent of spinal cord-derived cfDNA trended with preoperative neurologic status: 0.49% of total cfDNA was spinal cord-derived in ASIA A/B patients compared to 0.26% in ASIA C/D patients. We report the ability to quantitate spinal cord-derived cfDNA in the blood of patients with symptomatic ESCC. Future studies integrating spinal cord-derived cfDNA with ctDNA may provide an effective multimodal monitoring approach to detect impending neurologic injury in a high-risk population.

#029

CELL-FREE DNA SCREENING RESULTS BY STAGE OF MATERNAL MALIGNANCY

ZHAO E[1], MILLER K[2], JELIN A[2]
[1]Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA
[2]Division of Maternal Fetal Medicine, Department of Gynecology and Obstetrics, Johns Hopkins Hospital, Baltimore, Maryland, USA

Introduction: As cell-free DNA (cfDNA) screening is increasingly utilized for non-invasive fetal aneuploidy screening, the association between abnormal or nonreportable cfDNA screening results and maternal malignancy is recognized. However, the impact of tumor stage and tissue of origin on cfDNA screening remains unclear. We hypothesize that cfDNA screening is less likely to be impacted by early stage, localized solid tumors.
Methods: We present a single center, retrospective review of cases with a known, suspected, or incidentally diagnosed malignancy during pregnancy who underwent cfDNA fetal aneuploidy screening between 2015-2023. We queried patients using ICD9/10 codes for malignancy to identify patient charts for review. We excluded patients who did not have a confirmed malignancy during pregnancy or within 2 years post-partum, and those who did not undergo cfDNA testing. We extracted maternal history, cancer type/stage, cfDNA results, and fetal outcomes from the medical record. We used Fisher's exact test to analyze categorical variables.
Results: Fifty three cases of malignancy in pregnancy were identified, of which 25 patients underwent cfDNA screening. 9 patients had abnormal or nonreportable cfDNA results: 2 cases of stage IV rectal adenocarcinoma and 1 case each of untreated acute myeloid leukemia, stage II microinvasive ductal carcinoma of the breast, stage II chromophobe renal cell carcinoma, stage III rectal adenocarcinoma, stage IV breast carcinoma, stage IV Classic Hodgkin lymphoma, and stage IV serious ovarian carcinoma. 2/3 (67%) patients with blood cancers had abnormal cfDNA screening results, compared to 7/22 (32%) patients with solid tumors. Overall, the fraction of stage 0, I, II, III, and IV solid cancers with abnormal cfDNA screening results was 0/3 (0%), 0/4 (0%), 2/3 (67%), 1/4 (25%), and 5/8 (63%), respectively. Women with stage 2-4 cancers were more likely to have an abnormal cfDNA result than women with stage 0-1 cancers (p=0.02). No fetuses had a genetic abnormality.
Conclusions: Our data suggest that early stage, solid tumors are not reliably detected by prenatal cfDNA screening, whereas hematologic cancers and metastatic tumors are more likely to yield nonreportable or abnormal cfDNA results. It remains uncertain whether this is due to the cytogenetic composition of different tumors, or the varying levels of tumor shedding based on tumor type and/or stage. Larger patient cohorts are needed to further characterize the association of different tumor types and the pattern of cfDNA results. Ultimately, cfDNA may prove more useful in identifying certain types of undiagnosed maternal malignancy compared to other types.

#030

CIRCULATING, CELL-FREE METHYLATED DNA REVEALS CELLULAR SOURCES OF GRAFT INJURY AFTER LIVER TRANSPLANT

MCNAMARA ME[1], JAIN SS[1], OZA K[2], MURALIDARAN V[2], KILITI AJ[1], MCDEED AP[1], PATIL D[2], CUI Y[2], SCHMIDT MO[1], RIEGEL AT[1], KROEMER AHK[2], WELLSTEIN A[1]

[1]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA.

[2]MedStar Georgetown University Hospital, MedStar Georgetown Transplant Institute, Washington, DC, USA.

Molecular biomarkers to monitor changes after liver transplant are essential to reveal mechanisms of injury, guide clinical decision-making and improve patient outcomes. Current approaches have limited scope and are unable to differentiate between host and graft amongst the causes of graft injury. Here, we utilize circulating, cell-free methylated DNA released from dying cells to monitor cellular damages after liver transplant impacting the graft tissue as well as host organs. We expand existing cell-type-specific DNA methylation atlases curated from whole-genome bisulfite sequencing (WGBS) of healthy tissues to characterize liver cell-types relevant to injury progression and tissue repair. Liver cell-type-specific methylation blocks are validated through multi-omic data integration and found to be enriched in open chromatin and regulatory regions functionally important for the respective cell populations. Cell-free DNA (cfDNA) 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 of cell types. We profiled 135 blood samples collected from 44 liver transplant patients at serial time points before and after transplant. We found that liver transplant initially results in multi-tissue cellular damage that subsequently recovers in patients with graft acceptance during the first post-operative week. Further, we show that sustained elevation of hepatocyte and biliary epithelial cfDNA beyond the first week is indicative of early-onset graft injury. Most notably, cfDNA composition can differentiate amongst causes of graft injury. Thus, cell-free methylated DNA can detect cellular damages and prompt early intervention.

#031

PLASMA CELL-FREE DNA HISTONE METHYLATION ENABLES PHENOTYPIC AND CLINICAL SEGMENTATION OF METASTATIC PROSTATE CANCER

MUNZUR AD[1], SIPOLA J[1,2], SEO C[3], KWAN EM[1,4,5], PAREKH K[1], BERNALES CQ[1], DONNELLAN G[1], BLOISE I[6], VANDEKERKHOVE G[1,4], ANNALA M[1,2], DROR C[1,4], CHI KM[1,4], HERBERTS C[1], TAKEDA DY[3], WYATT AW[1,7]
[1]Vancouver Prostate Centre, Department of Urologic Sciences, University of British Columbia, British Columbia, Canada
[2]Prostate Cancer Research Center, Faculty of Medicine and Health Technology, Tampere University and Tays Cancer Center, Tampere, Finland
[3]Laboratory of Genitourinary Cancer Pathogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States
[4]Department of Medical Oncology, BC Cancer, Vancouver, British Columbia, Canada
[5]Department of Medicine, School of Clinical Sciences; Monash University; Melbourne, Victoria, Australia
[6]Instituto Brasileiro de Controle ao Cancer, Sao Paulo, Brazil
[7]Michael Smith Genome Sciences Centre, BC Cancer, Vancouver, British Columbia, Canada

Background: Epigenomic reprogramming frequently occurs in the context of metastatic castration-resistant prostate cancer (mCRPC), particularly during emergence of treatment resistance and neuroendocrine prostate cancer (NEPC). Existing clinical circulating tumor DNA (ctDNA) assays focus on genomic alterations but do not provide information on epigenome characteristics. Circulating plasma cell-free DNA (cfDNA) can remain bound to histones containing cell-of-origin posttranslational modifications—and can be analyzed via chromatin immunoprecipitation followed by sequencing (cfChIP-seq). Here, we tested the potential for cfChIP-seq to inform on biological and clinical subgroups of mCRPC.
Methods: We examined 63 cfDNA samples derived from 33 patients with mCRPC and 19 controls. Patients were selected to represent diverse clinical characteristics, including varied location and extent of metastases and pathologically-confirmed NEPC. Samples underwent H3K4me2 profiling (indicative of active gene promoters and enhancers) using cfChIP-seq. cfDNA samples and patient-matched white blood cells also underwent low-pass whole genome and deep targeted sequencing to provide insights into their genomic driver alterations. A custom statistical framework was used to account for variable ctDNA% between samples, which can confound comparisons of H3K4me2 activity between genes and/or patients.
Results: 51% of patients had >10 bone lesions, 20% had liver, 15% had lung, and 58% had bone metastases without visceral involvement. Frequent *TP53* (61% of patients), *RB1* (18%), and *PTEN* (19%; homozygous deletion only) disruption reflected clinically-aggressive disease. Transcription factors canonically linked to AR-driven adenocarcinoma (e.g. AR, FOXA1) and NEPC (e.g. EZH2, NANOG, POU5F1) were differentially enriched across multiple separate binary patient subgroups, including patients with bone versus liver-predominant disease, biopsy-confirmed NEPC, and presence of *RB1* deletions, suggesting an apparent axis of AR versus NEPC phenotypic segregation even in patients without biopsy-confirmed NEPC. *KLK3* (encodes prostate specific antigen [PSA]) promoter H3K4me2 count was strongly correlated with time-matched serum PSA ($p<0.01$). AR binding motif enrichment showed a positive correlation with PSA ($p<0.005$) while NEPC related motifs were negatively correlated ($p<0.05$). Leveraging a public pan-cancer ATAC-seq atlas, H3K4me2 density was highest in prostate cancer-specific open chromatin regions relative to other cancers. Interestingly we also observed epigenomic footprints of metastasis-induced normal tissue destruction in bulk cfDNA (e.g. strong lung lineage identity in a patient with extensive pulmonary metastases).
Conclusions: cfChIP-seq can capture biological distinctions in clinically stratified patients with mCRPC. cfChIP-seq may complement ctDNA profiling for discovery of predictive and prognostic biomarkers and detection of treatment resistance.

#032

EXPLORING EXTRACELLULAR VESICLES AS LIQUID BIOPSY BIOMARKERS FOR CLEAR CELL RENAL CELL CARCINOMA

LAKSHMINARAYANAN H[1], YIM K[2], PFAMMATTER S[3], BANAEI-ESFAHANI A[1], RUTISHAUSER D[1], CHAHWAN R[2], BOLCK H[1], MOCH H[1]

[1]Department of Molecular Pathology and Pathology, University of Zurich and University Hospital Zurich, Zurich, Switzerland

[2]Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland

[3]Functional Genomics Center Zurich, ETH Zurich University of Zurich, Zurich, Switzerland

Clear cell renal cell carcinoma (ccRCC) is the most lethal urological malignancy, with under 10% overall survival in patients with aggressive metastatic disease. At the clinic, 30% of patients exhibit metastasis, while an additional 30% develop metastasis as their disease advances. Currently, prognosis is based on histopathological characterization and there are no ccRCC-specific molecular biomarkers to predict metastatic dissemination or patient survival. Secreted, nanoscale, membrane-bound extracellular vesicles (EVs) are promising biomarkers that can be found in circulation, including blood. During ccRCC tumorigenesis, they are implicated in cell communication and metastatic progression. Here, we identify liquid biopsy-based EV-derived protein biomarkers in patient plasma for the prediction of prognosis in ccRCC patients. In our study, we define a ccRCC-specific EV signature using *in vitro* patient-derived cell culture (PDC) and commercial cell culture models. Following this, we will validate the diagnostic and prognostic potential of our ccRCC EV-derived protein signature in patient plasma. To this end, we used three ccRCC PDCs, one ccRCC commercial cell line (786-O), and a non-ccRCC control cell line (HeLa). EVs were isolated from media supernatant using ultracentrifugation and the morphology and concentration of isolated EVs were characterized using transmission electron microscopy (TEM) and nano flow cytometry (nFCM). Isolated EVs had typical cup-shaped morphology and ranged in size between 40-100nm. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), we further identified ~4600 proteins in isolated EVs across the different cell culture models. Functional annotation of proteins identified in common across the ccRCC models highlighted the enrichment of several known vesicular and associated proteins. Finally, from this data, we defined a ccRCC-specific EV-derived protein signature including proteins such as CD9, CD147, Cadherin-2 and MMP14 among other proteins, based on cellular localization and observed protein intensities. We will validate the prognostic and discriminatory potential of this signature in patient plasma samples, collected before and after surgery. This will allow us to develop a tool for the active surveillance of ccRCC patients using specific molecular biomarkers, and thereby help improve prognostication and cancer management in ccRCC patients who currently remain at risk for developing aggressive disease.

#033

EARLY DETECTION OF MALIGNANT AND PRE-MALIGNANT PERIPHERAL NERVE TUMORS USING CELL-FREE DNA FRAGMENTOMICS

SZYMANSKI JJ[1], SUNDBY RT[2], , PAN A[2], JONES PA[1,3], MAHMOOD SZ[2], REID OH[2], SRIHARI D[3], ARMSTRONG AE[4,5], CHAMBERLAIN S[6], BURGIC S[6], WEEKLEY K[6], MURRAY B[2], PATEL S[2], QAIUM F[1], LUCAS AN[2], FAGAN M[2], DUFEK A[2], MEYER CF[7], COLLINS NB[8], VAN TINE BA[4,5,6], PRATILAS CA[9,10], DOMBI E[2], GROSS AM[2], KIM A[11], CHRISINGER JSA[12], DEHNER CA[13], WIDEMANN BC[2], HIRBE AC[3,4,5,6], CHAUDHURI AA[1,3,4,14,15], SHERN JF[2]

[1]Division of Cancer Biology, Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri, USA

[2]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

[3]Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri, USA

[4]Siteman Cancer Center, Barnes Jewish Hospital and Washington University School of Medicine, St. Louis, Missouri, USA

[5]Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA

[6]Division of Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, USA

[7]Division of Medical Oncology, Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[8]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, Massachusetts, USA

[9]Division of Pediatric Oncology, Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD USA

[10]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[11]Center for Cancer and Blood Disorders, Children's National Hospital, Washington, DC, USA

[12]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, Missouri, USA

[13]Department of Anatomic Pathology and Laboratory Medicine, Indiana University, Indianapolis, IN, USA

[14]Department of Biomedical Engineering, Washington University School of Medicine, St. Louis, Missouri, USA

[15]Department of Computer Science and Engineering, Washington University in St. Louis, St. Louis, Missouri, USA

Purpose: Early detection of neurofibromatosis type 1 (NF1) associated peripheral nerve sheath tumors (PNST) could inform clinical decision-making, potentially averting deadly outcomes. NF1 is the most common heritable cancer predisposition syndrome and is characterized by a spectrum of benign, pre-malignant, and malignant nerve sheath tumors. Approximately 50% of patients with NF1 develop benign plexiform neurofibromas (PNs). A subset of PN evolving into pre-malignant atypical neurofibromas (ANs) and, ultimately, 8-15% of patients with NF1 developing cancerous malignant peripheral nerve sheath tumors (MPNSTs). MPNSTs account for the majority of NF1-associated mortality with a 5-year overall survival rate of only 20%. Differentiating between PN, AN, and MPNST remains clinically challenging as a result of insensitive clinical exams, overlapping findings on imaging, and tissue heterogeneity and sampling biases on biopsy. Biopsy also carries the risk of peripheral nerve injury, further complicating the diagnostic workup. Here we describe a cell-free DNA (cfDNA) fragmentomic approach that distinguishes non-malignant, pre-malignant, and malignant forms of NF1 PNST.

Methods: Blood plasma was collected from patients with PN (n = 69), AN (n = 35), MPNST (n = 60), and healthy controls (n = 21). Plasma cfDNA was isolated and whole genome sequencing (WGS) was performed to a target depth of 6x. cfDNA WGS reads were profiled for copy number alteration (CNA), bin-wise fragment length ratios, and end motifs. We deconvoluted end motifs and fragment lengths by non-negative matrix factorization (NMF) to identify distinguishing tumor-specific signatures. The ability of each method to differentiate tumor types was evaluated in one-versus-one (OVO) comparisons. In serial samples, cfDNA methods were compared to imaging and clinical outcomes.

Results: This study established the largest collection of cfDNA data for NF1 patients to date. cfDNA CNA

identified MPNST but could not distinguish between benign and pre-malignant states. In contrast, fragmentomic methods were able to differentiate pre-malignant states. Overall, fragment length NMF demonstrated the best performance in OVO comparisons (healthy vs. PN ROC AUC 0.84, PN vs. AN ROC AUC 0.75, AN vs. MPNST AUC 0.77). Fragmentomics also adjudicated AN cases suspicious for MPNST, correctly diagnosing samples noninvasively, which could have informed clinical management.
Conclusions: This study pioneers the early detection of malignant and pre-malignant peripheral nerve sheath tumors in NF1 patients using plasma cfDNA fragmentomics. This non-invasive approach surpasses current diagnostic limitations, facilitating timely interventions and personalized management for NF1 patients. Our findings have transformative implications for screening, surveillance, and treatment strategies within this high-risk population.

#034

CELLKEEP™ SLIDE ENHANCES RETENTION OF CTCs HARVESTED FROM PATIENT BLOOD SAMPLES USING THE PARSORTIX® SYSTEM.

