The Journal of Liquid Biopsy 1 (2023) 100010

Contents lists available at ScienceDirect

The Journal of Liquid Biopsy

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ISLB 2023 | Congress - Abstract Book Supplement

Oral Presentations

OP.01

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100011

OVARIAN CANCER IS DETECTABLE FROM PERIPHERAL BLOOD USING MACHINE LEARNING OVER T CELL REPERTOIRES

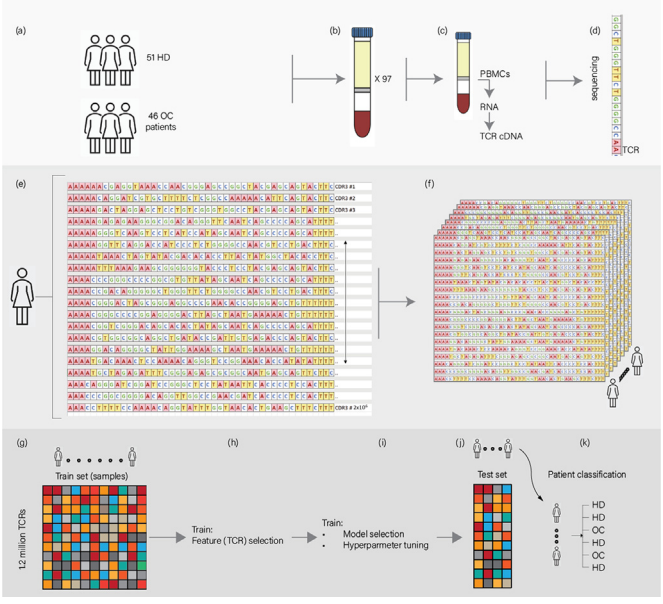
S. Efroni [1], A. Zilberberg [1], M. Zuckerbrot [1]. [1] *The Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel*

Introduction: The extraordinary diversity of T cell and B cells is critical for body maintenance. This diversity has an important role in protection against tumor formation. In humans, the T Cell Receptor (TCR) repertoire is generated through a striking stochastic process called V(D)J recombination in which different gene segments are assembled and modified, leading to extensive variety. In ovarian cancer, an unfortunate 80% of cases are detected late, leading to poor survival outcome. However, when detected early, approximately 94% of patients live longer than five years after diagnosis.

Methods: To determine whether the TCR repertoire obtained from peripheral blood is associated with tumor status, we collected blood samples from 97 women with or without ovarian cancer and obtained TCR information. We then used machine learning over these repertoires to perform supervised learning, to identify previously unseen samples as either coming from a patient or from a healthy donor.

Results: Using machine learning, we successfully stratified the two groups at high scores of Area Under the ROC of 0.9. These results show that we are able to associate peripheral blood TCR repertoire with the formation of ovarian cancer tumors.

Conclusions: Quantifying the TCR repertoire is a novel method to perform liquid biopsy. The use of the repertoires has been demonstrated over multiple uses. This work shows its utility and performance as a cancer biomarker.



OP.02

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100012

LONGITUDINAL PLASMA CTDNA TESTING IN RESECTED EARLY-STAGE NSCLC

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Introduction: Detection of circulating tumour DNA (ctDNA) in plasma perioperatively is associated with shorter recurrence free survival (RFS) in patients with early-stage lung cancer. ctDNA-Lung-Detect is an investigator-initiated prospective study of ctDNA detection and association with RFS in patients with early-stage non-small cell lung cancer (NSCLC) [NCT05254782].

Methods: Patients with clinical T<4cm N0 NSCLC planned for surgical resection at Princess Margaret/University Health Network underwent plasma ctDNA assessment before and after surgery (~1 month and 1 year). Minimal residual disease (MRD) was detected using the highly sensitive and specific tumor-informed RaDaR® assay (NeoGenomics, Durham, NC), testing up to 48 tumor-specific variants in plasma with a limit of detection 95 of 0.0011% variant allele fraction.

Results: From Aug 2021-Feb 2023, 129 patients were enrolled and 70 had sufficient tissue for ctDNA bespoke panel generation (Table 1). Preoperative ctDNA was detected in 17/69 (24.6%) patients (with a lower detection rate of 9/50 or 18% in pathologic stage I NSCLC). All but one patient (1/17; 94.1%) had ctDNA clearance with surgery at 1-month landmark timepoint. This patient with occult N2 disease remains disease-free radiographically on adjuvant targeted therapy, despite ctDNA persistence at 1 year. Currently, 4/46 patients with ≥1 year follow up have relapsed in plasma and clinically. A higher recurrence rate is seen in patients with detectable ctDNA pre-op (2/10; 20%) vs. undetectable (2/36; 5.6%). Patients with occult nodal involvement, larger tumor size, squamous histology, PD-L1 ≥1%, pleural or lymphovascular invasion, and high-grade tumors were more likely to have detectable pre-operative ctDNA.

Conclusions: In patients with clinical stage I NSCLC, 25% have detectable ctDNA pre-operatively using a highly sensitive tumor-informed assay. Surgical resection led to ctDNA clearance in 94% of cases. Updated data as of November 2023 will be presented, including an exploration of additional predictors of shedding versus non-shedding tumors.

	Patients with ctDNA results (n = 70)	Patients with preoperative ctDNA results (n=69)*		p-value
		Positive (n= 17)	Negative (n= 52)	
Age in years, median (range)	71 (51-85)	71 (62-85)	71 (51-85)	0.21
Sex				0.22
Female	46 (34.3%)	9 (52.9%)	36 (69.2%)	
Male	24 (65.7%)	8 (47.1%)	16 (30.8%)	
Invasive tumor size (mm), median (range)	20.5 (2-60)	26 (13-60)	19 (2 – 47)	0.006
Histology				<0.0001
Squamous	8 (11.4%)	7 (41.2%)	1 (1.9%)	
Non-squamous	61 (87.1%)	9 (41.2%)	51 (98.1%)	
Large cell neuroendocrine	1 (1.4%)	1 (5.9%)	0	
Pathology findings				
Visceral pleural invasion positive	13 (18.6%)	6 (35.3%)	6 (11.5%)	0.02
LVI positive	26 (37.1%)	11 (64.7%)	14 (26.9%)	0.005
STAS pattern present	31 (47.7%)	10 (66.7%)	21 (42.9%)	0.14
Grade				0.04
1-2	32 (49.2%)	4 (25%)	27 (56.3%)	
3-4	33 (50.8%)	12 (75%)	21 (43.8%)	
Oncogenic driver alterations				0.48
Actionable	37 (60.7%)	5 (50%)	32 (64%)	
Not actionable	24 (39.3%)	5 (50%)	18 (36%)	
PD-L1 TPS				0.048
< 1 %	28 (41.2%)	3 (17.7%)	25 (50%)	
1-49 %	28 (41.2%)	10 (58.8%)	17 (34.0%)	
≥ 50 %	12 (17.7%)	4 (23.5%)	8 (16%)	
T stage				0.01
T1mi	2 (2.9%)	0	2 (3.9%)	
T1a	5 (7.1%)	0	5 (9.6%)	
T1b	23 (32.9%)	3 (17.7%)	20 (38.5%)	
T1c	9 (12.9%)	3 (17.7%)	6 (11.5%)	
T2	1 (1.4%)	0	1 (1.9%)	
T2a	16 (22.9%)	5 (29.4%)	11 (21.2%)	
T2b	4 (5.7%)	2 (11.8%)	2 (3.4%)	
T3	9 (12.9%)	4 (23.5%)	4 (7.7%)	
T4	1 (1.4%)	0	1 (1.9%)	
N status				0.10
N0	62 (88.6%)	13 (76.5%)	48 (90.3%)	
N1	3 (4.3%)	2 (11.8%)	1 (1.9%)	
N2	5 (7.1%)	2 (11.8%)	3 (5.8%)	
Stage (AJCC 8th edition)				0.02
IA1	7 (10.0%)	0	7 (13.5%)	
IA2	23 (32.9%)	3 (17.7%)	20 (38.5%)	
IA3	7 (10.0%)	3 (17.7%)	4 (7.7%)	
IB	13 (18.6%)	3 (17.7%)	10 (19.2%)	
IIA	4 (5.7%)	2 (11.8%)	2 (3.4%)	
IIB	8 (11.4%)	3 (17.7%)	4 (7.7%)	
IIIA	8 (11.4%)	3 (17.7%)	5 (9.6%)	

ctDNA = circulating tumor DNA, PD-L1 = programmed death-ligand 1, TPS = tumor proportion score
Unknown/not documented: STAS pattern n=5; grade n=5; PD-L1 TPS n=2.
* One patient did not have a pre operative ctDNA samples drawn

OP.03
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100013
MOVING TOWARDS GLOBAL HARMONIZATION OF LIQUID BIOPSY FRAMEWORKS AND STANDARDS: THE BLOODPAC EXPERIENCE

L. Leiman [1], P. Febbo [2], R. Grossman [3], P. Kuhn [4], A. Martin [5], J. Vinson [6]. [1] *Blood Profiling Atlas In Cancer (BLOODPAC), Chicago, USA;* [2] *Illumina, San Diego, USA;* [3] *University of Chicago Center for Translational Data Science, Chicago, USA;* [4] *University of Southern California, Los Angeles, USA;* [5] *GSK, Waltham, USA;* [6] *Prostate Cancer Clinical Trials Consortium, New York, USA*

The Blood Profiling Atlas in Cancer (BLOODPAC) is a consortium focused on accelerating the development and validation of liquid biopsy assays. We have published four critical articles establishing standards and frameworks for assay validation and implementation which have seen substantial community uptake. Our membership is composed of over sixty organizations within academia, industry, government, and the nonprofit sector, all of which contribute to the BLOODPAC Data Commons (BPDC).

In one of our first publications, entitled "Minimum Technical Data Elements for Liquid Biopsy Data Submitted to Public Databases" (2020), we defined a list of attributes suggested for data collection to ensure that data can be pooled and analyzed across studies to further research efforts.

BLOODPAC's partnership with the US Food and Drug Administration (FDA) led to the publication of "Generic Protocols for the Analytical Validation of Next-Generation Sequencing-Based ctDNA Assays" (2020). Our open feedback period for this protocol has recently ended, and we are revising the document to ensure it remains relevant as the field evolves. We are also drafting a supplement covering blood tumor mutational burden (bTMB) applications and creating a new protocol for molecular residual disease (MRD) at the FDA's request. BLOODPAC has also advanced methods for inter-assay comparison through our study titled "Contrived Materials and a Data Set for the Evaluation of Liquid Biopsy Tests," published this year.

BLOODPAC's long-term goal is to empower the international liquid biopsy community to accelerate the development, validation, and adoption of assays for patient benefit. To that end, we are committed to collaborating with ISLB and other consortia in the field to educate assay developers, payers, and regulatory agencies on the agreed-upon standards and frameworks that the community has developed. As a first step towards this end, we recently convened a Global Regulatory working group charged with facilitating international regulatory standards for diagnostic testing with liquid biopsy. In the short term, members are working to obtain input on existing liquid biopsy validation protocols from our international partners. We look forward to serving as an educational resource and champion for the global liquid biopsy community.

OP.04
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100014
PERFORMANCE OF CIRCULATING TUMOR DNA (CTDNA) GENOMIC AND EPIGENOMIC PROFILING (GUARDANTINFINITY) IN THE TRESR AND ATTACC STUDIES

I. Silverman [1], J. Schonhoft [1], A. Yablonovitch [2], E. Lagow [2], M. Cecchini [3], B. Herzberg [4], J. Reis-Filho [5], E. Rosen [5], V. Rimkunas [1], T. Yap [6]. [1] *Repare Therapeutics, Cambridge, USA;* [2] *Guardant Health, Palo Alto, USA;* [3] *Yale School of Medicine, New Haven, USA;* [4] *Columbia University Cancer Center, New York, USA;* [5] *Medical Oncology, Memorial Sloan Kettering Cancer Center, New York, USA;* [6] *Investigational Cancer Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, USA*

Introduction: Commercially available first-generation ctDNA sequencing panels are limited in coverage and sensitivity, hindering their broader clinical utility for identification of biomarkers and response monitoring. Here, we evaluated the utility of a next-generation ctDNA sequencing platform in a clinical trial setting.

Methods: Samples were collected in two phase 1 studies evaluating ataxia telangiectasia and Rad3-related (ATR) inhibitor camonsertib in combination with gemcitabine, or PARP inhibitors talazoparib, niraparib, or olaparib (TRESR; NCT04497116 and ATTACC; NCT04972110). GuardantINFINITY, a combined genomic (>800 genes) and epigenomic (15 Mb) platform, was used to sequence cell-free DNA and PBMCs.

Results: Blood samples were drawn from 117 patients with ovarian (n=29), breast (n=18), pancreatic (n=18), prostate (n=16) and other (n=36) cancers, enrolled with pathogenic alterations in BRCA2 (n=41), ATM (n=32), BRCA1 (n=23), PALB2 (n=8) and other genes (n=13). Enrollment alterations were confirmed in 83% (94/114) excluding 3 patients enrolled with protein loss. 50% (5/10) of large deletions or rearrangements with sufficient circulating tumor fraction (cTF) were confirmed.

Among patients with breast, ovarian, prostate or pancreas cancers previously treated with a PARP inhibitor and/or platinum and harboring BRCA1/2 or PALB2 alterations, 36 reversions were detected in 31% (17/54) of patients. An additional CRC patient with a clonal biallelic sATM alteration had 2 missense reversions in cis. The most common reversion alteration type was short deletions (n=15), all of which showed evidence for microhomology.

One-hundred and six patients had evaluable paired samples at baseline and on-treatment (taken between 2–12 weeks on-treatment [median, 3 weeks]). Utilizing the mean variant allele frequency (mVAF) of the Guardant360 74-gene subset or methylation-based cTF, 72% or 86% were monitorable, respectively. In the subset of patients with matched PBMC sequencing (n=68), after PBMC-informed clonal hematopoiesis (CH) filtering, monitorability was reduced from 72% to 63% between G360 mVAF and G360 mVAF-CH, respectively. PBMC-informed CH filtering improved the correlation between variant-based and methylation-based monitoring (G360 mVAF: R=0.68, p=3.9e-7 vs G360 mVAF-CH: R=0.86, p=1.7e-12, Pearson correlation).

Conclusions: Comprehensive panel coverage aided in more precise diagnosis of complex alterations and identification of reversions. Methylation-based cTF allowed response monitoring in more patients and may mitigate signal contamination from CH.

Poster Presentations
Theme: Clinical Application of Liquid Biopsy
PP.01
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100015
A CELL-FREE DNA METHYLATION SIGNATURE TO DETECT METASTATIC BREAST CANCER IN BLOOD

I. Barragan [1,2], A. Gonzalez [1,3], J. Dubbelman [1], J. Onieva [1,3], A. Rodriguez-Casanova [4,5,6,7,8], N. Costa-Fraga [4,5,6,8], A. Diaz-Lagares [4,5,6,7,9], J. Pascual [1], B. Martinez [1], E. Alba [1]. [1] *Medical Oncology Service (Group of Translational Research in Cancer Immunotherapy), Regional and Virgen de la Victoria University Hospitals, Institute of Biomedical Research in Malaga and BIONAND Nanomedicine Platform (IBIMA BIONAND Platform), Malaga, Spain;* [2] *Group of Pharmacoepigenetics, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden;* [3] *Faculty of Medicine, Malaga, Spain;* [4] *Epigenomics Unit, Cancer Epigenomics, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), Complexo Hospitalario Universitario de Santiago de Compostela (CHUS,SERGAS), Santiago de Compostela, Spain;* [5] *Galician Precision Oncology Research Group (ONCOGAL), Medicine and Dentistry School, Universidade de Santiago de Compostela (USC), Santiago de Compostela, Spain;* [6] *Centro de Investigación Biomédica en Red en Cáncer (CIBERONC), Instituto de Salud Carlos III, Madrid, Spain;* [7] *Roche-Chus Joint Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), Santiago de Compostela, Spain;* [8] *Universidade de Santiago de Compostela (USC), Santiago de Compostela, Spain;* [9] *Department of Clinical Analysis, University Hospital Complex of Santiago de Compostela (CHUS), Santiago de Compostela, Spain*

Introduction: The early detection of metastatic disease is breast cancer is the most important remaining challenge with great impact in the patient survival. While classical protein markers like Carcinoembryonic Antigen, Cancer Antigen 15-3 and Cancer Antigen 125 have a limited sensibility of 50 % to detect metastasis in breast cancer patients, still scarce genetic variants can be associated specifically with the metastatic disease. Recent studies have identified DNA methylation signatures predictive of prognosis of breast cancer metastasis. This, together with the high tissue specificity informativity of DNA methylation, led us to evaluate the use of cell-free DNA methylation to mark metastatic breast cancer (MBC).
Methods: Cell-free DNA (cfDNA) was isolated from 3 ml of plasma from 8 MBC patients. Inclusion criteria restricted to MBC diagnosis by image. Genome-wide DNA methylation profiling was performed using 50 ng of end-repaired DNA in EPIC arrays. Public repository cfDNA DNA methylation profiles from patients with primary breast tumors and from healthy individuals were used as controls. IDAT files were processed, and differential methylation calculated using RnBeads.
Results: Genomically enriched DNA methylation variants in the metastatic versus the primary breast patients summed up to 3488 single CpG sites, 1233 hypomethylated and 2255 hypermethylated. Grouping the CpG sites per promoter or CpG island, we established a metastatic breast cancer signature of 6 genes: Glutathione peroxidase 1 (GPX1), Long Intergenic Non-Protein Coding RNA 2136 (LINC02136), Zinc Finger Protein 880 (ZNF880), AXIN 2 pseudogene (ENSG00000236555), Zinc Finger Protein 501 (ZNF501), and Succinate Dehydrogenase Complex Assembly Factor 3 (SDHAF3) (Figure 1).
All of them are hypomethylated in the metastatic scenario except for AXIN 2 pseudogene and BHLHE40. Interestingly, some show a plausible functional link to metastatic disease. For example, GPX1, hypomethylated in our metastatic patients, is involved in adhesion and other metastatic processes in breast cancer. Moreover, BHLHE40 is a suppressor of the main pharmacogene in cancer ATP-binding cassette subfamily B member 1 (ABCB1) and is hypermethylated in the metastatic patients.
Conclusion: A few milliliters of plasma can serve for the detection of DNA methylation marks specific to the metastatic tumor of breast cancer. This is a starting point to exploit the tissue of origin prediction potential of DNA methylation in the early detection of breast cancer metastasis.

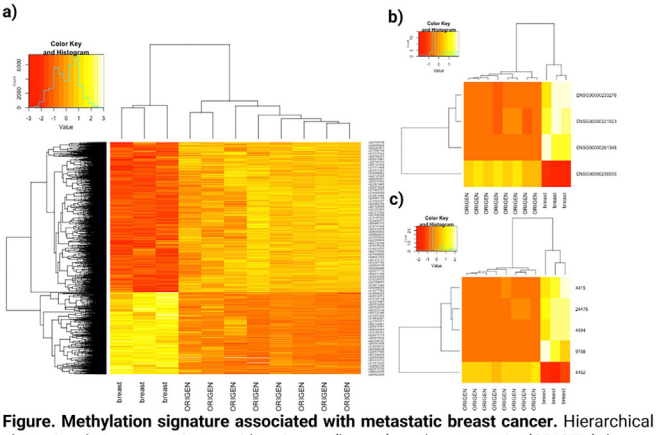

Figure. Methylation signature associated with metastatic breast cancer. Hierarchical clustering between patients with primary (breast) and metastatic (ORIGEN) breast cancer by **a)** CpG site, **b)** promoter and **c)** CpG island.

PP.02
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100016
CLINICAL UTILITY OF COMBINED PLASMA AND TISSUE NGS IN PATIENTS WITH NEWLY DIAGNOSED ADVANCED NON-SMALL CELL LUNG CANCER

H. Bote De Cabo [1,2], M. Siringo [3], E. García-Lorenzo [4], A. Castelo [1], E. Conde [2,5,6,7], S. Hernández [5,6], F. López-Ríos Moreno [2,5,6,7], L. Paz-Ares [1,2,6,7], J. Zugazagoitia [1,2,6,7]. [1] *Department of Medical Oncology, Hospital Universitario 12 De Octubre, Madrid, Spain;* [2] *Universidad Complutense de Madrid, Madrid, Spain;* [3] *Department of Medical Oncology, Policlinico Umberto I "Sapienza" University of Rome, Rome, Italy;* [4] *Department of Medical Oncology, Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain;* [5] *Pathology Department, Hospital Universitario 12 de Octubre, Madrid, Spain;* [6] *Research Institute Hospital 12 de Octubre (i+12), Madrid, Spain;* [7] *CIBERONC, Madrid, Spain*

Introduction: The incremental clinical value of performing both tissue and plasma next-generation sequencing (NGS) to detect actionable alterations (AA) at diagnosis in patients with advanced non-small cell lung cancer (aNSCLC) has not been comprehensively assessed.
Methods: We conducted a study with two aNSCLC cohorts, prospectively assessed with tissue and plasma NGS at diagnosis. Guardant360 CDx and FoundationOne Liquid CDx were used in cohort 1 (N=127) and cohort 2 (N=149), respectively. We considered AA according to ESCAT I-III levels. Undetectable ctDNA and unavailable tissue were considered non-informative results.
Results: AA were found in 50/127 (39%) and 65/149 (44%) patients in cohorts 1 and 2, respectively (Figure 1). In cohort 1, AA were detected only in plasma in 14/50 (28%) and only in tissue in 9/50 (18%). In the 14 cases with AA only detected in plasma, 3 had informative tissue results (1 EGFR and 1 KRAS G12C mutations detected in plasma but not in tissue). In the 9 cases with AA only detected in tissue, 6 had informative plasma results (1 ALK fusion, 1 ERBB2 amplification, 1 EGFR and 2 KRAS G12C mutations detected in tissue but not in plasma). In cohort 2, AA were detected only in plasma in 22/65 (34%) and only

in tissue in 15/65 (23%). In the 22 cases with AA only detected in plasma, 11 had informative tissue results (1 RET and 1 ALK fusions, 3 ERBB2 alterations, 2 KRAS G12C mutations detected in plasma but not in tissue). In the 15 cases with AA only detected in tissue, 6 had informative plasma results (3 EGFR mutations, 1 ROS1 fusion and 2 ERBB2 amplifications detected in tissue but not in plasma).
Conclusion: Due to data observed, plasma NGS should be considered when AA are not detected by tissue, and vice versa, in patients with newly diagnosed aNSCLC.

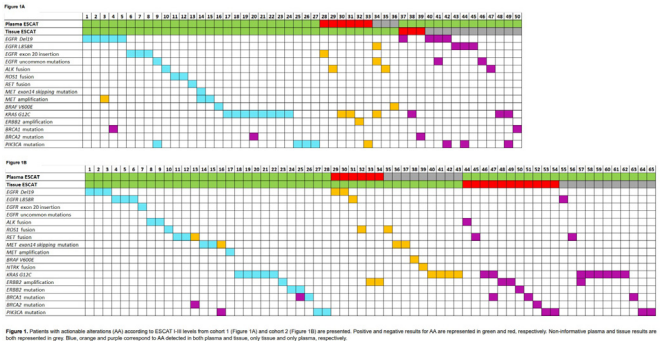

Figure 1. Patients with actionable alterations (AA) according to ESCAT I-III levels from cohort 1 (Figure 1A) and cohort 2 (Figure 1B) are presented. Positive and negative results for AA are represented in green and red, respectively. Non-informative plasma and tissue results are both represented in grey. Blue, orange and purple correspond to AA detected in both plasma and tissue, only tissue and only plasma, respectively.

PP.03
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100017
CLINICAL FEASIBILITY OF NGS LIQUID BIOPSY ANALYSIS IN NSCLC PATIENTS USING ONCOMINE PRECISION ASSAY WITH GENEXUS™ INTEGRATED SEQUENCER

S. Buglioni [1], E. Gallo [1], C. Ercolani [1], F. Rollo [1], L. Ronchetti [1], A. Palange [1], G. Americi [1], A. Di Benedetto [1], V. Di Noia [1], P. Visca [1]. [1] *IRCCS, Regina Elena National Cancer Institute, Rome, Italy*

Introduction: Analysis of tumor molecular profile can provide predictive information to guide treatment in NSCLC patients. Molecular characterisation obtained from tissue biopsy is often not feasible due to inadequate or anavailable of histological materials. Thus, liquid biopsy (LB) represents a practical alternative source for investigating tumor-derived alterations. The aim of this study was to investigate the feasibility of cell-free total nucleic acid (cfTNA) analysis in everyday practice, setting up a well defined "LB path" and using a sensitive and fast NGS approach in patients with NSCLC.
Methods: We performed cfTNA analysis of 20 cases selected on the basis of the availability of both tissue and plasma and trackable pathogenic mutation detected in the primary tumor. Extracted tumor tissue DNA/RNA and cfTNA were sequenced using the Oncomine Precison Assay gene panel and the Genexus integrated sequencer. Blood samples (16 mL) were collected at the time of tissue biopsy and cfTNA were isolated from plasma and subjected to the Genexus system for library construction, templating, sequencing, and data analyses.
Results: The sequencing resulted in median overall depth of 41,054× and median molecular coverage of 2,117×. When we compared the alterations detected on the tissue with those of the corresponding LB, we obtained an 80% concordance. In fact, in 16/20 cases the genomic alterations detected from tissue were concordantly detected from plasma. In the 4 discordant cases we detected EGFR exon 19 deletion, MET amplification, MET exon 14 skipping and ALK fusion only in tissue and not in the corresponding LB (Figure 1).
Conclusion: A well-defined "LB pathway" enabled us to achieve high concordance between tissue and LB in the identification of pathogenic alterations, even for fusions, identified directly on cfRNA. The present feasibility study allowed us to implement this NGS LB approach in routine diagnostics as a complementary tool for tumor genotyping.

Samples	Genomic alterations	Results
A1	EGFR p.L858R; TP53 p. H179R	
A2	EGFR p.L858R	
A3	EGFR p.E746_A750del	
A4	EGFR p.E746_A750del	
A5	EGFR p.L861Q; EGFR p.G719A	
A6	EGFR p.H773_V774ins; TP53 p.V216L	
A7	BRAF p.V600E	
A8	ERBB2 p. Y772_A775dup	
A9	KRAS p.G12C; TP53 p.Y220C	
A10	KRAS p.G12D	
A11	ALK fusion: EML4(13)-ALK(20)	
A12	ALK fusion: TPM3(7)-ALK(20)	
A13	ROS1 fusion: CD74(6)-ROS1(34); TP53 p.L194R	
A14	NTRK3 fusion: ETV6(4)-NTRK3(14)	
A15	RET fusion: CCDC6(1)-RET(12); TP53 pG266V	
A16	RET fusion: CCDC6(1)-RET(12)	
A17	RET fusion KIF5B(15)-RET(12)	
A18	MET exon 14 skipping; MET c.2942_1G>A	
A19	MET exon 14 skipping	
A20	MET amplification	

Legend

Concordant : alteration detected on both tissue and liquid biopsy

Discordant: alteration detected on tissue only

PP.04
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100018
A PLASMA MICRORNA SIGNATURE FOR EARLY DETECTION OF PAEDIATRIC CEREBELLAR ATROPHY CAUSED BY PMM2-CDG

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Introduction: Paediatric cerebellar atrophies (CA) are disorders that cause an irreversible loss of the cerebellar tissue. They are diagnosed in children, mostly when the symptoms have appeared and the damage is irreversible. Thus, a non-invasive early diagnosis method for paediatric CA is needed.
During the last few years, there has been a rising interest in miRNAs as non-invasive biomarkers, due to their important role as regulators of gene expression in many complex diseases and their remarkable stability in plasma.
Although CA currently has no cure, different strategies of treatment are under development, and the early diagnosis of this aetiology has the potential to improve the quality of life of the patients and facilitate familiar genetic counselling. The goal of this study is to find a minimally invasive plasma-based miRNA signature to differentiate PMM2-CDG patients (a specific subset of CA patients) from controls.
Methods: Plasma was isolated from 29 PMM2-CDG patients and 68 controls and divided into training and test sets. The concentration of different miRNAs in plasma was assessed using next-generation sequencing, and RNA-Seq reads were mapped against the known miRNome. Using multiple machine learning models, we built different signatures with different combinations of miRNAs. We also inferred the tissue of origin of the miRNAs present in the final signature.
Results: Overall, we detected a total of 1 030 distinct miRNAs, 107 genes expressed in all samples, and 6 genes differentially expressed (DE) in the control vs. CA samples in the training cohort. After evaluating the accuracy of the signatures obtained, we found one that showed excellent performance in detecting PMM2-CDG, with a sensitivity and specificity higher than 0.9 in both the training and test cohorts. Moreover, several DE genes and genes present in the final signature were brain or cerebellum-specific, linking the dysregulated genes to the diseased tissue.
Conclusion: This is the first study proposing the detection of plasma miRNAs as a non-invasive method for the early detection of PMM2-CDG. Our approach paves the way to a non-invasive diagnosis for children with presymptomatic cerebellar atrophy while highlighting the relevance of miRNA biomarkers for the diagnosis of PMM2-CDG.