GREAVES D[1], COTTINGHAM A[1], SPODE M[1], VARATHARAJAH A[1], CICCIOLI M[1], PAILHES-JIMENEZ A-S[1].
[1]ANGLE PLC, Guildford, United Kingdom

Background. Due to their rare nature efficient harvest, and deposition onto slides of circulating tumour cells (CTCs) from patient blood samples is paramount. However, industry standard microscopy slides such as Cytoslides, incur significant cell loss when used for sample processing downstream of CTC harvest using the Parsortix® system. To address this, ANGLE have developed the CellKeep™ slide. This research study compared the performance of CellKeep slides to Cytoslides.
Methods. Peripheral blood from six volunteers was drawn into Streck Cell-Free DNA tubes. At 96 hours post draw, blood was spiked with HCC1954 and Hs578T cells, then processed with the Parsortix® system (Research Use Only). Harvests were cytocentrifuged onto ANGLE CellKeep slides or Cytoslides then stained using ANGLE's IF assay for the detection of epithelial and mesenchymal markers, and PBMC exclusion markers. The above workflow was then used to process blood from eleven metastatic breast cancer (MBC) patients (unspiked) within 144 hours of collection. All stained slides were imaged using a BioView Allegro imaging system.
Results. Spiked cells: CellKeep slides demonstrated significantly higher percentage spiked cell retention ((final cell count / spiked cell count) × 100) (Mean values; HCC1954: CellKeep 49.6%, Cytoslide 12.6%; Hs578T: CellKeep 46.5%, Cytoslide 7.1%; $P<0.0001$), and greater harvest linearity than Cytoslides over the range of 0-500 spiked cells (CellKeep $R^2=0.95$, Cytoslide $R^2=0.50$). MBC patient blood samples: Sample positivity (≥1 CTC detected) was 55% (6/11) in CellKeep slides and 36% (4/11) in Cytoslides. Only 67% (4/6) patients CTC+ in CellKeep slides were also CTC+ with Cytoslides. In CellKeep slides 116 CTCs were identified (2-37 CTCs per sample: median 22, mean 19); Cytoslides showed 49 CTCs (4-22 CTCs per sample: median 7, mean 8). 100% (6/6) CTC+ donors showed ≥1 cluster with CellKeep slides (Total CTC clusters 20; Per sample: median 3, mean 3; cluster size range 2-20 CTCs); 100% (4/4) CTC+ donors showed ≥1 cluster with Cytoslides (Total CTC clusters 11; Per sample: median 1, mean 2; cluster size range 2-10). CTC phenotype was equivalent between slide types.
Conclusions. The ANGLE CellKeep slides outperformed Cytoslides in all tested parameters with contrived (percentage harvest, linearity) and patient derived samples (positivity rate, overall CTCs numbers, capture and preservation of CTC cluster morphology). The mesenchymal nature of identified CTCs also highlights the necessity of epitope-free CTC isolation systems for the processing of MBC patients' blood. Combining CellKeep slides with the Parsortix system boosts the potential utility of every sample processed.

#035

CHARACTERIZATION OF PLASMA AND CSF CELL-FREE DNA PROFILES IN PATIENTS WITH MULTIPLE SCLEROSIS AND PROGRESSIVE MULTIFOCAL LEUKOENCEPHALOPATHY USING CELL-OF-ORIGIN METHYLATION ANALYSIS

GAITSCH H[1], JANG MK[2], GAITÁN MI[1], LIN J-P[1], CORTESE I[3], AGBOR-ENOH S[2], and REICH DS[1].
[1]Translational Neuroradiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA
[2]Laboratory of Transplantation Genomics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA
[3]Experimental Immunotherapeutics Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD, USA

Recent advancements in epigenetic profiling, notably the publication of methylation atlases detailing tissue- and cell-specific methylation signatures, have set the stage for the use of liquid biopsy approaches in diseases without characteristic mutation profiles, including neurologic conditions with primarily inflammatory, demyelinating, and degenerative pathological features. Multiple sclerosis (MS) is a chronic neuroinflammatory and neurodegenerative disease affecting over two million people worldwide and is a major cause of neurological disability, particularly in young adults. Progressive multifocal leukoencephalopathy (PML) is a rare, severe demyelinating condition resulting from the reactivation of the JC polyomavirus in immunocompromised patients. In both conditions, there is a clinical need for relevant fluid biomarkers to predict/monitor disease progression and to assess treatment response. In this project, we aim to characterize and compare the plasma and cerebrospinal fluid cfDNA cell-of-origin compositional profiles in MS and PML patient samples with a focus on detecting CNS-derived cfDNA biomarkers that may be indicative of ongoing inflammation, demyelination, and neurodegeneration in these conditions. To this end, we have collected >150 biofluid samples from MS and PML patients seen in the NINDS Neuroimmunology Clinic, along with time-matched MRI scans and clinical data. MS samples include patients with relapsing-remitting and progressive disease phenotypes. PML samples include patients with inactive disease, active disease without immune reconstitution inflammatory syndrome (IRIS), and active disease with IRIS. When possible, longitudinal samples have been obtained, especially in PML. An additional >20 plasma samples from healthy volunteers (HV) have been collected. All blood samples were collected in cfDNA-stabilizing tubes (Streck) and processed within two hours of collection using a two-step centrifugation protocol, then plasma was frozen at -80°C. CSF supernatant was collected and frozen at -80°C. In pilot studies using HV, MS, and PML plasma samples, cfDNA was extracted using the QIAsymphony automated platform. Extraction efficiency was calculated using a known spike-in quantity of Lambda DNA. The quality and concentration of extracted cfDNA samples was assessed using size-distribution metrics (Agilent TapeStation) and ddPCR-based copy number variation assays. Isolated cfDNA from pilot studies demonstrated sufficient quality for downstream sequencing studies, and whole-genome bisulfite sequencing with subsequent cell-of-origin analysis, along with targeted methylation-sensitive ddPCR focusing on neuron- and glia-derived fragment detection, is planned for all samples.

#036

ANTIGEN REACTIVITY PROFILES PREDICT PROGNOSIS AFTER IMMUNOTHERAPY TREATMENT IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA

DO LW[1#], BEHRENS [1,2#], WANG L[1], REVSINE M[1], MAESTRI E[1], SARDOO AM[1], FORGUES M[1], JACOB A[1], CHANG CW[1], ARGEMI J[3,4], SOGBE M[3,4], SANGRO B[3], GRETEN TF[5,6], WANG XW[1,6]

[1]Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

[2]Surgical Oncology Research Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD, 20892, USA

[3]Clinica Universidad de Navarra, Pamplona, Spain

[4]Centro de Investigación Biomédica en Red (CIBER-EHD), Madrid, Spain

[5]Thoracic and GI Malignancies Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

[6]Liver Cancer Program, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

[#]Denotes equal contribution of authors

While immunotherapy has become a first line treatment strategy for advanced liver cancer, efficacy remains heterogenous across both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) prompting a focus on finding biomarkers of response. Phage immunoprecipitation sequencing (phip-seq) measures serum samples for reactivity to a comprehensive profile including ≥15K viral and bacterial strains, providing a unique profile of immunocapacity. Here, we have examined the ability for antigen reactivity profiles (defined using the viral and bacterial strain reactivity) to stratify prognosis following immunotherapy treatment in HCC and CCA patients. The patient population derived from the National Cancer Institute (NCI) Clinical Center with patients undergoing immune checkpoint inhibition therapy or tyrosine kinase therapy. We divided each cohort into a training and test set and used an XGBoost cox model to develop two models predicting all-cause mortality in patients with HCC (Training n=28, Testing n=7) and CCA (Training n=15, Testing n=14). The HCC and CCA models included 23 and 7 viral and bacterial strains which discriminated high vs. low survival, respectively, with two strains overlapping. The time dependent area under the curve (AUC) for the test set was 0.8 at 1 year in both models stabilizing to 0.6 (4 years) and 0.7 (1.5 years) in HCC and CCA models, respectively. Using the features from the models, we developed HCC and CCA antigen scores, respectively and tested the scores in the training/test set for HCC and CCA using cox-proportional hazard model with the HCC model significantly predictive of survival in the training set in the 4 year follow-up period (Hazard ratio [HR] Training [95% Confidence Interval {CI}]: 81.7 [9.88, 10690.96], p-value=9.91E-08; HR Testing [95% CI]: 2.36 [0.42, 12.96], p-value=0.36) and the CCA model significantly predictive of survival in both the training and test set in the 2 year follow-up period (HR Training [95% CI]: 41.38 [4.67, 5475.69], p-value=9.82E-05; HR Testing [95% CI]: 3.76 [1.02, 13.88], p-value=0.04). In both models, we found the antigen score remained associated with survival after adjustment for multiple clinical and sociodemographic parameters. We tested the HCC model in an external cohort (n=30) from the Clinica Universidad de Navarra (CUN) in Pamplona, Spain. We found the HCC model could validate in this cohort, though the results were slightly above our threshold for significance (HR Validation [95% CI]: 2.38 [0.98, 5.76], p-value=0.0549). Overall, these data suggest microbiome reactivity profiles could be unique non-invasive biomarkers of response to systemic therapy in liver cancer populations.

#037

CELL-FREE DNA TO PREDICT SEVERE EXACERBATIONS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

RICHERT ME[1,2], KONG H, BALASUBRAMANIAN S[1,2], JANG MK[1], ANDARGIE T[1], BROWER R[1], BLEECKER ER[3], BOWLER RP[4], CHRISTENSON S[5], MEYERS DA[3], O'NEAL WK[6], ORTEGA VE[3], WOODRUFF PG[5], MARTINEZ FJ[7], CURTIS JL[8,9], AGBOR-ENOH S[1,10]

[1]Genomic Research Alliance for Transplantation (GRAfT) and Laboratory of Applied Precision Omics, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, Maryland, USA.
[2]Department of Critical Care Medicine, NIH, Bethesda, Maryland, USA.
[3]Department of Pulmonary Medicine, Mayo Clinic, Phoenix, Arizona, USA.
[4]National Jewish Health, Denver, Colorado, USA.
[5]Division of Pulmonary, Critical Care, Sleep and Allergy, Department of Medicine, University of California at San Francisco, San Francisco, California, USA.
[6]Marsico Lung Institute, University of North Carolina, Chapel Hill, North Carolina, USA
[7]Division of Pulmonary and Critical Care Medicine, Joan and Sanford I. Weill Department of Medicine, Weill Cornell Medicine, New York, New York, USA.
[8]Division of Pulmonary and Critical Care Medicine, University of Michigan Health System, Ann Arbor, Michigan, USA.
[9]VA Ann Arbor Healthcare System, Ann Arbor, Michigan, USA.
[10]Department of Medicine, Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA.

Rationale: The number and severity of exacerbations are risk factors of morbidity and mortality in chronic obstructive pulmonary disease (COPD). Unfortunately, existing clinical approaches lack the precision to identify high-risk patients who may benefit from timely intervention to prevent exacerbations and improve outcomes. Because advanced COPD is accompanied by systemic tissue injury and metabolic exhaustion, we hypothesized that sensitive biomarkers of tissue injury such as nuclear cell-free DNA (ncfDNA) or of energy exhaustion such as mitochondrial cell-free DNA (mtcfDNA) are associated with exacerbations in COPD patients.
Methods: *Design:* This pilot study included n=39 healthy controls and n=145 participants from the Subpopulations and Intermediate Outcomes Measures in COPD Study (SPIROMICS) cohort. Half of the SPIROMICS cohort were enriched to include subjects with rapid spirometric disease progression. Among each of the SPIROMICS group, we documented the number of exacerbations, which were categorized as "severe" if requiring emergency room care or hospitalization. *Measure:* We measured plasma mtcfDNA and ncfDNA using digital droplet PCR. *Analysis:* ncfDNA and mtcfDNA were compared between groups with standard statistical models correcting for multiple comparisons. Cox regression was used to assess if ncfDNA or mtcfDNA predict the primary outcome, time from enrollment to first hospitalization.
Results: COPD subjects demonstrated higher concentrations of both ncfDNA [3,762 (2,620-5,184) vs. 2,382 (2,019-3,642), median (IQR) copies/mL, p=0.00028] and mtcfDNA [916,375 (359,300-2,335,442) vs. 90,659 (60,392-130,047, p<0.0001) than healthy controls. In total, 53% (n=77) of COPD participants experienced exacerbations, 61% (47/77) of which were severe. Numbers of total exacerbations per COPD participant correlated positively with ncfDNA levels but negatively with mtcfDNA levels (i.e., decreasing levels with more exacerbations). Higher mtcfDNA levels were associated with lower odds of experiencing severe exacerbations (OR 0.45 95% CI 0.22-0.89, p=0.027). Risk of time to first hospitalization correlated significantly with lower mtcfDNA (HR 2.05, 95% CI 1.07-3.91, p=0.03).
Conclusion: This pilot study indicates that the sensitive marker of metabolic exhaustion (mtcfDNA) is a potential predictor of the risk of severe COPD exacerbations. Follow-up studies in a larger cohort size are needed to further explore the prognostic performance of these markers, adjusting for relevant covariates. To further inform disease pathobiology, these follow-up studies should also profile the trends and tissue sources of injury associated to COPD exacerbation and downstream outcomes.

#038

MEASURING CYTOKINE LEVELS IN EXTRACELLULAR VESICLES ISOLATED FROM SERUM AND PLASMA IN HEALTHY BLOOD DONORS: A PILOT STUDY

SLATTERY K[1], KAUSS MC[1], RAVAL D[1], HSIEH E[1], ARMSTRONG TS[1], GUEDES VA[1]
[1]Neuro-Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Aging is a multifaceted process that starts at the molecular level and increases susceptibility to diseases, including cancer, and reduces physical and cognitive abilities. Aging-related processes have been associated with elevated levels of cytokines, which can be released in their soluble form or associated with extracellular vesicles (EVs). The potential of EV-associated cytokines as an aging biomarker is yet to be established. In this pilot study, we evaluated cytokine levels in serum, plasma, and serum and plasma-derived EVs in healthy volunteers. We aimed to compare levels of specific cytokines in distinct sample types to inform future studies to evaluate EV cytokines as biomarkers of aging. EVs were isolated using two types of size exclusion chromatography columns (Izon Science), qEV/35nm and qEV/70nm, which quantify particles in distinct size ranges. Each column was loaded with 500ul of serum or plasma, and EV fractions were concentrated using Amicon® Ultra-4/100kDa filters (Millipore). Ten cytokines (interleukin-1β [IL-1β], IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, interferon-γ [IFN-γ], tumor necrosis factor-α [TNF-α]) were measured using an immunoassay (V-PLEX/Cytokine Panel-1, MSD) in 6 sample types per participant (n=9): serum, plasma, serum-qEV35, plasma-qEV35, serum-qEV70, and plasma-qEV70. Participants were male (78%), white (78%), with a mean age of 57 (SD=18). Levels of IL-13 were higher in plasma than serum for qEV70 (p=0.008) and qEV35 (p=0.019). IL12p70 (p=0.0001) was higher in plasma-qEV70 than serum-qEV70. IL-13 was the most abundant cytokine in plasma, but along with IL-2 and IL-1β, was not detected in serum. Age correlated with IL12p70 (serum-qEV70, p=0.030, r=0.778), IL-2 (plasma-qEV35, p=0.019, r=0.770), and IL-6 (plasma, p=0.038, r=0.711). Here, cytokine levels varied depending on sample type. Future larger studies should evaluate the best sample types to analyze links between specific EV-associated cytokines and aging, and their potential as biomarkers in age-related conditions.

#039

COMPARISON BETWEEN LEVELS OF VASCULAR INJURY MARKERS IN EXTRACELLULAR VESICLES DERIVED FROM SERUM AND PLASMA IN HEALTHY VOLUNTEERS: A PILOT STUDY

KAUSS MC[1], SLATTERY K[1], RAVAL D[1], HSIEH E[1], ARMSTRONG TS[1], GUEDES VA[1]
[1]Neuro-Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Cognitive dysfunction is a common symptom in primary brain tumor (PBT) patients, as a result of the primary disease and damage caused by surgical procedures and radiation therapy leading to secondary pathological responses and inflammation in structures including the vasculature. Emerging evidence suggests a role for extracellular vesicles (EVs) in the pathogenesis of vascular damage. However, the potential of EVs as vascular injury biomarkers has been little explored. In this pilot study, we measured vascular injury markers in serum and plasma-derived EVs in healthy donor blood to evaluate quantitative differences between sample types, which will inform the design of biomarker development projects. EVs were isolated using size exclusion chromatography columns (qEV/35nm, Izon Science) from 500ul of serum or plasma. Collected EV fractions were concentrated using Amicon® Ultra-4/100kDa filters (Millipore). C-reactive protein (CRP), serum amyloid A (SAA), intercellular adhesion molecule-1 (sICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) were quantified using an immunoassay (V-Plex/Vascular Injury Panel-2, MSD). Four sample types (plasma, serum, plasma EVs, serum EVs) were analyzed per participant (n=9). Participants were male (78%), white (78%), with a mean age of 57 (SD=18). All markers were detectable in EVs derived from either plasma or serum. Median levels of all markers were higher in serum EVs when compared to plasma EVs, but these differences were not statistically significant. Levels of VCAM-1 and ICAM-1 were significantly correlated in serum EVs ($p<0.001$, $r=0.950$), but not plasma EVs ($p=0.097$, $r=0.600$). Our results suggest that both plasma and serum EVs are suitable for experiments to analyze markers measured in this study. However, the use of serum rather than plasma might confer advantages, which should be investigated in future studies with larger samples sizes. Our findings will inform projects that explore the potential of EVs as biomarkers in PBT and associated cognitive dysfunction.