PP.05
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100019
TGF-β MRNA EXPRESSION LEVELS IN EXTRACELLULAR VESICLES ARE ASSOCIATED WITH IMMUNOTHERAPY RESPONSE IN METASTATIC MELANOMA PATIENTS

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Introduction: Immune checkpoint inhibitors (ICIs) as anti-PD1 significantly increased survival in melanoma patients and became the standard treatment in the metastatic setting. However, several patients developed drug resistance and disease progression. Nowadays, no convincing biomarkers are present and able to predict ICIs response in melanoma. Therefore, the identification of predictive biomarkers is an important clinical challenge. Potential biomarkers such as PD-L1 expression and the transforming growth factor-β (TGF-β) have been evaluated because of their pleiotropic role in melanoma progression. In this context, liquid biopsy, including tumour-derived extracellular vesicles (EVs) carrying nucleic acids, may be used to evaluate these potential biomarkers. The present study aimed to investigate the possible biomarker role for ICIs response of TGF-β, IFN-γ and PD-L1 mRNAs in tumour-enriched EVs isolated from peripheral blood of patients with metastatic melanoma treated with first-line anti-PD1 antibodies. Radiomic analysis has also been performed in a subset of patients.
Methods: Blood samples were collected in EDTA tubes at baseline, and RNA was extracted from EVs for analysis of PD-L1, IFN-γ, and TGF-β mRNA levels using digital droplet PCR (ddPCR). Additionally, a biomarker-radiomic correlation analysis was performed on a subset of patients.
Results: Thirty patients with metastatic melanoma that received anti-PD1 antibodies as first-line therapy were enrolled in the present study. Patients with high TGF-β expression (cut-off fractional abundance [FA] >0.19) at baseline showed longer median progression-free survival (8.4 vs. 1.8 months; p=0.006) and overall survival (17.9 vs. 2.63 months; p=0.0009). Additionally, radiomic analysis exhibited that patients with high TGF-β expression at baseline presented smaller lesions (2.41 ± 3.27 ml vs. 42.79 ± 101.08 ml, p<0.001) and higher dissimilarity (12.01 ± 28.23 vs. 5.65 ± 8.4; p=0.018).
Conclusion: Although the present study examines a small cohort of patients, the results support the hypothesis that high TGF-β expression levels in EVs are associated with favourable responses to immunotherapy in patients with metastatic melanoma, suggesting its role as a potential predictive biomarker.

PP.06
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100020
HETEROCHROMATIN PROFILING IN LYMPHOMA PATIENT PLASMA FOR THE DISCOVERY OF NOVEL CHROMATIN VARIANTS

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Introduction: Lymphomas are driven by epigenetic aberrations that are reflected in differential chromatin states, represented by a network of histone modifications. Histone modifications in circulating cell-free chromatin (cf-chromatin) have recently been introduced as a class of liquid biopsy analytes detectable in plasma. To date, there are no works describing the comprehensive profiling of histone modifications linked to heterochromatin domains from cf-chromatin, which may be critical for understanding the epigenetic landscape of lymphomas using liquid biopsies.
Methods: To address histone modifications linked to heterochromatin domains, we developed a method for comprehensive profiling of H3K27me3 from cf-chromatin. We applied this to conditioned media from SU-DHL-6 (a diffuse large B-cell lymphoma (DLBCL) cell line), and plasma from 78 patients with B-cell lymphomas and 21 non-lymphoma controls. Plasma H3K27me3 profiles were sequenced to a mean of 142.3 million reads, using the Illumina NovaSeq platform. Counts from the sequencing profiles were summarized in 300bp bins, and normalized for library depth. We conducted differential analysis using edgeR. Finally, we assessed the fragment length differences between lymphoma and control plasma H3K27me3 profiles using Picard, and quantified the fraction of fragments 126-135 bp in length (representing the shortened mononucleosome) and 240-330 bp (representing the shortened dinucleosome), relative to the total fragment pool.
Results: Genome wide correlations between replicates of H3K27me3 from conditioned media cf-chromatin were highly concordant (Spearman correlation: 0.96-0.99). For the clinical cohort, mean plasma cfDNA concentrations were significantly different between lymphoma and controls (hLINE-1 quantification, lymphoma = 47.79ng/μL, control = 18.31ng/μL, Welch's t-test: $p = 0.011$). Within plasma cf-chromatin, we detected 79,873 differentially expressed regions ($p < 0.01$). Sequenced cf-chromatin fragments associated with H3K27me3 from lymphoma patient plasma were enriched in short fragments compared to controls (lymphoma = 19.2%, control = 16.2%, Welch's t-test: $p = 0.002$).
Conclusion: Here we report robust and reproducible profiling of heterochromatin states from cf-chromatin. Plasma H3K27me3 profiles reflect expression and topological changes that are unique to lymphoma samples. Altogether, we demonstrate that plasma H3K27me3 provides an alternative analyte for the discovery of novel biomarkers, expanding the toolkit of epigenetic liquid biopsy biomarkers for the future of cancer guidance.

PP.07
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100021
CLINICAL AND MOLECULAR UTILITY OF CELL BLOCK FROM PLEURAL EXUDATE CONCERNING CYTOLOGY AND SURGICAL BIOPSY

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Introduction: Pleural exudate is a beneficial sample for diagnosing primary neoplastic or metastatic and following pleural disease, avoiding more aggressive procedures.
Methods: Prospective observational study between January 2020 and December 2022. Patients with mononuclear predominant pleural exudates, with cytology, and submitted to pleural biopsy were included. A cell block was made from the exudate using the thrombin technique: centrifugation in a 50 ml falcon tube for 5 minutes at 1200 rpm to obtain a cell button. The supernatant was then decanted, and four drops of previously reconstituted thrombin and four drops of the same patient plasma were added. Second centrifugation was performed for 5 minutes at 1200 rpm, 1 ml of 10% buffered formaldehyde was added, and fixed for 2 hours. The diagnostic rate of each technique was compared with the surgical biopsy, considered as Gold Standard. Immunohistochemistry and NGS methods were performed on the cell blocks. A multiparametric analysis of the variables was performed.
Results: One hundred fifty-three patients with cytology and biopsy were recruited and underwent cell block. The biopsy was positive for neoplasia in 71/153 cases, with 46% of the cytology positive for neoplastic cells compared to 39% of the cell blocks. Of the suspicious cytology, 92% were neoplastic on biopsy. Of the suspicious cell blocks, 100% were neoplastic on biopsy. The concordance between cytology and biopsy was 68% (k=0.39), between cell block and biopsy, was 62% (k=0.31), and between cytology and cell block was 71% (k=0.57). The specificity for diagnosing neoplasia of cytology and cell block was 100%. The sensitivity for the diagnosis of neoplasia was 59% for both methods. Cell blocks were used for NGS, obtaining optimal results in all cases analyzed.
Conclusion: The cell block from pleural exudate is a less invasive tool with 100% specific for diagnosing neoplasia, with sensitivity equivalent to cytology, It allows the enrichment of % of tumor cells by macrodissection for NGS. This method is fundamental in cases where surgical biopsy is not accessible and feasible in a public hospital setting due to its low cost.

PP.09
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100022
GENOMIC PROFILING IN NSCLC: INSIGHTS FROM LIQUID BIOPSY

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Introduction: Liquid biopsy (LB) has emerged as a promising non-invasive, short turnaround time (TAT) approach for detecting and monitoring genomic alterations in many cancers. The aim of this study was to evaluate the clinical usefulness of NGS analysis of LB in the management of NSCLC patients.
Methods: This prospective study included 44 NSCLC patients divided in 4 clinical scenarios (CS): CS1 (tumor genotyping for therapy selection), CS2 (referall to rapid diagnostic program), CS3 (disease progression assessment and identification of resistance mechanisms), CS4 (monitorying treatment responce). For each patient, blood samples were collected and cell-free total nucleic acid (cfTNA) was isolated from 8 ml of plasma using Genexus™ Purification System (ThermoFisher Scientific). Analysis was conducted using Oncomine Precision Assay (OPA) with Genexus™ Integrated Sequencer. OPA panel analyzes 78 variants, including 45 mutations, 14 CNVs and 19 fusion variants, across 50 key gene for target therapy in NSCLC.
Results: NGS-based LB provided us with clinically relevant information in each clinical scenario:
CS1: In 6 patients with inadequate or unavailable tissue biopsy, NGS analysis of cfTNA allowed us to detect at least one actionable alteration in 2/6 cases (33%).
CS2: In 5 potentially "oncogene addicted" patients, in which LB was performed at the time of tissue biopsy, actionable alterations on cfTNA were detected by the OPA panel in 4 cases. All these patients started a targeted therapy before tissue results available.
CS3: In 11/14 patients with progression of disease to a target therapy, LB analysis confirmed the presence of the driver gene alteration detected on the primary tumour, while in 3/14 patients (21%) it revealed a second acquired resistant mutation.
CS4: In 19 patients serial blood collections were performed during therapy for monitoring therapeutic efficacy and response.
Conclusion: The NGS analysis of cfTNA, with a short TAT, could be used as complementary to tumor tissue biopsies to identify actionable mutations and it could assist clinicians to make more timely decision.

PP.10
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100023
UTILITY OF LIQUID BIOPSY FOR COMPREHENSIVE GENOMIC PROFILING IN A FIRST DIAGNOSTIC CONTEXT: A COMPARATIVE ANALYSIS WITH TISSUE BIOPSY

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Introduction: Liquid biopsy (LB) analyzed by NGS has emerged as a valuable tool in cancer care. By analyzing circulating tumor Nucleic Acids (ctNAs), LB offers a non-invasive alternative for detecting genetic alterations. In this study, we discuss the utility of LB for comprehensive genomic diagnosis when tissue biopsy is compromised.
Methods: Samples from two patient cohorts were collected and analyzed prior to the initiation of treatment: 11 patients harboring ALK and ROS rearrangements, and 26 with colorectal cancer (regardless of the KRAS status). Oncomine Precision Assay (Thermofisher Scientific) was performed. Due to the high cost of the technique, and the knowledge that the required sensitivity for this analysis is not as high as when analyzing minimal residual disease, we used a lower coverage depth than recommended, to adapt the determination to a context that closely resembles daily clinical practice.
Results: A correlation of 0.857 was found between ctNAs and tissue in both rearrangement and point mutation detection. The positive predictive value was 1, and the negative predictive value was 0.833. Only 2 patients from each cohort were false negatives, but alterations were detected in 2 samples with insufficient tissue and in another sample where the tissue tested negative. Moreover, one ctNA sample exhibited low-frequency (1.5%) NRAS alteration while tissue present no mutation. The clinical response time for ctNA analysis, under the cited conditions, is less than 72 hours, which is very close to tissue analysis.
Conclusion: In conclusion, our study highlights the efficacy and utility of LB as a valuable tool for the detection of mutations and translocations in cancer patients. Despite its lower sensitivity compared to minimal residual disease analysis, our approach offers an accurate and quick alternative for the identification of genomic alterations in the absence of tissue, with a real clinical approach.

COLORECTAL CANCER		
ID	TISSUE	LB (NGS)
OO1	G12V	G12V
OO2	WT	WT
OO3	G12R	G12R
OO4	G12V	G12V
OO5	NO	NO
OO6	A146P/T/V	A146T
OO7	G12D	G12D
OO8	G12D	G12D
OO9	WT	WT
O10	G13D	WT
O11	G12D	G12D
O12	WT	WT
O13	G12C	G12C
O14	G12D	G12D
O15	G12V	G12V
O16	G12D	G12D
O17	WT	WT
O18	NO	NO
O19	G12V	G12V
O20	A146P/T/V	WT
O22	WT	WT
O24	WT	WT
O25	WT	NRAS: p.Q61K
O26	BRAF: p.V600E	BRAF: p.V600E
O27	WT	WT

NON SMALL CELL LUNG CANCER		
ID	TISSUE	LB (NGS)
OO1	ALK imbalance	ALK IMBALANCE
OO2	ROS: EZR(10) - ROS1(34)	NOT DETECTED
OO3	ROS CD74(6) - ROS1(34)	ROS CD74(6) - ROS1(34)
OO4	SLC34A2(13) - ROS1(32)	SLC34A2(13) - ROS1(32)
OO5	SLC34A2(13) - ROS1(34)	SLC34A2(13) - ROS1(34)
OO6	EML4(6) - ALK(20)	EML4(6) - ALK(20)
OO7	EML4(6) - ALK(20)	NOT DETECTED
OO8	NO TISSUE (IHQ POS)	EML4(6) - ALK(20)
OO9	EML4(13) - ALK(20)	EML4(13) - ALK(20)
O10	EML4(13) - ALK(20)	EML4(13) - ALK(20)
O11	NO TISSUE (IHQ POS)	EML4(39) - ALK(20)

CONCORDANCE
NEW DETECTION
LACK OF TISSUE
DISCORDANCE

PP.11
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100024
UTILITY OF LIQUID BIOPSY FOR MOLECULAR PROFILING AND MONITORING IN METASTATIC COLORECTAL CANCER: A PILOT STUDY IN A REAL-WORLD COHORT

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Introduction: Liquid biopsy (LB) has emerged as a promising alternative for molecular profiling and monitoring of treatment response and early relapse detection in several tumors. Specifically, in metastatic colorectal cancer (mCRC) is of special interest in monitoring RAS mutational status during antiEGFR treatment. However, its utility out of clinical trials has not been studied deeply. In this pilot study, we aimed to assess the utility of LB in a real-world cohort of patients with mCRC from two hospitals in Cantabria, Spain.
Methods: Between 2021-2023 clinical information and 3 blood samples from 14 patients diagnosed with mCRC were collected: at the time of the diagnosis (sample 1, S1), after 4 (S2) and 8 months (S3) of treatment. Circulating tumor DNA (ctDNA) was analyzed using Idylla ctKRAS Mutation Assay (Biocartis). Additionally, concordance between ctDNA alterations detected in LB and those identified in matched tissue biopsies at the time of the diagnosis were evaluated.
Results: Our results demonstrate a concordance rate of 78,6% between RAS mutational status in LB and tissue samples. It should be noted that of the 3 RASm cases that could not be detected by LB, 2 had lung or peritoneal-limited metastases, but not in the liver, and the third one had a ctDNA concentration above the average ($p<0,005$), which can be explained by a possible germinal contamination. During follow up, RASm were still detected in S2, but they decreased in S3. One resistant mutation was found in the RASwt group.
Conclusion: Despite the small size of this cohort, our study confirm the valuable role of LB as a non-invasive technique in clinical practice both for diagnosis and follow-up of patients with mCRC, being capable of detecting mutational clearance and the presence of resistance mutations, even using a technology with intermediate sensitivity such as RT-PCR.

PP.12
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100025
A COMBINED MDETECT ASSAY FOR ALL SUBTYPES OF BREAST CANCER AND ITS USE IN MONITORING METASTATIC DISEASE

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Background: DNA methylation-based liquid biopsies offer many advantages over mutation-based assays for cancer detection and monitoring due to the high number of tumour specific CpG island methylation events throughout the genome combined with the high frequency of CpGs within these islands. This creates many observable events in each patient. The methylation DETEction of Circulating Tumour DNA (mDETECT) assay uses a targeted Next Generation Sequencing approach to identify methylation signatures in breast and other cancers. The triple negative breast cancer (TNBC) version of mDETECT previously demonstrated 93% sensitivity at 100% specificity for the detection of metastatic breast cancer.
Methods: An initial analysis of The Cancer Genome Atlas (TCGA) breast cancer methylation dataset indicated that the TNBC subtype had a significantly different methylation signature compared to Estrogen Receptor positive and HER2 positive subtypes. Therefore, we developed the TNBC assay separately from the assay for the rest of the breast cancer subtypes together (HR+) which was independently validated. The two tests have now been integrated into one breast cancer assay which detects all subtypes of breast cancer.
Results: Initial validation of the combined breast cancer test has been conducted on control samples, cell lines, and tumour samples. Numerous breast cancer plasma samples have also been collected for analysis. By providing a reliable and quantitative estimate of tumour burden in patients undergoing any type of treatment, this assay offers an ideal tool for the assessment of treatment response. A clinical study of patients undergoing a variety of treatments for metastatic breast cancer is being carried out to validate the use of the combined test. Blood is being drawn with standard-of-care throughout the first six months of treatment. Changes in mDETECT assessed tumour burden levels will be correlated with RECIST v.1.1 standard of care imaging, to determine if the mDETECT breast cancer assay can detect a response to treatment at an earlier timepoint than CT scans.
Conclusions: Measuring tumour burden will enable clinicians to make informed decisions about treatment selection, likely improving outcomes and prolonging patient's lives.

PP.13
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100026
NEXT GENERATION SEQUENCING IN LIQUID BIOPSIES: TOWARDS PERSONALIZED CANCER TREATMENT.

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Introduction: Liquid biopsies have advantages over tissue biopsies as they are representative of tumour status, non-invasive, and allow testing for monitoring treatment alterations. NGS is a valuable tool for personalized diagnosis, enabling accessible precision medicine. The aim is to evaluate the usefulness of liquid biopsies to detect targetable alterations by NGS.
Methods: 87 cancer patients without tissue available from HGUV were included. Total circulating free nucleic acids were isolated using MagMax Cell-Free Total Nucleic Acid Isolation Kit from 79 blood, 7 pleural fluid and 1 ascetic fluid samples. NGS was performed with Ion GeneStudio S5 Series using Oncomine Pan-Cancer Cell-Free Assay covering 52 genes. Variant calling was performed using Ion reporter Software. Statistical analyses were conducted using SPSS 23.0 software.
Results: The study included 87 patients, with 81,6% having lung cancer. Circulating-free nucleic acid isolation was successful in 96.5% of the cases, as only 3 patients had insufficient cfNA concentration for library preparation. Alterations (including CNVs and fusions) were found in 79.8% of patients (67/84), varying from 0 up to 6 alterations. Most patients showed one (44.6%) or two alterations (22.9%). Mutations were detected in 73.5% of patients, with one (44.6%), two (19.3%), three (7.2%) or four mutations (2.4%) each.
The most frequently mutated gene was TP53, found in a 47.6% of the patients (40/84), with 34 different variants. KRAS mutations were observed in 25% of patients, mainly the G12C variant. EGFR gene was mutated in 23.1% of patients, being L858R the most frequent variant. CNVs were found in 14.3% of patients, with 1 and 2 CNVs each. Amplified genes included CCND1, CCND3, CDK6, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, MET and MYC.
Gene fusions were found in only one patient (1.2%), which was in RET gene [KIF5B(15)-RET(12)]. This patient was eligible for a clinical trial with targeted therapy. Notably, druggable alterations were identified in 26 patients (38.8%), highlighting the potential of liquid biopsies as non-invasive tool for implementing targeted therapies in oncology clinical practice.
Conclusion: This study supports the therapeutic value of using non-invasive biopsies as fluid samples to conduct NGS studies that lead to personalized treatments for cancer patients.

PP.14
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100027
MUTATIONAL PROGNOSIS OF SURVIVAL BY LIQUID BIOPSY IN ADVANCED EGFRM NSCLC

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Introduction: The use of NGS in liquid biopsy not only allows molecular diagnoses to be performed in patients with cancer in whom it is not possible to obtain tissue, today this tool can help us determine mutational prognostic factors and predictors of survival and response especially in the management NSCLC with driver mutations.
Methods: Fifty-seven patients with EGFRm advanced NSCLC detected by NGS on liquid biopsy and treated with erlotinib from 2019 to 2021 were evaluated. NGS was performed with FoundationOne Liquid CDx at diagnosis and only pathogenic variants were used for analysis. We evaluated the impact of EGFR molecular

structure and concurrent genetic alterations (co-alt) on progression-free survival and overall survival.

Results: Of the 57 patients evaluated, 93% had a classic structure and 7% a PACC structure of the EGFR. Regarding concurrent genetic alterations, 86% presented at least one concurrent genetic alteration with a median of 2 (0 - 7 co-alt), of these, the most frequent were TP53 (59.6%), EGFR co-alt (35.1%), DNMT3A (17.5%) and CTNNB1 (12.3%).

The median PFS was 12.8 months, we found differences according to the classic structure vs. PACC (12,8m vs 5.9m, p=0.013), the number of concurrent alterations, at least 1 vs ≥2 (17.1m vs 9.3m, p=0.001) and at least 2 vs ≥3 (15.5m vs 7.5m, p =0.001) and the presence of CTNNB1 (5.6m vs 13.0m, p=0.003).

The median OS was 19.5 months, we found differences according to the classic structure vs. PACC (19.6m vs 4.1m, p=0.038), the number of concurrent alterations, 0 vs ≥1 (NR vs 16.7m, p=0.008), at least 1 vs ≥2 (29.3m vs 14.7m, p= 0.001) and at least 2 vs ≥3 (22.6m vs 15.3m, p=0.011) and in the presence of TP53 (15.8m vs 31.7m, p=0.002).

Conclusion: The use of NGS in liquid biopsy allows us to identify the structure and concurrent genetic alterations of the EGFR mutation. These findings may have clinical utility as predictors of response and prognosis of survival, with which we could individualize each patient and design treatment intensification strategies to obtain better results.

PP.15
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100028
NEGATIVE PREDICTIVE VALUE OF PRE-DIAGNOSTIC PLASMA CTDNA TESTING IN PATIENTS WITH SUSPECTED LUNG CANCER

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Introduction: In the ACCELERATE study, the use of plasma circulating tumor (ct)DNA testing before tissue diagnosis in a cohort of 150 patients with suspected advanced lung cancer accelerated time to treatment in those with and without actionable alterations.

Methods: We analyzed the negative predictive value of plasma ctDNA testing (InVision First Lung, Inivata) in patients with advanced non-squamous NSCLC in the ACCELERATE cohort.

Results: Of 90 patients with advanced non-squamous NSCLC that underwent plasma testing, 46 (51%) had Tier1 actionable drivers identified in plasma, 30 patients (33%) had non-actionable alterations, and 14 (16%) had no detectable ctDNA. Non-actionable alterations included KRAS non-G12C (n=9), STK11 (n=4), PIK3CA, PTEN mutations (1 each), ERBB2, MET or FGFR amplification (1 each) and TP53 mutations (12 isolated, 11 co-altered with other non-actionable alterations). Only one patient with a non-actionable mutation in plasma (TP53) had a driver alteration identified in tissue (ALK fusion). Of 14 patients with undetectable cfDNA in plasma, 6 had actionable alterations found in tissue (4 EGFR activating mutations, 2 ALK fusions). Excluding cases with undetectable ctDNA, the negative predictive value of plasma testing was 96.7% (29/30).

Conclusion: Plasma ctDNA testing before pathological diagnosis in patients with suspected advanced lung cancer can inform treatment decisions even in patients with non-actionable drivers in plasma. However, sensitivity and specificity of plasma assays for complex variants including fusions need to be further developed.

PP.16
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100029
CIRCULATING TUMOR DNA (CTDNA) DYNAMICS AND CLINICAL OUTCOME IN METASTATIC COLORECTAL CANCER (MCRC) PATIENTS (PTS) UNDERGOING FRONT-LINE CHEMOTHERAPY

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Introduction: The potential of monitoring circulating tumor DNA (ctDNA) dynamics to guide clinical decisions in metastatic colorectal cancer (mCRC) patients (pts) treated with I and II line systemic anti-cancer therapy (SACT) has not been widely tested.

Methods: 862 serial plasmas were collected 4-weekly from baseline (BL) until disease progression in 75 mCRC pts undergoing SACT. ctDNA was tested using a custom (RMH GI; 20 genes) or a commercial (Roche Avenio; 77 genes) ctDNA next generation sequencing (NGS) panel. White blood cells were sequenced to rule out clonal hematopoiesis. Whole exome sequencing (WES) was performed on tissue biopsies. ctDNA normalization was defined as ≥99% clearance after 1 month of therapy (Mo1) in the 3 variants with the highest allele frequency in BL ctDNA.

Results: 83 paired samples from 75 pts were available for analysis (for 8 pts, I and II line bloods were available). 12 pairs (14.4%) showed no variants in either BL or Mo1. In the remaining 71 comparisons (65 pts), 37 (52.1%) showed ctDNA normalization at Mo1. Among normalized pts there was a higher proportion of cases with a baseline ECOG performance status (PS) 0-1 (97.3% vs 82.4%, p = 0.0362) in comparison to non-normalized pts, whilst no other clinic-pathologic characteristics, including age, sex, prior primary tumor resection, sidedness, RAS/RAF genotype, type of regimen and number of metastatic sites were significantly associated with ctDNA dynamics. Pts with normalized ctDNA had significantly longer overall survival (OS), 45.6 months (95% confidence interval [CI]: 30.0 - not reached, 14 events) and progression-free survival (PFS), 13.9 months (95%CI: 11.2-18.3; 30 events) compared to non-normalized pts [OS = 22.6 months (95%CI: 16.6-31.2, 24 events) (Log-rank p = 0.01) and PFS = 10.7 months (95%CI: 7.53-13.8; 32 events) (Log-rank p = 0.036) respectively]. In addition, pts with normalized ctDNA had higher overall response rate (ORR) of 72.9% (27/37 responses; 95%CI: 53.0-84.1) compared to 38.2% (13/34 responses; 95%CI: 22.1 – 56.4) in non-normalized pts. In a multivariate model, ctDNA normalization was confirmed as an independent predictor of decreased risk of death (hazard ratio [HR] 0.47, 95%CI: 0.23-0.96; p = 0.04) and higher probability of achieving an objective response from front-line treatment (odds ratio [OR] 3.03, 95%CI: 1.08-8.49; p = 0.0351). Only 23/50 (46%) of variants detected in ctDNA were detected by WES in paired tissues in 12 pts for whom liquid/solid biopsy was available.

Conclusions: ctDNA monitoring represents an early indicator of benefit from systemic therapy in mCRC pts. A significant fraction of variants detected in ctDNA was not detected in paired tissues.

PP.17
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100030
GENE EXPRESSION SIGNATURES IN CONJUNCTIVAL FORNIX ASPIRATES OF PATIENTS WITH DRY EYE DISEASE ASSOCIATED WITH MEIBOMIAN GLAND DYSFUNCTION

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Background: Meibomian gland dysfunction (MGD) is one of the most common conditions in ophthalmic practice and the most frequent cause of evaporative dry eye disease (DED). However, the immune mechanisms leading to this pathology are not fully understood and the diagnostic tests available are limited. Here, we used the nCounter technology to analyze immune gene expression and develop diagnostic signatures in DED-MGD.

Methods: RNA was purified from 49 conjunctival cell samples of patients with MGD-DE (n=27) and asymptomatic controls (n=22) and converted to cDNA. A 14-cycle pre-amplication step was performed before using the Gene Expression Human Immune V2 panel (NanoString) which includes 579 target and 15 housekeeping genes. A machine learning (ML) algorithm was applied to design a signature associated with DED-MGD.

Results: Forty-five immune genes were found upregulated in DED-MGD vs. controls, involved in eight signaling pathways, IFN I/II, MHC class I/II, immunometabolism, B cell receptor, T Cell receptor, and T helper-17 (Th-17) differentiation. Additionally, statistically significant correlations were found between 31 genes and clinical characteristics of the disease such as lid margin or tear osmolarity (Pearsoń s r <0.05). ML analysis using a recursive feature elimination (RFE) algorithm selected a 4-gene mRNA signature that discriminated DED-MGD from control samples with an area under the ROC curve (AUC ROC) of 0.86 and an accuracy of 77.5%.

Conclusions: We have demonstrated that multiplexed mRNA analysis of tears may be an effective method to genetically characterize patients with MGD-DE. We have also been able for the first time to develop a genetic signature with 4 genes, namely interferon induced transmembrane protein 1 (IFITM1), major histocompatibility complex, class I, A (HLA-A), Cathepsin S (CTSS) and C-C Motif Chemokine Ligand 4 (CCL4), which analysis can aid MGD-DE diagnosis.