#040

SMALL EXTRACELLULAR VESICLES IN PROXIMAL POSTOPERATIVE BIOFLUIDS OF HPV+ OROPHARYNGEAL CANCER DRIVE M1 MACROPHAGE POLARIZATION VIA CGAS-STING PATHWAY ACTIVATION

XU Z[1], EARLAND N[2], KHALIL L[1], HARRIS PK[2], GERNDT SP[3], RAMIREZ RJ[2], SEMENKOVICH NP[2], WHITESIDE TL[4], CHAUDHURI AA[2], ZEVALLOS JP[1]

[1]Department of Otolaryngology-Head and Neck Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

[2]Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO, USA

[3]Division of Otolaryngology-Head and Neck Surgery, Allegheny Health Network, Pittsburgh, PA

[4]Departments of Pathology and Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Background: Human papillomavirus (HPV) infection is implicated in oropharyngeal squamous cell carcinoma (OPC). Our previous investigations revealed that postoperative biofluids (PPB) collected from the surgical drains in HPV(+) OPC patients contain tumor-derived cell-free HPV DNA, potentially influencing patient prognosis. Herein, we investigated the role of PPB derived small extracellular vesicles (sEVs) containing HPV DNA in mediating STING activation and modulating macrophage polarization within the tumor microenvironment (TME) of HPV(+) OPC.

Methods: PPB samples were obtained post-surgery from 31 HPV(+) and 10 HPV(-) OPC patients. sEVs were isolated by ultracentrifugation, Qiagen precipitation or size exclusion chromatography (SEC). Isolated sEVs were characterized using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and immunoblots. sEVs were pretreated with DNase to remove surface DNA, followed by total DNA extraction and quantification of HPV16 copy numbers using TaqMan qPCR. *In vitro* OPSCC cell media were used to isolate HPV(+) and HPV(-) sEVs. Human naive M0 cells were co-incubated with HPV(+) and HPV(-) sEVs to assess cGAS-STING pathway activation, IFNβ mRNA expression, TBK1 phosphorylation, and M1 macrophage polarization via flow cytometry.

Results: TEM and NTA confirmed vesicular morphology and size (70-150 nm) of PPB derived sEVs, which were CD63+, CD9+, and TSG101+ by immunoblots. HPV16 DNA was detected in 87% of PPB-derived sEVs in HPV(+) but not in sEV of HPV(-) OPC patients. A paired comparison of sEV and baseline PPB cfDNA revealed that 39.5±24.4% circulating HPV16 cfDNA was associated with sEVs. *In vitro* assays showed that uptake of sEVs carrying HPV DNA stimulated IFNβ1 mRNA expression and TBK1 phosphorylation in recipient M0 cells, an indication of cGAS-STING pathway activation. Moreover, HPV(+) sEVs significantly promoted M1 macrophage polarization, while HPV(-) sEVs inhibited polarization.

Conclusions: This study reports that sEVs in PPBs of HPV(+) OPC patients carry HPV16 DNA. Furthermore, HPV DNA-containing sEVs activate the cGAS-STING pathway in M0 macrophages, promoting their polarization to M1 macrophages. This finding suggests that HPV DNA-containing sEVs in PPB play a crucial role in antitumor immunity within the TME of HPV(+) OPC patients.

#041

CIRCULATING DIFFERENTIAL METHYLATION ALLELE FRACTION (DMAF): AN ALTERNATIVE TO DETECTION OF TUMOR-DERIVED CIRCULATING CELL-FREE DNA

CHEN T[1*], SRINIVASAN P[1], HAGHSHENAS E[1], BABIARZ J[1], NAKAMURA Y[2], YOSHINO T[2], ALESHIN A[1], KAWLI T[1] and REITER JG[1]

[1]Natera, Inc., Austin, Texas, USA

[2]Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, Kashiwa, Japan

Circulating tumor DNA (ctDNA) is a promising biomarker that reveals tumor-associated genetic change and epigenetic aberrations and several applications have been demonstrated, including early cancer detection, treatment monitoring, and recurrence detection. Recent studies show that differential methylation patterns can be used as an alternative tool to predict ctDNA fraction. In this study, we evaluated the correlation of circulating DMAF with variant allele fraction (VAF) in colorectal cancer (CRC) patients. 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 (Signatera™) were included in this analysis and ctDNA VAF (%) was calculated. We performed deep methylation sequencing (Illumina Novaseq) across multiple target regions using two independent target sets. DMAF was calculated computationally, which estimates the fraction of differential methylation alleles for circulating cell-free DNA across target regions. 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 unstable n=10) patients, the DMAF correlation was 0.9 and 0.915, respectively. The correlation between two target sets evaluating DMAF with differing numbers of targeted regions was high (0.89). These data indicate that DMAF is a promising alternative assay to quantify the fraction of tumor-derived circulating cell-free DNA in order to assess tumor burden and monitor disease response.

#042

COMBINING METHYLATION MARKERS FOR EFFICIENT, BLOOD-BASED MULTI CANCER DETECTION

BANG-CHRISTENSEN SR[1], MARGOLIN G[1], GRIMM SA[2], BENNET BD[2], PATHAN S[1], FEDKENHEUER K[1], FARNEY SK[1], LI, JL[2], ELNITSKI L[1]

[1]The National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA
[2]Integrative Bioinformatics Support Group, Biostatistics and Computational Biology Branch, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA

The ability to detect several types of cancer using a non-invasive test holds the potential to revolutionize oncology screening. Tumors are known to shed DNA into the bloodstream, and since the tumor DNA is marked by aberrant methylation patterns, this can be exploited for their detection through a simple blood sample. We hypothesized that targeted design of a collection of DNA methylation markers could detect circulating tumor DNA (ctDNA) from multiple cancer types in one test. We obtained methylation values from >4,000 tumor samples spanning 14 cancer types as presented in The Cancer Genome Atlas (TCGA) and used 2,700 normal blood samples as reference. By screening for CpG sites with extreme and opposite values in tumor vs healthy blood samples, we identified a panel of 8 targeted methylation sites located across the genome which robustly detected the presence of tumors considering all 14 cancer types with an overall sensitivity of 91.4%. The 8-marker panel was initially validated in independent methylation array datasets from 7 out of 14 tumor types. Except for kidney tumors, where detection level was only 20%, the panel showed a sensitivity of 69-93% across the tumor types. Since methylation values from array data focusing on single CpG sites is predicted to lack the sensitivity needed to detect cancer signal in dilute samples such as blood, we applied targeted methylation sequencing when testing our panel in plasma samples. The sequencing data covered 120 bp surrounding the original CpG site, however, by including all sequenced CpGs we could report both methylation densities and epiallelic frequency which has previously been shown to boost efficiency of ctDNA detection in blood. In a cohort of liver cancer patient samples (n=12) and samples from healthy individuals (n=13), receiver operating characteristic analysis showed that one of the markers had an area under the curve value of 0.885, and that the liver samples had a significantly higher fraction of methylated reads compared to normal samples ($p = 0.001$, Wilcoxon rank sum test with continuity correction). When applying all 8-markers the assay successfully captured presence of tumor in 10/12 liver cancer plasma cases (sensitivity = 83%), with specificity among healthy controls at 100%. While further validation in other tumor types is still in progress, these results demonstrate the utility of the 8-marker panel in detecting ctDNA from tumors and distinguishing its presence from healthy plasma samples.

#044

GLYCOME PROFILING WITH A DNA-BARCODED ANTIBODY LIBRARY

BHAKTA A[1], MARGLOUS S[1], GILLMANN K[1], GILDERSLEEVE J[1]
[1]Chemical Biology Laboratories, National Cancer Institute, Frederick, Maryland, USA

Surface glycans are known to differ between cancer and noncancer cells, as well as between different types of cancer cells, but the limitations of current methods for studying surface glycosylation have led to a gap in this area of research. Methods commonly used for studying proteins cannot be applied to carbohydrates and existing technology for studying glycans such as mass spectrometry or lectins are often low throughput or less specific. Anti-glycan antibodies provide a potential alternative with their standardized structure, diverse targets, and greater potential specificity. One promising application for anti-glycan antibodies is the creation of a DNA-barcoded antibody library for use in a variety of sequencing techniques. DNA-barcoded antibodies allow the sequencing of surface glycans on both a bulk and single cell level, and have the potential to be used simultaneously with RNA sequencing. This library of antibodies is created using a standardized expression system and their targets are determined using glycan microarrays containing diverse potential glycan targets. Following production and classification, the anti-glycan antibodies are conjugated with DNA barcodes. These barcoded antibodies can then be used during sequencing to detect the presence and prevalence of specific glycans on the surface of cells. Preliminary sequencing done with SW480 (derived from a primary tumor in a patient with colorectal cancer) and SW620 (derived from a lymph node metastasis in the same cancer patient) cell lines have yielded promising results. Barcoded antibody replicates with the same target but different barcodes display strong linear correlations, suggesting that the barcoded antibodies associate with glycans as predicted and are sequenced reliably. Comparisons between the two cell lines also suggests that sequencing with the barcoded antibodies can detect differences in the surface glycomes of different cell types. Furthermore, UMAP clustering analysis suggests the presence of subpopulations within these cell lines that may have different surface glycosylation patterns which can be detected by the barcoded antibodies. Experiments are currently being done to verify the findings of these sequencing experiments using flow cytometry. This DNA-barcoded anti-glycan antibody library provides a novel method of studying the surface glycosylation of cells with the potential for eventual applications in cancer diagnostics.

#045

IMPACT OF DELAYED PROCESSING ON CFDNA QUANTITY AND QUALITY USING STRECK CFDNA TUBES: LIQUID BIOPSY CONNECT PILOT STUDY

BESS J[1], HOPKINS D[1], DIAZ MAYORAL N[2], COOLEY H[2], DE PALATIS A[2], WONG D[3], ELASHOFF D[6] , WEI F[3], LI F[3], WILLIAMS M[4], KARLOVICH C[4], GREENBANK E[4], WEINSTEIN OSHINSKY S[1], ABNET C[1], HORNER MJ[1], BLACK A[1], BROTZMAN M[1], WYATT K[1], SORBARA L[5], YOUNG M[5], GAUDET M[1], WENTZENSEN N[1]

[1]Division of Cancer Epidemiology and Genetics (DCEG), National Cancer Institute (NCI), NIH, MD, USA.
[2]BioProcessing Laboratory, Frederick National Laboratory for Cancer Research, MD, USA.
[3]School of Dentistry, University of California, Los Angeles (UCLA), Los Angeles, CA,USA.
[4]Molecular Characterization Laboratory, Frederick National Laboratory for Cancer Research, MD, USA.
[5]Division of Cancer Prevention (DCP), National Cancer Institute (NCI), NIH, MD, USA.
[6]School of Medicine, University of California, Los Angeles (UCLA), Los Angeles, CA, USA.

Background: Interest in cell-free DNA detection for multi-cancer early detection and tumor monitoring is growing. Prospective evaluations of liquid biopsy assays are critical, and knowledge gaps in standard collection and preparation practices must be addressed for adequate assay execution. The NCI Connect for Cancer Prevention Cohort Study (Connect) aims to study new approaches in early detection. A pilot study was conducted with the STRECK Cell-Free DNA BCT tube to determine the impact of processing time on cfDNA quantity and quality utilizing two complementary quality control assays.
Methods: 4 STRECK DNA BCT tubes were drawn from 40 donors. Plasma samples were analyzed, with half processed immediately and the other half stored for five days before processing. Two QC assays were performed to compare results at the two processing time points. The Alu EFIRM assay measured Alu element concentration (copies/avg). The Cell-free DNA ScreenTape assay evaluated Qubit DNA concentration (µg/mL) and %cfDNA. Descriptive statistics and paired sample t-tests were performed to compare mean concentrations at the two processing time points, with and without identified samples with cytolysis.
Results: Median, mean, and range were recorded for each assay measure (Table 1). A reduction in cfDNA quantity was observed at the 5-day processing time point. Five 5-day processing samples were identified with high DNA quantity and a high Alu element concentration. The five samples experienced hemolysis and clotting as a result of handling and processing at odds with standard protocol. There was a significant difference between the mean DNA quantities of the processing time points following exclusion of the five samples. There was no change in significance between the mean Alu concentration and %cfDNA following exclusion (Table 2).
Conclusions: Both assays successfully detected samples with high nuclear DNA, a major contaminant for cfDNA assays, and identified a reduction in cfDNA at the 5-day processing time point. Proper handling and processing procedures are necessary to avoid cell lysis and maximize cfDNA quality and yield. This pilot establishes a starting point for future evaluations of liquid biopsy tube types, processing delays, and preanalytical cfDNA quality variables.

Table 1. Descriptive Statistics by Processing Time

Assay Measure	Median		Range		Mean	
	0 Days	5 Days	0 Days	5 Days	0 Days	5 Days
Alu EFIRM (copies/avg)	13647.70	12252.42	20469.09	231989.99	12264.71	29736.71
Qubit Concentration (ng/mL)	0.19	0.17	0.54	5.98	0.21	0.51
Percent cfDNA (%cfDNA)	76.10	67.80	27.10	46.90	76.90	65.90

Table 2. Paired T-Test Results

Assay Measure	Including 5 Samples				Excluding 5 Samples			
	t Stat	t crit	df	p	t Stat	t crit	df	p
Alu EFIRM (copies/avg)	-2.01	2.03	36	0.05	-0.37	2.04	30	0.71
Qubit Concentration (ng/mL)	-1.61	2.02	39	0.11	4.21	2.03	34	0.00
Percent cfDNA (%cfDNA)	5.62	2.02	39	0.00	7.80	2.03	34	0.00

#046

BRAIN SPECIFIC MRNAS IN BLOOD EXTRACELLULAR VESICLES ARE POTENTIAL BIOMARKERS FOR DETECTING TRANSCRIPTIONAL CHANGES IN THE BRAIN

SMIRNOVA L[1], OSBORNE LM[2], PAYNE JL[3] AND SABUNCIYAN S[4]
[1]Center for Alternatives to Animal Testing, Bloomberg School of Public Health, Johns Hopkins University
[2]Departments of Obstetrics & Gynecology and of Psychiatry, Weill Cornell Medicine
[3]Department of Psychiatry and Neurobehavioral Sciences, University of Virginia
[4]Department of Pediatrics, Johns Hopkins School of Medicine

The inaccessible nature of the brain is a fundamental problem for treating brain disorders. Recent efforts to develop peripheral biomarkers for the brain have focused on isolating neural derived extracellular vesicles (EV) in body fluids by targeting EV surface proteins such as L1CAM and NCAM that are assumed to be specific for neural tissue. In our current work, we demonstrate the feasibility of using the mRNA content of EVs along with tissue specific mRNA expression information available in publicly available databases to determine the tissue of origin of EV mRNAs in blood. The approach of characterizing the mRNA cargo of EVs is a more specific measure of transcriptional activity in neural tissue since L1CAM and NCAM are expressed at high levels in multiple non-neural tissues. Our approach identified 181 brain specific mRNAs that are found in blood. Gene set enrichment analysis revealed that these EV mRNAs are implicated in mood disorders, schizophrenia, epilepsy and various other brain diseases. The EV mRNA level of 13 of these brain-specific transcripts is associated with postpartum depression, raising the possibility that they can be used to infer the state of the brain. In order to evaluate their biomarker potential, we used brain organoids differentiated for 3 and 9 weeks to investigate the extent to which EV mRNA levels reflect transcriptional changes in the brain. Although mRNA levels inside EVs and cells were not identical, they correlate and suggests that inferring transcriptional changes in the brain using EV mRNA levels is possible. Our findings establish the potential of EV mRNAs as biomarkers for brain pathology and bring them to the forefront of biomarker development efforts in psychiatric and neurological diseases.

#047

TRACING THE CLONAL DYNAMIC OF METASTATIC CASTRATION-RESISTANT PROSTATE CANCER OVER IMMUNOTHERAPY USING CIRCULATING TUMOR DNA

LI C[1], BAJ A[1], SEO CY[1], TERRIGINO NT[1], BRIGHT JR[1], HENNIGAN ST[1], KING IM[1], WILKINSON S[1], TROSTEL SY[1], FIGG WD[1], DAHUT WL[1], LEE JM[2], TAKEDA DY[1], KARZAI F[1], AND SOWALSKY AG[1]
[1]Genitourinary Malignancies Branch, National Cancer Institute, Bethesda, Maryland, USA
[2]Women's Malignancies Branch, National Cancer Institute, Bethesda, Maryland, USA

Human cancer tissues are known to release their own DNA into body fluids such as plasma. These DNA molecules derived from tumors, collectively known as circulating tumor DNA (ctDNA), contain genetic information about disease progression at the time of sampling. Importantly, ctDNA has recently been utilized for capturing the whole-body disease profile and when sampled at various time points over the treatment, can inform the dynamic of clonal heterogeneity and its contribution to therapy failures. Metastatic castration-resistant prostate cancer (mCRPC) is a class of cancerous diseases with a high degree of clonal heterogeneity and high mortality which is largely due to the frequent emergence of therapy resistance. Therefore, reconstructing the clonal history for mCRPC diseases using ctDNA has great potential in identification of novel and targetable molecular mechanisms that drive mCRPC progression. To assess this possibility, we hypothesize that tumor clones that are responsible for therapy resistance carry distinct genomic profiles, which are captured in plasma ctDNA and could be computationally resolved through genomic sequencing and clonal reconstruction. To address this question, we obtained plasma cell-free DNA (cfDNA), a mixture of ctDNA and other tissue-derived DNA, from 38 individuals treated with combination PD-L1 and PARP inhibition involved in a recent clinical trial (NCT02484404). Whole-genome sequencing (median coverage: 139x) was performed using cfDNA and fragmented buffy coat DNA as germline control. cfDNA samples with a tumor fraction of lower than 10% were excluded. Various computational strategies were used to evaluate the potential impact of genomic alterations and model the clonal structures of diseases. Through this approach, we observed a negative association between cfDNA tumor fraction and therapy response. Importantly, diseases that failed to respond to the treatment often involved germline and somatic alterations to various DNA damage and repair genes (including *TP53*, *CDK12*, and *ATM*) and greater genomic rearrangements. By tracing genetic alterations over the course of treatment, evidence of clonal persistence was frequently detected that was associated with drivers of genomic damage and repair. Additionally, therapy failures tended to associate with greater changes in clonal architectures inferred using cfDNA. Multiple clonal evolutionary patterns leading to therapy failures were observed and will be discussed during the poster presentation. Our novel findings from this study will fill in a critical gap of knowledge about the complex genetic mechanisms driving mCRPC immunotherapy resistance.

#048

DEVELOPMENT OF AN EPIGENETIC PANEL FOR UROLOGICAL CANCERS

BRIKUN I[1], FECZKO J[2], FREIJE D[1]
[1]APR Biosciences Inc., 9800 Connecticut Dr., Crown Point, IN 46307
[2]Northwest Health-Porter, 85 East US Hwy 6, Valparaiso, IN 46383

Asymptomatic cancer screening will need to be performed on tens of millions of people at regular intervals to achieve the goal of early detection. It will require accurate, cost-effective diagnostic tests that are easy to explain to physicians and patients. Liquid biopsies that are based on nucleic acids are subject to high sampling error and multiple markers will be needed to overcome it. We previously analyzed urine DNA from patients undergoing prostate biopsies with a panel of 32 epigenetic markers. We were able to predict disease status using the number of positive markers with near perfect sensitivity and specificity. Furthermore, the study showed that accurate diagnosis can be achieved with a smaller number of markers and that many combinations of markers can yield equivalent results. The PCA panel included several CpG islands associated with the homeobox gene clusters HOXA, B, C, and D which were frequently recovered in urine DNA. Analysis of the HOX markers on Gleason 3 (GS3) and Gleason 4 (GS4) prostate tumors shows that their methylation is common in GS3 tumors and nearly homogeneous in GS4. This suggests that they are acquired early and progressively during tumorigenesis. Their inclusion in the PCA panel made it easier to overcome tumor heterogeneity and improved the panel's sensitivity and specificity when analyzing circulating DNA. Methylation of the HOX genes has been reported for other cancers. It raised the possibility that they can serve as the basis of a multicancer diagnostic test. In this study, we investigated the methylation of the PCA panel in bladder and kidney tumors and expanded the analysis to additional homeobox genes. The results show that some PCA markers are also methylated in bladder and/or renal cancers including HOXA7, HOXD3c, ADCY4, and GPR62 while others are PCA specific such as AOX1, CYBA, GSTP1, and HOXD8. Other homeobox genes are also methylated in one, two or all three cancers such as HOXA3, HOXA9, HOXC4, GSC, LHX9, and NKX2-2. A cost-effective diagnostic test for urological cancers can be achieved using a small number of commonly methylated markers supplemented with markers with biased or restricted methylation patterns to identify the tissue of origin.

#049

NOVEL CD47 INTERACTING CARGO PROTEINS OF EXTRACELLULAR VESICLES FROM T LYMPHOBLAST AND PROSTATE CARCINOMA CELLS IDENTIFIED USING SINGLE VESICLE IMAGING AND MASS SPECTROMETRY

KAUR S[1], JENKINS LM[2], ROBERTS DD[1]

[1]Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, USA

[2]Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, USA

CD47 is a ubiquitously expressed membrane protein that functions as a receptor for thrombospondin-1 and the counter receptor for signal regulatory protein alpha in phagocytes. CD47 is widely expressed on extracellular vesicles (EVs) that contain a distinct population of RNAs. CD47 colocalizes predominantly with CD81 and α4β1 integrin on Jurkat T cell-derived EVs but not with EVs bearing CD63 or CD9. CD47 and its cytoplasmic adapter ubiquilin-1 regulate which RNAs are packaged into T cell EVs via physical interactions with components of the exportin-1/Ran nuclear export complex and its known cargos. Here, we report that disruption of CD47 in Jurkat T lymphoblast and PC3 prostate carcinoma cells impairs the sorting of filamin A and α4β1 integrin into EVs. Targeted mass spectrometry and coimmunoprecipitation analyses indicate that CD47 indirectly interacts with filamin A either via ubiquilin-1/Exportin-1 or β1 Integrin. Filamin A may thereby play an important role in CD47-dependent sorting of protein and RNA cargoes into specific subsets of EVs.

Conclusions: CD47 and ubiquilin-1 interact with filamin A, which is known to interact with the cytoplasmic domain of β1 integrins to regulate integrin function. Less filamin A and α4β1 integrin sort into EV in the absence of CD47, suggesting that CD47 promotes filamin A and integrin sorting into EV, mediated through ubiquilin1 and/or exportin-1.

#050

ENHANCED CELL-FREE DNA EXTRACTION FROM LARGE WHOLE URINE VOLUMES: IMPLICATIONS FOR PROSTATE CANCER DETECTION

JAFARI N, SAENZ J, LEE L, HERNANDEZ C, DUNNIGAN A, and SAIDIAN M
nRichDX, Irvine, California, USA

Introduction: Urine, as a non-invasive source of genomic materials, offers a promising avenue for prostate cancer detection. However, the extraction of cfDNA from urine is challenged by its low concentration, necessitating the development of more efficient extraction methods. This study aimed to enhance DNA extraction from large whole urine volumes, thereby improving the sensitivity and reliability of prostate cancer detection. This enhanced extraction method eliminates the analyte yield-lowering steps often used by other extraction methods – schemes like pre-extraction volume concentration, splitting samples into separate extractions, sample transfers between plasticware, and eluate pooling. These schemes lower rare analyte yields; thus, liquid biopsy applications require better sample prep methods.
Methods: This study collected fifty-milliliter whole urine samples from 12 healthy donors. After collection, the samples were preserved with nRichDX whole urine preservative. Total (cellular and cell-free) spiked with LANCAP cells containing the PTEN mutation—a genetic alteration commonly associated with prostate cancer. The nRichDX Revolution Max20 cfDNA Isolation Kit was employed to extract DNA from the samples. The replicate samples were also extracted with the Zymo Quick-DNA Urine Kit. The actionable recovery of DNA molecules was determined by quantitative real-time polymerase chain reaction (qPCR) mutation detection assay using TaqMan.
Results: Quantitative assessments revealed a notable increase in DNA concentration, surpassing the yields obtained through standard extraction methods by a significant margin for the nRichDX Revolution Max20 cfDNA Isolation Kit. The yield was consistent across all the samples. The actionable DNA molecules were statistically significantly higher than those recovered using the Zymo Quick-DNA Urine Kit, as evidenced by Ct values calculated on qPCR mutation detection.
Conclusion: The study's findings underscore the efficacy of the enhanced DNA extraction protocol using the nRichDX Revolution Isolation Kit in processing large urine volumes. This kit is designed to process larger volumes of up to 50 mL in a single extraction step, thus potentially recovering higher yields and quality of DNA compared to other kits, which typically require the splitting and pooling of samples, leading to lower yields and possibly compromised DNA integrity. This advancement holds significant promise for the non-invasive detection of prostate cancer, enabling a more sensitive and reliable diagnostic approach. By improving the yield and quality of DNA extracted from urine, this method paves the way for the broader application of liquid biopsy techniques in prostate cancer management, potentially facilitating earlier detection and more effective disease monitoring.

#051

DETECTION OF MINIMAL RESIDUAL DISEASE IN LYMPH PREDICTS RECURRENCE IN HPV-NEGATIVE HEAD AND NECK CANCER PATIENTS

LAZARE S. S.[1], GU Z. [1], WHITFIELD D. [1], EARLAND N.[2,3], HARMON A. [1], LONG M. [1], HARRIS P.[2], XU Z.[4], RAMIREZ R.[5], GERNDT S.[6], PACULA M. [1], FRANCIS M. S. [1], ZEVALLOS J. P.[7,8], CHAUDHURI A. A.[2,3,9,10,11], WINCKLER W.[1]

[1]Droplet Biosciences, Inc., Cambridge, Massachusetts

[2]Division of Cancer Biology, Department of Radiation Oncology, Washington University School of Medicine, St. Louis, Missouri

[3]Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri

[4]Department of Otolaryngology-Head and Neck Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania

[5]Department of Otolaryngology-Head and Neck Surgery, Washington University School of Medicine, St. Louis, Missouri

[6]Division of Otolaryngology-Head and Neck Surgery, Allegheny Health Network, Pittsburgh, Pennsylvania

[7]Department of Otolaryngology-Head and Neck Surgery, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania

[8]Hillman Cancer Center, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania

[9]Department of Computer Science and Engineering, Washington University in St. Louis, St. Louis, Missouri

[10]Department of Genetics, Washington University School of Medicine, St. Louis, Missouri

[11]Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, Missouri.

Locoregional cancer relapse remains a major cause of failure in head and neck squamous cell carcinoma (HNSCC), particularly for HPV-negative patients whose 2-year locoregional failure rate is up to 50%. There is unmet need for an accurate diagnostic test that predicts risk of recurrence prior to adjuvant therapy selection. We present a novel proximal assay for minimal residual disease (MRD) profiled in lymphatic exudate collected via surgical drains ("lymph"). Lymph, plasma, and blood were collected from 39 HPV-negative HNSCC patients postoperatively at 24 hours along with resected tumor. Cell-free DNA was extracted from lymph and plasma and sequenced using the TruSight Oncology 500 panel to a depth of >100 million reads. Somatic mutations were identified by exome sequencing tumor and blood. All patients had >1 year clinical follow-up, 18 patients showed disease recurrence (REC) and 21 had no evidence of disease (NED). Tumor-specific variants were force-called in lymph and plasma using a custom pipeline. Patients were considered MRD positive if the mean variant allele fraction (mVAF) was greater than 0.015% (the estimated limit of detection). The Kaplan-Meier (KM) estimator with log-rank test and Cox proportional-hazards model were used for survival analyses. Logistic regression model was performed with 5-fold cross-validation. KM survival analyses showed lymph accurately predicts recurrence (sensitivity (SN) = 78%, specificity (SP) = 67%; p = 0.003, Hazard ratio (HR) = 4.7), while plasma was not significant (p = 0.92) at this postoperative timepoint. Performance was enhanced for locoregional relapse (SN = 92%, SP = 67%; p = 0.001, HR = 13.9. N=33). We also observed accurate recurrence prediction in the 19 N0 patients (node-negative by pathology) (SN = 83%, SP = 69%, p = 0.03, HR = 7.7) Lymph MRD outperformed individual pathology features (extranodal extension, perineural invasion, lymphovascular invasion, and nodal disease status) for recurrence prediction. A logistic regression model combining lymph MRD with these 4 high-risk pathologic features showed superior performance over lymph alone or pathology alone (SN = 89%, SP = 67%; p = 0.0007, HR = 8.6). ctDNA analysis of lymph from surgical drains represents a novel proximal MRD. Postoperative lymph significantly outperforms plasma for prediction of recurrence in HPV-negative HNSCC. Accurate MRD identification in patients with lower risk pathologic features suggests that lymph MRD testing has potential to augment traditional pathology and provide more personalized adjuvant treatment decision-making in patients with HPV-negative HNSCC.

#052

DENTIFYING ACTIONABLE GENOMIC ALTERATIONS IN NON-SMALL CELL LUNG CANCER THROUGH THE ANALYSIS OF EXTRACELLULAR VESICLES IN PLASMA

MILOSEVIC J[1], HE H[1]*, ZHENG S[2]*
[1]Captis Diagnostics Inc, Pittsburgh, PA.
[2]Carnegie Mellon University, Biomedical Engineering, Pittsburgh, PA.

Lung cancer is the second most common cancer worldwide, accounting for 2.2 million new cases and 1.8 million deaths in 2020. Personalized targeted therapy based on actionable molecular markers has completely transformed the therapeutic landscape in NSCLC, which has improved considerably the overall survival. However, ~50% of NSCLC patients with tissue biopsy inaccessible or inadequate, limiting biomarker testing and access to targeted therapies. The availability of a wide range of highly effective targeted therapy options for NSCLC justifies the significance of developing non-invasive and cost-effective detection assays for guiding targeted therapy selection in NSCLC. Extracellular vesicles (EVs) are lipid-bilayer-enclosed vesicles of sub-micrometer size that are secreted by virtually all cell types. They contain hundreds of different proteins, thousands of intact RNA species, and double-stranded DNA (dsDNA) fragments that sample the entire human genome. Recent findings further corroborate the potential role of EVs in early cancer screening, cancer diagnosis, treatment selection and monitoring. In this study, we used lipid nanoprobe (LNP) for EV isolation from plasma and droplet digital PCR assay for detecting actionable genomic alternation to aid targeted therapy selection in NSCLC. Specifically, we extracted EV associated DNA/RNA from plasma first, and then we used the individual ddPCR assays for detecting KRAS mutations, EGFR mutations, and ALK fusions from isolated EVs associated nucleic acids, respectively. We identified KRAS mutations G12C/G12S/G13C/G12A/Q61H, EGFR L858R mutation and exon 19 deletion, and ALK-EML4 gene fusion variant 3 from 12 NSCLC patients that matched the mutation status obtained from patient tissue specimens. Only in 1 out 13 patients, EV mutation status did not match the mutation status from the tissue specimen. This data indicated that clinical actionable gene mutations are present in EVs associated nucleic acids isolated from plasma samples of NSCLC patients. We anticipate that this novel non-invasive EVs-based actionable genomic alterations detection test would efficiently assist physicians/oncologists in selecting optimal targeted therapies for cancer treatment, as recommended by National Comprehensive Cancer Network (NCCN) guidelines for NSCLC.

#053

DISSECTING THE CIRCULATING PROTEOME IN NF1-ASSOCIATED PERIPHERAL NERVE SHEATH TUMORS

SUNDBY RT[1], ZHANG X[1], SHAHSAVARI S[1], ANNOR GK[1], and SHERN JF[1]
[1]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Background: Malignant peripheral nerve sheath tumors (MPNST) are aggressive cancers with a poor prognosis that, in neurofibromatosis type 1 (NF1), frequently arise from within their benign plexiform neurofibroma (PN) or pre-malignant atypical neurofibroma (AN) precursors. We previously published that copy number analysis (CNA) with size selection of cell free DNA (cfDNA) non-invasively distinguishes PN from MPNST. Our published assay, however, has limited sensitivity for pre-malignant AN when intervention would be most impactful. Previous publications have demonstrated utility for circulating proteins in detecting early-stage disease and tumors with low mutational burden. **We hypothesize that PN, AN and MPNST have unique circulating proteomic signatures that can non-invasively distinguish pre-malignant disease states.**
Methods and Findings: Candidate circulating proteins were identified using publicly available MPNST RNAseq data (Lee et al, 2014), revealing 324 genes significantly overexpressed in MPNST. Forward selection of these candidate genes against the Human Protein Atlas for genes encoding secreted proteins retains 64 candidate proteins. 35 of these secreted proteins are cancer associated. Candidate proteins were included in a panel of pre-validated and pre-optimize DNA-oligonucleotide labeled dual recognition antibodies (Olink, Boston, MA) for proximity extension assay (PEA). Using minimum normalized protein expression (NPX) difference of 0.8 and p value cutoff of 0.05, ANOVA of PEA from 40uL/subject of plasma from healthy volunteers (n = 10), PN (n = 29), AN (n = 25) and MPNST (n = 54) identified differentially expressed proteins: 29 differentiating PN and healthy, 7 distinguishing PN and AN, 65 differentially expressed between AN and MPNST, and 134 proteins differing between PN and MPNST. Finally, proteins identified to have significantly different expression between disease state in PEA, e.g. DLK1, map specifically to malignant cell clusterss in unmatched NF1 scRNAseq UMAP.
Conclusions: In this early-stage study, we demonstrate that (1) NF1 disease states have distinct secreted proteins profiles that are detectable in peripheral blood using PEA and (2) proteins that are differentially detected in plasma between MPNST and other disease states map to malignant cell subpopulations in unmatched scRNAseq data. Validation in a larger cohort is required, however, this study suggests that proteomics alone or integrated with an orthogonal cfDNA approach may be a viable circulating biomarker for low-mutational burden pre-malignant and early stage NF1 disease. Earlier definitive detection of AN and MPNST would improve clinical outcomes by enabling patients to receive therapeutic intervention while disease burden is still minimal and marginal resections remain curative.