PP.18
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100031
IMPACT OF REFERENCE MATERIALS FOR ANALYTICAL PERFORMANCE EVALUATION OF LIQUID BIOPSY NGS ASSAYS

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Introduction: Liquid biopsy (LB) enables the non-invasive analysis of genetic tumor variants in circulating free DNA (cfDNA) in plasma to support personalized treatment. The majority of cancer patients present with actionable tumor variants with variant allele frequencies (VAFs) <1%. Therefore, accurate analytical validation of LB assays at the lower end of the measurement scale is mandatory, especially regarding the In Vitro Diagnostic Regulation (IVDR). Selection of appropriate reference materials is the first and key prerequisite for this purpose. We tested three commercially available reference materials for their suitability to be used for LB NGS assay validation. First, background noise was determined, which is critical for accurate assessment of specificity and sensitivity. Second, the similarity of the reference materials to native cfDNA was evaluated.
Methods: NA24385-based wild-type (WT) cfDNA reference materials from SensID, Coriell, and SeraCare and 19 patient samples were analyzed using Duplex Sequencing-based LB NGS assay.
Results: To investigate the background noise of the three different WT reference materials, which are important for the evaluation of specificity, the number of variants <0.1% VAF was determined and compared to patient samples. In both SensID and Coriell materials, <0.1 variants/kb were detected, whereas in SeraCare materials, 16.9 variants/kb were detected. For comparison, <0.1 variants/kb were detected in the 15 patient samples. Accordingly, both the SensID and Coriell materials showed very low background noise at low VAFs, which are in the same range as the signals observed in patient samples and are therefore highly suitable for specificity determination.
To test the closeness of agreement with native cfDNA, DNA profiles and library yields of reference materials were compared to patient samples. The SeraCare cfDNA reference material more closely resembled native cfDNA than SensID and Coriell materials, allowing for more accurate development of experimental protocols.
Conclusion: In summary, careful consideration of commercially available reference materials is required for performance evaluation of LB NGS assays. While reference materials with well-defined variants are preferable for determining specificity, reference materials that closely resemble native cfDNA aid in the development of experimental protocols.

PP.20
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100032
HIGH SENSITIVITY DETECTION OF SPECIFIC ULTRA LOW-FREQUENCY SOMATIC MUTATIONS FOR MINIMAL RESIDUAL DISEASE (MRD) MONITORING

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Introduction: Minimal residual disease (MRD) monitoring tracks the abundance of any malignant cells remaining in the body after therapeutic intervention. Currently, circulating tumor DNA (ctDNA) is a promising biomarker for MRD diagnosis. Due to low abundance of ctDNA and unique somatic variants developed from each individual, MRD diagnosis requires personalized NGS assays with high sensitivity and specificity.
Methods: To address this need and empower accurate assessments of MRD, Twist Bioscience has developed the MRD Rapid 500 Panels. This product enables customers to design, manufacture and ship fully personalized MRD panels (up to 500 targets) in as little as six days. To demonstrate the detection sensitivity of Twist Rapid 500 MRD panels, we designed five custom MRD panels which specifically target somatic variants found in Breast, Lung, Colorectal Cancer, Melanoma and Renal Cell Carcinoma. Each of these MRD panels were designed to include 197 targets, with 3-5 variants per tissue origin and a selection of passenger mutations. Probe sequences of each panel were designed to incorporate the variant allele in the test sample set. To create the sample set, we blended synthetic variant sequences with fragmented cell line gDNA (NA12878) to form a contrived specimen which approximates the profile of cell-free and circulating tumor DNA. Five frequency levels were created with average variant allele frequencies (VAFs) of 0% (WT), 0.01%, 0.05%, 0.1% and 2%. Libraries were prepared with UMI adapters and target enrichment was performed using the MRD panels. They were sequenced on both Illumina NextSeq and PacBio Onso platforms.
Results: With a sequencing depth of 80,000x, variant calling results revealed that an average of 20 SNV targets can be detected with confidence in the 0.01% VAF samples for each MRD panel, clearly distinguishable from the WT control samples. In addition to demonstrating the accuracy of variant calling by targeting the alternate allele, we showcase the utility of targeting a large number of variants for the detection of an MRD signature at very low levels (e.g. 0.01% VAF).
Conclusion: In summary, the performance of the Twist MRD Rapid 500 Panels showed high detection sensitivity of ultra low-frequency somatic mutations.

PP.21
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100033
GLIOMA PATIENT MANAGEMENT: UTILIZING MGMT METHYLATION IN EXTRACELLULAR VESICLE-BASED LIQUID BIOPSY

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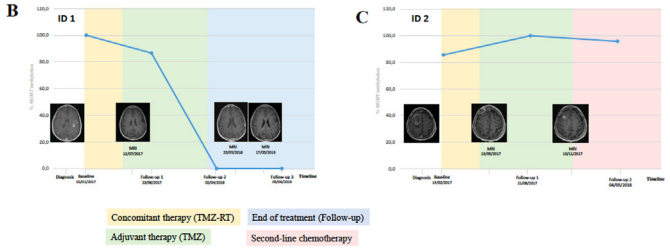
Introduction: Glioblastoma (GB) is a highly aggressive central nervous system tumor with a poor prognosis. O6-methylguanine-DNA methyltransferase (MGMT) methylation (mMGMT) is the only well-established predictive and prognostic biomarker in GB patients, linked to improved treatment response and survival. However, current methods for monitoring mMGMT status have been unsuccessful. Small extracellular vesicles (sEVs) offer a potential breakthrough as they could revolutionize clinical diagnosis through liquid biopsy.
Methods: In this prospective exploratory study, we assessed the sensitivity and specificity of sEV-based liquid biopsy for determining mMGMT status compared to tissue samples. Additionally, we explored the association between sEV-DNA methylation and overall survival and its utility in patient follow-up.
Results: For the first time in liquid biopsy, we successfully evaluated MGMT promoter methylation (mMGMT) in sEV-DNA, obtaining concordant results with tissue analysis. Our liquid biopsy approach achieved an impressive sensitivity of 85.7% in detecting mMGMT, the highest reported to date. Furthermore, we demonstrated the power of sEV-DNA liquid biopsy for disease monitoring and detecting disease progression in glioblastoma patients, providing critical prognostic insights.
Conclusion: Accurate detection of molecular markers is crucial to avoid missed treatment opportunities and misdiagnoses leading to ineffective therapies. The findings from this study present a significant advancement in sEV-DNA-based liquid biopsy, highlighting the heterogeneity of tumor tissue and its potential as a promising tool for identifying nervous system biomarkers, particularly in gliomas.
This research was funded by PI18/050; PI21/0145, Cofinanciado por Unión Europea EU; DTS20/029; JR21/03; FI19/061; CA22/00002; MICINN (PLEC2021-08034), GHETTI2021P.

A

Patient ID	*MGMT* tumour methylation	Extent of initial resection	*MGMT* sEV-DNA methylation (Baseline)	*MGMT* sEV-DNA Methylation (Follow-up 1)	*MGMT* sEV-DNA methylation (Follow-up 2)	MGMT sEV-DNA methylation (Follow-up 3)
1	M (78.0%)	Biopsy	M (100.0%)	M (86.7%)	U (0.0%)	U (0.0%)
2	M (81.6%)	Complete resection	M (85.6%)	M (100.0%)	M (95.8%)	n.a.
12	U (0.0%)	Complete resection	U (0.0%)	U (0.0%)	U (0.0%)	n.a.
28	M (87.7%)	Subtotal resection	M (100.0%)	M (97.4%)	n.a.	n.a.
32	M (47.2%)	Subtotal resection	M (81.6%)	M (99.5%)	n.a.	n.a.
34	M (79.4%)	Complete resection	M (38.1%)	U (0.0%)	n.a.	n.a.
40	U (0.0%)	Complete resection	M (100.0%)	U (0.0%)	n.a.	n.a.
41	U (0.0%)	Subtotal resection	U (0.0%)	U (0.0%)	n.a.	n.a.

M: *MGMT* methylated, U: *MGMT* unmethylated, n.a.: Not available



PP.22

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100034

DETECTION OF ACTIVATING MUTATIONS IN LIQUID BIOPSY OF EGYPTIAN SOLID TUMOR PATIENTS USING TARGETED NEXT GENERATION SEQUENCING: A PILOT STUDY

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Introduction: Cancer is a genetic disorder caused by accumulation of molecular alterations guiding the acquisition of the malignant phenotype. The number of newly described cancer-associated genes has grown exponentially since the emergence of next generation sequencing (NGS) technology. Breast cancer (BC) is the most common cancer among Egyptian females. Glioblastoma multiforme (GBM) is the most common aggressive primary brain tumor.

Method: We aimed to identify activating mutations in liquid biopsy of 8 female BC patients & 7 Egyptian GBM using targeted NGS technology (MiSeqDx) as well as the microsatellite instability (MSI) status was tested using BAT25, BAT26 and NR27 markers on the Agilent Bioanalyzer.

Results: Among BC patients, 3/8 (37.5%) were TNBC, 4/8 (50%) patients were luminal B and only one patient was luminal A. TP53 p. (Pro72Arg) missense mutations were the most common mutation found in 75% of patients. PIK3CA p.(Ile391Met), VEGFR2/KDR p.(Gln472His) missense mutations were detected in 25% of patients each. Two patients revealed APC gene missense mutation with p.(Ile1307Lys) & p.(Glu1317Gln) variants. Only one patient showed ATM p.(Phe858Leu) gene mutation and one showed FGFR3 p.(Ala719Thr) variant. Regarding microsatellite instability (MSI) status, 2/8 (25%) patients were MSS, 3/8 (37.5%) patients were MSI-LO and 3/8 (37.5%) patients were MSI-HI.

Six out of 7 (85.7%) GBM patients were grade IV and only one were grade II with 4 substitution – missense mutations were detected: drug responsive TP53 (p.Pro72Arg) variant was detected in 6 patients (85.7%). KDR (p.Gln472His) variant was noted in 4 patients (57.1%) as a result of substitution at c.1416A>T. Two patients revealed KIT (p.Met541Leu) variant which results from substitution at c.1621A>C. Only one patient showed mutation in JAK3 gene which was (p.Val718Leu) variant resulting from c.2152G>C substitution. Regarding MSI status, four cases (57.1%) were MSI-LO & three cases (42.9%) were MSI-HI.

Conclusion: It is essential to use & validate minimally invasive liquid biopsy samples for driver mutations detection by NGS especially in patients with inoperable disease or bone metastasis.

This work should be extended in larger patient series with comparison of genetic mutations in liquid-based versus tissue-based biopsy and longer follow-up periods for better personalized oncology practice.

PP.23

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100035

MOLECULAR INTELLIGENCE TARGETING DRIVER ACTIVATING MUTATIONS IN CORRELATION WITH GUT MICROBIOME IN EGYPTIAN COLORECTAL CANCER

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Introduction: Assessment of genetic mutations in CRC patients is an essential element in the era of personalized cancer treatment. Also, the gut microbiome identification has expanded revealing new links established between microorganism & CRC development.

Methods: We aimed at evaluating the incidence of activating genetic mutations together with the gut microbiome in correlation with different clinicopathological factors among a group of Egyptian CRC patients. Targeted next-generation sequencing is used for detection of activating genetic mutations in a panel of 50 genes using liquid biopsies together with testing microsatellite instability in those patients. Also, identification of gut microbiota using conventional microbiological and parasitological techniques were used.

Results: Twenty four Egyptian CRC patients were enrolled with 1/4 of our patients were metastatic. Missense mutations were detected in 7 genes as follows: TP53, PIK3CA, KDR, KIT, APC, FGFR3 & MET. Twelve patients (50%) had MSI-low, 6 patients (25%) had MSI-high and 5 patients (20.8%) had MSI stable. PIK3CA, TP53, KIT & KDR missense genetic mutations were found in 6, 11, 3 & 2 MSI-Low patients, respectively and in 2, 3, 0 & 2 MSI-High patients, respectively. Ten out of 16 examined patients revealed mixed GIT flora, 3 patients had E. Coli, 2 patients showed Klebsiella spp. and only 1 patient revealed Proteus spp. Fecal occult blood was detected in 62.5% of patients and H. pylori antigen was found in only 37.5% patients. Blastocystis hominis cysts were detected in only 36.4% of examined patients.

Conclusion: We aimed at understanding the disease genetic mutational status as well as contributing environmental stress factors such as gut microbiome dysbiosis and their interplay with patient genetic variations. NGS is a laboratory cornerstone of precision oncology treatment with liquid biopsy could be used as a noninvasive tool overcoming the challenges posed by pathology samples.

PP.24

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100036

CIRCULATING TUMOR CELLS AS A NON-INVASIVE GLIOBLASTOMA DIAGNOSTIC AND PROGNOSTIC TOOL

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Introduction: Circulating tumor cells (CTCs) detection in the blood of glioblastoma (GBM) patients can facilitate the assessment of tumoral information without the need for invasive approaches. Our aim is to use GBM CTCs to obtain knowledge about the tumor and improve the current GBM diagnosis and prognosis.

Methods: We collected primary, matched recurrent GBM and healthy controls blood samples. CTCs were enriched from whole blood controls with Parsortix Cell Separation System and analysed on DEPArray system. CTCs were recognized by immunofluorescence with GFAP.

The CTCs have been counted for all the samples. Copy Number Aberrations (CNAs) analysis was performed on CTCs. To compare CTCs genetic background with the same patient's primary and recurrence tissues, whole exome sequencing (WES) was realized on tumor tissues.

Recurrence of GBM was diagnosed according to the RANO criteria. We assessed tumor volume of contrast enhanced (CE) at the time of the CTCs collection.

Results: We found CTCs in GBM patients newly diagnosed at the first surgery (86%) but also at recurrence (100 %). Cell counting demonstrated in GBM patients' blood a statistically significantly higher number of CTCs compared to healthy controls (average of cells number 15.8 vs 2.3). Interestingly, recurrent GBM patients showed more CTCs than primary GBM patients (average 9 vs 22.7). CNAs analysis confirmed the tumor status of the selected CTCs. Some of GBM classical mutations (in HTERT promoter, BRAF, PI3KCA and EGFR genes) have been searched both in the tumor tissues and in the CTCs. HTERT was found mutated in CTCs of one patient's tumor carrying mutant HTERT and wild type IDH1/2. We also included three IDH1 mutant glioma patients and one BRAFV600E mutant GBM patient in order to track these in CTCs.
Conclusion: We found CTCs in GBM patients newly diagnosed and at recurrence. Several factors may affect CTCs release such as tumor volume, CE and the CTCs genetic background, which seems to be heterogeneous. CTCs detection by our methodology could be helpful in the clinical setting for diagnostic, prognostic and theranostic purposes and for patients monitoring. Prospective study is ongoing in order to assess CTCs variations on and after therapy.

PP.25
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100037
DETECTION OF CIRCULATING TUMOR CELLS IN PERIPHERAL BLOOD OF PEDIATRIC OSTEOSARCOMA PATIENTS

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Introduction: Osteosarcoma (OS) is the most common primary bone tumor in children, adolescents, and young adults. Treatment for high-grade OS involves neoadjuvant chemotherapy, surgical tumor removal, and postoperative chemotherapy. Detecting circulating tumor cells (CTCs) in patients' blood offers a non-invasive method for diagnosis, monitoring treatment response, and assessing disease progression. Our aim is to investigate CTCs in OS patients before and after neoadjuvant chemotherapy.
Methods: We enrolled seven patients aged 5-18 years diagnosed with high-grade OS. Blood samples were collected at diagnosis, along with a biopsy tissue (PRE-BIO), and during surgery. CTCs were enriched using the Parsortix tool, and immunofluorescence staining was performed using epithelial (E-CTCs) and mesenchymal (M-CTCs) markers. The CTCs were then recovered using the DEPArray System. Chromosomal copy number alterations (CNA) analysis was conducted on the CTCs. In one patient, M-CTCs and E-CTCs were tested for the presence of a somatic FLT4 gene mutation observed in the parental tumor. Formalin-fixed paraffin-embedded metastatic tissue was available for this patient and screened for the FLT4 mutation.
Results: Mean M-CTCs/ml and E-CTCs/ml in pre- and post-chemotherapy blood samples were 93 and 17, and 1 and 2, respectively. Percentages of CTC types varied among patients and before and after therapy. The GR patient showed the most significant decrease in M-CTC numbers, going from 567/ml to 0/ml after chemotherapy. CNA was observed only in CTCs, not in CD45+ leukocytes. The somatic FLT4 mutation was found as homozygote in M-CTCs and as heterozygote in E-CTCs, PRE-BIO sample, and metastatic tissue.
Conclusion: Conclusion: Both mesenchymal (M-CTCs) and epithelial (E-CTCs) circulating tumor cells were detected in blood samples from patients with osteosarcoma (OS). The confirmation of the tumoral origin of all CTCs, along with the presence of the same mutation in CTCs, biopsy tissue, and metastatic tissue, is particularly noteworthy. These findings provide compelling evidence supporting the utilization of CTC investigation in the clinical management of OS patients. However, further clinical data are required to gain a comprehensive understanding of the role played by these different types of CTCs in OS.

PP.26
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100038
CELL-FREE (CFDNA) BASED EVALUATION OF KRAS MUTATION PROFILE IN SOLID TUMORS WITH FOCUS ON G12C VARIANT

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Introduction: We report the incidence of KRAS mutations from molecular diagnostics, liquid and tissue biopsies across multiple solid tumors, as well as feasibility of using a liquid first approach, with focus on KRAS G12C.
Methods: We retrospectively reviewed treatment-naive and previously treated solid tumors of all stages that underwent next-generation sequencing (NGS) at Datar Cancer Genetics with multi gene panels, including KRAS.
4,463 cfDNA samples (>17,000X coverage) and 2,587 tumor tissue DNA (ttDNA) samples (>1000X coverage) were analysed, of which 554 had both tissue and liquid analysis. In a subset of samples (n=55), genomic DNA (gDNA) was isolated from white blood cells (WBCs) and evaluated by NGS to rule out clonal hematopoiesis of indeterminate potential (CHIP) as the source of KRAS mutation.
Results: KRAS variants were detected in 10% (n=449) of all cfDNA samples and 13.6% (n = 351) of all ttDNA samples. KRAS-G12C accounted for ~10% of all KRAS mutated cfDNA samples and ~11% of all KRAS mutated ttDNA samples. Evaluation of cfDNA showed 69% concordance for detection of KRAS variants and 99% concordance for detection of KRAS wild type, in comparison to ttDNA based determinations. In 5 samples where KRAS variants were detected in cfDNA but not ttDNA, one was a case of rectal adenocarcinoma post Panitumumab (Vectibix®) progression and another was a case of lung adenocarcinoma post Osimertinib (Tagrisso®) treatment.
Conclusion: KRAS variations are detected using molecular testing in ~13% of all solid tumors. Liquid biopsy assessments are a safe and potentially more efficient diagnostic approach to ascertain the status of KRAS in solid tumors particularly when tumor tissue assessments may not be feasible.

Table 1 Comparison of cfDNA and tissue based analysis of KRAS mutation subtypes across KRAS mutant solid tumors.

Sample Type	p.G12C	p.G12A	p.G12D	p.G12S	p.G12V	p.G13D	p.G13S	p.Q61H
cfDNA (All tumors)	10%	5%	35%	2%	21%	8%	0%	4%
Tissue (All Tumors)	11%	6%	37%	2%	19%	8%	0%	3%
cfDNA - Lung	20%	2%	32%	1%	19%	8%	1%	2%
Tissue - Lung	39%	3%	22%	0%	15%	3%	0%	4%
cfDNA - Colon	11%	4%	28%	2%	22%	15%	0%	5%
Tissue - Colon	7%	4%	39%	3%	18%	19%	0%	1%
cfDNA - Pancreas	3%	4%	53%	0%	26%	0%	0%	5%
Tissue - Pancreas	1%	2%	54%	0%	23%	4%	0%	4%
cfDNA - Ovary	0%	43%	0%	0%	43%	0%	0%	0%
Tissue - Ovary	0%	20%	13%	13%	40%	0%	0 %	0%

PP.27
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100039
BLOOD-BASED CIRCULATING TUMOR CELLS AND CELL-FREE DNA MONITORING FOR DYNAMIC RECURRENCE RISK PREDICTION IN MELANOMA

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Introduction: Detecting cancer recurrence through surveillance plays a crucial role in the ongoing care of cancer survivors. After undergoing curative-intent surgery for patients diagnosed with stage II or III melanoma, the detection of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) in the bloodstream could be a strong predictor of early disease recurrence and its outcome. This study aimed to explore the prognostic value of CTCs and cfDNA as a real-time indicator for early recurrence and to assess the effectiveness of the surgical procedure.
Methods: Peripheral blood samples were taken from 50 patients with melanoma in stage II/III (Stage II: 24, Stage III: 26) for the analysis of CTCs, cfDNA levels, and ctDNA mutation status. Samples were obtained at the initial assessment (T0) and subsequent follow-up visits (T1, T2, T3) during routine clinical examinations. CTC analysis was conducted using the CellSearch® Circulating Melanoma Cell assay. Additionally, cfDNA was extracted and quantified, and a mutation panel was used to evaluate the ctDNA mutation status.
Results: At pre-surgery, 41.7%, 54.0%, and 50.0% of the patients tested positive for CTC, cfDNA level, and ctDNA mutations, respectively. The Mann-Whitney test showed a significant association of all three analytes with disease recurrence (CTC: P=0.0130; cfDNA level: P= 0.0472; ctDNA mutations: P= 0.0156). Among

the patients who tested positive for CTCs, 70% (N=14/20) had clinical recurrence, whereas the CTC negative group only had a 21.4% (N=6/8) recurrence rate. Furthermore, the group with ctDNA mutations showed a significantly higher recurrence rate of 60.9% (N=14/23) in contrast to the ctDNA mutation negative group, which had a recurrence rate of 21.7% (N=5/23). Overall, there was also a notable distinction in post-surgery CTC levels between the groups that experienced recurrence and those that did not (P=0.0092).

Conclusion: Pre- and post-surgery measurements of circulating biomarkers (CTCs, cfDNA levels, and ctDNA mutation status) are associated with melanoma recurrence risk and they have potential for surveillance monitoring. Incorporation of liquid biopsy-based multi-analytes with other tumor molecular features may improve prediction of recurrence.

PP.28
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100040
EVALUATION OF PARSORTIX PR1 AND PX+ SYSTEMS FOR THE ISOLATION OF CIRCULATING TUMOUR CELLS IN PATIENTS WITH RESECTABLE LUNG CANCER

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Introduction: Recurrence rates for non-small cell lung cancer (NSCLC) even after curative-intent surgery, remain high at 24% in stage I NSCLC and 50% overall. Circulating tumour cells (CTCs) are emerging as a promising prognostic biomarker in lung cancer (LC) and CTC enumeration may be used to identify patients who are at a high risk of disease recurrence. The Parsortix system is a microfluidic label-independent technology that isolates CTCs (epithelial and/or mesenchymal) based on their size and resistance to compression. In this study, the Parsortix PR1 and a new beta test version, the Parsortix Plus (PX+) were evaluated.

Methods: The recovery rate of both technologies were assessed using spike-in experiments. Healthy donor blood was spiked with H1975 cells, a NSCLC adenocarcinoma cell line and processed through PR1 and PX+ in parallel. Furthermore, peripheral blood samples were prospectively collected from 20 early-stage LC patients at surgery and on subsequent follow-ups (3 days and 6 weeks after surgery). 10 patient blood samples were processed through the PR1 and PX plus systems respectively for CTC isolation. Captured CTCs were identified by immunofluorescent in-cassette staining (using the PR1) using a combination of epithelial (pan cytokeratin, EpCAM) and mesenchymal markers (vimentin).

Results: There was no statistical difference in the recovery rate between PR1 and PX+ systems from the spike in experiments. This was an expected result as both systems use the same method of CTC capture. A trend was also observed between patients' CTC counts and their clinical-pathological data such as tumour size and the type of surgical procedure.

Conclusion: Our results show that the PR1 and PX+ systems can effectively capture CTCs from patients with resectable LC. The isolated CTCs displayed heterogeneity in EMT (epithelial-to-mesenchymal transition) status, and they also correlated with the clinicopathological characteristics of the patients. The PX+ has a faster CTC isolation time and requires less manual handling than the PR1. However, the PR1 system facilitates fixing and in-cassette staining of captured CTCs while the PX+ isolates and harvests CTCs for downstream processing such as staining, sequencing or culturing of patient CTCs.

Keywords: Circulating tumour cells, non-small cell lung cancer, epithelial-to-mesenchymal transition, Parsortix PR1 and Parsortix Plus

PP.29
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100041
HIGH LEVEL OF CIRCULATING TUMOUR DNA AT DIAGNOSIS CORRELATES WITH DISEASE SPREADING AND DEFINES MULTIPLE MYELOMA PATIENTS WITH POOR PROGNOSIS

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Introduction: Multiple Myeloma (MM) is a plasma cell (PC) disorder characterized by the presence of skeletal involvement at the time of diagnosis. Recently, cell-free DNA (cfDNA) has been proven to resume the heterogeneity of spatially distributed clones.

Aims: to evaluate cfDNA at diagnosis and during follow-up in correlation with imaging data to possibly define the integration of this approach with molecular bone marrow (BM) and whole-body residual disease assessment.

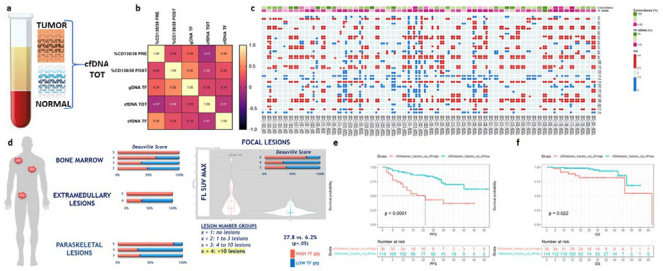
Methods: A total of 162 newly diagnosed MM patients were screened at baseline with 18F-FDG PET/CT, and molecularly assessed by Ultra Low Pass-Whole Genome Sequencing (ULP-WGS). For each pts, ULP-WGS was used to characterize both the neoplastic PC clone(s) in the BM (gDNA) and the cfDNA.

Results: Genomic profile comparison between cfDNA and BM revealed an overall concordance (56/62 = 90.3%) strictly dependent from the cfDNA tumor fraction (TF). Interestingly, high cfDNA TF levels (ROC-determined cut-off: > 12% cfDNA TF values; range: 4.4-84.3%) correlates with different MM tumor mass markers, particularly for b2-microglobulin (p = <0.001), albumin (p = 0.006) and total bone marrow plasma cells (p = <0.001) (Fig1a-b-c).

Strikingly, patients with high cfDNA TF were more likely to present with paraskeletal (PS) and extramedullary (EM) lesions as compared to pts with low TF (PS lesions: 13/27 vs. 13/69, p = 0.005; PS Deauville score (DS) ≥ 4: 11/27 vs 10/69, p = 0.003; PS SUV MAX: p = 0.007. EM lesions: 4/27 vs. 3/69, p = 0.09; EM lesions DS ≥ 4: 4/27 vs. 3/69, p = 0.067; EM SUV MAX: p= 0.008) (Fig. 1d).

Finally, high cfDNA TF at diagnosis predicted for poorer prognosis as compared with low cfDNA TF (PFS at 20 months: 67% vs. 86%, p=0.05; OS at 20 months: 90% vs. 100%, p=0.04) (Fig. 1e-f).

Acknowledgements: AIRC IG2019, AIL BOLOGNA ODV, Banca di Bologna



PP.30
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100042
LIQUID BIOPSY ANALYSIS SHOWS MAGI2 GENE AS POTENTIAL NEGATIVE PROGNOSIS BIOMARKER IN NSCLC PATIENTS

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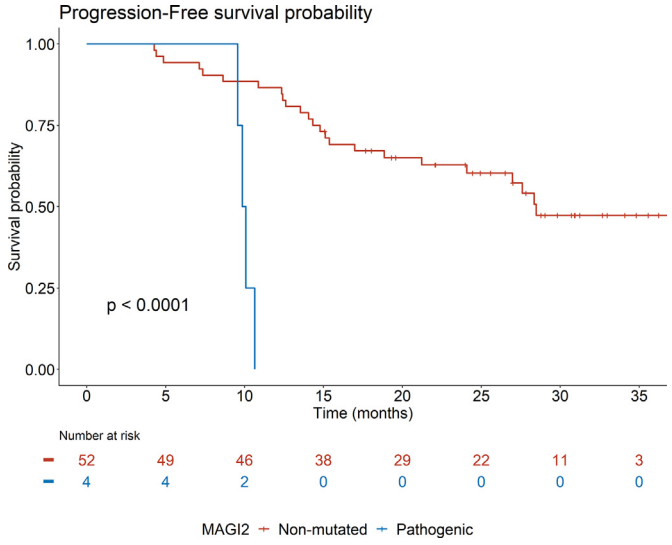
Introduction: Neoadjuvant chemoimmunotherapy is the new standard of treatment for locally-advanced potentially resectable NSCLC patients. However, molecular landscape from patients included in this type of clinical trials has not been well studied. The aim of this analysis is to obtain a baseline molecular profiling from patients of NADIM II clinical trial using liquid biopsies samples analyzed by Next Generation Sequencing.

Methods: A total of 56 peripheral blood samples from stage IIIA-B NSCLC patients were collected before neoadjuvant treatment. 38 belonged to experimental arm and 17 to control arm. Cell-free DNA (cfDNA) from plasma samples was isolated using QIAamp-Circulating-Nucleic-Acid Kit and analyzed using the hybrid capture-based TruSight-Oncology-500 ctDNA. Variant filtering was performed using a pipeline developed in our laboratory. Clinical significance of the detected variants was assessed in different databases (ClinVar;Varsome). Finally, we carried out a statistical analysis in order to identify genes associated with poor prognosis. P-values were adjusted by false discovery rate (FDR).