#054

CELL-FREE DNA METHYLATION SIGNATURES: BRIDGING THE GAP IN ALS BIOMARKER DISCOVERY

JIN Y[1], MA W[1], NAVIAUX RK, SIDDIQUE T[3], JIN P[1]
[1]Emory University, Atlanta, GA, USA
[2]University of California, San Diego, CA, USA
[3]Northwestern University, Evanston, IL, USA

Cell-free DNA (cfDNA) in plasma carry epigenetic signatures specific to their tissue or cell of origin. Altered methylation patterns in circulating cfDNA have emerged as valuable tools for noninvasive cancer detection, prenatal diagnostics, and organ transplantation assessment, which could also be promising for diagnosing neurodegenerative diseases, which often progress slowly and have a lengthy asymptomatic period. However, understanding the cfDNA methylation changes in neurological diseases remains limited. Amyotrophic lateral sclerosis (ALS), an adult-onset neurodegenerative disorder, is characterized by the progressive degeneration of motor neurons in the brain and spinal cord. Approximately 5-10% of ALS cases are familial (FALS), showing a genetic predisposition, such as the hexanucleotide repeat expansion in the C9ORF72 gene. The remainder is considered sporadic (SALS), with a polygenic and multifactorial etiology. Here, we conducted whole genome bisulfite sequencing (WGBS) on plasma samples from ALS patients and age/sex-matched healthy controls across two cohorts (Cohort 1, n=18; Cohort 2, n=6). We identified between 1045 and 2342 differentially methylated regions (DMRs), highly enriched in the regulatory regions marked by histone modification and transcription factor binding. The genes associated with DMRs are involved in ALS-related pathways, such as endocytosis and axon guidance. Comparative analysis revealed 129 DMR-associated genes shared across both cohorts, implicating these pathways in ALS. Remarkably, 33-35% of these DMR-associated genes showed differential expression in spinal cord segments of ALS patients compared to controls, with over 20 genes significantly correlated with disease duration, highlighting their importance in ALS progression. Furthermore, by comparing our findings with single-nuclei multi-omics datasets from the frontal cortex of C9orf72 patients and controls, we found approximately 400 DMRs co-localized with differentially accessibility regions in excitatory neurons and microglia from C9 patients. Many DMR-associated genes were differentially expressed in excitatory neurons, astrocytes, and microglia. To better understand the contributions of various tissues/cells to the cfDNA pool in ALS patients, we performed cfDNA deconvolution using type I/II methylation markers. This analysis showed that ALS patients had a higher contribution of liver and intestine in cfDNA and a lower proportion of T-cell and B-cell than controls. Our data indicate that DMRs identified in circulating cfDNA can reflect altered chromatin states and gene expression in the ALS brain in a cell-type-specific manner. Methylation analysis of cfDNA represents a powerful approach for ALS assessment, revealing disturbed regions, genes, and the proportional contributions of different tissues/cells to the plasma. This technique holds promise for clinical application in biomarker discovery across a broad spectrum of neurodegenerative disorders.

#055

ULTRA-SHORT TRANS-RENAL CIRCULATING TUMOR DNA IN URINE FOR NON-INVASIVE LIQUID BIOPSY OF HPV+ OROPHARYNGEAL CANCER

BHAMBHANI C[1], KANG Q[1], HOVELSON DH[2,3], SANFORD E[1], OLESNAVICH M[1], DERMODY SM[4], WOLFGANG J[1], TUCK KL[1], BRUMMEL C[4], BHANGALE AD[4], HE K[1], GUTIERREZ MG[5], LINDSTROM R[1], LIU CJ[2,6], TUCK M[1], KANDARPA M[1], MIERZWA M[7,8], CASPER K[4,8], PRINCE ME[4,8], KRAUSS JC[1], TALPAZ M[1], HENRY NL[1,8], GIRALDEZ MD[1,9], RAMNATH N[1], TOMLINS SA[2,6,10], SWIECICKI PL[1,8], BRENNER JC[4,8,11], TEWARI M[1,8,12,13]*

[1]Department of Internal Medicine, Division of Hematology/Oncology, University of Michigan, Ann Arbor, MI, USA

[2]Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, MI, USA [3]Department of Computational Medicine & Bioinformatics, University of Michigan, Ann Arbor, MI, USA [4]Department of Otolaryngology, University of Michigan, Ann Arbor, MI, USA

[5]Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA [6]Department of Pathology, University of Michigan, Ann Arbor, MI, USA [7]Department of Radiation Oncology, University of Michigan, Ann Arbor, MI, USA [8]Rogel Cancer Center, University of Michigan, Ann Arbor, MI, USA

[9]Instituto de Biomedicina de Sevilla, IBiS/Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla; Seville, Spain

[10]Department of Urology, University of Michigan, Ann Arbor, MI, USA

[11]Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA

[12]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA

[13]Center for Computational Biology and Bioinformatics, University of Michigan, Ann Arbor, MI, USA

Detection of “trans-renal” cell-free tumor DNA (TR-ctDNA), which transits from the bloodstream into urine, holds promise for urine-based detection of virtually any cancer type, including non-urologic cancers. This approach could also overcome some of the limitations of blood ctDNA testing, including the limited blood volume that can be sampled (e.g., in studies requiring high sampling frequency and/or volume to study ctDNA kinetics), and the need for phlebotomy services that can limit patient access to testing, especially for underserved populations. However, some studies have reported that this approach has low sensitivity and optimal methods of TR-ctDNA analysis remain to be defined. Using single-stranded urine DNA sequencing to study TR-ctDNA in a range of cancer types, we discovered that TR-ctDNA fragments are predominantly ultra-short (<50 bp) and therefore likely to be missed by conventional ctDNA assays designed to measure plasma ctDNA. To test this hypothesis, we developed an ultra-short DNA-compatible ddPCR assay for detection of TR-ctDNA originating from human papillomavirus-associated oropharyngeal squamous cell carcinoma (HPV+ OPSCC) as a test case. We found that an unconventional ddPCR assay designed to detect ultra-short fragments (42 bp), had a greatly improved sensitivity of TR-ctDNA detection compared to a conventional ddPCR assay which was designed for longer amplicons (77 bp). During clinical validation, we found that TR-ctDNA from urine samples was concordant with plasma ctDNA for cancer detection, even for early stage HPV+ OPSCC patients. Notably, in many cases the TR-ctDNA was obtained from urine samples shipped by patients who were up to 300 miles away from our testing lab. Furthermore, by comparing voids from HPV+ OPSCC patients over different days, we found that TR-ctDNA exhibits relatively low day-to-day variability, which was in a range similar to what has been reported for plasma ctDNA. Lastly, as a proof- of-concept for post-treatment surveillance, we found that urine TR-ctDNA-based longitudinal monitoring for HPV+ OPSCC in a case series of four patients who experienced cancer recurrence showed detectable TR-ctDNA earlier than imaging. In conclusion, our study shows how ultra-short DNA-based analysis could help realize the full potential of urine-based TR-ctDNA cancer diagnostics. This has implications for facilitating access-to-care through at-home specimen collections and for urine-based detection of a wide variety of non-urologic cancer types.

#056

HIGA N[1], KUHN P[1,2], BERRY JL[1,3], XU L[1,3]

[1]Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[2]Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA, USA
[3]The Vision Center at Children's Hospital Los Angeles, Los Angeles, CA, USA

HIGH PURITY CTDNA AS A MODEL SYSTEM: EVALUATING ICHORCNA PERFOMANCE IN SILICO FOR IMPROVED LIQUID BIOPSY ACCURACY, COVERAGE EFFECTS, AND SIZE SELECTION EFFICIENCY

Background: Cell-free DNA fragmentomic features are increasingly being investigated to improve detection of circulating tumor DNA (ctDNA). Current understanding of differentiating features of ctDNA versus non-tumor fragments is largely derived from comparisons between plasma samples of cancer patients and healthy donors. However, patient samples contain a mixture of both ctDNA and non-tumor fragments. This makes it difficult to validate novel ctDNA-specific features and perform analytical studies for new detection methods. ichorCNA, a robust liquid biopsy tool, offers comprehensive genomic insights, including copy number alterations and tumor fraction (TFx) information. Drawing on the shorter fragment size of ctDNA, incorporation of size selection with ichorCNA may further enhance sensitivity for detecting genomic alterations and ctDNA. Leveraging the unique enrichment of ctDNA in retinoblastoma aqueous humor (AH), this study aims to utilize high-purity ctDNA reference samples to evaluate the performance of the ichorCNA analysis method.
Methods: One blood sample with 0% TFx and one diagnostic AH sample with >99% TFx from a retinoblastoma patient were used for this study. Samples underwent cfDNA extraction followed by library preparation and whole genome sequencing. Blood and AH reads were downsampled and combined to produce three replicates of synthetic admixtures at 0.1x, 0.5x, 1x, and 2x unique coverage and 0.01-0.5 TFx. Size filtering was also performed to select fragments ≤150 base-pairs. TFx was determined by ichorCNA with either standard or optimized (to detect low TFxs) parameters.
Results: TFx analysis using standard parameters resulted in an LoD of 5-6% TFx with accurate quantification above this level, regardless of sample coverage. With optimized parameters, the LoD improved to 3% TFx, though absolute quantification was more accurate with 0.5x coverage or higher (median absolute deviation from expected TFx: 0.1x=2.6% vs <1% for 0.5x, 1x, and 2x). Size selection resulted in augmented TFx estimates that maintained a consistent relationship with the original TFx values across 0.5x, 1x, and 2x coverage. This relationship could be approximated using polynomial curve fitting (R-squared=0.97).
Conclusions: Increased coverage above 0.1x did not improve the LoD of ctDNA detection. However, 0.5x coverage or higher resulted in accurate TFx quantification and was most suitable for analyses with size selection. The relationship between amplified TFx values after size selection and the original TFxs could be applied to predict the true TFx for size selected samples. Overall, this study demonstrates the utility of the AH liquid biopsy as a biological model system for testing the performance of ctDNA analyses.

#057

MULTIPLE METHYLATED MARKERS OF MALIGNANCY: A NON-INVASIVE, MULTI-CANCER DIAGNOSTIC

PATHAN, SK[1], BANG-CHRISTENSEN SR[1], FEDKENHEUER K[1], MARGOLIN G[1], GRIMM SA[2], BENNET BD[2], JIANG-LIANG, LI [2], FARNEY SK[1], ELNITSKI L[1]

[1]The National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA

[2]Integrative Bioinformatics Support Group, Biostatistics and Computational Biology Branch, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina, USA

Invasive testing is often required to diagnose cancer. Here, we describe a cost-efficient, non-invasive test that can detect multiple cancers. DNA methylation, frequently disordered in malignancy, is a potential multi-cancer biomarker. We hypothesized that a panel of methylation probes can be used to detect circulating tumor DNA in cell-free DNA from human plasma. The panel of methylation probes was developed using methylation data from The Cancer Genome Atlas (TCGA), which contained 4050 tumors and 646 healthy samples across 14 different cancer types. CpG probe sites were selected if an absolute median and mean methylation beta value difference between tumor and healthy samples was above 0.4, and with significant *p*-values using both *t*-test and Wilcoxon rank sum tests after Bonferroni-correction. From this data, 3 probes were selected in 9 cancer types, and 11 probes were selected in 8 cancer types, yielding 14 probes as potential multi-cancer markers. When analyzing these individual probes in silico in various cancer types (n= 200 samples) compared to healthy tissue samples (n=20 samples) from the TCGA dataset, sensitivities ranged from 66-100%, depending on the probe and the cancer type, with highest sensitivities and specificities for head and neck squamous cell cancer, lung adeno- and squamous cell carcinoma, and rectal adenocarcinoma. Since these samples provide methylation data from tissue biopsies only, validation of the probes in liquid biopsies, where tumor DNA is far more dilute, is crucial. We collected 16 hepatocellular carcinoma (HCC) plasma samples, 24 pancreatic adenocarcinoma (PAAD) plasma samples, and 13 healthy plasma samples to assess differential methylation at our 14 multi-cancer probes. When comparing PAAD to healthy samples, 5 of the 14 probes were found to be significantly hypermethylated. The area under the curve (AUC) in the ROC analysis for the probe with highest sensitivity and specificity was 0.86. All of the pancreatic cancer samples were positive for at least one of the 14 probes. In the HCC plasma, the AUC was 0.88 for the probe with the highest sensitivity and specificity; 10 out of 12 cancer samples were detected by at least one probe. The probes with highest AUC in PAAD plasma and HCC plasma were also the top performing probes in the TCGA analysis. Overall, these 14 probes show promise as a non-invasive multi-cancer diagnostic.

#058

DROPLET DIGITAL PCR SEQUENCING OF CELL-FREE DNA FROM CEREBROSPINAL FLUID FOR DETECTION OF HOTSPOT MUTATIONS IN GLIOMA

TARTARO M[1], NANDAKUMAR S[1,3,4], HERTZ C[1], HOLLE B[1], HILL K[7], LAPSHINA M[3], FARINA A[5], ARCILA M[8], MELLINGHOFF I[1,2,6], MILLER A[9]

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA
[2]Department of Neurology, Memorial Sloan Kettering Cancer Center, New York, NY, USA
[3]Marie-Josée and Henry R. Kravis Center for Molecular Oncology, Memorial Sloan Kettering Cancer Center, New York, New York.
[4]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York.
[5]Integrated Genomics Operation, Memorial Sloan Kettering Cancer Center, New York, New York.
[6]Department of Pharmacology, Weill Cornell Medical College, New York, NY, USA
[7]Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY, USA
[8]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY, USA
[9]Department of Neurology, NYU Langone Health, New York, NY, USA

Sequencing of circulating tumor DNA (ctDNA) from cerebrospinal fluid (CSF) has the potential to enhance diagnosis, prognosis, and longitudinal monitoring for glioma patients through a minimally invasive approach. We previously detected somatic mutations from gliomas in CSF samples using MSK-IMPACT, a 505-gene FDA-authorized next-generation DNA sequencing panel and detected tumor-derived mutations in 50% of samples of patients with high-grade gliomas (HGG). To investigate whether we could enhance sensitivity of CSF ctDNA sequencing, we sequenced residual DNA libraries from 43 samples submitted for MSK-IMPACT in our larger cohort using droplet digital PCR (ddPCR), an amplicon-based DNA sequencing platform, with previously validated probes. These residual libraries were prepared from the same sample aliquots as those submitted for MSK-IMPACT and each sample submitted for ddPCR contained a median of 18ng of DNA library (range, 0.0153-18.0ng). Of the 7 samples positive for IDH1 R132H, TERT C228T, or TERT C250T mutations in MSK-IMPACT, we found that ddPCR accurately called each mutation (7/7, 100%), confirming sensitivity of ddPCR in CSF ctDNA. For the 36 samples in which MSK-IMPACT did not detect hotspot mutations, we sequenced samples with probes specific for 7 hotspot mutations and found that ddPCR rescued calls in 41.9% of mutations (18/43). Specifically, we found that ddPCR rescued hotspot mutations such as BRAF V600E (2/3, 66.7%), IDH1 R132G/H (3/8, 37.5%), TERT C228T (11/26, 42.3%), and TERT C250T (2/4, 50%). Overall, our results suggest that combined-modality sequencing of CSF ctDNA with MSK-IMPACT and ddPCR may provide a more comprehensive, minimally invasive methodology to molecularly profiling glioma.

#059

A PIPELINE FOR THE IDENTIFICATION OF DISEASE-SPECIFIC GENETIC BIOMARKERS USING NGS SEQUENCING DATA OF CFDNAS IN HUMAN PLASMA

VITTORINI-ORGEAS A[1], GRABUSCHNIG S[2], SENSEN CW[1]
[1]The Hungarian Centre of Excellence for Molecular Medicine (HCEMM kft.), Szeged, Hungary
[2]Graz University of Technology, InsKtute of ComputaKonal Biotechnology, Petersgasse 14, 8010, Graz, Austria

Circulating cell-free DNAs (cfDNAs) are DNA fragments released into the blood plasma/serum by different tissues. They can be isolated from a routine blood draw that is a cost-effective, fast and non- invasive procedure. While their presence is detectable under healthy conditions, there are solid evidences of their association with various clinical conditions. The availability of a higher number of samples and high-throughput sequencing technologies have enabled the production of massive amounts of complex genomic data. This explains the great interest for cfDNAs in clinical settings, because of their potential role as biomarkers. This potentiality can be exploited if supported by a computational platform that simplifies the execution of the analysis and makes it reproducible and shareable across different platforms. To address the complexity of this analysis, we propose a computational pipeline that automatically chains together multiple modules, each one executed by an independent open-source software tool. The pipeline starts with the raw data pre-processing necessary to remove residual adapters and filter the low-quality reads. It is followed by the composition analysis where the average sample composition is calculated and expressed in terms of specific target regions of human DNA. Next, a machine learning algorithm is followed by a MANOVA analysis that evaluates in an iterative manner the statistical significance of the output. This module produces a subset of those genomic target regions that perform best at predicting the health outcome. The last module runs through the original set of sequencing data and compares the composition of each entry against the composition represented by the subset of target regions deriving from the previous module. The final result is a list of nucleotide sequences that have been identified as the subset of best performing indicators of a clinical condition based on the analysis above described. Thanks to the containerization technology and workflows managers this pipeline can be shared and executed with the same functionality across different platforms, and its installation process is automated.