Results: We identified four pathologic variants in MAGI2 in four different plasma samples; two from experimental arm and other two from control arm. MAGI2 is a membrane-associated-guanylate-kinase which recently has been directly related to PTEN signaling pathway. Three of the variants were single

nucleotide variation and one deletion (c.1457A>G,p.H486R; c.3680G>C,p.R1227T; c.1538C>G,p.P513R; c.74delA,p.E25Gfs*8). All the patients who had tumors with those alterations had an incomplete pathological response after neoadjuvant treatment, and half of them were diagnosed with squamous histology. The survival analysis showed that patients with tumors harboring pathogenic alterations in MAGI2 might had higher risk of disease progression and death (HR = 9.90, 2.74-35.8; P< 0.001, FDR p-value=0,0722 and HR = 6.99, 1.89-25.92; P = 0.00363, FDR p-value=0,5518).

Conclusions: Molecular profiling of liquid biopsies suggests that pathogenic mutations in MAGI2 gene might be a potential bad prognostic factor for patients with potentially resectable stage IIIA-B NSCLC.



PP.31
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100043
LIQUID BIOPSY MONITORING IN BRAF-V600E MUTATED NSCLC PATIENTS TREATED WITH DABRAFENIB PLUS TRAMETINIB: A PROSPECTIVE, EXPLORATIVE, MULTICENTRIC STUDY. LIBRA_STUDY (GOIRC-03-2020)

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Background: Dabrafenib plus trametinib represent the standard treatment of advanced BRAF-V600E mutated non-small cell lung cancer (NSCLC) patients. However, little is known about the implementation of circulating biomarkers monitoring as tool for predicting response to therapy and progression of disease (PD). Liquid biopsy (LB) represents a feasible and non-invasive method for detection and longitudinal monitoring of BRAF-V600E mutation in cell-free DNA (cfDNA).

Methods: In the multicentric LiBRA study 25 Italian centers were involved. Plasma samples were collected before starting treatment (baseline time-point), every 4 weeks during the first 16 weeks of treatment and every 8 weeks until PD. Semi-automatic extractor Maxwell (Promega) was used to obtain cfDNA from 1 ml of plasma and digital droplet PCR (ddPCR) was performed to monitor BRAF-V600E mutation in all plasma samples.

Results: To date, 41 BRAF-V600E mutated NSCLC patients treated with dabrafenib plus trametinib as first-line therapy were enrolled in 17 Italian centers. Overall, 25 (60.9%) patients were males and all patients had adenocarcinoma. Median age at treatment initiation was 69 years (range, 40-82), and 31/41 (70.7%) patients were current/former smokers. At the present time, a total of 131 plasma samples from 32 patients were collected at different time-points. 14/32 (43.8%) evaluable patients had a baseline plasma sample positive for BRAF-V600E mutation on circulating tumor DNA (ctDNA) and were defined as shedders, with a median variant allele frequency (mVAF) of 6.3%. 10/13 (76.9%) evaluable baseline shedders became ctDNA negative after 4 weeks of treatment (T1 time-point). Until now, three patients experienced PD and all of them were positive for BRAF-V600E mutation with a mVAF at the PD time-point of 39.7% (mVAF at T0=10.3%).

Conclusion: LB might be a promising approach to predict the probability of response and PD to first-line dabrafenib plus trametinib in BRAF-V600E NSCLC patients. This ongoing study will continue to monitor the presence of BRAF-V600E mutation in plasma samples and this data will be correlated with outcome measures (analysis scheduled for october). Furthermore, positive ctDNA plasma samples, both at baseline and at PD, will be analyzed through NGS platform in order to explore putative resistance mechanisms to dabrafenib plus trametinib.

PP.32
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100044
CIRCULATING TUMOR DNA MONITORING IN ADVANCED MUTATED MELANOMA TREATED WITH IMMUNE CHECKPOINT INHIBITORS: A PILOT STUDY

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Introduction: In recent years novel systemic therapies, such as immune checkpoint inhibitors (ICI) and BRAF/MEK targeted therapies, have emerged for treating advanced melanoma. Metastatic cancers release circulating tumor DNA (ctDNA) into the bloodstream that can be isolated and analyzed as a prognostic and predictive tool for patients' clinical outcomes. To this aim, we longitudinally monitored ctDNA assessing its role as a biomarker of clinical response in patients with advanced melanoma sequentially treated with ICI.

Methods: Patients with diagnosis of advanced mutated melanoma (BRAF or NRAS) candidates for anti-PD1-based ICI therapy (as monotherapy or combined with an anti-CTLA-4) were prospectively enrolled in the LIQUID-MEL study at the University Hospital of Parma. Mutations were identified on diagnostic tissue according to clinical practice. Plasma samples were collected prior to starting ICI (T0), at first radiological revaluation (T1), longitudinally, and at progressive disease (PD). MagCore extractor (Diatech Labline) was used to obtain ctDNA from 4 ml of plasma and digital droplet PCR (ddPCR) was performed to monitoring BRAF/NRAS mutations (BRAF V600K/E, NRAS Q61R/L/H, NRAS G12X-13X) in T0, T1 and TPD plasma samples.

Results: From August 2019 to November 2022 twenty-three patients were enrolled in the LIQUID-MEL study. The median age was 63 years (range 47-87), 61% were males. Sixty-two plasma samples were collected from the entire cohort during the study period, and 50 (23 T0, 17 T1, 10 PD) ctDNA samples were extracted and analyzed with ddPCR. Overall, 5/23 (21.7%) patients were positive for BRAF or NRAS mutation at T0, defined as shedders. We found that shedder patients at baseline had shorter progression-free survival than non-shedders (p=0.017, 3.9vs0.7 months). Shedding status also correlated with PD as best response (p=0.038). Among shedders, only 1 obtained ctDNA clearance after ICI-treatment initiation, but no correlation with best response was found due to the small sample size of the cohort. At PD, 7/10 (70%) patients had positive ctDNA.

Conclusions: From our preliminary analyses, we can speculate that ctDNA can play a predictive role in clinical outcomes of patients with melanoma treated with ICI. In daily practice, liquid biopsy could be a non-invasive tool helping in patients' clinical management.

PP.33
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100045
CLINICAL UTILITY OF LIQUID BIOPSY IN THE DIAGNOSIS AND MONITORING OF PATIENTS AFFECTED BY PROS SYNDROMES

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Introduction: PIK3CA-related overgrowth spectrum (PROS) is a group of rare diseases that cause overgrowth in various parts of the body due to mosaic mutations in the PIK3CA gene. Diagnostic techniques rely on identifying the mutation in affected tissue biopsies while confirming its absence in healthy tissue. However, these methods can only genetically characterize a limited number of patients, as they are an invasive procedure and carry a high risk. Liquid biopsy has already demonstrated its effectiveness in other fields, such as oncology. Therefore, it is proposed as a minimally invasive alternative to enhance and accelerate the diagnosis of PROS-affected patients.
Objective: This study aims to detect PIK3CA mutations using Next Generation Sequencing (NGS) in cell-free DNA (cfDNA) from the plasma of patients exhibiting PROS clinical features.
Methodology: In collaboration with the Dermatology Department of CHGUV, this study included 11 patients with clinical features associated with the PROS spectrum. Blood samples were collected from all patients in EDTA tubes and processed to obtain plasma. Circulating free nucleic acids were then extracted from the plasma and subsequently sequenced using the Oncomine™ Pan-Cancer Cell Free Assay panel.
Results: cfDNA isolation yielded successful amounts of DNA from patient`s plasma samples. Through liquid biopsy, mutations in c.2176G>A p.(E726K) with a MAF of 1.69% and 0.61% were identified in two patients, and a mutation in c.1258T>C p.(C420R), with a MAF of 0.35% was detected in a third patient. The total turnaround time to get the results was seven working days. Correlation of liquid biopsy results with tissue results was assessed, demonstrating the potential of liquid biopsy for diagnosis. Mutations found in tissue biopsies, such as c.1133 G>A p.(C378Y), could not be detected in liquid biopsy due to it not being covered by the sequencing panel. Thus, a specific panel has been designed to comprehensively sequence the PIK3CA gene, allowing for the detection of a wider range of mutations associated with PROS.
Conclusions: Our data shows that Next Generation Sequencing liquid biopsy analysis of cfDNA is an effective tool for PI3KCA-related overgrowth spectrum syndrome mutations diagnosis, which is necessary for personalized treatment with PI3KCA inhibitors.

PP.34
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100046
EXPLORING THE ROLE OF CK+/CD45+ (DUAL-POSITIVE) CIRCULATING CELLS IN PATIENTS WITH EARLY BREAST CANCER (EBC)

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Background: Circulating tumor cells (CTCs) have been associated with poor outcome in patients with EBC. However, just 20% of EBC patients have ≥1 CTCs highlighting the need of further cell-based biomarkers for prognostic stratification. Recent evidence showed clinical significance of those CTCs co-expressing cytokeratin and CD45 (i.e. dual-positive, DPs) in metastatic breast cancer (BC). The distribution and clinical significance of DPs in EBC is unexplored. The aim of this study was to investigate the association of DPs with recurrence in EBC.
Methods: This study retrospectively analyzed 48 patients with EBC treated at Northwestern University between 2016-2021. Blood samples were collected at diagnosis (T0) and close to surgery (T1, end of neoadjuvant or before starting adjuvant treatment). Samples were processed using the CellSearch® system. CTCs and DPs were manually enumerated. The association between DPs and event-free survival (EFS, from blood draw to recurrence or death) was tested.
Results: Among 48 female patients, 90% had stage III BC, 26 were diagnosed with inflammatory BC (54%), 23 (48%) had HER2+BC, and 9 (19%) had triple-negative BC. Forty-two and 38 patients received neoadjuvant and adjuvant therapy, respectively. Median follow-up was 30 months. T0 sample was available for 40 patients: median DP count was 0 (range 0-39). At T0, 6 patients had ≥2 DPs and had shorter EFS (27 vs 44 months, p=0.2) as compared with those with <2 DPs. T1 sample was available for 26 patients of which 6 (23%) had ≥2 DPs: these patients experienced a shorter EFS (18 months vs not reached, p=0.04). Interestingly, at both timepoints only 2 patients had both ≥2 DPs and >1 CTCs and experienced a particularly short EFS (11 and 6 months at T0 and T1, respectively).
Conclusions: In this study the detection of ≥2 DPs in patients with EBC, both at diagnosis and close to surgery, was associated with early disease recurrence. The combined use of DPs and CTCs in the early setting could increase sensitivity of cell-based liquid biopsy approach, helping to better define patient prognosis.

PP.35
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100047
CFDNA NGS FOR IDENTIFICATION OF PRIMARY AND ACQUIRED RESISTANCE IN PATIENTS WITH LUNG CANCER AND EGFR MUTATIONS

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Rational: Despite the clinical efficacy of EGFR tyrosine kinase inhibitors (TKIs) in patients with advanced-stage non-small cell lung cancer (NSCLC) harboring an activating EGFR mutation, mechanisms of resistance inevitably develop because of intratumoral molecular heterogeneity. Liquid biopsy (LB) serves as an ideal tool to investigate intratumoral heterogeneity. As a non-invasive method capable of detecting molecular dynamic changes that occur under the pressure of cancer-directed treatment at various sites of disease, liquid biopsy may capture tumor heterogeneity more efficiently than tissue biopsy. The aim of this study is to describe mechanisms of primary and acquired resistance to first line (1L) TKI identified by LB.
Methods: This study included data from patients with advanced NSCLC harboring an EGFR mutation who were treated with 1L TKIs at Mount Sinai Hospital and Tel Aviv Sourasky Medical Center. Patients were included if they had next generation sequencing of circulating tumor DNA assessed prior to starting 1L TKI and at progression of disease using institutional preferred liquid biopsy platform.
Results: A total of 48 patients were included in this analysis. Overall, 8% of patients were identified as primary refractory, harboring baseline MET amplification (4%) or mutations in RAS (2%) or PI3K (2%). Among patients who received 1L first- or second-generation EGFR-TKIs (37.5%), 42% acquired an EGFR T790M mutation at the time of progression, 8% had de novo MET amplification and 8% had acquired RAS mutation. 44% of patients did not display any known acquired mechanisms of resistance.
Among patients treated with 1L osimertinib (62.5%), 12% had de novo PI3K mutations at time of progression, 8% MET amplifications, 6% RAS mutations, and 3% RET fusions. New FGFR fusion, BRAF V600E or EGFR C797S mutation were seen in 2% each. 65% of patients did not have any identifiable mechanisms of resistance.
Conclusions: The present results confirm the role of liquid biopsy in the investigation of intratumoral heterogeneity and identification of primary and acquired mechanisms of resistance.

PP.36
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100048
A NARROW NGS PANEL IN THE ANALYSIS OF KRAS P.G12C MUTATION IN LONGITUDINAL SERIES OF NSCLC PATIENTS: A PILOT STUDY

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Introduction: KRAS p.G12C hot spot mutations have rapidly modified the therapeutic paradigm for clinical stratification of Non-Small Cell Lung Cancer (NSCLC) patients. NSCLC patients exhibiting p.G12C mutation highlighted an objective response rate of 37.1% (95% confidence interval [CI]) with a median progression-free survival of 6.8 months (95% CI) and median overall survival of 12.5 months (95% CI). Interestingly, in a not negligible percentage of NSCLC patients (20.0-40.0%) target drugs against p.G12C mutation failed in clinical benefit due to intrinsic or acquired resistance caused by cellular, molecular, and genetic mechanisms. Here, we investigate the role of a narrow NGS panel

evaluating p.G12C mutant allele fraction (MAF) variations in a longitudinal series of liquid biopsy specimens from NSCLC patients. (1,2)

Methods: A median of 3.8 (ranging from 2.0 to 8.0) longitudinal liquid biopsy specimens from n=27 p.G12C positive NSCLC patients on basal tissue specimens were retrospectively selected. For each case, cfDNA was purified by using an automatized system. Each sample was screened with a customized targeted NGS panel able to deeply analyze hotspot mutations in seven druggable genes, including p.G12C exon 2 KRAS hotspot mutation.

Results: Overall, all specimens were successfully analyzed with NGS panels. In details, p.G12C molecular alterations were detected in 29 out of 107 (27.1%) samples. A median MAF value of 16.2% (from 0.1 to 73.5%) was observed; among them 14 out of 29 (48.2%) samples showed p.G12C mutation with >5% MAF levels. Interestingly, manual inspection of raw data detected p.G12C alteration with MAF levels <5% in remaining cases. Variation in terms of p.G12C allele frequency was listed in table 1.

Conclusion: High-sensitive NGS systems represent a reliable technical option to evaluate p.G12C MAF variation in longitudinal series of liquid biopsy specimens.

ID	KRAS p.G12C %						
	S1	S2	S3	S4	S5	S6	S7
AMG_02	52.11%	44.56%	69.24%				
AMG_03	1.98%	ND	ND	ND			
AMG_07	ND	ND	ND	3.31%			
AMG_10	1-79%	ND					
AMG_12	15.67%	3.80%	5.61%				
AMG_13	21.11%	11.98%					
AMG_15	ND	ND	ND	ND	ND	ND	2.05%
AMG_17	ND	ND	0.15%	ND			
AMG_18	ND	ND	ND	24.20%	73.48%		
AMG_19	ND	2.05%					
AMG_20	3.60%	ND	ND				
AMG_21	ND	25.29%					
AMG_22	ND	1.95%	40.00%				
AMG_24	7.42%	ND	1.70%	1.69%	1.20%	17.85%	
AMG_25	3.01%	1.64%					

Table 1: MAF value variation in *KRAS* p.G12C positive liquid biopsy samples from NSCLC patients by using a narrow NGS panel. Particularly, 15 out of 29 (51.8%) samples harbouring MAF level <5% resulted p.G12C positive after manual inspection of raw data.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100049
EVALUATION OF CIRCULATING TUMOUR DNA DURING THE CLINICAL FOLLOW-UP OF MELANOMA PATIENTS

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Introduction: Melanoma is a highly aggressive form of skin cancer, and despite treatment advances, disease recurrence remains a significant concern. Detecting relapse early and improving outcomes requires regular monitoring of patients after surgical resection. Liquid biopsy shows promise for monitoring cancer patients as a non-invasive method. We aim to evaluate the clinical utility of circulating tumour DNA (ctDNA) detection in the follow-up for melanoma patients.

Methods: We conducted a prospective cohort study involving patients with melanoma undergoing clinical follow-up. Patients were categorized based on their clinical behaviour during follow-up as (A) disease-free patients, (B) relapsed patients, (C) patients with surgically removed disease (D) and persistent disease patients. Peripheral blood was collected every 3-6 months. After plasma isolation and cfDNA extraction, the presence of ctDNA was evaluated by digital-droplet PCR (ddPCR).

Results: We collected 404 plasma samples from 219 patients encompassing all stages of the disease (N=10 stage I, N=58 stage II, N=103 stage III and N=35 stage IV). BRAF mutant melanomas (mel.BRAF+) account for 40.2% of patients (88/219). Until now, screening of BRAF mutations at codon V600 (p.V600E, p.V600K, p.V600R) has been performed in 90 plasma samples from 30 mel.-BRAF+ patients.

ctDNA was detected in 33.3% (30/90) samples with a mean concentration of 0.71 copies/µl (range: 0.33-9.43). Levels of BRAF mutation (copies/µl) differ among the different clinical subgroups of patients (A subgroup = 0.04±0.13, B subgroup = 1.47±3.03, C subgroup = 0.00±0.01), and D subgroup = 0.25±0.40). We found differences in the concentration of BRAF mutation in samples from clinically disease-free individuals compared to affected melanoma patients (0.10±0.45 vs 0.57±1.92 copies/µl; p=0.047). We detected ctDNA in samples from 66.6% (4/6) of persistent disease patients and in 50% (9/18) of disease-free patients during the clinical follow-up.

Conclusion: Cell-free DNA-based test, such as screening of BRAF mutations, shows potential as a non-invasive tool for monitoring patients with resected melanoma. However, multimodal tests, including additional genes, might improve the detection of ctDNA in certain clinical subgroups.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100050
NON-INVASIVE ASSESSMENT OF PATHOLOGICAL COMPLETE RESPONSE AND MINIMAL RESIDUAL DISEASE THROUGH PLASMA METHYLOMICS IN PATIENTS WITH TRIPLE POSITIVE BREAST CANCER

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Introduction: Neoadjuvant chemotherapy (NACT) is recommended for early HER2-positive breast cancer (BC). The availability of accurate biomarkers for the non-invasive assessment of treatment response and minimal residual disease (MRD) would allow for the design of trials of escalation or de-escalation in patients with and without molecular evidence of disease, respectively. Cell-free DNA (cfDNA) methylation has emerged as a potential game-changer in the panorama of liquid biopsy for its analytic robustness, low limit of detection, and the possibility of inferring tumor tissue of origin. Cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq) offers a cost-effective, comprehensive whole-genome analysis of cfDNA methylation, overcoming the limitations of traditional bisulfite-based methods and targeted panels.

Methods: cfMeDIP-seq was performed on plasma cfDNA collected from patients with triple-positive BC at the end of NACT (T1) and after surgery (T2). cfDNA differentially methylated regions (DMRs) between patients with pathological complete response (pCR) and with residual disease at surgery (RD) were assessed on samples collected at T1. DMRs between patients who relapsed (R) after surgery and patients free of disease (No-R) at their last follow-up were assessed on samples collected at T2.

Results: cfMeDIP-seq was performed on plasma samples collected from seven women with triple-positive BC matched for age, menopausal status, and type of NACT. Three patients had RD and relapsed at a median of 19.3 months from surgery, while four patients achieved pCR and have not relapsed at a median of 77.6 months from surgery. We identified 28 DMRs (adjusted p-value < 0.05) between patients with pCR and patients with RD, including the transcription factor binding sites (TFBSs) of POLR2A and FOXA1 and the gene body of PRODH, and 50 DMRs between R and No-R patients, including the TFBSs of GATA1, GATA3, POLR2A, MAFF, and MAFK.

Conclusions: cfMeDIP-seq is a promising technique for the non-invasive assessment of response to treatment and MRD in early BC. Its validation is warranted in larger prospective studies.

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KRAS MUTATIONS, POTENTIAL BIOMARKERS FOR THE IDENTIFICATION OF NON-SMALL-CELL LUNG CANCER PATIENTS THAT MAY BENEFIT FROM IMMUNOTHERAPY-BASED TREATMENTS

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Introduction: Immunotherapy-based treatments have become the standard of care for most advanced non-small-cell lung cancer (NSCLC) patients who are not candidates for targeted therapies. However, some patients experience disease progression in the short term and do not benefit from these treatments. Nowadays, there is no optimized methodology to identify patients at a high or low risk of disease progression. Thus, we performed a study analyzing tumor molecular alterations before treatment with immunotherapy to identify possible biomarkers that could predict patient outcomes.

Methods: Liquid biopsy samples from 88 stage IV NSCLC patients were collected before first-line treatment with immunotherapy or chemo-immunotherapy. Circulating-nucleic acids were analyzed by next-generation sequencing using the Oncomine Pan-Cancer Cell-Free Assay on an Ion S5 sequencer. All-variants files were obtained from the Ion Reporter analysis platform and variant filtering was performed using an optimized script developed in our laboratory. The tumor response was evaluated with PET-CT and CT imaging according to the RECIST v. 1.1 criteria. Survival statistical analysis was performed using RStudio and Stata version 17.0.

Results: We were able to detect genetic alterations in 80.68% (n=71) of liquid biopsy samples. Specifically, we found 193 variants including 172 single-nucleotide polymorphisms (SNPs, 88.66%), 17 insertions and deletions (InDels, 8.77%), 3 Multiple Nucleotide Polymorphism (MNP, 1.54%), and 1 fusion. KRAS was among the most prevalent mutated genes, finding pathogenic or likely pathogenic mutations in 23 of the patients (26.14%). Particularly, we found variants in codons 12, 13, and 61; being p.G12C the predominant KRAS alteration (39.13%). Survival analysis showed that patients with KRAS variants had an improved progression-free survival (PFS) compared to non-KRAS mutated patients (Log-Rank p-value of 0.046), with a Hazard Ratio of 0.54 (95% CI 0.29-1, p-value of 0.049). Indeed, the median PFS for patients with wt tumors was 5.29 months, while it increased to 12.39 months in KRAS mutated patients.

Conclusion: Molecular profiling of liquid biopsies at baseline identified alterations that could impact on patient outcomes. Our data suggest that the presence of KRAS pathogenic/likely pathogenic variants may be a biomarker of decreased risk of progression in stage IV NSCLC patients treated with immunotherapy or chemo-immunotherapy.

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ASSOCIATION OF IDH1 MUTATION DETECTED IN CIRCULATING-FREE DNA WITH SURVIVAL OUTCOME IN PATIENTS AFFECTED BY GLIOMA

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Introduction: Circulating-free DNA (cfDNA) is a useful tool to investigate the molecular profile of tumors. Since cfDNA release is correlated with tumor biological characteristics, its evaluation in glioma is still challenging. Recently, the IDH1 mutation status has been recognized as a prognostic biomarker of tumor molecular evolution allowing an easier and more accurate risk stratification of glioma patients. The present study aimed to evaluate the detection of IDH1 mutation in cfDNA and its correlations with survival and clinical characteristics of glioma patients.

Methods: Six mL of blood were drawn in EDTA tubes from glioma patients and centrifuged to collect plasma. CfDNA was extracted from plasma and IDH1 p.R132H mutation analysis was performed on a digital droplet PCR. Statistical analysis was performed to investigate the prognostic power of such mutation and any association with clinical factors.

Results: Sixty-seven patients were enrolled in the present study. A concordance between the IDH1 mutation status evaluated in tissue and plasma samples was found (p=0.0004). In tissue samples, there was a statistically significant difference in terms of median overall survival (OS) in patients carrying vs. not carrying IDH1 mutation, being 138.8 vs. 25.6 months, respectively (p<0.0001). Similarly, the presence of IDH1 mutation was found to be associated with longer OS in cfDNA samples (116.3 months vs. 35.8, p=0.016). In univariate Cox regression analysis, significant correlations were observed between OS and IDH1 mutation, age, and tumor grade.

Conclusion: The present study supports the utility of liquid biopsy to detect molecular alterations in brain tumors. The identification of IDH1 mutation through liquid biopsy plays a central role as an important prognostic biomarker for glioma patients, being associated to OS.

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INTEGRATING IDYLLA TM ALONGSIDE REAL-TIME PCR AND NGS FOR INVESTIGATION OF GENE FUSIONS IN PLEURAL EFFUSIONS OF LUNG ADENOCARCINOMA

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Introduction: Pleural effusion (PE) from patients with advanced non-small cell lung cancer (NSCLC) has been proved valuable for molecular analysis, especially when tissue sample is not available however, simultaneous detection of driver fusions in PE is still challenging. Novel IdyllaTM GeneFusion assay, is emerging as a stand-alone test in tissue samples for laboratories lacking NGS facilities. In this study, we investigated Idylla GeneFusion assay in the evaluation of ALK, ROS1, RET and NTRK1/2/3 and MET ex14 skipping mutations also in pleural effusion and compared its performance with routine reference methods RealTime (RT)-PCR-based and NGS.

Methods: Four patients with advanced lung adenocarcinoma and pleural effusion were enrolled. Three harbored ALK fusion and one RET fusion. Pleural effusions were centrifugated at 1500 rpm for 5 min. Four ml of supernatant were extracted using Promega Maxwell® automatic system with the LV ccfDNA KIT. cfRNAs were tested for ALK and RET fusion by RT-PCR (LUNG-RT48, ENTROGEN), NGS (Oncomine Lung Cell-Free Assay) and IdyllaTM GeneFusion assay. 150ul of the same supernatant from PEs were centrifugated again at 15000rpm for 10min and pellet resuspended in 80 ul of RNA Later solution (Idylla A); other 150ul were simply added with 50ul of RNA Later solution (Idylla B).

Results: As shown in the table ALK and RET fusions were identified in all samples in ctRNA by RT-PCR and NGS . Idylla Method failed to identified fusions in ctRNA however detected fusions in unextracted samples: 3 out of 4 Idylla A samples and only in 1 out of 4 Idylla B sample.

Conclusions: Our data show that pleural effusion are suitable material for investigating fusion alterations. IdyllaTM GeneFusion, although indicated for investigation of tissue samples, successfully identified fusions also in unextracted PE supernatants.

Case n.	histology	%neoplastic cells*	Conc. (ng/ul)	RT-PCR ctRNA	NGS ctRNA	Idylla ctRNA	Idylla A§	Idylla B§
1	ADK**	5	2,5	ALK+	ALK+	failed	ALK+	failed
2	ADK	95	128	RET+	RET+	failed	RET+	RET+
3	ADK	5	17	ALK+	ALK+	failed	ALK+	failed
4	ADK	60	10,4	ALK+	ALK+	failed	failed	failed

* % was evaluated in cytoblock sample obtained from PE
** ADK: lung adenocarcinoma
§ see methods

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ANALYTICAL PERFORMANCE OF TRUSIGHT™ ONCOLOGY 500 CTDNA V2: IMPROVED SENSITIVITY FOR SMALL NUCLEOTIDE VARIANTS WITH REDUCED DNA INPUT REQUIREMENTS

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Introduction: Liquid biopsy combined with NGS is emerging as standard of care in oncology for comprehensive genomic profiling (CGP) of solid tumors. TruSight™ Oncology 500 ctDNA version 2 (TSO 500 ctDNA v2, Illumina, San Diego, CA; Research Use Only) is an NGS-based assay that utilizes the same gene panel as TSO 500 ctDNA (targeting 523 cancer-related genes), with similar sensitivity, but using lower cell free DNA (cfDNA) input. The TSO 500 ctDNA v2 assay incorporates unique molecular identifiers (UMIs) for error suppression, and the workflow utilizes a recommended input of 20ng of cfDNA into a single hybrid capture workflow resulting in a decreased hands-on time.
Methods: Libraries generated from patient cfDNA, commercially available cfDNA-like reference materials, and nucleosome (npDNA) prepared cell lines harboring variants of interest were sequenced on the NovaSeq™ 6000 instrument (Illumina) and analyzed with the DRAGEN TSO500 ctDNA Analysis Software v2.1. The software detects small nucleotide variants, gene amplifications and deletions, gene rearrangements and determines a Tumor Mutational Burden (TMB) score and Microsatellite Instability (MSI) status. Assay sensitivity was evaluated using titrations at specific variant allele frequencies (AF), or specific tumor fractions. Specificity was evaluated testing cfDNA replicates from healthy donors. Assay precision was evaluated testing a panel of samples across multiple operators, reagent lots and sequencing instruments.
Results: TSO 500 ctDNA v2 consistently detected key pathogenic small variants at AF $\leq$0.2% with 20ng input. Detection of indels and gene fusions with >95% sensitivity was observed at AFs $\leq$0.4%. Testing of reference samples demonstrated sensitive detection of 1.25-fold change gene amplifications and 0.8-fold change gene deletions. Testing of cfDNA from samples from healthy normal donors showed that TSO 500 ctDNA v2 can achieve a >99.999% assay specificity. Precision evaluation showed >99% precision for targeted small DNA variant calls.
Conclusion: results demonstrate >99.999% analytical specificity with high analytical sensitivity to AFs ranging at 0.2%-0.4% depending on variant class; performance was achieved using a lower recommended input of 20ng of cfDNA, utilizing a workflow that reduces total turn-around time and hands-on time while still delivering all key biomarker data available from the TSO 500 gene panel.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100055

DIRECT SALIVA ANALYSIS BY MALDI MASS SPECTROMETRY: A WORKFLOW SUITABLE FOR FUTURE CLINICAL APPLICATIONS

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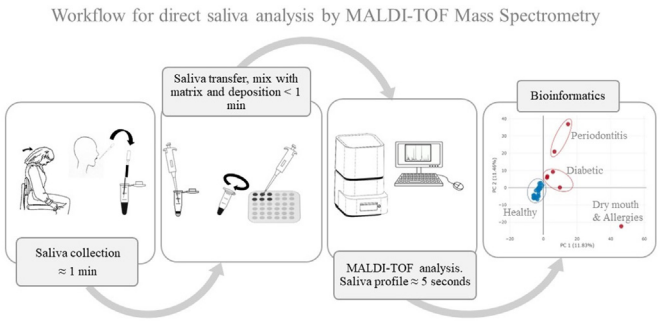
Saliva is an ideal diagnostic fluid for its non-invasive ease of collection, and correlation with various diseases. Despite the recognized and potential diagnostics applications using saliva, there remain unresolved challenges hindering its use in clinical laboratories such as the lack of standardized preparation protocols. Matrix-Assisted-Laser-Desorption-Ionisation-Time-Of-Flight mass spectrometry (MALDI-TOF MS), the gold standard technique in clinical laboratories for microbial identification, is a rapid and easy to use technology that holds strong potential for wider clinical applications. To explore its viability for disease diagnostics, we developed and evaluated a novel 2-minute direct saliva preparation workflow for MALDI-TOF MS (see image), saliva profiles were generated in the mass range m/z [2000-20000] using a linear MALDI-TOF mass spectrometer (Ascend diagnostics, UK).
Technical repeatability and biological variability were evaluated using 160 saliva samples collected from 4 healthy individuals for 20 days. Data were evaluated using CloverBiosoft software (Spain). Saliva profiles were correlated using the Pearson correlation coefficient (PCC) heatmap and the coefficient of variation in signal intensity (CV) was calculated for the 150 most common molecules. Results showed good repeatability and reproducibility with an average PCC of 0.98 for technical replicates, 0.94 for intra-individual biological variation, and 0.90 for inter-individual biological variation. The CV was below 5% for technical replicates and under 30% for biological replicates for over 100 common molecules.
Lastly, saliva profiles of 14 healthy individuals were compared with those of 4 individuals diagnosed with medical conditions. Unsupervised analyses, including PCA and hierarchical clustering, revealed distinct clustering of presumed 14 healthy individuals' saliva profiles and clear separation from profiles obtained from individuals with confirmed medical conditions. PCC between each of the healthy individuals and those with specific medical conditions were below 0.55. In conclusion, our proposed method represents a promising advancement toward the creation of reliable prediction models for disease diagnostic using saliva.



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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100056

HETEROGENEITY OF SOMATOSTATIN RECEPTORS ON CIRCULATING TUMOR CELLS IN NEUROENDOCRINE TUMORS AND MELATONIN'S POTENTIAL IN NON-SMALL-CELL LUNG CANCER

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Introduction: Neuroendocrine tumors (NET) exhibit overexpression of somatostatin receptors (SSTR) that can be targeted for therapy. However, the heterogeneity of SSTR expression poses challenges in defining and tracking its variations. We propose measuring SSTR expression on circulating tumor cells (CTCs) as a means to investigate heterogeneity and monitor changes during therapy. Additionally, we review the therapeutic actions of melatonin and its potential for future research in non-small-cell lung cancer (NSCLC) treatment.
Methods: MCF-7 cells were transfected with SSTR2 or SSTR5 and introduced into donor blood samples for analysis using CellSearch. Optimal antibody concentration and exposure time were determined, and flow cytometry was employed to assess assay sensitivity. Clinical evaluation involved analyzing blood samples through CellSearch and performing SSTR2/5 immunohistochemistry on matched tissue samples. DNA karyometry was used in combination with CTC analysis for a more comprehensive investigation.
Results: Flow cytometry confirmed the sensitivity of CellSearch for detecting SSTR on CTCs, unaffected by somatostatin analog presence up to 100 ng/ml. Among 31 NET patients, 68% exhibited CTCs, with 33% showing heterogeneous SSTR2 or SSTR5 expression. The majority (87%) had SSTR-positive tumors confirmed by molecular imaging techniques. The study also explores the

potential of melatonin as a biomarker and its effects on NSCLC.

Conclusions: CTCs from NET patients express detectable levels of SSTR2 and SSTR5, making them potential biomarkers for evaluating SSTR-targeted therapies. Melatonin, with its diverse anticancer properties, including anti-proliferative, anti-metastatic, and immunomodulatory effects, holds promise as a supplementary therapy for NSCLC. However, further research is needed to understand its mechanisms fully and assess its potential in combination with conventional treatments.The discordance between CTC and tissue expression of SSTR highlights potential implications for therapy response and disease control. Evaluating SSTR expression on CTCs may aid in patient management. A multicenter prospective trial is investigating the relationship between SSTR2/5 expression on CTCs and progression-free survival in midgut NET patients receiving Lanreotide Autogel treatment. This longitudinal study aims to provide novel insights into CTCs as pharmacodynamic markers in the disease, shedding light on their role and clinical significance.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100057

CIRCULATING TUMOR CELLS (CTCS), MELATONIN, AND UNOS: NEW FRONTIERS IN CANCER DIAGNOSIS AND TREATMENT

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Introduction: Cancer, a significant global health challenge, requires early, accurate diagnosis for effective treatment. Currently, detection is reliant on invasive tissue biopsies, which are limited in their capacity to monitor disease progression and can cause patient discomfort. The demand for minimally invasive, repeatable biomarkers for disease tracking is critical. Liquid biopsy, which involves the capture and analysis of tumor-related biomarkers in bodily fluids, has emerged as a promising alternative. It offers advantages such as minimal invasiveness, low reagent consumption, and compatibility with routine clinical procedures.

Methods: The liquid biopsy approach primarily analyses circulating tumor cells (CTCs), circulating tumor nucleic acids (ctNAs), and tumor-derived extracellular vesicles (exosomes). These biomarkers, shed from the tumor into bodily fluids, provide information on the cancer's stage and progression. The research detailed here uses various technologies to detect and study these biomarkers. We employed an FDA-approved CellSearch assay and cutting-edge microfluidic platforms to improve CTC isolation purity and recovery levels. For a more comprehensive analysis, we combined this with "DNA Karyometry" to automatically detect CTCs, staining the detected cells with specific fluorescent dyes for further examination.

Results: Through these methods, we discovered vital correlations between biomarker levels and different types of cancer. For instance, in prostate cancer patients, certain biomarkers like tensin homolog and survivin were found at higher levels. Interestingly, non-coding RNAs (ncRNA) in extracellular vesicles (EVs) showed elevated concentrations in the plasma of cancer patients. Moreover, our study revealed a lack or significant decrease of Universal Nitric Oxide Synthase (uNOS) in certain cancers and an increase in membrane expression of MT1 and MT2 receptors in the CTCs of all investigated malignant tumors.

Conclusion: Our research underscores the potential of liquid biopsy in cancer diagnosis and management. The observed correlations between CTCs, novel biomarkers such as melatonin and uNOS, and various cancers suggest a promising future for these techniques. They could serve as potent clinical markers for cancer monitoring, providing insights into its biological status and aiding in predicting treatment efficacy. However, crucial questions persist, necessitating continued research into the information consistency from different biomarker sources and the real-world impact of liquid biopsy techniques on patient outcomes.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100058

PROGNOSTIC AND PREDICTIVE NON-INVASIVE BIOMARKERS IN CUTANEOUS MELANOMA TREATED WITH ANTI PD-1 ANTIBODIES

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Introduction: Despite significant advancements in melanoma treatment with immune checkpoint inhibitors (ICI), limitations arise from primary and acquired resistances. Identification of predictive biomarkers to ICI is still under investigation.

Objective: Evaluate predictive and prognostic biomarkers to anti PD-1 antibodies, in patients with advanced cutaneous melanoma.

Methodology: Blood samples were collected before treatment and at revaluation (3-6 months). 28 plasma analytes levels were measured by multiplexed bead-based immunoassays using MILLIPLEX® HSTCMAG-28SK, HCKP1-11K and HCKP2-11K kit. Parametric and non-parametric tests were employed for statistical analysis. Receiving Operating Curve (ROC) method was used to determine predictive power of analytes. For survival analysis, Cox regression and Kaplan Meier with log-rank test were performed. P-value <0.05 was considered statistically significant.

Results: 18 patients with advanced cutaneous melanoma were evaluated (mean age 70 years, [range 37-87]). At baseline, responders had significantly lower median sCD276 levels (2170.40 pg/ml) than non-responders (3368.3988 pg/ml) ($p=0.046$). ROC curve analysis determined a cut-off concentration of 2077,16 pg/ml associated with 87.5% sensitivity, 70 % specificity, 70% PPV and 87.5% NPV, to predict response to anti-PD-1 treatment with a 0.788 AUC ($p=0.041$). At response evaluation, responders had significantly higher median sCD28 levels (195.85 pg/ml) than non-responders (65.84 pg/ml) ($p=0.015$). Moreover, those patients with high levels of sCD28 at response evaluation had also a durable clinical benefit (DCB) (185.72 pg/ml) than those with non-DCB (63.18 pg/ml) ($p=0.005$). Besides, the presence of hepatic metastases was found to be related to high levels of sMICB and low levels of sICOSL ($p=0.035$ and $p=0.032$, respectively). The secretion of sMICB, sMICA, sCD276, sPD1, sIL6 and ITGA6 was associated to prognosis based on Cox regression analysis (Z-score > 1.5), so their absolute regression coefficients from a multivariate model were used to calculate a immunogenic score. Patients with high sMICB, sCD276 and sIL6 and low levels of sPD1 and sMICA, have shorter overall survival (OS) [19.53 vs. not reached, $p=0.012$] and PFS [2.83 vs. not reached, $p=0.006$].

Conclusions: sCD28 and sCD276 might be used as immunotherapy predictive biomarker. The proposed scored might be used as prognostic biomarker. These preliminary results should be validated in a larger cohort.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100059

NANOSCOPIC CANCER BIOMARKERS CHARACTERIZATION OF EXTRACELLULAR VESICLES IN LIQUID BIOPSY

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Introduction: Liquid biopsy has emerged as a groundbreaking strategy for cancer diagnosis and prognosis prediction, characterized by its minimally invasive nature and the ability to detect cancer-related biomarkers in biofluids. Extracellular vesicles (EVs) are nano-sized lipid bilayer particles constitutively released from cells known as mediators of cell-to-cell communication. EVs have diverse biological activities, ranging from roles in homeostasis to cancer progression. They have been shown to have great potential as liquid biopsy; unfortunately, the clinical applicability EVs have not been fully investigated. This is because EVs are extremely heterogeneous in size, and molecular composition, thus making them very difficult to characterize with currently available tools. Here, we developed an integrated workflow based on single molecule localization microscopy (SMLM) to characterize individual EVs from patients with advanced cancer. We demonstrate that SMLM can provide unique insights about EVs subpopulations based on EVs size and biomarker distribution.

Methods: Plasma from healthy and advanced cancer patients was diluted in PBS (1:50) and loaded on slides with capture monoclonal antibodies (CD9; CD63; CD81; etc..) for SMLM imaging. Captured EVs were stained using a mix of anti-tetraspanin Ab (CD9 + CD63 + CD81) labelled with AF647 fluorophores. Single

molecule imaging (dSTORM) was performed using Abbelight SMART-kit buffer on a SAFe360 Abbelight super-resolution module mounted on an Nikon Ti2 inverted microscope. Single molecule localization in 2D was performed on the RAW data using Abbelight Neo_Analysis Software, and localization clusters corresponding to labelled EVs were extracted using DBSCAN clustering algorithms.

Results: SMLM was used to generate EVs high-resolution images. The quantification of biomarker distribution in each patient's sample revealed variations in the size and number of clusters between the healthy controls and cancer patients. These findings support the great potential of SMLM technology to detect and characterize individual EVs in biological clinical samples.

Conclusions: The association of tetraspanin labeling with SMLM imaging enables the detection of differences in plasma samples obtained from healthy individuals versus those with various cancers. Further studies are ongoing to refine this technology and make it suitable for use for clinical implementation.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100060

REFINEMENT OF MRD ASSESSMENT IN HAIRY CELL LEUKEMIA BY INTEGRATED MOLECULAR AND FLOW CYTOMETRY ANALYSIS

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Introduction: Circulating tumor DNA (ctDNA) is revolutionizing the landscape of precision oncology, guiding cancer profiling, treatment and showing potential to mirror disease evolution. Hairy cell leukemia (HCL) is a chronic lymphoproliferative B-cell disorder sustained by BRAF V600E mutation. Currently, measurable residual disease (MRD) assessment in HCL is based on cytology and flow cytometry (FC). However, diagnostic accuracy could be hampered by the small numbers of HCs circulating into the peripheral blood (PB) of patients with complete responses, or 'dry tap' bone marrow (BM) aspirations. Molecular testing to BRAF V600E has been introduced both on cells and ctDNAs, but feasibility in the MRD setting is still controversial. Herein, we evaluated the sensitivity and specificity of a combined FC/digital PCR (dPCR)-based approach for HCL longitudinal tracking in a real-world clinical scenario.

Methods: BM aspirates and/or PB samples were sequentially collected from HCL patients (n=6) at different time points. Plasma was isolated by a double-spin centrifugation. Clinical-grade B-cell and HCL-specific antibodies were used for FC. HCs were sorted according their immunophenotype, and BRAF V600E frequency was assessed in paired cell and plasma samples with dPCR.

Results: Fifteen paired (cell and plasma) samples were collected upon 2 years of follow up. Five out 6 (83%) positive patients for FC were also scored positive by ctDNA analysis (PPV: 100%, NPV: 90%). Liquid biopsy data were confirmed on circulating HCs, either in bulk or sorted, for all but one cases. Discrepancy referred to a patient with no traces of the disease according to FC and very low (0.014%) abundance of BRAF V600E into the bloodstream, thus highlighting the improved sensitivity of our approach. Moreover, dPCR on circulating HCs recovered 2 samples previously scored ctDNA-negative but positive to FC, while none of FC negatives were scored positive by molecular analysis, strongly confirming the robustness of our investigations. Overall, a concordance rate between FC and molecular analysis of 93% was observed. Importantly, our approach enabled HCs detection in patients with no clinical signs of the disease, raising additional questions about their persistence.

Conclusions: Ultra-deep molecular analysis, including liquid biopsy, are robust companion tools for MRD assessment in HCL.

PP.49

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100061

NGS TESTING ON PLASMA-BASED CTDNA SAMPLES AT KEY CLINICAL TIMEPOINTS IN ADVANCED NSCLC: HANDLING OF BLOOD SAMPLES AND REAL-WORLD EXPERIENCE

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Introduction: Targeted therapy has improved clinical outcomes for non-small cell lung cancer (NSCLC) patients. Although tissue is not always informative, it still represents the gold standard for molecular genotyping. Circulating tumor DNA (ctDNA) is a candidate biomarker for molecular profiling both at diagnosis and treatment failure. However, ctDNA shedding is affected by heterogeneous clinical behavior. Here, we present our real-life experience of using liquid biopsy into clinical practice.

Methods: PaStients with a histological diagnosis of advanced NSCLC were enrolled in this prospective study at the University Hospital of Parma. Only patients with an oncogene addiction (EGFR, KRAS-G12C, BRAF-V600E, HER2 exon 20 insertion; ALK, ROS1, RET fusions, MET exon 14 skipping and MET amplification) and candidate to a Tyrosine Kinase Inhibitor treatment (TKI), were considered. Tissue specimens were analyzed at diagnosis according to clinical practice. Plasma specimens were collected at onset (baseline), longitudinally and progressive disease (PD) of every subsequent line of treatment with TKIs. ctDNA samples were analyzed using Avenio Expanded kit panel (Roche, Swisse). Feasibility of ctDNA testing in terms of percentage of detected driver gene alterations, as well as the concordance rate between tissue and plasma samples at diagnosis, were investigated.

Results: From November 2015 a total of 96 advanced NSCLC patients were enrolled. The median age was 64 years (range 30-87) and 66% were females. 102 ctDNA samples were collected among baseline (77) and PD (25) timepoints and, at present, 70 were analyzed with NGS. Driver alteration was detected in 31/70 (44%) patients. Specifically, the detection rate was higher for DNA-based molecular alterations (EGFR, KRAS-G12C, BRAF-V600E, MET amplification, HER2 exon 20 insertion) 93%, rather than for the RNA-based molecular alterations (ALK, ROS1, RET fusions, MET ex14 skipping) 31%. Besides, concordance rate between tissue and plasma at diagnosis was 38%. Notably, we found that driver detection in rearranged samples is affected by analysis timing. Indeed, the detection efficiency in samples stocked for more than a year is 16% versus 61%.

Conclusion: Our results demonstrate the usefulness of ctDNA testing in clinical practice as well as the appropriate handling of plasma samples in the pre-analytical phase.

PP.50

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100062

PROGNOSTIC SIGNIFICANCE OF CIRCULATING TUMOUR CELLS AND DISSEMINATING CIRCULATING TUMOUR CELL CLUSTERS IN EPITHELIAL OVARIAN CANCER

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Cancer cells that transit from primary tumours into the blood circulatory system are known as circulating tumour cells (CTCs). Research has highlighted the difficulties with detection of CTCs from ovarian cancer patients using EpCAM-based techniques, with classical EpCAM-CTC enumeration alone having limited prognostic significance. This study aims to isolate ovarian cancer CTCs using Parsortix technology.

Blood specimens [n=127] were prospectively collected from 62 newly diagnosed epithelial ovarian cancer patients since December 2020. Samples were taken pre and post-neoadjuvant chemotherapy, pre-surgery and during cytoreduction surgery from the central ovarian vein and at 1 year following diagnosis. CTCs were isolated using Parsortix microfluidic device and immunophenotyped [DAPI, CD45, CK7/panCK/EpCAM and CD42b] by immunofluorescence and confocal microscopy.

68% of patients recruited had >1 CTC detected [CTC range of 1–22 cells per 7.5 ml of blood]. CTCs were present in 74% of ovarian vein [n=22] samples [CTC range of 1–2475 cells]. Patients with >2 CTCs had higher CA125 levels. Median PFS was significantly lower at 11.5 months in those with CTCs CTCs compared to 21 months with no CTCs. Higher CTC counts did not correlate with PLR, NLR, or platelet count. No statistical difference was seen between pre and post neoadjuvant CTC counts, however, a number of poor chemotherapy responders, or those who only received chemotherapy had persistent CTC levels following treatment at all timepoints. Large CTC clusters were significantly isolated from the ovarian vein compared to peripheral blood and were cloaked by CD42b+ platelets and immune cells with 3D confocal imaging of CTC clusters performed at single-cell resolution. Follow up of treatment responders and 1 year follow up is ongoing to establish the further clinical significance of single CTC and CTC clusters detection in primary surgery and neoadjuvant setting in epithelial ovarian cancer.

We report that the use of expanded CTC-ID markers and Parsortix CTC

enrichment, single CTC and CTC clusters can be isolated from ovarian cancer patients. We show that CTCs isolated from the ovarian vein during surgery are abundant for the precursor metastasising cells that are CTC-clusters, and may show utility for monitoring treatment response and prognosis in the adjuvant setting.

PP.51
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100063
MULTILAYER CHARACTERIZATION OF PLASMA EV CONCENTRATION AND CARGO ALLOWS TO DIAGNOSE AND MONITOR GLIOBLASTOMA BY LIQUID BIOPSY

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Glioblastoma (GBM) is a lethal brain tumor in adults. Its diagnosis and profiling rely on neuroimaging and brain biopsy, that have limited specificity and sensitivity. The molecular evolution of the tumor can't be monitored by tissue sampling due to the surgical risks. The major clinical need is the identification of circulating GBM biomarkers, that can be assessed non-invasively by liquid biopsy. Extracellular Vesicles (EVs) secreted by GBM cells cross the blood brain barrier and harbor tumor-derived molecules. The possibility to analyze plasma EV concentration and cargo longitudinally makes them an attractive tool for GBM detection and characterization.
We isolate EVs from 2mL of plasma by Size Exclusion Chromatography (SEC), which is fast and requires minimal equipment. We measured EVs concentration by Tunable Resistive Pulse Sensing (TRPS) in plasma from 50 primary GBM patients, with respect to 100 healthy subjects and 50 patients with other brain pathologies, and longitudinally during treatment until relapse.
Proteomic analysis on GBM and healthy EVs will allow the identification of a GBM protein signature to support the EV-based diagnosis of GBM. We will profile the EV proteome at different time points after primary tumor resection, to monitor the stages of the disease, and to identify patient-specific druggable targets.
We isolate EV-DNA by the SelectEV kit. We will characterize matched tissue and EV-DNA by targeted droplet digital PCR and shallow NGS to assess if EVs carry mutations and large chromosomal aberrations present in the parental tumor.
EVs are enriched in pre-operative plasma from GBM patients, and can be used as biomarker of GBM presence.
The analysis of EV proteins is feasible. We annotated >900 proteins including EV-specific proteins as well as proteins previously associated with GBM pathology.
EV-DNA was isolated from 2mL of GBM plasma and the yield is suitable to detect mutations and copy number variations.
These results highlight the potential of EV-based liquid biopsy for GBM diagnosis and molecular profiling. The minimal invasiveness and easiness to isolate plasma EV fit with clinical requirements. The multi-layered analysis of plasma EV concentration and cargo will improve the personalisation of GBM care for every time-point of the disease.

Clinical Trials Including Liquid Biopsies

PP.52
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100064
LIQUID BIOPSY-BASED RISK STRATIFICATION AND INTENSIFICATION OF TREATMENT IN EGFR MUTANT ADVANCED NSCLC (L-BRITE)U

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Background: Documentation of ctDNA clearance at various time points after targeted therapy correlates with better progression free survival (PFS). Serial plasma ctDNA monitoring may provide risk stratification strategy by selecting the poor risk and a window of opportunity of treatment escalation which has not been formally tested. Patients of advanced NSCLC with EGFR mutations are treated with TKIs alone and Osimertinib is preferred over first generation TKIs. Recently, combination of chemotherapy or VEGF inhibitors with TKIs have shown promising results. In this trial, we are evaluating the role of combining chemotherapy in patients with residual ctDNA after gefitinib monotherapy for initial 9-12 weeks, in terms of 6 months PFS rates.
Methods: This is a phase II single centre, single arm, open-label study in treatment naïve metastatic NSCLC with sensitising EGFR mutations (exon 19,21or 18) detected on liquid biopsy. We excluded patients with poor PS(ECOG PS 3 or 4), and symptomatic brain metastases. Treatment consists of gefitinib monotherapy for 9-12 weeks followed by the addition of 4 cycles of carboplatin and pemetrexed followed by pemetrexed maintenance in patients with residual ctDNA. Serial ctDNA monitoring using ddpCR is planned at 3 monthly intervals. Treatment is continued till disease progression or intolerance. Response evaluation is being done by contrast enhanced CT every 3 months using RECIST v1.1.
The primary endpoint of the study is to evaluate 6-month Progression-free survival (PFS) rate by adding chemotherapy to gefitinib in patients with residual CtDNA after gefitinib monotherapy with the presumption that it would improve 6 month PFS rates in these patients from 40% to 65%. The secondary endpoints are to evaluate PFS in patients on gefitinib monotherapy who have cleared ctDNA on, overall survival (OS), time to develop T790M resistance mutation in plasma ctDNA, safety, and quality of life. A Simon's two-stage design has been utilized. If we observe ≥6 responses among the first 11 evaluable patients, a further 20 patients will be accrued for a total of 31. The null hypothesis will be rejected if at least 17 patients are progression free at 6 months. Active enrolment is ongoing and interim analysis is planned.

PP.53
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100065
LONGITUDINAL TRACKING OF ALK-POSITIVE LUNG CANCER FROM PLASMA USING CIRCULATING-TUMOR RNA AND CIRCULATING-TUMOR DNA IN THE BRIGHTSTAR CLINICAL TRIAL

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Background: Detection of gene fusions in ALK fusion-positive lung cancer remains a challenge, particularly in liquid biopsies. Tissue biopsies using RNA-based assays have shown improved sensitivity for fusion detection. To address this, we hypothesized that combining circulating tumor RNA (ctRNA) assessment with circulating tumor DNA (ctDNA) in a liquid biopsy assay would improve fusion detection. In addition, we expected gene fusion and mutation detection to correlate with clinical outcomes. Here, we present updated outcomes data from the ASCO 2023 meeting.
Methods: We retrospectively analyzed 86 samples from 34 patients enrolled in the BRIGHTSTAR clinical trial (NCT03707938). Samples were collected at baseline (N = 28), before LCT after 8 weeks of brigatinib treatment (N = 29), after LCT (< 3 weeks; N = 25), and at progression (N = 4). We used a targeted next-generation sequencing (NGS) assay that assesses both ctDNA and ctRNA (LiquidHALLMARK, Lucence Health, Palo Alto) to detect ALK fusions and mutations in 80 genes. Up to 5 mL of plasma was analyzed per sample.
Results: At baseline, ALK fusions were detected in 15/28 patients (54%), 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 completely eliminated after LCT. Overall, the inclusion of ctRNA analysis resulted in a 36% relative increase in ALK fusion detection compared to ctDNA analysis alone. The absence of ALK fusion detection at baseline was associated with numerically longer progression-free survival (not reached versus 27.6 months; p = 0.052; HR = 0.24; 95% CI: 0.05-1.14) and an increase in ALK fusion allele frequency as detected by ctDNA was associated with an increased risk of progression (HR = 1.15; 95% CI: 1.04-1.28; p = 0.0077).
Conclusions: We highlight that ALK fusions can be reliably detected in the plasma of lung cancer patients and that the detectability of ALK fusions correlates

with survival outcomes. The analysis of ctRNA in addition to ctDNA improves the sensitivity of fusion detection and is a promising strategy in this setting.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100066

FOLLOWING THERAPY RESPONSE THROUGH LIQUID BIOPSIES IN METASTATIC COLORECTAL CANCER – THE PATIENT'S EXPERIENCE

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Background: Liquid biopsies (LBs) have emerged as a promising technique in oncological research. Although research has focused on LBs' analytical specifications and applications, it is essential to consider the perspective of patients. Therefore, we developed a questionnaire to explore patients' experiences.
Methods: The lead-in FOLICOLOR trial (Following therapy response through liquid biopsies in metastatic colorectal cancer patients - NCT04735900) included patients with metastatic colorectal cancer starting first-line treatment. Patients underwent bi-weekly LB monitoring for 18 months or until disease progression. However, LB results were not communicated to patients or their physicians. At end of study, patients received a questionnaire about the benefits and drawbacks of CT-scans and LBs, along with free-text fields for suggestions.
Results: Out of the fifteen trial participants, nine (60%) completed the end-of-study questionnaire. The reasons for not completing the questionnaire were refusal and death. Five patients (55.56%) preferred follow-up based on LBs over follow-up with CT-scans (and one patient favoured both LB and CT). Interestingly, all nine patients stuck to their preference, even if LBs could be performed more frequently than CT-scans (LBs every 2, 4, or 8 weeks vs. CT every 8 weeks). Reasons for selecting CT-scans over LBs included reduced hospital visits and greater trust in this method. On the other hand, the preference for LBs was driven by decreased fear and shorter time spent in the hospital. Other reported advantages of LBs included the possibility of testing at other locations than hospitals and fear of CT-scan radiation. However, one patient reported a preference for a combination of CT-scans and LBs, finding both methods tolerable.
Conclusions: While this data provides valuable insights, it is important to note that it originates from a small and highly selected patient cohort. Further investigation is necessary to determine why some patients did not prefer LBs over CT-scans. It is hypothesized that education and knowledge about LBs play an important role in shaping patients' opinions. Therefore, we have initiated a follow-up study at the Antwerp University Hospital, wherein all patients providing LBs receive in-depth interviews and questionnaires to study their experience, knowledge, and trust in different follow-up methods.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100067

IMPACT OF PROPHET ASSAY IN CHANGING PHYSICIAN'S THERAPEUTIC DECISION-MAKING FOR CHECKPOINT IMMUNOTHERAPY IN NSCLC (NON-SMALL CELL LUNG CANCER)

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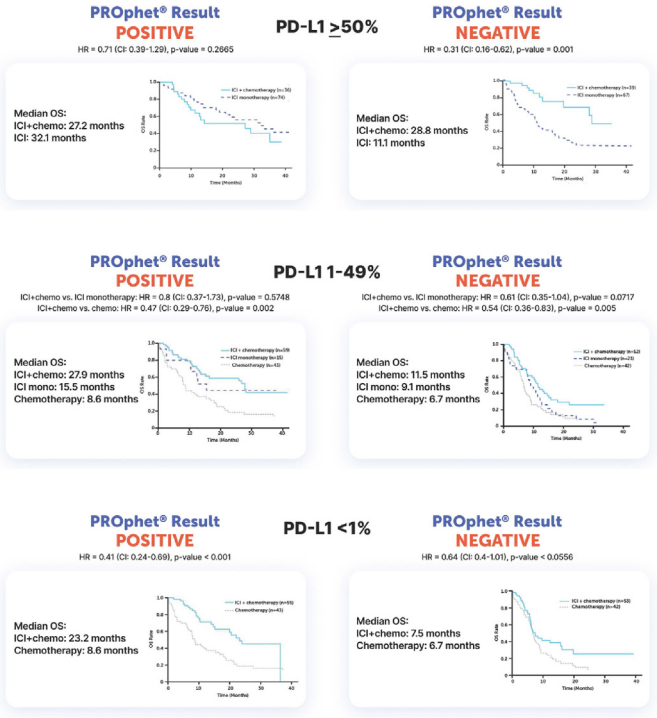
Introduction: Immune Checkpoint Inhibitor (ICI) based regimens are approved for 1st line treatment of patients with metastatic, EGFR/ALK/ROS1 wild-type NSCLC. Guidelines are insufficient in differentiating which patients with PD-L1 ≥50% should receive ICI monotherapy. There is an unmet need for biomarkers predictive of improved survival with monotherapy vs combination. The PROphet NSCLC plasma proteomic assay, both analytically and clinically validated, is designed to inform this therapeutic decision.
Methods: 100 randomly selected oncologists virtually reviewed 3 newly diagnosed metastatic NSCLC cases with PD-L1 scores and were asked to recommend an approved 1st line regimen. They then watched an online webinar about the PROphet NSCLC assay. Post webinar, the same 3 cases were presented with the addition of a PROphet result, and physicians again recommended a 1st line regimen. Responses were compared to assess the impact of knowledge about the PROphet test.
Results: Treatment changed in 39.6% (238/600) of PROphet-tested cases, with 94% of physicians changing treatment in at least 1 case. In the PD-L1≥50%, PROphet POSITIVE group, which predicts high clinical benefit from ICI monotherapy, there was an increase in the recommendation of ICI monotherapy from 60% to 89%. For the PD-L1 L1≥50%, PROphet NEGATIVE group, where poorer survival for ICI monotherapy is anticipated, the recommendation for monotherapy dropped from 60% to 9%. In the PD-L1 <1%, PROphet NEGATIVE group, predicted to have no additional benefit from ICI combined with chemotherapy, 35% of patients were spared toxicity from ICI compared to 11% in PROphet untested cases.
Conclusion: The addition of PROphet to PD-L1 significantly impacted therapeutic decision-making in 1st line NSCLC cases. As shown in the figure, PROphet identifies those predicted to have overall survival benefit from ICI monotherapy vs combination vs chemotherapy, both improving the probability of efficacy and reducing toxicity in some patients.