#060

THE PATH TO TRANSLATE GENOMIC FINDINGS INTO A PILOT CLINICAL TRIAL OF A CELL FREE DNA PLATFORM FOR EARLY DETECTION AND MONITORING of MMR DEFICIENT CANCERS

MAENG H[1], SONG Y[2], CALZONE K[3], FASAYE G[3], LIU Y[3], BERZOFSKY J[1], SHOEMAKER R[4]
[1]Vaccine Branch, Center for Cancer Research, NCI, Bethesda, MD, USA
[2]Vaccine, Immunity and Cancer Directorate, Frederick National Laboratory for Cancer Research, Frederick, MD, USA
[3]Genetics Branch, Center for Cancer Research, National Cancer Institute
[4]Division of Cancer Prevention, National Cancer Institute, Bethesda, MD, USA

Lynch syndrome (LS) is a hereditary cancer syndrome most frequently associated with but not limited to colorectal cancer (CRC) and endometrial cancer. It is caused by germline pathogenic or likely pathogenic (P/LP) variants in DNA mismatch repair (MMR) genes, and the deficient MMR (dMMR) status leads to numerous genomic variations. The most characteristic genomic features of dMMR cancers include microsatellite instability (MSI-H) and frameshift mutations (FSM) at coding mononucleotide repeats (cMNRs). Others have reported recurrent FSMs in these regions of dMMR cancer tissue in driver and non-driver genes which may reflect critical changes during the development of premalignant lesions to invasive cancer. For carriers of LS, the consensus is to offer counseling and surveillance in attempts to decrease cancer-related mortality. However, there is no strong evidence that certain measures will reduce the mortality except CRC. Financial burden, lack of consensus in surveillance guidelines and psychological stress are some of frequently mentioned challenges. Thus, there is a need for developing a sensitive biomarker platform that can help guide clinical management. An FSM-based gene panel of 122 FSM targets was developed and characterized using targeted next generation sequencing (NGS) and cultured MSI-H CRC-derived cfDNA. Blood-derived cfDNA collections from patients with dMMR cancer, healthy LS carriers and healthy volunteers were used as controls. Preliminary results support high sensitivity and specificity for this biomarker panel.As part of an interdivision collaboration, a pilot clinical trial is under development to help validate this technology as a biomarker for early detection and monitoring of dMMR cancer. The endpoints of the study include the feasibility of the platform, focused on biospecimen logistics and sensitivity and specificity. The association between findings of the biomarker and clinical events, e.g. (pre)malignant lesions, will be explored. The study will include healthy volunteers, healthy LS carriers, individuals with history of somatic or germline dMMR cancer in remission, and patients with active dMMR cancer. Each cohort will be followed by clinical and laboratory exams, and review of outside medical records if any. Biospecimen (serum/plasma, urine, and stool) will be curated for a batch testing using the FSM-based cfDNA biomarker platform. The study will focus on evaluating the feasibility and utility of the platform as a clinical research endpoint. The long-term vision is to develop a tool that can aid informed decision making between concerned individuals and the medical team in a preventive setting or monitoring of cancer status.

#061

COVID-19 PROTEINS IN EXTRACELLULAR VESICLES ARE LINKED TO IMMUNE, COAGULATION, AND CARDIOVASCULAR COMPLICATIONS AND POOR OUTCOMES IN SEVERE DISEASE PATIENTS

DE MIGUEL PEREZ D[1,2], ARROYO-HERNANDEZ M[3], LASALVIA S[4], GUNASEKARAN M[2,5], CARDONA AF[6], KAUSHAL S[2,5], ATANACKOVIC D[7], SAHOO S[4], ARRIETA O[3], and ROLFO C[1,2].

[1]Center for Thoracic Oncology, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[2]Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA.

[3]Thoracic Oncology Unit, Instituto Nacional de Cancerología (INCan), Mexico City, Mexico.

[4]Department of Cardiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[5]Departments of Surgery and Pediatrics, Ann and Robert H. Lurie Children's Hospital of Chicago, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA.

[6]Clinical and Translational Oncology Group, Clínica del Country, Bogota, Colombia.

[7]Transplant and Cellular Therapy Program, Department of Medicine, University of Maryland School of Medicine and Greenebaum Comprehensive Cancer Center, Baltimore, MD, USA.

Introduction: The severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) causes the coronavirus disease 2019 (COVID-19) which has been associated with more than 7 million deaths since 2019. Initially, severe COVID-19 cases were associated with elevated IL-6 and prompted the evaluation of the anti-IL6 inhibitor Tocilizumab as a treatment. Extracellular vesicles (EVs) play an important role in intercellular communication and immune response and could be critical during SARS-COV-2 infection and being potentially used as biomarkers.

Methods: This retrospective study enrolled patients with severe COVID-19, approximately three-quarters received Tocilizumab and one-quarter considered controls. Blood samples were collected at hospitalization before treatment, at day ~7, and day ~15. Overall survival (OS) was calculated as the time before enrollment until death or last follow-up. EVs were isolated by ultracentrifugation and dynamic expression of spike (S) 1, S2, and nucleocapsid (N) proteins was evaluated by immunoblot and normalized against CD9, a commonly expressed EV marker. Plasma antibody levels of spike S1, S2, N, and receptor binding-domain (RBD) were measured by ELISA. EV expression of S2 and CD9 were evaluated by imaging flow-cytometer and by dual immunogold staining. Additionally, samples from 3 healthy patients were included as negative control in the analyses.

Results: Thirty-nine patients were enrolled in this study, including a total of 103 longitudinal blood samples during the 3 timepoints. Expression of SARS-COV-2 proteins was observed in EVs by western-blot and S2 expression was validated by immunogold and imaging flow-cytometry in COVID-19 patients, revealing an enrichment in CD9+ EVs. Tocilizumab was administered to 29 patients, which showed a reduction in EV SARS-COV-2 proteins in comparison to 10 controls, but did not improve the OS. However, increased EV S2 during follow-up was associated with shorter OS (HR=5.28, *p*=0.022) and decreased S2 and N antibody titers. Increased EV SARS-COV-2 proteins also correlated with elevated CD3+CD8+ cells and coagulation and cardiac abnormalities such as high fibrinogen, C reactive protein, systolic and diastolic pressure.

Conclusion: We confirmed by 3 different methodologies that plasma EVs from patients with COVID-19 carry SARS-COV-2 proteins and their dynamics can be indicative of poor outcomes and hematological and cardiovascular complications. These results suggest that EVs might play a role in COVID-19 infection and be used as predictive biomarkers for outcomes and potential long-term consequences. Further research is warranted to better understand this process.

#062

PLASMA EXTRACELLULAR VESICLES AND RADIOMICS PREDICT RESPONSE TO IMMUNE-CHECKPOINT INHIBITORS IN PATIENTS WITH NON-SMALL CELL LUNG CANCER.

DE MIGUEL-PEREZ D[1,2], MAMINDLA P[3], RUSSO A[2,4], AK M[3,5], GUNASEKARAN M[2,6], DEL RE M[1], BUEMI F[4], HIRSCH FR[1], CARDONA AF[7], ARRIETA O[8], NAING A[9], KAUSHAL S[2,6], ADAMO V[4], COLEN R[3,5] and ROLFO C[1,2].

[1]Center for Thoracic Oncology, Tisch Cancer Institute and Icahn School of Medicine, Mount Sinai, New York, NY, USA.

[2]Marlene and Stewart Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD, USA.

[3]Hillman Cancer Center, University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

[4]Medical Oncology Unit, A.O. Papardo & Department of Human Pathology, University of Messina, Italy.

[5]Department of Radiology, University of Pittsburgh, Pittsburgh, PA, USA.

[6]Departments of Surgery and Pediatrics, Ann and Robert H. Lurie Children's Hospital of Chicago, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA.

[7]Clinical and Translational Oncology Group, Clínica del Country, Bogota, Colombia.

[8]Thoracic Oncology Unit, Instituto Nacional de Cancerología (INCan), Mexico City, Mexico.

[9]Department of Investigational Cancer Therapeutics, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA.

Introduction: Immune-checkpoint inhibitors (ICIs) have showed unseen efficacy in the treatment of patients with advanced non-small cell lung cancer (NSCLC). However, usually, only a subset of them experience clinical benefit. This is caused by the fact that tissue PD-L1, the most commonly used biomarker, presents low accuracy. PD-L1 is also expressed in plasma extracellular vesicles (EVs), where it can also directly inhibit the anti-tumor immune response or bind to specific ICIs antibodies in the circulation. EVs also contain immune suppressive factors as TGF-β, playing a critical role in the tumor microenvironment. Furthermore, radiomics analysis of computerized tomography (CT) images has demonstrated to have high potential as a predictive biomarker. Our objective was to investigate the role of these minimally invasive biomarkers at predicting the response to ICIs in patients with NSCLC.

Methods: Retrospective plasma samples before treatment and after 3rd cycle of ICIs, as well as radiomic features in pre-treatment CT scan images, from 27 patients with advanced/metastatic NSCLC were analyzed. Durable responses were determined after treatment cycle 6-8 by CT following RECISTv1.1. Plasma EV were isolated by serial ultracentrifugation and expression levels of PD-L1 and TGF-β were evaluated by immunoblot and ELISA. A total of 400 radiomic features were evaluated in target and non-target lesions.

Results: Durable response was associated with lower pre-treatment levels of EV TGF-β (p=0.038) and decreasing levels of ΔEV PD-L1 during treatment (p=0.048) but not with tissue PD-L1 tumor proportion score (TPS)(p>0.05). The individual predictive models for the analyzed biomarkers showed that a model including 6 specific radiomic features was the most sensitive and accurate biomarker with 50% sensitivity at 90.9% specificity, followed by ΔEV PD-L1. Interestingly, the combination of the pre-treatment biomarkers radiomics and EV TGF-β achieved a high accuracy (77.8%) with 68.8% sensitivity and 90.9% specificity, which was improved when TGF-β was substituted by on-treatment ΔEV PD-L1, reaching a final 82.5% accuracy, with 75.0% sensitivity and 90.9% specificity.

Conclusion: Combination of pre-treatment EV TGF-β and radiomics is a potential biomarker for high accurate prediction of response to ICIs in patients with NSCLC. Moreover, on-treatment dynamics of EV PD-L1 can add longitudinal information that increases the sensitivity and accuracy of this prediction. If validated, the combination of minimally-invasive biomarkers in plasma EVs and CT scan images could outperform and/or complement the current standard-of-care tissue PD-L1 to predict responders to ICIs.

#063

BASELINE LOW EXTRACELLULAR VESICLE MIRNA-30C AND PRESENCE OF AUTOPHAGIC CTCS PREDICT CHEMORADIOTHERAPY RESISTANCE AND POOR OUTCOMES IN PATIENTS WITH LOCALLY ADVANCED NON-SMALL CELL LUNG CANCER

DE MIGUEL-PEREZ D[1,2,3], ORTEGA FG[2], GUERRERO TEJADA R[4], PETERSON CB[5], RUSSO A[6], CARDONA AF[7], LORENTE JA[2,3], EXPÓSITO HERNÁNDEZ J[4], SERRANO MJ[2,8], and ROLFO C[1].

[1]Center for Thoracic Oncology, Tisch Cancer Institute, Mount Sinai Medical System & Icahn School of Medicine, Mount Sinai, New York, NY, USA.

[2]GENYO, Centre for Genomics and Oncological Research, Pfizer/University of Granada/Andalusian Regional Government, Liquid biopsy and Cancer Interception group, PTS Granada, Granada, Spain.

[3]Laboratory of Genetic Identification, Legal Medicine and Toxicology Department, Faculty of Medicine, University of Granada, Granada, Spain.

[4]Radiation Oncology Department, Virgen de las Nieves University Hospital, Ibs Granada, Granada, Spain.

[5]Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA.

[6]Medical Oncology Unit, A.O. Papardo & Department of Human Pathology, University of Messina, Messina, Italy.

[7]Luis Carlos Sarmiento Angulo Cancer Treatment and Research Center (CTIC) / Foundation for Clinical and Applied Cancer Research (FICMAC) / Molecular Oncology and Biology Systems Research Group (Fox-G), Universidad El Bosque, Bogotá, Colombia.

[8]Integral Oncology Division, Virgen de las Nieves University Hospital, Granada, Spain.

Introduction: Concomitant chemotherapy and radiotherapy is the standard treatment for patients with unresectable locally advanced non-small cell lung carcinoma (LA-NSCLC), however, resistance is developed early and outcomes are poor in these patients due to the lack of predictive biomarkers. Autophagy up-regulation has been proposed as a mechanism of resistance to chemoradiation and liquid biopsy has emerged as a minimally invasive source of biomarkers such as circulating tumor cells (CTCs) and extracellular vesicles (EVs) which have a promising potential as minimally-invasive tissue surrogates to reveal tumor status over time. Here, we aim to investigate the role of autophagic CTCs and EV miRNAs as biomarkers for autophagy activation and development of chemoradiation resistance in advanced NSCLC patients.

Methods: Prospective blood samples from 38 LA-NSCLC patients were collected before, during and after chemoradiotherapy. CTCs were immunomagnetically isolated and the autophagic marker LC3B was analyzed. EVs were isolated by ultracentrifugation and specific EV-miRNAs were evaluated. Autophagy, viability, and miR-30c levels were analyzed in chemoradiation *in vitro.*

Results: Low pretreatment levels of EV-miR-375, miR-200c, miR-30c identified non-responders with an area under the curve of 86%. Decrease of these EV miRNAs levels were associated with an increase of autophagic CTCs during treatment ($p<0.05$). Low EV miR-30c and presence of autophagic-CTCs were also independent predictive biomarkers for shorter relapse-free survival and overall survival. Then, *in vitro* experiments revealed that chemoradiation activated autophagy and decreased levels of miR-30c. When miR-30c mimics were added, treatment-induced autophagy was inhibited and cellular viability was decreased, while the opposite process was observed when adding the miR-30c inhibitor. Moreover, when miR-30c was packaged and added to these cells undergoing chemo or radiotherapy, autophagy was drastically reduced in comparison to controls.

Conclusion: These results demonstrated that EV-miRNAs, in particular miR-30c, were involved in autophagy modulation as a mechanism of resistance to chemoradiotherapy manifested also by the presence of autophagic-CTCs. Thus, EV miR-30c and autophagic CTCs are promising biomarkers for the stratification and monitoring of stage III NSCLC patients undergoing chemoradiation, who could also potentially benefit from novel combinatorial therapies with autophagy inhibitors.

#064

DEVELOPMENT OF IMAGING MASS CYTOMETRY AS A TOOL TO CHARACTERIZE CIRCULATING TUMOR CELLS IN PRE-CLINICAL MOUSE MODELS

PORE M[1], BALAMURUGAN K[2], MALLORY P[1], BREEN D[1], CARDAMONE A[1], NEWKIRK C[1], MCKENNETT L[2], SHARAN S[2], BOCIK W[1], and STERNECK E[2]

[1]Imaging Mass Cytometry Laboratory, Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, Inc., National Cancer Institute, Frederick, MD, USA

[2]Laboratory of Cell and Developmental Signaling, Center for Cancer Research, National Cancer Institute, Frederick, MD, USA

Metastasis is the primary cause of most cancer-specific mortality. Among circulating tumor cells (CTCs), CTC clusters are particularly associated with progression, poor prognosis, and treatment resistance. The number of CTC clusters surges after biopsy and surgery as well as during neo-adjuvant treatment. Therefore, CTC clusters may represent a novel target for the prevention of distant metastases, and the development of methods for their functional characterization is imperative toward assessing and achieving this goal. Though many advancements in techniques to detect, isolate, and analyze CTCs have been made in recent years, most technologies cannot specifically address the differences between single CTCs versus CTCs in clusters. This objective is particularly challenging with regard to the quantitative analysis of proteins. Through metal-tagged antibodies, multiplex imaging mass cytometry (IMC) allows the simultaneous detection of more than 40 proteins in situ. Thus, we set out to advance this technology for mechanistic studies of CTC clusters in pre-clinical xenograft mouse models. IMC can be used on relatively small volumes of mouse blood (<100 µl) in a unique single step process for the detection and characterization of CTC clusters. We have developed a panel of antibodies (>25) that target human proteins such as cytokeratins, epithelial-mesenchymal transition markers, signaling molecules, transcription factors, and indicators of cell death. For optimization, we are using human cancer cell lines representing a spectrum of epithelial-mesenchymal phenotypes and spiking into mouse blood. For data analysis, we will employ the open-source Bodenmiller pipeline, consisting of cell segmentation software, single-cell marker quantification, and R-based downstream analyses for unsupervised cell-type clustering. Additionally, we aim to supplement our workflow with recently released Visiopharm software, which provides a more supervised approach for single-cell phenotyping. Ongoing efforts include analyzing CTCs in liquid biopsies of mice with xenograft tumors of human cell lines expressing fluorescent reporters, followed by patient-derived breast cancer xenografts, known to generate CTC clusters. Taken together, our effort in developing this tool for the detection and functional characterization of single CTCs versus CTC clusters in pre-clinical models will shed light on the biology of this elusive but critical cancer cell population, and lead toward a better assessment of the efficacy and clinical utility of therapeutic drugs.