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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100068

THE PROGNOSTIC VALUE OF THE CIRCULATING-TUMOR DNA IN HIGH-GRADE SEROUS EPITHELIAL OVARIAN CANCER: EXPERIENCE FROM MITO-16A/MANGO-OV2A/ CLINICAL TRIAL

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Background: High-grade serous epithelial ovarian cancer is a highly deadly, complex and heterogeneous disease. Despite in the last decades large efforts have been focused on the identification of molecular biomarkers with predictive and prognostic relevance, to date none of these is used in the clinical practice. In this scenario, the use of liquid biopsy, and in particular the analysis of circulating tumor DNA (ctDNA) could be considered as an achievable strategy to assess the prognostic role of ctDNA at the time of diagnosis and to infer the dynamic changes of tumor clonal architecture over the time.
Material and Methods: 172 eligible patients enrolled with the frame of the MITO-16A/MaNGO-OV2A (EUDRACT number: NCT01706120) clinical trial, for whom plasma sample was collected before chemotherapy, were selected for the study. For each plasma sample, circulating-free DNA (cfDNA) was purified and an

untargeted shallow whole genome sequencing libraries prepared (HyperPlus, Roche). Libraries were sequenced (Illumina Sequencing System Platform, mean coverage 0.5X) and ichorCN algorithm (Adalsteinsson et al., Nature Communication) was used to infer the amount of ctDNA (Tumor Fraction, TF), as described by Paracchini et al., Clinical Cancer Research.

Results: In 168 out of 172 plasma samples analyzed, the TF was detectable and it ranged from 2.4% to 47%. To assess the prognostic relevance of ctDNA, TF percentages were initially correlated using Cox-model univariate analysis with patients' overall survival (OS) and Progression-Free Survival (PFS). Results indicate a significant association of TF levels with both OS and PFS ($p<0.001$). The prognostic relevance of TF was also confirmed in multivariate analysis, where, considering CA-125, residual tumor, stage of disease, histology and age as co-variables, TF maintained its statistical significance in PFS (HR = 2.1, CI 95%, p-value < 0.003).

Conclusion: These preliminary results confirm:

i) liquid biopsy as a feasible approach to intercept the total amount of ctDNA (Tumor Fraction) in
plasma samples

ii) TF levels calculated before chemotherapy treatment is a prognostic factor for HGS-EOC patients

Composite Biomarkers Using Liquid Biopsy

PP.57

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100069

CIRCULATING PD-L1 AND TIM3 AS PROGNOSTIC MARKERS IN PATIENTS WITH ADVANCED NON-SMALL CELL LUNG CANCER TREATED WITH IMMUNE CHECKPOINT INHIBITOR

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Introduction: Immune checkpoint blocks (ICBs) improved overall survival (OS) of patients with advanced non-small cell lung cancer (NSCLC). In addition to the PD1/PD-L1 pathway, other immune checkpoints (ICs) such as CTLA4, TIM3 and LAG3 are currently used or under evaluation as immunotherapeutic targets. Tumor PD-L1 expression is the only approved biomarker, but its predictive power is often unsatisfactory; therefore, reliable biomarkers are warranted. Here, we investigated the prognostic role of circulating forms of four ICs in patients with advanced NSCLC receiving ICB using an automated platform.

Methods: A single-center retrospective study was conducted on 183 patients with advanced NSCLC receiving first-line pembrolizumab alone (PA=98; Tumor PD-L1 ≥50%) or with chemotherapy (P + C=85; Tumor PD-L1 <50%). For each patient, we collected plasma samples at baseline and in 63 cases (PA=27; P + C=36) also at progression. Plasma levels of PD-L1, TIM3, LAG3 and CTLA4 were assessed by ELLA platform (Bio-Techne) using customized multiplex plates. Multivariable Cox regression analyses including gender, age, histology were performed using Stata software (v.17, StataCorp).

Results: Within our global population, 68% were men and median age was 70 years; adenocarcinoma was the most frequent histotype (83%). Higher circulating levels of PD-L1 (>101 pg/mL) and TIM3 (>1963 pg/mL) were independently associated with shorter OS (HR=3.49, 1.85-6.58 p-value<0.001 for PD-L1; HR=2.74, 0.50-4.98 p-value=0.001 for TIM3) and progression-free survival (PFS; HR=2.74, 1.58-4.75 p-value<0.001 for PD-L1; HR=2.05, 1.21-3.48 p-value=0.008 for TIM3) in patients treated with the combo. Notably, their combination identified a subset of patients (PD-L1&TIM3> cutoff values) with more than three-fold and five-fold risk of progression and death, compared to those in whom both biomarkers were below their cutoffs (p-value<0.001). Furthermore, both markers significantly increased upon progression after chemo + pembro (paired t-test p-value=0.001). Conversely, a significant association between LAG3 levels >674.5 pg/mL and longer PFS (HR=0.55, 0.34-0.89 p-value=0.014) was found in patients treated with anti-PD1 alone, but not with P + C.

Conclusion: PD-L1 and TIM3 emerge as prognostic markers in advanced NSCLC patients with low expression of tumor PD-L1. Validation of our cutoffs is currently ongoing in independent patient cohorts. If the test is validated, it could easily be transferred to the clinical setting.

PP.58

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100070

NETWORK ANALYSIS IDENTIFIES CIRCULATING MIR-155 AS PREDICTIVE BIOMARKER OF TYPE 2 DIABETES MELLITUS DEVELOPMENT IN OBESE PATIENTS: A PILOT STUDY

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Introduction: Obesity is the main risk factor for many non-communicable diseases. In clinical practice, unspecific markers are used for the determination of metabolic alterations and inflammation, without allowing early diagnosis or the characterization of subjects at higher risk of complications. Circulating microRNAs represent an attractive approach for early screening to identify subjects affected by obesity most at risk of developing connected pathologies. The aim of this study was the identification of circulating microRNAs able to identify obese patients at higher risk of type 2 diabetes (T2D).

Methods: Obese subjects without (n=12, OB) and with T2D (n=10, OBDM) and a cohort of healthy, age- and BMI-matched subjects (n=9) were recruited in the Policlinico Umberto I Hospital (Rome, Italy). Circulating microRNAs were isolated from whole plasma and extracellular vesicles and analysed with the miRNA v3 expression assay (Nanostring). The microRNAs identified by using the weighted gene co-expression network analysis (WGCNA) were analysed by means of RT-qPCR in a second cohort of OB (n=16) and OBDM (n=9) patients.

Results: The expression data of circulating microRNAs derived from OB, OBDM and healthy donors were combined with clinical data, through the network-based methodology implemented by the WGCNA. The six circulating microRNAs overexpressed in OBDM patients were evaluated in a second cohort of subjects, confirming the overexpression of miR-155-5p in OBDM patients. Interestingly, the combination of miR-155-5p with serum levels of IL-8, Leptin and RAGE was useful to identify OB patients most at risk of developing T2D.

Conclusion: These results suggest that miR-155-5p is a potential circulating biomarker for T2D and that the combination of this microRNA with other inflammatory markers in OB patients can predict the risk of developing T2D.

PP.59

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100071

COMPARISON OF TUMOR-ASSOCIATED MIRNAS IN EPCAM+, CD47+ AND CD29+ EXTRACELLULAR VESICLES (EVS) IMMUNOMAGNETICALLY ENRICHED FROM CANCER PATIENTS' PLASMA

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Background: EVs act in physiological and pathological conditions as mediators of intercellular communication by transporting a variety of molecular cargos. The emerging role of EVs, secreted by neoplastic cells, and miRNAs, in cancer progression makes them a promising diagnostic and prognostic tool in liquid biopsy. EVs-associated miRNAs are able to modulate oncogenic or tumor suppressor pathways, and several tumor-specific miRNA signatures have been identified. CD47 and CD29 are cancer-related markers expressed on EVs surface. Here we adapt a previously described target-based method to enrich patients' plasma EpCAM+, CD47+ and CD29+ EVs and investigate tumor-associated miRNAs in the obtained subpopulations.

Methods: Blood from n=7 stage III/IV cancer patients (n=4 prostate; n=3 breast) and n=3 healthy donors (HD) was collected in CellRescue™ tubes. Parallel enrichments of CTCs and plasma EpCAM+, CD47+ and CD29+ EVs were performed with CellMag™ Epithelial CTC Kit and custom ferrofluid-conjugated antibodies (MSB)1;2. CTCs were enumerated at CTAII®. Plasma EVs were visualized by Transmission Electron Microscopy and analyzed formiRNAs by Real-time PCR (TaqMan™ Advanced miRNA Human Serum/Plasma panel). A procedure for the functional analysis of tumor-associated miRNA transcriptional signatures is under development, aiming to: extract miRNA targets (miRTarbase; Ingenuity pathways analysis); identify miRNA-specific functional networks and

miRNA hubs associated with tumorigenesis; define a significance score of the association.

Results: One patient had CTCs (n=68). Extracellular microvesicles were observed at TEM in three samples enriched with all captures. Endogenous hsa-miR-16-5p was detected in five patients and two HDs, with lower abundance in EpCAM+ EVs. One control and two patients were screened for n=188 miRNAs. CD29 capture showed similar results to what observed with CD47 capture2: ≈50% of detection rate in both patients and HD EVs (versus ≈30% in patients and ≈17% in HD with EpCAM) and only ≤9% of patient-specific miRNAs (versus >50% with EpCAM). The three captures showed minimal overlap of patient-specific miRNAs.

Conclusions: Preliminary analysis on cancer patients' plasma EVs enriched with EpCAM-/CD47-/CD29- based captures indicates the presence of circulating EVs subsets having different abundance and carrying distinct miRNAs potentially involved in cancer development.

PP.60

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100072

LIQUID BIOPSIES: UNCOVERING INSIGHTS THAT DRIVE DEVELOPMENT AND COMMERCIALIZATION IN THE CLINIC

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Introduction: Liquid biopsies represent a groundbreaking technology with the potential to revolutionize cancer care. In the clinical setting, they offer promising applications such as early detection, personalized treatment guidance, and real-time monitoring of disease progression. While the widespread adoption of liquid biopsies for population-wide screening is still a subject of ongoing discussion, their use in selecting and monitoring patients receiving immunotherapy has gained traction, enabling the advancement of precision oncology. This shift is fueled by the increasing prevalence of a simple and non-invasive blood sample collection method, which renders traditional, painful, and potentially risky tissue biopsies less necessary. Consequently, next-generation sequencing (NGS)-based liquid biopsy testing options are rapidly being developed by commercial laboratories. Many of these laboratories are diligently pursuing regulatory approval, seeking companion diagnostics (CDx) claims through the Federal Drug Administration (FDA) in collaboration with their pharmaceutical co-developers. However, a major challenge for widespread acceptance lies in securing reimbursement from governmental and commercial payors, that is being actively addressed by these laboratories.

Methods: A comprehensive synthesis of qualitative research and analysis of quantitative survey data was collected during the American Society of Clinical Oncology (ASCO) annual meeting in June 2023.

Results: Key product specifications utilized in the development of liquid biopsies, along with technologies such as circulating tumor cells (CTCs), cell-free DNA (cfDNA), and circulating tumor DNA (ctDNA) are summarized. Additionally, the potential advantages and disadvantages of Next-Generation Sequencing (NGS)-based liquid biopsies, specifically in relation to their commercial use and application in clinical settings have been identified. The greatest disadvantage that liquid biopsies face, appears to be sensitivity. Furthermore, the survey found that ctDNA is the most often used technology and that 84% of respondents indicated that not having an FDA clearance is not a barrier for use in the clinic. Also, 80% of survey respondents indicated that market access and reimbursement are the greatest barriers to acceptance in the market.

Conclusion: This comprehensive analysis of the liquid biopsy market offers valuable insights into the product features and challenges that impact the progress and commercial viability of NGS-based liquid biopsies within the clinical setting.

PP.61

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100073

IMPRESS: IMPROVED MULTIPLEX METHYLATION PROFILING USING RESTRICTION ENZYMES AND SMMIP SEQUENCING FOR HIGHLY SENSITIVE MULTI-CANCER DETECTION

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Introduction: DNA methylation is an ideal candidate for cancer biomarkers. Despite recent advancements, a low-cost, bisulfite-free detection method for multiplex analysis is still needed. Therefore, we designed IMPRESS, combining methylation sensitive restriction enzymes (MSREs) and single molecule molecular inversion probes (smMIPs).

Methods: DNA is digested using four MSREs (HpaII, HpyCH4IV, AciI and HinP1I). As such, methylated CpGs remain intact, while unmethylated CpGs are cleaved. Then smMIPs that are designed for CpGs with ≥50% methylation in cancer and ≤20% methylation in normal samples and blood, capture the methylated targets. After multiplex PCR, purified pools are sequenced using NGS.

We validated IMPRESS by designing a multi-cancer detection assay for eight of the most lethal cancers worldwide. 1,791 CpG sites were first validated in 35 whole blood, 111 tumor and 114 normal fresh frozen tissue samples. A classifier model with 358 sites was built predicting tumor status for each sample. Lastly, characterization experiments were performed to prove utility in cfDNA.

Results: We present the successful development of our technology. Validation experiments proved the repeatability with a correlation coefficient of r= 0.99. The final classifier model to discriminate tumor from normal tissue samples had 95% sensitivity and 91% specificity. Comparison to ddPCR resulted in equal sensitivity and specificity of IMPRESS (cvAUC=0.92) and ddPCR (cvAUC=0.90) for tumor detection.

Importantly, we show the potential of the technology to use cfDNA from liquid biopsies as input samples. DNA input can be as low as 5 ng. It shows comparable results as inputs of 100 ng DNA. This low amount of DNA input makes our technique well suited for use in liquid biopsies. Normal cfDNA samples (5ng) were already successfully sequenced with IMPRESS. Lastly, we determined the detection limit using a dilution series from 100% methylated DNA to 0% methylated DNA. The limit was found at 2.29% ± 1.20%, indicating that we can successfully detect down to 2% DNA methylation.

Conclusion: In conclusion, we successfully created a low-cost, innovative DNA methylation detection technology. By combining IMPRESS with a new biomarker panel, we made a highly sensitive assay to discriminate tumor from normal tissue samples, with important potential for use in liquid biopsies.

PP.62

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100074

LIQUID BIOPSY CHARACTERIZATION OF CTCS AND THCS ISOLATED IN PATIENTS WITH UC

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Background: Urothelial cancer (UC) is one of the most frequent tumors worldwide. This oncological disease requires extensive follow-up due to its high recurrence rates. Advance and metastatic disease lowers the survival rate close to 6% and is the major cause of UC-related deaths. The presence of circulating tumor cells (CTCs) provides alternative mechanisms to better understand the metastatic process. In this context, the presence of CTCs and/or tumor hybrid cells (THCs) in blood has been correlated with worse prognosis for survival and resistance to conventional therapies. The existence of CTCs that, additional to being positive for EpCAM or CK, co-express CD45 and/or myeloid markers has been reported and defined as tumor hybrid cells (THCs).

Methods: We have generated THCs in vitro by co-culturing UC cell lines with human macrophages and evaluated their molecular features by flow cytometer and RT-qPCR. We had also carry out cytomic analysis of PBMCs of UC patient's treated with immunotherapy (IO) using Spectral Flow Cytometry and multiplex cytokine assay (Legenplex) in a longitudinal experimental design.

Results: The evaluation of UC THCs in vitro revealed an enrichment for stemness markers such as NANOG and SOX2. We also observed an increased expression of PDL1. In order to find out whether these cells correlate with clinical parameters and to assess changes in the immune landscape of patients treated with BCG and BCG+IO we evaluated 15 surface markers in blood samples form patients at different time points. The analysis reveals the presence of this circulating cells. The correlation of these cells counts with the response to therapy as well as the immune modulation followed by the cytokine profiling will be discussed.

Conclusions: Liquid biopsy have proven to be the best way to obtain a general

picture of tumor behaviour and we have seen that combining soluble biomarkers to CTCs and THCs could be a better way to obtain more clinically relevant information.

PP.64
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100076
CHARACTERIZATION OF PLASMA DERIVED CELL FREE NUCLEIC ACID-BASED MARKERS FOR DISEASE MONITORING IN ACUTE MYELOID LEUKEMIA

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Introduction: The aim of this study was to strengthen the diagnostic utility of circulating tumor DNA (ctDNA) in identifying genetic alterations in Acute myeloid leukaemia (AML). In parallel, immunological phenotypes of AML patients at diagnosis are an important determinant of outcomes to standard-of-care therapies. An attempt to identify cell free RNA based biomarkers in conjunction with genetic alterations in ctDNA as a dual pronged minimally invasive approach was explored.
Methods: Bone marrow (BM) and peripheral blood (PB) were collected from de novo AML patients (n=10) at the time of initial diagnosis. Genomic DNA (gDNA) was isolated from BM-MNCs and ctDNA was isolated from matched PB. Detection of mutation was performed on ctDNA and gDNA by targeted NGS based on 141-gene panel. IDH1/2 mutations were validated in both ctDNA and gDNA by ddPCR. In parallel, RNAseq was performed to identify immune signatures and data analysis was performed using appropriate softwares.
Results: Of the 141 genes sequenced by the NGS platform, similar profiles of mutations were obtained in gDNA and ctDNA with a high concordance of VAF assessment (r=0.97; p value=0.00002). Five samples harboring IDH1 R132H/IDH2 R140Q tested positive by ddPCR with implications in targeted therapy and AML disease monitoring. RNAseq analysis showed significant alteration of gene expression in 73 gene targets. Majority of the downregulated genes (n=58) were involved in TCR signaling pathways suggesting an overall immunosuppressive BM microenvironment. Of the top upregulated genes, guanylate binding protein 4 (GPB4) transcripts were significantly upregulated in all the samples whereas HOPX, regarded as a stem cell marker, was altered in few cases.
Conclusion: Our study demonstrated that ctDNA is a reliable sample in AML and could complement mutation detection in the BM. CtDNA based genotyping upfront may facilitate selection of patients for targeted therapies and AML disease monitoring. In the absence of a consensus on T-cell functional status in AML, immunological phenotypes of AML patients at diagnosis might further stratify these patients. The diverse biological profiles of AML patients might predict the efficacy and design of novel prospective trials and contribute to better clinical outcomes.

Liquid Biopsy Beyond ctDNA

PP.08
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100077
COMPREHENSIVE ANALYSIS OF PREANALYTICAL PARAMETERS IN CFDNA FROM LUNG, COLON, AND BREAST CANCER PATIENTS

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Introduction: Circulating tumor DNA refers to the portion of cfDNA drained from cancer cells, with less than 146 bp in length, and represents 10-0.01%. Its detection requires a technology that provides adequate precision and sensitivity. The project was 80% funded by FEDER and 20% by the Ministry of Science and Innovation/Andalusian Health Service.
The study aims to analyze ctDNA preanalytical parameters in lung, colon, and breast cancer in early and metastatic stages.
Methods: Ninety samples of 30 mL of blood were recruited in EDTA tubes to perform NGS analysis, 15 samples per stage and tumor, 86% of the samples from University Hospital Virgen de las Nieves (Granada, Spain), and 14% from University Hospital Virgen del Rocio (Seville, Spain) origin. The sample was processed to obtain plasma within 4 hours of extraction (20 - 22ºC) in darkness to avoid hemolysis. The first centrifugation was performed for 10 min at 1800g to separate the plasma from the cell fraction of the blood. The second centrifugation was performed at 16000 g for 10 min to remove all cellular debris. The cfDNA was purified with Maxwell® RSC System (cfDNA LV kit) and quantified with TapeStation 2200 Agilent (High Sensitivity D1000 kit) and Invitrogen™ Qubit (dsDNA HS Assay Kit).
Preanalytical variables were analyzed to find differences between early primary and metastasis tumors as regards sex, age, blood, and plasma volume, using a two-way ANOVA and posterior Fisher's Least Significance Difference (LSD) over significant variables.
Results: Figure 1 shows the preanalytical variables LSD test result. Most differences are found at the tumor level, but the cancer stage favors the total input at the metastatic compared to early stage.
Conclusion: We found a relationship between Plasma/Blood ratio/concentration and tumor that impacts the total input for NGS where colon > lung > breast and metastasic > early.

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	Factor/Variable	Age	Plasma/Blood	Tape DNS HS-D1000 (ng/ul)	Tape DNA 100-300pb HS-D1000 (%)	Mean Fragment Size (bp)	Total Input (ng)
1	Tumor	0.212	0.012	0.001	<0.001	0.036	0.008
2	Stage	0.657	0.163	0.525	0.881	0.812	0.019
3	Tumor*Stage	0.010	0.413	0.416	0.045	0.094	0.144

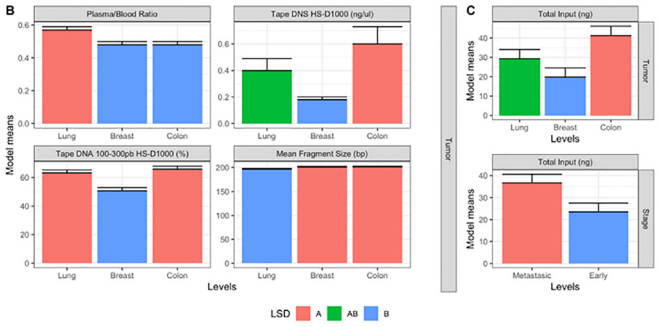

Figure 1: Fisher's Least Significant (LSD) Test Results. A) Test result table where each cell shows the multiple comparison-adjusted False Discovery Rate (FDR). B) and C) presents the model-adjusted means with the standard error bars, for the experimental factor (tumor or stage) where the LSD group assignment (A, AB, or B) is in colors. The means that share a letter are not different (FDR > 0.05).

PP.65
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100078
OPTIMIZATION OF CTCS AND THCS ISOLATION FROM HUMAN BLOOD BASED ON GENESIS TECHNOLOGY AS A STRATEGY OF UROTHELIAL CARCINOMA MONITORING

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Urothelial cancer (UC) is the ninth most common cancer worldwide, accounting for more than 200,000 deaths annually. Current treatments for UC pose a clinical challenge due to high recurrence rates, incompatibility with conventional treatments due to patient co-morbidities, and the absence of effective alternative therapies. Therefore, it is necessary to search for new therapies that are effective for patients, as well as early diagnostic and prognostic methods that can detect

the disease early and follow its development. It has been demonstrated that Circularting Tumor Cells (CTCs) and Tumor Hybrids Cells (THCs) serve as a prognostic tool and could be a method of monitoring the disease. The limited presence of this type of cells in the blood makes it difficult to obtain, study and characterize them.

By using the Genesis System (BioRad), a new technology that allows to separate CTCs and THCs from the rest of the blood components by size, we are able to carry out cellular characterizations, which allow us to differentiate and define the types of CTCs and THCs presented in patient's blood.

To reach this main objective, we are optimizing this technology to be able to use its full potential. Thus, we have isolated tumor cells from UC cell lines with GFP expression spiked in blood of healthy donors. We have been able to successfully isolate these cells with a recovery rate of 80%, being able to establish viable cultures after separation. In addition, these tumor lines when co-cultured with macrophages isolated from the peripheral blood, using Ficoll-Paque gradient, form cell fusions that define viable in vitro THCs cells. In this way, we will evaluate this technology for effectively recovered THCs by the Genesis System.

In conclusion, the optimization of CTCs and THCs isolation will allow us to effectively characterize and culture both cell types, which is essential to evaluate their correlation to disease prognosis and possible new targeted therapies.

PP.66

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100079

SEE.D®, A NOVEL AUTOMATED PLATFORM TO STANDARDIZE BLOOD SAMPLE PROCESSING FOR CELLS ON SBS® SLIDES AND PLASMA FOR LIQUID BIOPSY

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Introduction: The pre-analytical phase of liquid biopsy for cancer detection is not standardized among different laboratories, causing around 50% of analytical errors in diagnosis. The current clinical practice relies mostly on chemically stabilized blood samples shipped to clinical laboratories, risking sample degradation or manual errors in blood processing.

Tethis launches See.d®, a pre-analytical platform for standardized and automated preparation of fresh blood samples at the point of collection.

Methods: Fresh whole blood samples from 20 healthy donors (HDs) were collected in 10 mL K2EDTA tubes and automatically processed by See.d® obtaining two outputs: a) plasma collected in a dedicated vial and b) whole White Blood Cells (WBCs) fraction adhered and fixed on proprietary nanocoated SBS® slides.

Genomic contamination and cfDNA quality of plasma samples were analysed, as well as different quality parameters of WBCs fraction upon bright field and immunofluorescence staining.

The analytical performance of See.d® was evaluated by spiking into HDs' blood both reference synthetic DNA and in vivo labelled model cancer cells.

Results: Each blood sample tube produces 2.3 mL of plasma and 10 SBS® slides containing a monolayer of around 2.5x106 WBCs each.

Compared to a manual standard protocol, plasma from See.d® allowed to extract an equivalent amount of cfDNA/ml of plasma, with negligible contamination of genomic DNA, even after storage and shipment-mimicking conditions. Moreover, we reported a comparable recovery of spiked synthetic DNA between See.d® produced plasma and manual one.

SBS® slides provided by See.d® showed high cell adhesion efficiency and homogenous distribution. The intact cellular morphology and optimal hematopoietic staining quality were preserved also after slides storage and shipment mimicking. Moreover, we observed a high recovery rate of spiked cancer cells from See.d® processing compared with manual one.

Conclusion: See.d® automates and standardizes the preanalytical steps of liquid biopsy in blood samples: cells on SBS® slides and plasma are stable and can be shipped to analytical laboratories for multianalyte liquid biopsy assays (CTCs counting, cellular and soluble biomarkers, cfNA etc.). Tethis is currently performing studies for the introduction of the platform in specific clinical applications (i.e., metastatic breast cancer, prostate cancer) and its IVDR certification.