#066

MULTIPARAMETRIC CHARACTERIZATION OF SINGLE EXTRACELLULAR VESICLES FROM PLASMA

JIANG N[1], SAFTICS A[1], PURNELL B[1], BERES B[1], GHAELI I[1], ROMANO E[1], RESTO C[2], SEEWALD VL[2], VAN KEUREN-JENSEN K[3], and JOVANOVIC-TALISMAN T[1*]

[1]Department of Cancer Biology and Molecular Medicine, Beckman Research Institute, City of Hope, Duarte, CA, USA.

[2]Deprtment of Population Sciences, City of Hope Comprehensive Cancer Center, Duarte, CA, USA.

[3]Center for Alzheimer's and Related Dementias, NIA, NIH, Bethesda, MD, USA.

One enticing source of biomarkers for liquid biopsies are membrane delimited nanoparticles called extracellular vesicles (EVs). EVs harbor molecular signatures of their origin cells and they can rapidly report on changes to cellular status. Additionally, EVs have excellent stability and they are abundant in biofluids. As such, EVs can be readily and frequently sampled from biofluids. However, certain challenges remain when moving EVs forward as a platform for liquid biopsies. EVs are not only small but also highly heterogeneous in terms of size, shape, and molecular content. Moreover, in biofluids, they are found as a mixture from different cell types. Altogether, the research community urgently needs new methodologies to rigorously characterize desired EV subpopulations – i.e., EVs from specific cell types or diseases*.* We recently developed a new approach to rigorously probe individual EVs called Single Extracellular VEsicle Nanoscopy (SEVEN). By combining affinity isolation with super-resolution microscopy, SEVEN sensitively quantifies the number, size, shape, molecular cargo content, and heterogeneity of EV subpopulations. To rapidly quantify EVs from specific subpopulations, we further optimized preanalytical protocols and data analysis. We show: 1) the yield of rare EVs is increased when the preanalytical isolation protocol is robustly optimized; and 2) data analysis is enhanced when EVs are classified with the support of machine learning. We then assessed EVs from four types of cultured HER2-positive breast cancer cell lines, where we specifically pulled-down EVs enriched in either HER2 or tetraspanins, transmembrane proteins commonly found on EV membranes. Compared to the tetraspanin-enriched EVs, the HER2-enriched EVs were larger and more elongated, indicating a unique population. We also used our approach to probe EVs from patient biofluids: HER2-enriched EVs from HER2-positive breast cancer patients (vs. triple-negative breast cancer patients) were significantly more abundant and had significantly higher content of tetraspanins. Altogether, SEVEN revealed unique characteristics of HER2-enriched EVs from cultured cells and complex biological fluid. The approach thus is well poised to support more precise therapeutic decisions.

#067

ADVANCE PROSTATE CANCER DETECTION THROUGH EPIGENOMIC PROFILING OF CFDNA

ADIL M[1,2 *], HANRATTY B[1 *], MUSTAFI P[1], MITTAL C[1], RICHARDS H[1], COLEMAN L[1], PATEL RA[1], DOEBLEY A[1], PATTON R[1], CRUIKSHANK AE[1], GALIPEAU P[1], DUMPIT R[1], ROUDIER MP[1,2], LOW J[1], DE SARKAR N[3], MONTGOMERY RB[2], COREY E[2], MORRISSEY C[2], NELSON PS[1], HA G[1†], HAFFNER MC[1†]

[1]Fred Hutchinson Cancer Center

[2]University of Washington

[3]Medical College of Wisconsin

*These authors contributed equally

† Co-senior authors.

Introduction: Metastatic castration-resistant prostate cancer (mCRPC) is a heterogeneous disease which can be classified into clinically relevant subtypes based on the expression of genes, such as the androgen receptor (AR) or neuroendocrine markers. Neuroendocrine prostate cancer (NEPC), characterized by gain of stem-like and neuroendocrine features and lack of AR expression is a clinically aggressive variant. Due to the absence of adequate biomarkers, NEPC is usually detected at a very advanced stage. There is mounting evidence that molecular subtype changes seen in NEPC are enforced by widespread epigenetic alterations, in particular DNA methylation changes. In this study, we aim to devise a novel DNA methylation-based assay for molecular subtyping and disease monitoring from cell-free DNA (cfDNA).

Methods: We analyzed genome wide methylation patterns in 56 prostate cancer patient-derived xenograft (PDX) and 128 mCRPC tumors using array- and sequencing-based assays. We integrated DNA methylation at promoters, gene bodies and transcription factor binding site (TFBS) to determine the landscape of methylation alterations at key lineage specific genes. Using whole genome methylation derived from tissue with matching expression data we developed a deep learning framework to predict gene expression directly from tissue or cfDNA. Using key marker genes, the model was then used to discern tumor molecular phenotypes from tissue and cfDNA in three independent cohorts of mCRPC patients using whole genome bisulfite sequencing and low-pass Enzymatic Methyl-Seq (EM-seq).

Results: We observed a tight association between promoter, gene body and TFBS methylation with gene expression. Inferring gene expression from methylation for lineage specific markers such as AR, KLK3, ASCL1, INSM1, SRRM4 and DLL3 we classified molecular subtypes from both tissue and cfDNA. Additionally, for AR and ASCL1, we identified core sets of TFBSs whose differential methylation allowed for accurate assay-independent molecular subtype quantification. Applying the optimized quantitative model to mCRPC patients who underwent comprehensive tissue sampling by rapid autopsy we observed accurate subtype classification from both tissue samples and cfDNA for all cases. A similar analytical performance was observed in additional clinical mCRPC cohorts with cfDNA.

Conclusion: Whole-genome methylation analysis of cfDNA allows for the prediction of gene expression patterns in tumor tissues, enabling non-invasive tumor subclassification and assessment of therapeutic targets.

#068

ENRICHMENT OF TUMOR-ASSOCIATED FRAGMENTOMIC FEATURES DETECTED IN CELL-FREE DNA WITH SONOBIOPSY IN GLIOMA PATIENTS

CHAUHAN PS[1], ALAHI I[1], YUAN J[2], XU L[2], HARRIS PK[1], CHIEN CY[2], FADERA S[2], STARK AH[2], CHEN H[2,3], LEUTHARDT EC[3] and CHAUDHURI AA[1]

[1]Department of Radiation Oncology, Washington University School of Medicine, Saint Louis, MO, USA.
[2]Department of Biomedical Engineering, Washington University in St. Louis, Saint Louis, MO, USA.
[3]Department of Neurosurgery, Washington University School of Medicine, Saint. Louis, MO, USA.

Introduction: Blood-based liquid biopsy for brain cancer offers a noninvasive alternative to invasive surgical biopsies. However, the presence of the blood-brain barrier (BBB) impedes the release of brain tumor-derived molecular biomarkers into the bloodstream for sensitive molecular diagnosis. Focused ultrasound (FUS)-enabled blood-based liquid biopsy (sonobiopsy) locally opens the BBB and enriches for circulating tumor-specific biomarkers for the molecular diagnosis of brain cancers. Here, we evaluate the enrichment of tumor-associated fragmentomic features with sonobiopsy in patients with glioma.

Method: Blood samples were collected from fourteen patients enrolled prospectively at our institution between April 2022 and February 2024 with median age of 63 years (range 36 -74 years). Among them, 12 were glioblastoma (GBM) patients and two were of low-grade glioma patients. Enzymatic Methyl-seq (EM-Seq) was applied to plasma cell-free DNA (cfDNA) isolated from five patients at pre-FUS (5 minutes) and post-FUS (5, 10 and 30 minutes) timepoints. In parallel, plasma from 9 healthy adults and tumor tissue obtained from five glioma patients was also subjected to EM-Seq. In addition, DNA obtained from the tumor was also analyzed by whole genome sequencing. We then quantified fragmentomic features such as ratio of short fragments (< 120bp) to long fragments (140-250 bp), median fragment length, and coefficient of variation (CV) of the fragment length distribution at the transcription start sites (TSSs) of all protein-coding genes. Tumor fraction (TFx) levels based on genome-wide copy number alterations in plasma cfDNA was also estimated using ichorCNA.

Results: Among the five glioma patients, three had GBM and two had low-grade glioma. All the three GBM patients were IDH1 wildtype, two low-grade glioma patients were IDH1 R132H mutated, and four patients were positive for TERT mutations based on analysis of resected tumor tissue. Comparing FUS-induced fragmentomic features between pre-FUS and post-FUS samples, the ratio of short to long fragments was significantly higher in post-FUS samples ($p < 0.0001$). Similarly, the median fragment size was significantly shorter in post-FUS samples compared to pre-FUS ($p < 0.0001$), and post-FUS samples had significantly higher cfDNA fragment size CVs at protein-coding TSSs than pre-FUS ($p < 0.0001$). Futhermore, TFx levels in plasma cfDNA were consistently higher in post-FUS samples compared to pre-FUS.

Conclusion: Our analysis of cfDNA fragmentation patterns in glioma patients strongly suggests that post-FUS cfDNA is more fragmented that pre-FUS, consistent with enrichment of tumor DNA. These data support the continued investigation of sonobiopsy for noninvasive molecular diagnosis of brain cancers.

#069

MOLECULAR RESIDUAL DISEASE (MRD) DETECTION BY CIRCULATING TUMOR DNA IN PATIENTS WITH UROTHELIAL AND RENAL CELL CANCERS

CHAUHAN PS[1], BEDNAR R [2], HASHMI A[3], SEMENKOVICH NP[4], LI R [5], CHAUDHURI AA[1], and AGARWAL G[2]
[1]Division of Cancer Biology, Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO, USA
[2]Division of Urology, David Pratt Cancer Center, Mercy Hospital, 607 S New Ballas Rd, St. Louis, MO, USA
[3] Radiation Oncology, Stanford Medicine, Stanford University, Palo Alto, CA, USA
[4]Endocrinology and Molecular Medicine, Medical College of Wisconsin, Milwaukee, WI, USA
[5]Department of Urologic Oncology, Moffitt Cancer Center, Tampa, Florida, USA

Introduction: Patients with urothelial carcinoma as well as renal cell carcinoma often present with high-risk of recurrent disease post-surgical resection, requiring very close surveillance or adjuvant therapy when indicated. Traditional imaging may fail to detect early recurrences, while adjuvant therapies pose significant toxicity risks and overtreatment. Currently we use stage and grade of disease to help determine the risks of recurrence, however these tools are not always reliable predictors. This study aims to explore circulating tumor DNA (ctDNA) molecular residual disease (MRD) profiling after surgical resection for improved prognostic accuracy and earlier recurrence detection, potentially changing the treatment landscape.
Methods: We conducted a retrospective chart review on patients who underwent nephrectomy for renal cell carcinoma and cystectomy or nephroureterectomy for urothelial carcinoma with a total of 123 patients eligible for review. Our cohort included 59 patients with renal cell carcinoma, 58 patients with urothelial carcinoma, and additional cases spanning various types including renal sarcoma, adrenal, penile and prostate cancers with ages ranging from 35-94 years. We followed up with the patients for an average of 12 months post-surgery, with a follow-up range of 3-23 months. Postoperative blood samples were collected for ctDNA analysis using the tumor-informed Signatera assay.
Results: In the renal cell carcinoma cohort, 7 out of 59 (12%) patients had positive postoperative ctDNA and 6 of those developed metastatic disease, out of which one patient died. The most frequently altered genes detected in tumor tissue were *VHL* (39%), *PBRM1* (20%) and *BAP1* (12%). In those that were metastatic, ctDNA provided disease response information and helped with selecting second- and third-line treatments based on targetable mutations found during ctDNA analysis. In the 58 urothelial carcinoma patients, the most common somatic mutations detected in tumor tissue were *TP53* (36%), *ARID1A* (16%), *KDM6A* (16%) and *PIK3CA* (14%). Among those with postoperative MRD detected via ctDNA, 18 (33%) patients who had positive ctDNA developed metastatic disease and 5 of them died. Similarly, therapeutic response was able to be monitored with ctDNA and also helped to select systemic therapy. In both cohorts, ctDNA-negative patients remained alive and disease free.
Conclusions: This is among the largest real-world uses of ctDNA MRD testing in renal and urothelial cancers. Our findings underscore ctDNA's prognostic value and its utility in therapeutic monitoring and treatment selection, highlighting its potential to precisely guide clinical management in the future.

#070

URINE CELL-FREE DNA FRAGMENTOMICS TO DETECT GENITOURINARY MALIGNANCIES AND PREDICT PATHOLOGICAL RESPONSE IN BLADDER CANCER

CHAUHAN PS[1], ALAHI I[1], PANDA A[2], SHENG J[3], NATHAN C[3], MUELLER R[1], NAWAF C[4], SHIANG A[4], HARRIS PK[1], QAIUM F[1], KIM EH[3], REIMERS MA[5], SMELSER W[3], SMITH ZL[3], and CHAUDHURI AA[1]

[1]Department of Radiation Oncology, Washington University School of Medicine, Saint Louis, MO, USA.
[2]University of Chicago, Chicago, IL, USA
[3]Division of Urology, Department of Surgery, Washington University School of Medicine, St. Louis, MO, USA.
[4]Cedars-Sinai Medical Center, Los Angeles, CA, USA
[5]Division of Medical Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

Background: Liquid biopsy approaches analyzing cell-free (cf) DNA from plasma have the potential to revolutionize disease detection and monitoring. However, the sensitivity of these approaches for MRD detection has remained subpar. Here, we analyzed urine, a noninvasive and genitourinary-relevant analyte, from patients with genitourinary (GU) cancers and utilized an approach that incorporates copy-number derived tumor fraction as well as fragmentomic profiling to sensitively detect MRD and predict pathologic complete response (pCR).
Methods: A total of 150 patients with GU malignancies and 34 healthy adults were enrolled onto this study. We acquired urine preoperatively from 90 bladder cancer (BC) patients (67% muscle-invasive) who underwent cystectomy and 18 patients with renal-cell cancer (RCC) who underwent nephrectomy. We also collected urine samples from 42 metastatic prostate cancer (mPC) patients. We performed ultra-low pass whole genome sequencing (ULP-WGS) of urine cfDNA from all 150 patients and 34 healthy adults. Tumor fraction (TFx) level based on genome-wide copy number alterations was estimated using ichorCNA. In subset of 51 BC patients, we inferred fragment size and genomic coverage of urine cfDNA using Picard Tools. Then, we computed the short-to-long (S/L) fragment ratio for genomic bins in each sample. The S/L ratio per bin was derived by partitioning the genome into 100 kb bins and evaluating the ratio of GC-corrected short fragments (50-150 bp) to long fragments (151-250 bp) within each bin. A normalized genome-wide S/L score was calculated by averaging across all bins.
Results: Comparing TFx across the GU cohort, BC had the highest median TFx (4.4%) followed by RCC (3.7%) and mPC (2.7%). In our cohort of 90 BC patients, 43% of patients achieved pCR (*n* = 39) while 57% had residual disease detected in their surgical sample (no pCR; *n* = 51). Patients with no pCR had significantly higher copy number-derived TFx in urine compared to patients with pCR (median 11.8% vs 2.3%, $p < 0.0001$). TFx achieved an AUC of 0.89 for classification of no pCR versus pCR with sensitivity of 65% and specificity of 87%. In a subset of 51 low-risk individuals (healthy and BC pCR), urine cfDNA median fragment sizes were significantly longer than BC patients with no pCR (median 177 bp vs. 156 bp, $p = 0.002$). Healthy and BC pCR individuals also had significantly shorter S/L ratio scores than no pCR BC patients (mean 0.98 vs. mean 1.5, $p = 0.007$). Genomic bins with maximally differential S/L ratio between BC patients with pCR versus no pCR were enriched for genes involved in immune signaling (chemokine signaling, leukocyte chemotaxis, cellular response to LPS) suggesting biological relevance.
Conclusions: Copy number-derived tumor fraction inference and genome-wide fragmentomics of urine cfDNA can be used to predict pCR in bladder cancer patients, and could have broader applicability across GU malignancies.

#071

CELL-FREE DNA AS A NOVEL BIOMARKER FOR DISEASE PROGRESSION AND RESPONSE TO TREATMENT IN HUTCHINSON-GILFORD PROGERIA SYNDROME

THAIVALAPPIL AA[1], CABRAL WA[1], TAVAREZ UL[1], ERDOS MR[1] AND COLLINS FS[1]
[1] Molecular Genetics Section, Center for Precision Health Research, National Human Genome Research Institute, NIH, Bethesda, MD, USA

Hutchinson-Gilford Progeria Syndrome (HGPS) is a premature aging disorder that affects tissues of mesenchymal origin. Most individuals with HGPS harbor a de novo c.1824C>T (p.G608G) mutation in the gene encoding lamin A (LMNA), which activates a cryptic splice donor site resulting in production of a toxic protein termed "progerin". Clinical manifestations include growth deficiency, lipodystrophy, cardiovascular defects and bone dysplasia. Currently lonafarnib, a farnesyltransferase inhibitor, is the only FDA-approved treatment for progeria. For development of new therapeutics, a reliable biomarker is needed to demonstrate qualitative or quantitative efficacy of disease progression or treatment response in preclinical or clinical trials. We have developed a novel liquid biopsy approach to characterize phenotypic progression in two HGPS mouse models, as assessed by plasma concentration of cell-free DNA (cfDNA). cfDNA are short circulating DNA fragments released into the bloodstream through cellular breakdown and active DNA release. With a High Sensitivity DNA chip, we observed elevations of cfDNA in heterozygous and homozygous HGPS mice compared to age-matched counterparts, including a 107% increase in cfDNA levels of 12 week homozygotes compared to wild type. Digital droplet PCR (ddPCR) amplification of short and long interspersed retrotransposable elements (LINES/SINES) within the cfDNA sequences correlated with plasma cfDNA concentrations. Additionally, ddPCR provided a secondary validation of cfDNA trends, demonstrating a 119% elevation in homozygote cfDNA compared to wild type at 12 weeks. Quantification of plasma cfDNA via LINE/SINE copy number also greatly improved the sensitivity of the assay, enabling quantification with as little as 5uL of murine plasma. Validation of cfDNA levels as a clinical biomarker for therapeutic response was achieved by demonstrating quantitative reductions in plasma LINE and SINE copy number up to 56% at 8 weeks from mice treated in vivo with a locus-specific DNA base editor that corrects the mutation and partially rescues the HGPS phenotype. Thus, plasma cfDNA has the right properties to serve as a reliable biomarker for disease progression and treatment response in mouse models and can now be tested in human trial samples.