PP.67

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100080

EXTRACELLULAR VESICLES CARRY SARS-COV-2 PROTEINS ASSOCIATED WITH LOWER ANTIBODY PRODUCTION, CARDIOVASCULAR COMPLICATIONS, AND WORSE OUTCOMES IN PATIENTS WITH SEVERE COVID-19

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Introduction: Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) which has been associated with almost 7 million deaths and is still classified as a worldwide pandemic. Severe COVID-19 cases were associated with elevated IL-6, thus, the anti-IL6 inhibitor Tocilizumab was evaluated 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, being potentially used as biomarkers.

Methods: Patients with severe COVID-19 were enrolled in this retrospective study, approximately two-thirds received Tocilizumab vs. one-third considered controls. Blood samples were collected at hospitalization before treatment, at day 7, and day 15. Overall survival (OS) was evaluated at 30 days after discharge. EVs were isolated by ultracentrifugation and dynamic expression of spike (S) 1, S2, and nucleocapsid (N) proteins was evaluated by western-blotting and normalized against CD9. Plasma antibody levels of spike S1, S2, N, and receptor binding-domain (RBD) were measured by ELISA. Additionally, EV expression of S2 and CD9 were evaluated with the Amnis Imaging flow-cytometer and by dual immunogold staining.

Results: Thirty-four patients were enrolled, from which 29 had longitudinal blood collection 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, revealing an enrichment in CD9 + EVs. Tocilizumab was administered to 21 patients, which showed a reduction in EV SARS-COV-2 proteins in comparison to 13 controls, but did not improve the OS. However, increased EV S2 at day 7 was associated with shorter OS (HR=1.81, p=0.028) and decreased RBD, S1, and S2 antibody titers. Increased EV SARS-COV-2 proteins also correlated with elevated CD8 + cells and coagulation and cardiac abnormalities such as high fibrinogen, C reactive protein, systolic and diastolic pressure, or longer QTC prolongation.

Conclusion: Plasma EVs from patients with COVID-19 contain SARS-COV-2 proteins that could reveal poor outcomes and cardiovascular complications. These results suggest that EVs could 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.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100082

CIRCULATING TUMOR CELLS OF NON-SMALL CELL LUNG CANCER (NSCLC) PATIENTS. A POTENTIAL NEW LIQUID BIOPSY BIOMARKER

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Introduction: Protein O-glycosylation abnormalities are found in most carcinomas. Several clinically useful biomarkers detecting these changes have been developed. Synthesis of mucin-type O-glycans starts in a reaction catalyzed by a complex enzyme family (UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferases; GalNAc-Ts), which has at least 20 members in humans. We previously found that GalNAc-T13 isoenzyme is a strong predictor of poor clinical outcome in neuroblastoma(1) and breast cancer patients(2). Given that GalNAc-T13 is also expressed in NSCLC(3), this work aimed to evaluate GALNT13 expression in circulating tumor cells (CTC) as a new potential liquid biopsy biomarker in NSCLC patients.
Methods: GALNT13 expression in CTC was evaluated by a nested RT-PCR method in 119 NSCLC patients from Hospital Maciel, Montevideo. Results were correlated with clinical-pathologic data and patients' survival.
Results: No expression of GALNT13 was found in 20 healthy individuals' samples and the sensitivity of the method was established at 0.01%. GALNT13-positive CTCs were found in 72/119 (60.5%) lung cancer patients. Concerning histological types, GALNT13 expression was detected in adenocarcinoma patients (36/62; 58.1%), as well as squamous cell carcinomas patients (20/33; 60.6%). Relating to stages, the enzyme was found in 13/19 (68.4%) of patients with stages I/II, 16/33 (48.5%) of stages IIIA/IIIB, and 43/69 (62.3%) of stages IIIC/IV. In this preliminary evaluation, we attained three years of follow-up. In early-stage patients (I/II) 6/13 (46.1%) of GALNT13-positive died, in contrast to none of the negative ones (0/6). In intermediate stages (IIIA/IIIB) 10/16 (62.5%) of GALNT13-positive and 8/17 (47.1%) of negative patients died. Finally, in advanced stages (IIIC/IV) death occurred in 31/43 (72.1%) of GALNT13-positives and 20/24 (83.3) of negative patients.
Conclusion: In this study, we suggest that GALNT13 expression in CTC could be a potential new biomarker in liquid biopsy for lung cancer patients.
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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100083
PROSPECTIVE ON-TREATMENT DYNAMICS OF CIRCULATING EXTRACELLULAR VESICLES IN THE FIRST-LINE SETTING OF PATIENTS WITH ADVANCED NON-SMALL CELL LUNG CANCER

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The interrogation of extracellular vesicles (EVs) content might be complementary for response assessment. We aimed to describe whether the serial characterization of EVs could longitudinally reflect response and resistance to first-line treatments in advanced NSCLC.
From February 2020 to May 2022, treatment-naïve patients with advanced NSCLC receiving osimertinib, alectinib or pembrolizumab ± platinum-based chemotherapy were prospectively enrolled at the University Hospital of Palermo, Italy. Blood samples were collected into K2 EDTA tubes early in the morning with fasting required. We isolated EVs from plasma using either ultracentrifugation or exoEasy Maxi Kit (Qiagen, USA). EVs were characterized by using either Western Blot (WB) or Dynamic Light Scattering (DLS) to assess the size distribution and the number of vesicles (R90) or Bradford assay for the quantification of protein content (cfEV). According to the radiologic response, cfEV and R90 kinetics were evaluated in patients from baseline (T0) to the first radiologic restaging (T1). A 20% cfEV and R90 increase were used as the cutoff point for median progression-free survival (mPFS) analysis.
Among 27 consecutive patients with no other medical conditions or concomitant medications, isolated EVs were first characterized via WB revealing CD9 and Alix. Secondly, EVs were determined via DLS showing an average size ranging from 183 to 260 nm. Thirdly, the mean cfEV value at T0 and at T1 time was 1.26 and 1.49 μg/ml, respectively (p=0.021). Within the cfEV responsive group, 13 patients had improved mPFS (25.2 months, 95% CI: 14.9–35.5) when compared to 11 cfEV non-responders (8.3 months, 95% CI: 3.6-12.9)(p=0.07). Namely, cfEV responders receiving single-agent pembrolizumab experienced a statistically improved mPFS (25.2 months, 95% CI: 11.7-38.8; p=0.04) compared to patients receiving pembrolizumab plus chemotherapy (6.1 months, 95% CI: 1.1-11.1; p=0.9). EGFR-positive cfEV responders showed an improved mPFS (35.1 months, 95% CI: 14.9–35.5) as compared to cfEV non-responders (20.8 months, 95% CI: 11.2–30.4) (p=0.06). In the EGFR-mutated subgroup, four patients with R90 decreasing levels are still responding whereas one patient with R90 increasing level had a rapidly progressive disease.
The quantitative changes of EVs correlated with survival in the real-time monitoring of first-line patients, mostly in patients receiving single-agent pembrolizumab.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100084
TOTAL RNA-SEQUENCING FROM PLASMA CELL-FREE RNA TO IDENTIFY PREDICTIVE/PROGNOSTIC BIOMARKERS IN METASTATIC CASTRATION-RESISTANT PROSTATE CANCER PATIENTS

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Introduction: Metastatic castration-resistant prostate cancer (mCRPC) is a heterogeneous disease currently lacking an optimal patient selection strategy and a validated sequence of therapies. Therefore, there is an urgent need to identify non-invasive biomarkers to guide treatment selection for personalized medicine. RNA profiling has emerged as a tool to investigate potential liquid biopsy biomarkers in human biofluids. In this study, we evaluated a method to capture total RNA content deriving from plasma and identify potential biomarkers for mCRPC.
Methods: Plasma cell-free RNA purification was performed using miRNeasy Serum/Plasma Advanced Kit (Qiagen). DNase treatment and ethanol precipitation were performed for all samples. Sequencing libraries were generated using SMARTer Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian (Takara Bio). A low reads depth sequencing was used for quality check of RNA content and to assess feasibility of the approach. 75bpx2 sequencing was performed on NextSeq 550 platform (Illumina).
Results: Twenty-nine mCRPC plasma samples collected and stored between 2017 and 2022 and 4 healthy donors as control were sequenced. The mean concentration of extracted RNA was 498 pg/μl with a mean RNA Integrity Number of 2. No difference in RNA yield and profile was observed between long (2017) and short-term storage samples (2022). A mean libraries concentration of 32 ng/μl was obtained with an average size of 300 bp. On average, 16M reads were produced, obtaining a mean of 6.8M unique reads per sample. The mean of reads uniquely mapping on hg19 was 20%. Among these, 16% were mRNA, 11% intronic, 21% intergenic. Ribosomal RNA was not totally removed, with a mean of 29% of reads remaining in the samples.
Conclusion: Despite enormous interest in extracellular nucleic acids, RNA-sequencing methods for total RNA content outside cells are rare and not standardized. In this study, we found a method to identify plasma non-invasive RNA biomarkers in mCRPC samples, retrieving coding and non-coding RNAs. Case series enlargement and differential gene expression analyses are ongoing.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100085
FULLY AUTOMATED CELL-FREE DNA EXTRACTION FROM UP TO 8 ML PLASMA ENABLES SENSITIVE MUTATION DETECTION WITH DIGITAL PCR AND NGS

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Cell-free DNA (cfDNA) is extracellular DNA found in body fluids, crucial for cancer research, but its low abundance requires efficient purification. The new EZ1&2 ccfDNA process uses magnetic beads to purify cfDNA from 1-8 ml human plasma in 35-75 minutes without pre-treatment. Up to 24 samples can be processed simultaneously, providing eluates ready for downstream applications like

qPCR, dPCR, fragment length analysis, and NGS.
To evaluate its performance, healthy donor plasma was processed using the EZ1&2 ccfDNA Kit. cfDNA yield and quality were assessed using Qubit, qPCR, dPCR, and fragment length analysis, demonstrating efficient extraction without inhibitory effects. The kit's ability to detect variants with allele frequencies (VAF) below 0.5% was confirmed using dPCR. Additionally, the new QIAseq Targeted cfDNA Ultra Panel was tested with eluates from the EZ1&2 ccfDNA kit, successfully detecting low VAF using NGS.
Results showed that the EZ1&2 ccfDNA Kit efficiently isolated ccfDNA from 1-8 ml plasma with elution volumes of 75 µl or 40 µl, while maintaining compatibility with various analytical methods like Qubit, qPCR, dPCR, fragment length analysis, and NGS. The fully automated extraction method exhibited equal or superior performance compared to alternative methods, enabling the detection of VAFs as low as 0.1% in dPCR and NGS.
In conclusion, the novel fully automated system offers a robust and rapid cfDNA extraction solution, processing up to 24 samples in just 35 minutes. Its performance matches or surpasses alternative methods, particularly in detecting rare mutations, making it a valuable tool for research applications.

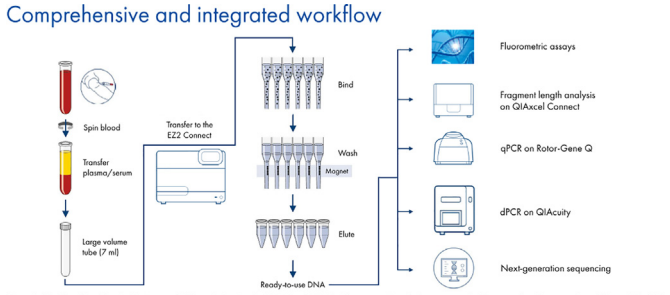

Figure 1. Workflow from blood collection to cfDNA analysis using the EZ1&2 ccfDNA Kit. After generation of plasma, up to 4 ml are transferred into one Large Volume Tube (LVT; 2 LVTs with 4 ml each used for 8 ml protocol) and cfDNA is automatically extracted on the EZ2 Connect instrument, ready-to-use for downstream analyses like fluorometric quantification, fragment length analysis, qPCR, dPCR and NGS applications.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100086
DEVELOPMENT OF AUTOMATED CFDNA EXTRACTION FROM VARIOUS PLASMA VOLUMES AND IMPLICATIONS ON SENSITIVITY AND REPRODUCIBILITY OF QPCR AND DPCR ANALYSIS

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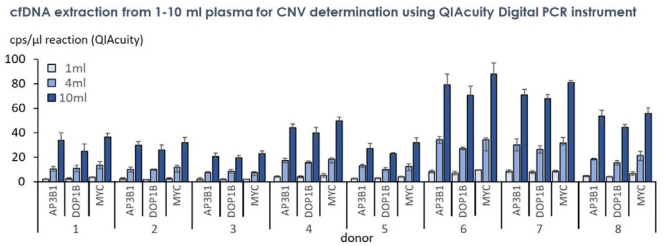
Introduction: Cell free DNA (cfDNA) is a key analyte for liquid biopsy samples. Due to the very low concentrations of cfDNA found in plasma, sensitivity of the downstream applications and its implication for the result reporting are of highest importance. Here, we analyzed cfDNA from 1 ml liquid biopsy samples for RHD detection In addition, we detected copy number variations (CNVs) in cfDNA samples using QIAcuity Digital PCR (dPCR) platform with sample input volumes ranging from 1 ml to 10 ml.
Methods: CfDNA was extracted using an automated bead based method on the QIAsymphony SP instrument using 1 ml to 10 ml input volume range. The extracted cfDNA was analyzed by qPCR for a house keeping gene and RHD (Devyser) as well as by dPCR for different CNVs using the QIAcuity dPCR instrument.
Results: Use of a dedicated kit for cfDNA extraction and subsequent qPCR assays allow RHD determination from 1 ml plasma. dPCR provided highly sensitive and reproducible detection of CNV in samples independent of plasma extraction volumes using dPCR. In parallel, linearity in cfDNA recovery for different plasma input volumes ranging from 1-10 ml as well as linearity in cfDNA detection by dPCR for different template loading volumes was shown.
Conclusion: Standardization of pre-analytical workflows for downstream applications with a clinical implication shall be considered to ensure reliable and reproducible results as well as improved sensitivity for cfDNA analysis from liquid biopsies.



PP.74
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ANALYSIS OF IMMUNOREGULATORY GENES EXPRESSION IN PBMCS AS POTENTIAL PREDICTIVE AND PROGNOSTIC BIOMARKERS IN NSCLC PATIENTS TREATED WITH IMMUNOTHERAPY

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Introduction: Given the need for accurate biomarkers to identify Non-Small Cell Lung Cancer (NSCLC) patients who will benefit from treatment with immunotherapy, we have analysed the expression of 8 immunoregulatory genes in peripheral blood mononuclear cells (PBMCs). From these genes, we would like to highlight that we have previously described the prognostic value of CD5 and CD6 expression levels in early-stage NSCLC patients (https://doi.org/10.21037/tlcr-19-445).
Methods: In this study we have included 52 advanced-stage NSCLC patients treated with first-line anti-PD1 alone or in combination with chemotherapy (anti-PD1 + QT) and 13 healthy controls. We have analysed by RTqPCR the expression levels of CD5, CD6, CD247, CD4, CD8, PD1, PDL1 and PDL2 in PBMCs from pretreatment samples and samples taken at first response assessment (FR). Statistical analyses were conducted using Rstudio.
Results: It was found that healthy controls presented higher expression levels of CD5 and CD6 ($p<0.001$ in both cases) than NSCLC patients.
We analysed the predictive and prognostic role of these genes according to treatment:
Regarding NSCLC patients treated with anti-PD1 in monotherapy (n=28), we found that higher expression levels of CD5 and CD6 at FR were associated with durable clinical benefit (DCB, p=0.008 and p=0.014, respectively), and improved progression-free survival (PFS, p=0.040 and p=0.041, respectively). Regarding changes produced during treatment, we calculated ratios of gene expression between FR and pretreatment samples and found that patients with DCB increased the expression levels of CD5, CD6 and CD8 in PBMCs throughout treatment (p=0.045, p=0.029, and p=0.032, respectively).
In the subgroup of patients treated with anti-PD1 + QT (n=24), higher pretreatment expression levels of PDL1 were associated to improved PFS (p=0.044) and a tendency to improved overall survival (OS, p=0.086). Furthermore, higher pretreatment expression levels of CD247 were associated to DCB (p=0.035) and a tendency to improved prognosis (PFS, p=0.112 and OS, p=0.044).
Conclusion: These results highlight the potential of PBMCs as a useful tool to assess immunoregulatory genes expression levels as non-invasive predictive and prognostic biomarkers for NSCLC patients treated with immunotherapy alone or in combination with chemotherapy.
Study supported by AECC Valencia Scientific Foundation, CB16/12/00350 and PI18/00266 (ISCIII) and AICO/2021/333 (GVA).

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100088

TCR-β REPERTOIRE AS A SOURCE OF BIOMARKERS FOR ANTI-PD1 TREATMENT ALONE OR IN COMBINATION WITH CHEMOTHERAPY IN ADVANCED-STAGE NSCLC PATIENTS

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Introduction: Despite immunotherapy has clearly improved Non-Small Cell Lung Cancer (NSCLC) patient outcomes, there is still a lack of efficient biomarkers to identify which patients will respond to treatment. In this study we have characterized peripheral T-cell receptor β chain (TCR-β) repertoire as a tool to identify predictive and prognostic biomarkers for NSCLC patients treated with immunotherapy.

Methods: Our study included 52 advanced NSCLC patients treated with anti-PD1 alone or in combination with chemotherapy (anti-PD1 + QT) in the first-line setting, from which PBMCs have been collected pre-treatment (PRE) and at first response assessment (FR). Additionally, PBMCs from 12 healthy controls were also analysed. TCR-β libraries were prepared starting from Peripheral Blood Mononuclear Cells (PBMCs) with Oncomine TCR-β SR (RNA) assay, sequenced on Ion GeneStudio S5 Series and analysed using Ion Reporter Software (Thermofisher Scientific). Statistical analysis was conducted on Rstudio.

Results: It was found that healthy controls present a higher number of clones and Shannon diversity index than NSCLC patients ($p<0.001$ and $p=0.046$).

In the subgroup of patients treated with anti-PD1, patients who present durable clinical benefit (DCB) have a higher frequency of certain V and J genes. As an example, higher frequency of TRBV20-1 in PRE and 1E samples was associated to DCB ($p=0.006$ and $p=0.029$), and a tendency to improved progression-free survival. In addition, patients with DCB presented a higher number of shared clones between FR and PRE samples ($p=0.023$), as well as an increase in the number of clones and higher frequency of convergent TCR in FR than in PRE samples ($p=0.005$ and $p=0.029$).

In the subgroup of patients treated with anti-PD1 + QT, higher frequency of TRBV4-1 in PRE and FR samples correlated to DCB ($p=0.041$ and $p=0.030$), and a tendency to improved prognosis.

Conclusion: These results support the use of peripheral blood TCR-β repertoire as a tool to assess and monitor NSCLC patient response to immunotherapy. Furthermore, we have found an increased frequency of certain V and J genes in patients responding to treatment, which might be due to clonotypes recognizing tumour-antigens.

Study supported by AECC Valencia Scientific Foundation, CB16/12/00350 and PI18/00266 (ISCIII), and AICO/2021/333 (GVA).

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100089

YES1 AS A POSSIBLE LIQUID BIOPSY BIOMARKER IN LUNG CANCER

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Introduction: Liquid biopsy is currently used to detect mutation profiles in non-small cell lung cancer (NSCLC) patients with the goal of monitoring response to tyrosine kinase inhibitors (TKIs). In small-cell lung cancer (SCLC), the clinical utility of liquid biopsy is still under investigation. "Omic" techniques have identified novel potential targets in both NSCLC and SCLC, and liquid biopsies could help in the monitoring of circulating levels of such targets. YES1 is a non-receptor tyrosine kinase belonging to the Src family, overexpressed in both NSCLC and SCLC, which promotes tumor growth and metastasis. Inhibition of YES1 with the multikinase inhibitor dasatinib or with more specific drugs results in tumor growth inhibition. In the present study we have analyzed expression of YES1 in plasma-derived exosomes from NSCLC and SCLC patients and mouse models.

Methods: Blood samples from lung cancer patients or healthy donors were used. Exosomes were isolated by untracentrifugation and characterization was performed by Western blots using exosomal markers and electron microscopy. Animal models included the H209 SCLC cell line implanted into nude mice.

Results: We were able to detect YES1 protein in plasma-derived exosomes from NSCLC and SCLC patients, where significantly higher levels were observed in comparison with healthy individuals. The AUROC curves were 0.75 ($p=0.002$) and 0.66 ($p=0.02$) for NSCLC and SCLC patients, respectively. Exosomal YES1 levels were significantly correlated with tumor size ($p<0.05$), YES1 tumor expression and YES1 tumor gene amplification in NSCLC ($p<0.01$). High exosomal YES1 levels were significantly associated with worse progression-free survival (PFS) ($p<0.05$) in SCLC patients and values were close to significance in NSCLC patients. In a small cohort of SCLC, YES1 protein was found in circulating tumor cells (CTCs), suggesting that YES1 protein could also be a biomarker in CTCs. In NSCLC and SCLC animal models, exosomal YES1 levels were associated with tumor growth and were reduced upon treatment with YES1-targeted drugs. We will also present data on the functional role of YES1 in plasma exosomes, using preconditioning metastatic assays.

Conclusions: Although these findings need validation in alternative cohorts, YES1 could serve as a liquid biomarker in lung cancer, in particular to monitor response to Src-related drugs.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100090

USE OF CIRCULATING EXTRACELLULAR VESICLES AND CTDNA FROM LYMPHATIC FLUID EXUDATE AS MARKERS FOR MINIMAL RESIDUAL DISEASE IN MELANOMA PATIENTS

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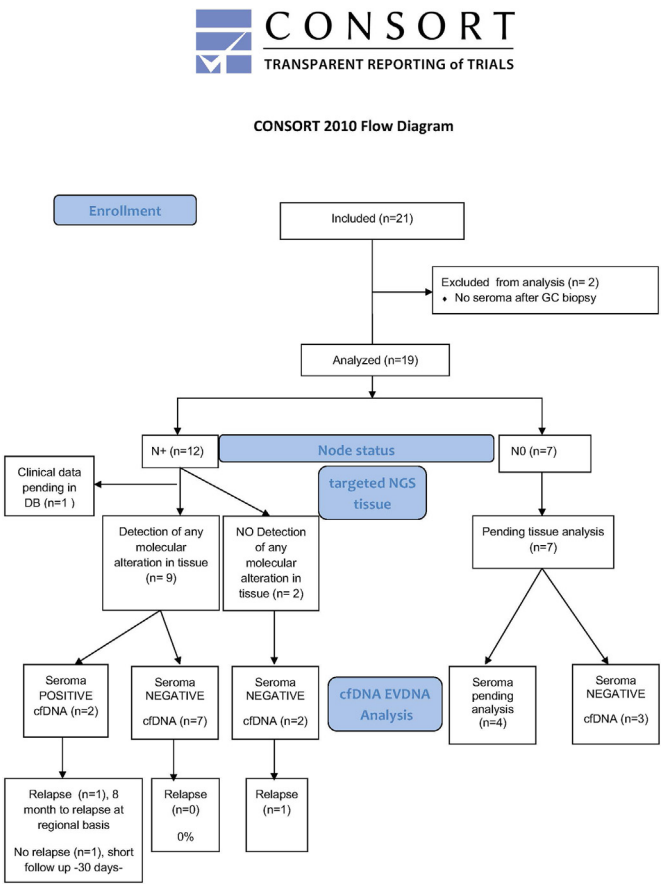
The aim of this study was to evaluate the potential of analyzing circulating extracellular vesicles (EVs) and circulating free DNA (cfDNA) from lymphatic fluid exudate as surrogate markers for minimal residual disease in melanoma patients who underwent lymphadenectomy. The study compared the pros and cons of using EVs and cfDNA from lymphatic fluid exudate with those of using plasma samples. Methods involved the purification of cfDNA and nucleic acids from EVs using a differential ultracentrifugation protocol or filtration kits using EDTA tubes-derived plasma. Molecular analysis was conducted through targeted Next Generation Sequencing (NGS) on primary tumors, cfDNA and EVs obtained from plasma and seroma samples of stage III melanoma patients who had undergone lymphadenectomy.

Results showed that the DNA pattern in plasma was smaller in size compared to seroma, but the total yield was higher in seroma. However, neither fraction showed detectable RNA. Out of the 19 enrolled patients, regional recurrence occurred in two cases, with one showing molecular disease detection (BRAFV600R) in the seroma sample, while mutations were not detected in the other case using our customized panel. Seven patients who tested negative for molecular detection in seroma remained disease-free. One patient tested negative for mutations in both seroma and plasma, but had no relapse after 3 months of follow-up.

These findings suggest that the presence of tumor mutations in seroma-derived EVs or cfDNA obtained post-lymphadenectomy could predict a higher risk of relapse in melanoma patients. Importantly, detection of BRAF mutations in EV-DNA and cfDNA from seroma exhibited similar sensitivity supporting that both fractions are equally useful in this study. Recruitment for the study will continue, and patients will be followed up for a minimum of 24 months or until melanoma relapse occurs.

We would like to express our gratitude to the Grupo Español Multidisciplinar del Melanoma (GEM) for providing funding for this project. Additionally, we extend our heartfelt appreciation to the professionals, hospitals, and patients involved, who generously contributed their time and participation, making this project possible.



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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100091

PULMONARY MICRORNA PROFILING IN A RAT MODEL OF VENTILATOR-INDUCED LUNG INJURY

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Introduction: Diffuse alveolar damage (DAD) is the histological correlate of the acute respiratory distress syndrome (ARDS). MicroRNAs (miRNAs) regulate gene expression and have been described in different models of acute lung injury. The objective was to determine if DAD development is associated to a specific phenotype and miRNA expression profile in a rat model of ventilator-induced lung injury (VILI).

Methods: Male Sprague–Dawley rats were anesthetized and ventilated for 2.5 h using two ventilatory strategies: 1) protective strategy with low tidal volume (Vt, Vt= 9 ml/kg; PEEP=5 cm H_2O, n= 10), and 2) injurious strategy with high tidal volume (Vt= 25ml/kg; PEEP= 0 cm H_2O, n = 16). We assessed pulmonary histological changes, gas exchange, inflammation in serum and lung (ELISA), apoptosis (TUNEL), and alveolo-capillary permeability in the lung (IgM and total protein in bronchoalveolar fluid, BALF). We measured miRNA expression in rat lungs by NGS and performed a pathway enrichment analysis by REACTOME and miRNET network. Statistical analysis: one-way ANOVA and pearson correlation, p value < 0.05 was considered statically significant.

Results: Seven of 16 rats in the high Vt group and none in the low Vt group developed DAD. Compared with rats without DAD, rats with DAD showed: 1) higher peak inspiratory pressure (PIP) and lower compliance 2) lower PaO_2/FiO_2 ratio; 3) increased concentration of cytokines in lung tissue and serum, 4) enhanced alveolo-capillary permeability; (5) increased apoptosis and lower caspase-3 activity in lungs; and (6) different miRNA expression profile in the lungs ; (7) miR 132-5p expression correlated with some cytokines concentration in serum and lungs, total protein in BALF, caspase-3 activity in lungs and PIP at the end of the experiment. Pathway enrichment analysis showed that the miRNAs differentially expressed were involved in pathways related to the immune response, inflammation, and apoptosis.

Conclusions: The DAD phenotype was characterized by higher PIP, impairment of oxygenation, acute systemic and pulmonary inflammation, increased permeability and apoptosis in the lung, and differential miRNA expression in the lung as compared to those without DAD. The miRNAs differentially expressed between groups are involved in pathways relevant in the pathophysiology of VILI and ARDS.

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AN END-TO-END CFRNA-SEQ PIPELINE FOR EARLY CANCER DETECTION FROM LIQUID BIOPSY

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Introduction: Liquid biopsies have become increasingly important diagnostic tools for early cancer detection due to their high sensitivity and minimal invasiveness.

Plasma cell-free RNA (cfRNA) is a rich source of information, including biomarkers for early cancer detection.

cfRNA-based biomarker identification requires a robust and reproducible in vitro workflow for RNA isolation and next generation sequencing (NGS), combined with high-performance in silico pipelines for NGS data processing and analysis. As the cfRNA-based liquid biopsy field is still developing, technical limitations remain to be overcome for the generation and analysis of high quality cfRNA-Seq data. Resolving these issues will improve the robustness and reproducibility of this approach and generate high confidence biomarkers for early cancer diagnosis.

Here we describe a combined in vitro and in silico pipeline that overcomes these challenges to generate and analyse high quality cfRNA-Seq data.

Methods: We obtained plasma from various donors and tested different protocols at key steps in the in vitro pipeline, including blood collection, RNA isolation, DNase treatment and library preparation. These approaches were evaluated quantitatively and qualitatively at the RNA and cDNA library levels, including sequencing of the cDNA libraries.

We built a custom in silico pipeline to process the cfRNA-Seq data. Using a variety of Quality Control (QC) statistics, including several innovative metrics developed in-house, we performed an extensive comparative analysis of the cfRNA profiles obtained across the in vitro conditions tested.