#072

BROAD RANGE CELL-FREE DNA ASSESSMENT OF LUNG CANCER IN INDETERMINATE PULMONARY NODULES

IRENE CHOI[1], NEETI SWARUP[1], JORDAN CHENG[1], JASON HYUN[2], RAGHURAMAN RAMAMURTHY[2], CHEUK Y. TANG[2], DAVID T.W. WONG[1]
[1]UCLA School of Dentistry, Los Angeles, California
[2]AIOnco, San Francisco, California

Background: Significant advancements in Liquid Biopsy (LB) and noninvasive early detection have been made by application of cell-free DNA (cfDNA). A notable development is the identification of a class of ultrashort cfDNA (uscfDNA) fragments (40-70 bps) in addition to a class of short cfDNA (scfDNA) fragments (<120 bps) demonstrates promising potential as a biomarker to differentiate between cancerous and control groups. Identifying the malignancy of indeterminate pulmonary nodules is imperative as lung cancer, a leading cause of cancer-related deaths globally, is accountable for 2.2 million new cases and 1.8 million deaths annually worldwide. Utilizing plasma-based LB screening initiatives offers a minimally invasive approach to identify malignancy of lung cancer within indeterminate pulmonary nodules (IPN).
Methods: We have developed a single-stranded sequencing strategy, BRcfDNA-Seq, which permits the inclusion of uscfDNA and scfDNA in addition to routinely observed ~167bps mononucleosomal cfDNA (mncfDNA). Current work focuses on preferential extraction of low molecular weight nucleic acids and single-stranded library preparation and sequencing, broad range cell-free DNA- Sequencing (BRcfDNA-Seq), to obtain uscfDNA, scfDNA, and mncfDNA, for disease detection. Sequencing data was bioinformatically processed: paired-end reads merged (BBmerge), aligned with BWA-mem, deduplicated using SRSLY UMI, and blacklisted portions removed. This was followed by analysis for non-mutational features like fragmentomics, occurrence of G-Quad structures, Motif sequences at the end of cfDNA sequences, genomic element peak formation, like exon fragments, are recently described non-mutation metrics which have demonstrated evidence of clinical relevance. With non-mutational multi-modal analysis of uscfDNA and scfDNA obtained using BRcfDNA-Seq, specifically exon fragments, were employed to develop classifiers in these IPNs into two cohorts: one identified as malignant (n=19) and one identified as benign (n=19).
Results: We observed a significant difference between the two classifications in a dataset of 38 benign and malignant IPN samples. CCCA and CCAT sequences were shown to be significant 4-mer endmotifs which differentiates into benign and malignant groups. Additionally, with the use of exons identified by limma ($p<0.001$), we were able to accurately classify samples into benign and malignant groups. Here, we present an XGBoost classifier model performing with 96.77% accuracy with the extraction of significant exons, for instance, GPR35's, ZNF594-DT's, and IGHGP's.
Conclusion: Results demonstrated clinical relevance of BRcfDNA-Seq for plasma-derived cfDNA in assessment of malignancy of lung indeterminate pulmonary nodules. These non-mutational multi-modal features of cfDNA maybe indicative of local and systemic factors associated with disease affecting cfDNA.

#073

UNVEILING DIAGNOSTIC BIOMARKERS FOR COLON ADENOCARCINOMA: A PROTEOMIC STUDY OF HUMAN EXTRACELLULAR VESICLES AND PARTICLES

KIM HS[1,2], LYDEN D[1]

[1]Departments of Pediatrics, and Cell and Developmental Biology, Drukier Institute for Children's Health, Meyer Cancer Center, Weill Cornell Medicine, New York, NY

[2]Yonsei Cancer Center, Division of Medical Oncology, Department of Internal Medicine, Graduate School of Medical Science, Brain Korea 21 FOUR Project, Yonsei University College of Medicine, Seoul, South Korea

There is an unmet medical need for circulating biomarkers to enhance early identification of colon adenocarcinoma (COAD). We examined the proteomic profile of extracellular vesicles and particles (EVP) from colon tissue explants and plasmas in COAD. The study addresses the constraints of standard serum carcinoembryonic antigen (CEA) testing and invasive procedures such as colonoscopy. To find possible EVP-based tumor diagnostic markers in COAD patients, we investigated EVPs from tumor and adjacent non-tumor tissues, as well as preoperative and postoperative plasma. During the discovery phase, we performed a proteomic analysis on 50 COAD patients using liquid chromatography-tandem mass spectrometry (LC-MS/MS), discovering approximately 50 markers from tissue explants and plasma. These markers were validated in an independent cohort of 104 people, which included 84 COAD patients and 20 healthy controls. We used ELISA to quantify 11 selected EVP proteins. Notably, seven of these proteins showed substantial differences in expression between preoperative and postoperative plasma, indicating their diagnostic value. These seven proteins also exhibited a significant increase in preoperative plasma compared to healthy controls, indicating their potential as diagnostic markers. The study expanded to assess the diagnostic accuracy of these seven proteins, known as "EV-7 levels," at various stages of COAD. Their performance was compared to serum CEA levels and plasma cfDNA mutation rates determined by an FDA-approved liquid biopsy assay. Compared to the serum CEA level, EV-7 levels showed higher detection rates in the early stages of COAD, particularly Stages I and II. Among the 84 individuals, 73 (86.9%) had EV-7 levels that exceeded the cutoff. In the validation cohort, EV-7 levels were more reliable throughout the COAD stages than serum CEA levels or cfDNA mutation rates. CEA values exceeding 5 ng/ml were 27%, 45%, 42%, and 64% for Stages I–IV, respectively. The detection rates of somatic mutations in cfDNA were 60%, 80%, 75%, and 75%. EV-7 levels were 73%, 100%, 88%, and 84%, respectively, with Stages I and II having the highest detection rates of the three approaches. ROC curve analysis for EV-7 levels in Stages I–IV revealed statistically significant results, with AUC values of 0.913, 1.000, 0.985, and 0.984, respectively. Our findings suggest that the seven EVP proteins have diagnostic potential in the plasma of early-stage COAD patients. Integrating these biomarkers into current diagnostic methods has the potential to improve early cancer detection, offering promising implications for clinical translation and application.

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PERFORMANCE OF NUCLEIC ACID-STABILIZING BLOOD COLLECTION TUBES FOR EV-BASED LIQUID BIOPSIES AND EV REPERTOIRE ANALYSES

CHIDESTER S[1], PLEET ML[2], SAVAGE J[1], COOK S[1], WELSH JA[1], GHIRAN I[3], CAMPHAUSEN K[4], ALDAPE K[1], JONES JC[1]
[1]Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland, USA
[2]Viral Immunology Section, Neuroimmunology Branch, National Institute for Neurological Disease and Stroke, National Institutes of Health, Bethesda, Maryland, USA
[3]Department of Anesthesia, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA
[4]Radiation Oncology Branch, National Cancer Institute, Bethesda, Maryland, USA

Introduction: Extracellular vesicles from blood are excellent sources of biomarkers for disease and treatment response. With the rapid evolution of circulating DNA and RNA liquid biopsy protocols during the past decade, blood collection tubes have been developed to stabilize blood at the point of collection for later processing and analysis. The performance of these sample-stabilizing blood collection tubes for analysis of EV repertoires based on EV surface markers has not been defined. The purpose of this study was to evaluate and compare the performance of Streck DNA Blood Collection Tubes (DNA BCT), RNA (RNA Complete), and Nucleic Acid (NA) tubes for collection of blood samples for evaluation of EV subsets based on EV proteins detected with EV flow cytometry following collection and processing.
Methods: Samples and metadata were collected following 2023 MIBlood-EV Guidelines (Lucien et al, JEV 2023). Blood collection tubes included serum SST, plasma EDTA, Streck DNA BCT, Streck RNA Complete, and Streck NA tubes. Each of these Streck tube types were processed per manufacturer-specified protocols, including centrifugal platelet removal step(s). EV enumeration was performed by RPS and NTA following size exclusion chromatography (qEV, Izon). EV surface protein repertoire analysis was performed in a flow cytometric multiplex, bead-based assay (Miltenyi Biotec), using calibrated fluorescent scales and MPA-PASS (Welsh JA, et al. Cell Rep Methods, 2021).
Results: Principal Component Analysis of EV repertoire marker profiles from these different blood collection tube types using MPA-PASS highlighted that a major distinction occurs with blood samples from Streck DNA BCT, due to an elevated platelet-derived EV profile (CD42a, CD62P). This distinction is less prominent in blood samples collected with Streck RNA Complete and Streck NA tubes.
Summary/Conclusion: Sample stabilization tubes allow blood to be transported between centers and stored for extended periods by mildly fixing blood cells and extracellular vesicles. Our results demonstrate that use of Streck NA tubes may be advantageous to use in clinical EV surface marker flow cytometry studies. However, this study also identifies some artifacts associated with stabilization tubes (especially DNA BCT) that should be considered when selecting blood collection methods for new EV biomarker trials.

CELLBIOPSY BASED COMPREHENSIVE MOLECULAR PROFILING OF CTC IN HER2 TESTED BREAST CANCER PATIENTS REVEALS NOVEL MEDIATORS OF EARLY DISEASE PROGRESSION AND THERAPEUTIC RESISTANCE

UTTARWAR M[1], SHAFI G[1], RAMESH A[2], BHARDE A[2], KHUTALE G[2], ANDHARI S[2], DESHPANDE R[2], JADHAV B[2], PRAJAPATI S[2], HARIRAMANI K[2], BASAVALINGEGOWDA M[2], KHANDARE J[1]
[1]OneCell Dx Inc, Cupertino, CA,USA.
[2]OneCell Diagnostics, Pune, India.

Problem statement: Obtaining tissue biopsy sample representing tumor is challenging. Also, it may not be adequate when the disease progresses to metastasis. While genomic profiling of liquid biopsy (primarily ctDNA) provides insights at genomic level, CellBiopsy based comprehensive molecular profiling offers an avenue to delve into genomic, transcriptomic, proteomic, and epigenomic dimensions of CellBiopsy based single circulating tumor cells (sCTCs). These additional 'multi-omics' analyses hold promise in identifying subpopulations with predisposition for driving disease progression, metastasis, and treatment resistance.
Background: Early recurrence of various cancer types, including breast cancer, stands as critical endpoint in clinical management. While recurrences can be managed effectively, they pose significant stress, financial burden, and elevate mortality risk if not detected promptly. Late diagnosis and rapid disease progression are key contributors to heightened mortality rates, compounded by genomic heterogeneity. Multiomics studies conducted on circulating CellBiopsies present promising opportunities for identifying disease progression, recurrence, clonal evolution, tumor heterogeneity, opening avenues for innovative diagnostic and prognostic strategies.
Expected outcomes: With continuously evolving nature of cancer, it is imperative to understand their molecular profiles. Molecular profiling of CellBiopsy in HER2 positive breast cancer patients to detect novel mediators of early disease progression and therapeutic resistance.
Methodology: Live sCTCs/CTC clusters were isolated from peripheral blood of 12 Breast Cancer patients using OncoRadar™ technology. Transcriptome and comprehensive genomic profiling (1080 genes) were performed using Illumina platform. In addition, presence/absence of surface protein biomarker(s) was determined using fluorescence imaging.
Outcomes achieved: First, genomic profiling of CellBiopsy revealed complex mutational patterns compared to paired traditional liquid biopsy. While all CellBiopsy based sCTCs had at least two actionable variants, most of mutations (92%) were found in the PIK3CA-MTOR pathway, paired ctDNA was negative for 25 % patients (3/12). Next, RNAseq technology was utilized for transcriptome analysis. This analysis enabled categorization of patients based on clinical outcomes. Furthermore, results indicate that focused profiling of CTC transcriptomes could potentially serve as predictive indicator for treatment response/therapy resistance. Protein expression of HER2 was detected by fluorescence microscopy using fluorescently labelled anti-HER2 antibodies. Based on fluorescence intensity CTCs were binned as HER2 negative for no detectable fluorescence signal or weakly or strongly positive based on low or high fluorescence signal.
Conclusion: Overall, cell proliferation pathways are particularly enriched in CellBiopsy based CTC profiling compared to ctDNA. Thus, combination of CellBiopsy with ctDNA offers a platform for early detection of disease progression and therapeutic resistance in breast cancer.

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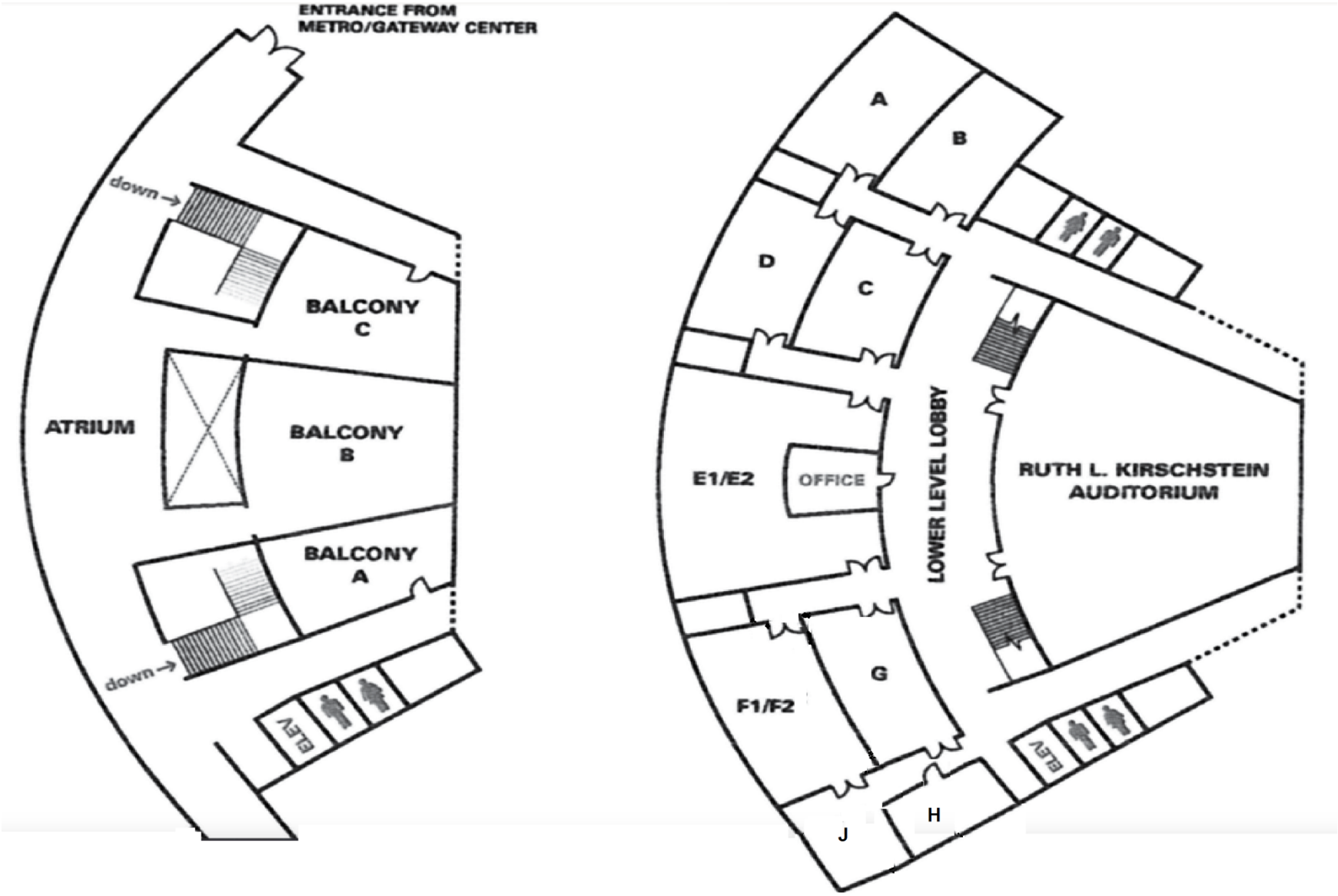
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