Results: We identified the optimal protocol at each stage of the in vitro pipeline for the generation of high quality cfRNA-Seq libraries. Data analysis using our in silico pipeline revealed that the optimised pipeline steps improve cfRNA-Seq data quality to a level suitable for confident biomarker identification.

Conclusions: We have developed a pipeline that overcomes the technical challenges that have prevented the generation and analysis of high quality cfRNA-Seq data.

Bioinformatics pipeline optimization was crucial to obtain relevant QC metrics that allowed selection of the optimal protocol at each stage.

This pipeline has great potential for the identification of high confidence biomarkers for early cancer detection, which will accelerate treatment and result in more favourable patient outcomes.

Liquid Biopsy in Early Detection for Cancer

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100093

ENHANCED BLOOD SAMPLING FOR ULTRA-SENSITIVE DETECTION OF CTDNA AND CTCS IN NEOADJUVANT-TREATED EARLY BREAST CANCER PATIENTS

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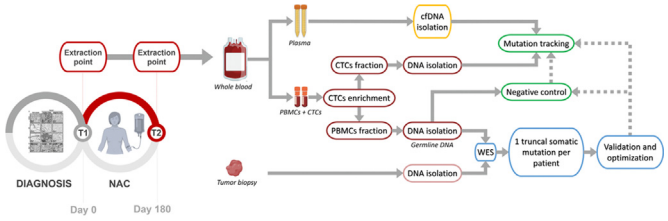
Introduction: Breast cancer (BC) patients often experience relapse due to residual disease (RD) after standard treatments. Liquid biopsy offers a non-invasive alternative to detect tumor components in blood, but low concentrations pose challenges. Next-generation sequencing shows promise but requires complex processes. Exploring increased blood volumes could address detection limitations.
Methods: A total of 133 plasma and blood-cell samples were collected, optimizing high-volume processing. Dual ctDNA and CTC detection employed a single droplet-digital PCR (ddPCR) assay per patient.
Results: Disease presence (ctDNA and/or CTCs) was detected in 100% of pre-treatment samples. Post-treatment samples exhibited limits of detection (LOD) of 0.007% for ctDNA and 0.261 CTCs/mL of blood, surpassing the performance of previous investigations. ddPCR demonstrated enhanced sensitivity at ultra-low concentrations compared to an NGS panel. In patients without a complete pathological response, 100% accurate prediction of RD was achieved. The model utilizing ctDNA dynamics achieved an AUC of 0.92 for predicting NAC response. This methodology required a median of 70 mL of blood in pre-treatment and post-NAC samples for ctDNA and CTCs detection.
Conclusions: This study employed a liquid biopsy method with increased blood draws, significantly improving ctDNA and CTC detection. These findings offer a promising tool for investigating clinical strategies aimed at reducing NAC or avoiding surgery in cases of negative blood RD.



Schema illustrating the study workflow, starting with blood sample collection at BC diagnosis (T1) and a subsequent sample after NAC and before surgery (T2). The diagram also depicts sample processing steps.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100094
UTILITY OF ULTRA-LOW-PASS WHOLE GENOME SEQUENCING IN BILE CFDNA FOR THE MANAGEMENT OF PATIENTS WITH BILIARY STRICTURES

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Introduction: The accurate differentiation between benign and malignant biliary strictures is still challenging. Endoscopic retrograde cholangiopancreatography is used for therapeutic and diagnosis purposes, allowing bile collection. We already demonstrated that mutational analysis in bile cfDNA accurately discriminates the benign and malignant origin of strictures. Here we investigate the utility of ultra-low-pass whole genome sequencing (ULP-WGS) in bile cfDNA as a cost-effective approach for early malignancy detection and for the differential diagnosis of cholangiocarcinoma (CCA) and pancreatic ductal adenocarcinoma (PDAC).
Methods: Bile samples from patients with biliary strictures (n=47) with an initial pathological diagnosis of benign, indeterminate or malignant origin were used. After follow-up, 44 of them resulted malignant. Eight bile samples from healthy donors were used as controls. Bile cfDNA was analyzed by ULP-WGS-0,2x and ichorCNA analytical approach was applied to calculate the tumor fraction and to evaluate large-scale copy number alterations (CNA). Mutations in bile cfDNA were analyzed by a NGS panel (Bilemut assay).
Results: We first compared the performance of ULP-WGS in paired bile and plasma samples from patients with malignant biliary stricture. Only 1/8 plasma samples tested positive, while CNA were found in 6. Given the better performance of ULP-WGS in bile, we analyzed CNA in bile samples collected from patients with a final diagnosis of malignancy and Bilemut positive (n=20). A TF>10% was detected in 75% of patients. Regarding specificity, bile from healthy donors were all negatives. In patients with an initial pathological diagnosis of not malignant stricture, that on follow-up resulted malignant, ULP-WGS showed a sensitivity of 37,5% (9/24), compared to 87,5% of Bilemut. The 3 patients with final diagnosis of benign stricture were negative. A CNA signature that discriminates CCA from PDAC malignant strictures with an accuracy of 0.8636 was identified.
Conclusion: Evaluation of CNA in bile cfDNA by ULP-WGS was feasible. A TF>10% was detected in 55% of the patients with a final diagnosis of malignant stricture. ULP-WGS showed a poor overall performance compared to Bilemut (93%), however it could represent a low-cost first screening strategy to diagnose malignancy in patients with biliary stricture. Its utility to discriminate CCA/PDAC requires further studies.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100095
PD-L1 AND BRAFV600E EXPRESSION ON CTCS FROM EARLY-STAGE COLORECTAL CANCER PATIENTS

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Background: The presence of BRAFV600E in colorectal cancer (CRC) is associated with the CMS1 phenotype, known as the "immune" phenotype, and occurs in 8% to 10% of metastatic CRC. Expression of PD-L1 in tumor cells also allows them to potentially evade the immune system. Correlation between PD-L1 and BRAFV600E was found in CRC with a prognostic role when absent. Expression of BRAFV600E and PD-L1 is especially important in circulating tumor cells (CTCs) as they are the ones with the potential to metastasize.
Methods: CTCs were isolated from 31 CRC patients of which 67.7% (21/31) were early-stage (stages I-IIa). CTCs were collected at baseline for all patients and 1 month after surgery for 22 patients of which 72.7% (16/22) were early stage. CTC enrichment was done on the semiautomatic microfluidic system IsoFlux based on EpCAM positive selection and phenotypic characterization for Cytokeratin (CK), BRAFV600E, PD-L1, and CD45 was done in an imaging flow-cytometry system -ImageStream- using our previously developed protocol.
Results: CTCs were isolated in 45.2% (14/31) CRC patients at baseline being more prevalent in advanced (50%; 5/10) than in early (42.9%; 9/21) stages, although not statistically significant (p=0.98). The median numbers of CTCs between baseline and follow-up were not significantly different (p=0.55). Heterogeneous phenotypes were observed in the CTCs with the most frequent CTC subpopulation both at baseline and in follow-up being PD-L1+/BRAFV600E+ (47.4%). Interestingly, the subpopulation PD-L1+/BRAFV600E- was only identified in the follow-up (7.3%), suggesting that the potentially metastatic CTC population was fully removed after surgery. Regarding changes in the CTC populations, two main groups were identified: those losing and those gaining double PD-L1+/BRAFV600E+ expression. CTC clusters were also identified in combination with CD45+ cells.
Conclusions: CTCs can be isolated and phenotypically characterized using IsoFlux and ImageStream in combination. CTC characterization using PD-L1 and BRAFV600E may identify potentially malignant CTCs in early-stage CRC patients.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100096
EARLY NON-SMALL CELL LUNG CANCER HETEROGENEITY AND RECURRENCE ASSESSED BY TISSUE NEXT-GENERATION SEQUENCING GENOTYPING AND CIRCULATING TUMOR CELL EZH2 CHARACTERIZATION

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Introduction: Non-Small Cell Lung Cancer (NSCLC) is the most common lung neoplasm comprising 85% of all cases with a poor 5-year survival rate. Surgical resection is the preferred treatment in early-stages, but a high percentage of them relapse due to the presence of minimal residual disease (MRD) and disseminated tumor cells. Identifying this heterogeneity is essential for stratifying patients with high risk of relapse and selecting the best treatment options, what can be achieved using Next-Generation Sequencing. Combining this with the evaluation and phenotyping of Circulating Tumor Cells (CTCs), is interesting for understanding the clinical evolution of these patients. Here we focus on EZH2 expression on CTCs, since its association with poorer outcomes in NSCLC.
Methods: Seventy-six early-stage NSCLC patients were recruited. Tissue biopsies (tumoral/peritumoral) were extracted during surgery and blood samples were extracted before (CTC1) and one month after it (CTC2). Genomic DNA from tissue-matched biopsies was extracted and sequenced from 27/76 patients (due to availability of specimens) using targeted-based sequencing with a customized 50-gene panel. CTCs presence was evaluated in all patients and their EZH2 protein expression was characterized at CTC1 and CTC2.
Results: Regarding NGS, 23/27 patients presented filtered variants and 143 of them were detected. The top 5 most mutated genes were: TP53, FLT1, MUC5AC, EGFR and NLRP3. Pair of genes with co-occurring mutations were: CD163-TLR4, FGF10-FOXP2, ADAMTSL3-FLT1, ADAMTSL3-MUC5AC and MUC5AC-NLRP3, and mutually exclusive mutations occurred in the pair RIN3-TP53. CTCs analyzed in all patients showed a significant decrease in the number of them between CTC1 and CTC2 ($p<0.0001$). CTCs+ patients with high EZH2 expression at CTC1 had 87% greater risk of death than those with low expression ($p=0.0180$).
Conclusions: The combination of both molecular profiling of the tumor and CTCs characterization represents a potential complementary tool to study the tumor heterogeneity and to facilitate patient stratification in early-stage NSCLC.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100097
HIGH-THROUGHPUT SECRETOME PROFILING OF EARLY BREAST CANCER

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Introduction: Non-invasive early cancer diagnosis is a highly sought-after goal in translational research. Circulating proteins in plasma are promising biomarkers with clinical potential. However, detecting proteomic features associated with small tumors holds technical and biological challenges. Advanced high-throughput technologies might overcome these limitations. Here, we present preliminary results from the RENOVATE trial study, where we assessed circulating proteins in early breast cancer (eBC) patients using two cutting-edge proteomics approaches.
Methods: The RENOVATE trial (NCT04781062) enrolled women with suspect breast lesions $\leq$ 2 cm. Before confirmation biopsy, they were asked to donate blood samples (detailed information on the standard operating procedures in Ravera et al., BMJ, 2021). Plasma-EDTA samples from 31 cancer, 29 benign, and 20 healthy donors, matched by age, body mass index (BMI), menopausal and smoking status, were assessed with aptamer-based proteomics arrays (SomaScan Somalogic®, 7596 features). Approximately 370 proteins were reassessed on the same biospecimens, leveraging an antibody-DNA linker technology based assay (Oncology panel, Olink®). Variance analysis was performed using ANOVA. Statistical tests with a p-value < 0.01 were considered significant. Proteomics signatures were identified using a penalized linear model (glmnet R package). Correlation between the two assays was evaluated using Intra-class correlation (ICC).
Results: Out of 7596 proteins, 29 showed a significant difference across the 3 groups. The majority of the detected proteins are involved in differentiation, migration, and cell death pathways. The two platforms shared 300 targets; 99% (n=296) passed the quality control step. The two used methodologies displayed moderately consistent results with 22% of probes (n=66) with an ICC index $\geq$ 0.5.
Conclusions: In the present study, we performed a comprehensive analysis of human circulating proteins. We detected proteins associated with early BC, associated with relevant pathways involved in cancer development. Results were validated using an orthogonal technology.
Despite the potential impact in early diagnosis, the application of advanced methodologies is constrained by costs, and the majority of detected biomarkers have not yet been validated or implemented by standardized routine assays. We are currently working to transfer our findings to low-throughput, low-cost methods.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100098
NOVEL AND NON-INVASIVE TOOL FOR IMMUNOTHERAPY-RESPONSE PREDICTION IN NON-MUSCLE-INVASIVE BLADDER CANCER PATIENTS BASED ON MIRNAS AND CYTOKINE DETECTION IN URINE

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Introduction: Bladder cancer (BC) represents a clinical and social challenge due to its high incidence and recurrence rates, as well as the scarce advances in effective disease management. For non-muscle-invasive BC, the best therapeutic alternative has proven to be intravesical immunotherapy based on BCG instillation. This provokes severe inflammation that, in responder patients, elicits an anti-tumor immune response. However, in clinical practice, there is no way to predict which patients will benefit from this therapy and about 50% suffer inflammatory reactions without the expected clinical outcome. Liquid biopsy, and especially urine-based methods, represents the best non-invasive approach to personalize therapy for BC patients. Here, we present the selection and urine evaluation of microRNAs and cytokines with good predictive capability for BCG immunotherapy response, alone or in combination with anti-PD-L1 therapy.
Methods: Ncounter miRNA expression panel was used to identify DE miRNAs from primary tumor of responder (R) and non-responder (NR) patients and miRNA-specific RT-qPCR was performed to validate it in urine samples. Identification and validation of cytokines with predictive capacity in urine was performed by LEGENDplex and ELISA analysis, respectively.
Results: We identified 26 DE miRNAs able to distinguish tumor samples from R vs NR patients. Some selected miRNAs were evaluated in urine samples and finally we selected 7 miRNAs that together efficiently discriminate between R and NR patients with an ROC curve with an AUC=1 ($P = 0.0004$) with a

specificity and sensitivity of 100%. Moreover, we have identified 3 cytokines with predictive capacity in urine samples with AUC > 0.85-0.9. Evaluation of these biomarkers in a small validation cohort validates this excellent prognostic result. In order to improve our system, we will study the combined predictive capacity of both type of biomarkers, miRNAs and cytokines, in this validation cohort.

Conclusions: Dysregulation of urine miRNA and cytokine expression allows discrimination between R and NR BC patients to immunotherapy prior to treatment, becoming the first accurate urine-based tool for this urgent unmet clinical need.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100099

DETECTION OF SOMATIC ALTERATIONS IN PERITONEAL LAVAGES OF EARLY-STAGE ENDOMETRIAL CARCINOMA PATIENTS

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Background: Surgical resection remains the best treatment option for early-stage endometrial carcinoma (EC) patients (p). However, some p recur even after curative resection. Peritoneal lavages may be performed during surgery but little data is available about their potential as liquid biopsies. This proof-of concept study aimed to determine if somatic mutations can be detected in peritoneal lavages.

Methods: Surgical biopsies were collected from 89 early-stage EC p of endometrioid (EEC, n=76), serous (SC, n=12) and undifferentiated (UC, n=1) subtypes. Molecular characterization of biopsies was performed using a custom, 55-gene NGS panel (GeneReader, Qiagen). Paired lavages were obtained from 53 p, all with negative cytology. The cfDNA was purified from the lavages using an automatic extractor (QIAsymphony, Qiagen) and the concentration of DNA was measured using the Qubit® Fluorometer. Hotspot mutations identified in paired biopsies were tested in cfDNA using custom NGS panels (n=21) and/or a sensitive Taqman assay (n=47) for KRAS codons 12, 13, and 61 and PIK3CA codons 542, 545 and 1047.

Results: NGS of tumor biopsies revealed different mutation patterns according the EC subtypes. For EEC, the most prevalent mutations were observed in PTEN (65%), PIK3CA (51%), KRAS (30%), TP53 (28%), BRCA2 (10%), ATM (10%), ARID1A (9%) and POLE (7,8%). For SC, TP53 was detected in 100% of p, followed by PIK3CA (50%), MSH6 and MUTYH (8% both), while the p with UC showed BRCA1, BRCA2, PIK3CA and TP53 mutations. In the case of peritoneal lavages analyzed by Taqman, KRAS and/or PIK3CA mutations were detected in 15/47 (32%) cases; mutations were uniformly concordant with those detected in tissue. NGS analysis was performed in cfDNAs from 21 p, with evaluable results in 17 cases. Mutations were detected in 4/17 (24%) samples. Allelic fractions ranged from 0.1% to 47%. The association of mutation detection in lavages with clinical parameters will be presented at the meeting

Conclusions: Mutation analysis in peritoneal lavages from surgically resectable EC tumors is feasible. Sensitive techniques such as Taqman or narrow NGS panels should be preferred.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100100

HIGHLY SENSITIVE DETECTION OF CTDNA IN EARLY BREAST CANCER USING PLASMA-SEQSENSEI™ AND DROPLET DIGITAL PCR WITH INCREASED BLOOD VOLUMES

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Introduction: Breast cancer (BC) is the most common tumour in women, and early detection is crucial for better outcomes. Liquid biopsy is a promising non-invasive method for monitoring cancer. However, detecting tumour components in liquid biopsies is challenging due to low concentrations in localized tumours. This study used an ultrasensitive next-generation sequencing panel called Plasma-SeqSensei™-Sysmex (PSS) designed specifically for BC. Higher plasma volumes were utilized to enhance sensitivity. The goal of this study was to test this technology while improving the detection and monitoring of ctDNA in early-stage BC.

Methods: We analysed 47 samples of plasma from 16 BC patients at pre-, post-neoadjuvant chemotherapy (NAC) and during follow-up. ctDNA detection were performed using the commercial NGS panel PSS and compared with the gold-standard droplet-digital PCR (ddPCR Bio-Rad).

Results: Herein, ctDNA was detected in 50% of pre-treatment samples by PSS, demonstrating the panel's potential to detect ctDNA. After NAC, ctDNA detection decreased to 1 out of 9 plasma samples, with a minimum mutant allele frequency (MAF) of 0.08%. Comparing the ultrasensitive NGS-based PSS and ddPCR technologies, we found a strong correlation in mutant molecule levels in positive samples. Notably, NGS identified 6 exclusive mutations in plasma, including 3 validated tumour mutations and 3 classified as clonal haematopoiesis of indeterminate potential (CHIP) mutations.

Conclusion: This technology showcased exceptional sensitivity in detecting ctDNA, enabling the identification of mutations below a VAF of 0.1%. These results highlight the superiority of PSS in ctDNA detection over other NGS solutions. Moreover, PSS offers the ability to uncover novel mutational profiles in BC patients, allowing for a comprehensive analysis of the mutational landscape, surpassing the limitations of tracking a single mutation using ddPCR assays.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100101

EPIC ARRAY STUDY IN LIQUID BIOPSY IDENTIFIES A NON-INVASIVE EPIGENETIC SIGNATURE FOR EARLY DIAGNOSIS IN NON-SMALL CELL LUNG CANCER

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Introduction: Early detection is a key factor for increasing lung cancer patients' survival and there is growing interest in the development of non-invasive molecular biomarkers. Since epigenetic alterations have been shown to play an important role in early-stage lung carcinogenesis, epigenetic biomarkers are emerging as a promising approach to improve clinical management. We have previously identified a novel epigenetic signature of 4 genes (BCAT1, CDO1, ZNF177, and TRIM58) in lung tissue and recently validated BCAT1 in free circulating DNA (cfDNA) for the early detection of lung cancer. However, the main objective is, using state-of-the-art omics technology, to identify an epigenetic model directly in to non-invasive plasma samples (liquid biopsy).

Materials and Methods: We conducted a prospective study from October 2019 to July 2021. cfDNA was purified from patients diagnosed in the pneumology service of Hospital La Fe in Valencia (see Figure 1). 75 patients were included, although only 58 patients met the inclusion criteria (36 patients with non-small cell lung cancer and 22 with non-neoplastic lung disease). We characterized the DNA methylation global profile of ctDNA using the novel Infinium Epic 850k DNA methylation platform and took advantage of bleeding edge statistical methods for the analysis of high dimensional data, such as penalized regression to identify a novel epigenetic signature with diagnostic value.

Results: We identified the minimum number of CpGs characterizing both groups, we performed. Thus, using an elastic net penalized multinomial logistic regression with disease status as response variable and all CpGs as potential predictors of epigenetic changes able to discriminate between lung cancer vs control groups. A profile consisting of only 19 CpG was enough to correctly classify 100% of the samples. The results of this supervised analysis are depicted in a clustered heatmap. Currently, we are validating the DNA methylation values by the ultrasensitive method digital droplet PCR of selected CpGs to generate an epigenetic model with high diagnostic performance.

Conclusion: The application of epigenomic technology in liquid biopsies is valid to identify an epigenetic model to classify lung cancer patients versus control donors with potential diagnostic value.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100102

TECHNOLOGICAL PLATFORM DEVELOPMENT FOR EARLY CANCER DETECTION, TOWARDS A FOCUSED MULTI-CANCER DETECTION

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Introduction: Early cancer detection is widely recognized as a life-saving opportunity. However, it is crucial to consider that cancer comprises a diverse group of diseases that share molecular bases while exhibiting variations in diagnosis, prognosis, and treatment based on their anatomical origin. In light of this perspective, developing technologies that enable early detection of these pathologies, in a clinically meaningful manner, becomes highly pertinent.

Methods: We accomplished a bioinformatic platform capable of identifying methylation-based biomarkers for different tumor types using open access databases. Our research has successfully revealed these biomarker's presence in DNA extracted from tissue samples obtained from patients diagnosed with colorectal cancer (CRC) and hepatocellular carcinoma (HCC). To facilitate detection, we performed our proprietary qPCR-based technology, allowing the simultaneous identification of multiple biomarkers. Additionally, we utilized one of our exclusive biomarker panels to detect CRC in 40 plasma samples from healthy individuals and cancer patients, employing ddPCR.

Results: Initially, we identified 275 candidate CpG sites for CRC and 96 CpG sites for HCC. Employing different classification criteria, we tailored distinct detection panels for each tumor type (between 3 and 6 biomarkers per panel). Through our qPCR-based technology, we successfully validated our bioinformatic platform by detecting individual and combined biomarkers in 40 tissue samples collected from CRC patients and 20 from HCC patients.

After biomarker panels validation, we engineered an early-stage prototype diagnostic test ddPCR-based (initially for CRC). This prototype allows the detection of a three biomarker panel in circulant DNA samples. To further verify its accuracy, we validated the prototype using plasma samples obtained from 20 CRC patients and 20 healthy individuals. Our results demonstrated an overall AUC of 0,91, accompanied by a 80% sensitivity (95% CI: 58.40% to 91.93%) and a 90% specificity (95% CI: 69.9% to 98.22%).

Conclusion: Through our validation process, we successfully achieved a PCR-based technology capable of detecting methylation-based biomarker panels in liquid biopsies. Although the initial panel was validated in CRC patients, the versatility of our technology allows it to detect different tumor types panels simultaneously, with the potential to be implemented in a focused multi-cancer test.

PP.90

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100103

PROGNOSTIC POTENTIAL OF CANDIDATE MIRNAS IN LUNG CANCER: INSIGHTS FROM TISSUE AND LIQUID BIOPSIES

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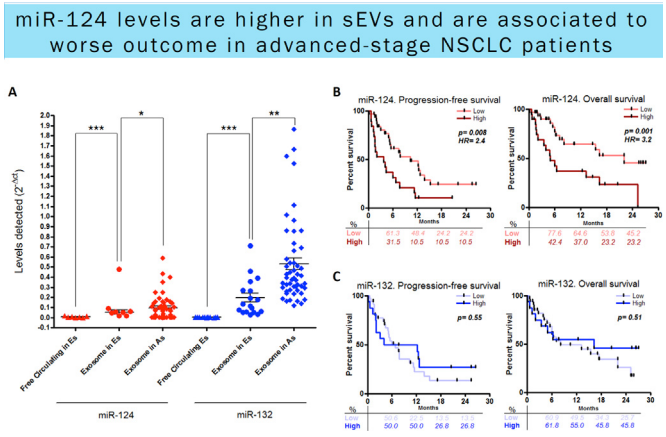
Background: Lung cancer, the most prevalent cancer type, requires novel biomarkers to predict disease development and optimize clinical decisions. miRNAs have emerged as promising oncological biomarkers for tissue and liquid biopsy samples. We evaluated the prognostic significance of 7 candidate miRNAs identified through a microarray screening study in early and advanced non-small cell lung cancer (NSCLC) patients.

Methods: We collected 120 samples from 88 patients, including tissue and plasma from early-stage patients, and alternative cohorts. miRNA fractions were isolated using specific kits. qRT-PCR with Taqman probes assessed the miRNA levels. Survival analyses integrated clinicopathological data. An in silico analysis identified miR-124 target genes in 2952 NSCLC patients.

Results: Tissue exhibited higher detection of all miRNAs compared to plasma, except for miR-124. In early-stage patients, lower miR-10 and higher miR-124 and miR-7 in plasma were significantly associated with relapse and exitus. Exosomal samples showed superior miR-124 and miR-132 detection compared to free circulating samples, with significantly increased levels in advanced-stage patients. Exosomal miR-124 predicted PFS and OS in advanced-stage NSCLC. In silico analysis suggested miR-124 may regulate TFAP2A, KPNA4, and SPOCK1, genes with potential oncogenic roles in NSCLC.

Conclusion: Plasma miR-124 holds promise as a predictive biomarker for PFS and OS in early and advanced NSCLC. Its anti-oncogenic activity may involve blocking the downregulation of oncogenes, such as TFAP2A, KPNA4, and SPOCK1. Our findings provide valuable insights for lung cancer prognosis using tissue and liquid biopsies.

This research was funded by PI18/00050; PI21/00145, cofinanciado por la unipn europea EU, RTC2016-5314-1, JR21/00003; FI19/00061; CD22/00040 and Fondos FEDER; Caixa-Impulse Validate program, CI20-00182 and Fundación Mutua Madrilena, AP180852022.



PP.91

THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100104

DEVELOPMENT OF A HORIZONTAL DATA INTEGRATION CLASSIFIER FOR NON-INVASIVE EARLY DIAGNOSIS OF BREAST CANCER: PRELIMINARY RESULTS FROM THE RENOVATE TRIAL

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Introduction: Liquid biopsy represents a powerful tool for early detection of cancer. Early breast cancer (eBC) diagnosis is often suboptimal in terms of accuracy and requires invasive tests such as biopsy. The assessment of circulating biomarkers to differentiate benign lesions (BL) from BC, together with radiomics, is of great potential in BC management.

Methods: The RENOVATE clinical trial (NCT04781062) is an academic prospective translational study investigating the accuracy of the combined assessment of multiple circulating analytes (cf-methylated DNA, copy number variations, proteins) together with radiomic variables for the non-invasive differential diagnosis of eBC. The RENOVATE trial recruitment criteria are reported in Ravera et al., BMJ, 2021. For each participant, we collected one K2EDTA tube (BD Vacutainer™), one Tempus™ Blood RNA tube (Applied Biosystems™) and two PAXgeneBlood ccfDNA tubes (BD Vacutainer™), together with a 40 ml urine bottle cup. Cf-methylated DNA is currently being assessed using PAXgene plasma samples through cfDNA immunoprecipitation and high throughput sequencing (cfMeDIP-Seq).

Results: The accrual started in January 2021 and ended in June 2022. During one year and a half recruitment, we collected and stored samples from 366 women. Of these, 87 patients were not considered eligible due to prespecified exclusion criteria. We identified 115 patients with a benign lesion and 64 patients with eBC. Additionally, as healthy controls, we collected 100 samples from women with negative mammography.

We extracted cfDNA from 4 ml PAXgene plasma samples using QIAamp MinElute ccfDNA Kit (Qiagen). The obtained average cfDNA quantity was 15,45 ng (median = 13,5; IQR = 10.84 - 19,18) for BL and 25,5 ng (median = 19,55; IQR = 14.45-25,15) for eBC (Mann-Whitney test p-value = 0.06).
Conclusions: The overall goal of the present study is to develop a horizontal data integration classifier for the non-invasive differential diagnosis of eBC. Such aim would be practice-changing in the management of BC. Besides the planned analyses, our study has generated a unique sample biobank to benchmark novel, transformative liquid biopsy methods.

PP.19
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100105
IMPACT OF LIQUID BIOPSY PREANALYTICS ON CFDNA FRAGMENTATION ANALYSES

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Background: Liquid biopsy for non-invasive disease monitoring of cancer patients is progressing towards clinical practice. So far, the main focus is on circulating tumor DNA (ctDNA) analysis, targeting actionable somatic hotspot variants to guide treatment decisions. Recent studies indicate the potential of the untargeted detection of lowest levels of ctDNA based on fragmentation analysis of total circulating free DNA (cfDNA). Here we performed a pilot study to evaluate the impact of preanalytics on cfDNA fragmentomics.
Methods: Blood samples from 10 healthy individuals and 3 patients with metastatic colorectal cancer (CRC) were collected in EDTA tubes at the partner site in Granada. To investigate the impact of sample shipping and cfDNA isolation on cfDNA fragmentation, cfDNA was isolated from each plasma sample using manual and automated extraction methods. The effects of different preanalytical workflows on sensitivity and specificity of fragmentation-based ctDNA detection were evaluated using the whole-genome sequencing (WGS)-based LIquid biopsy Fragmentation, Epigenetic signature, and Copy Number Alteration analysis (LIFE-CNA), previously developed by the partner site in Munich.
Results: Plasma cfDNA concentration in samples collected from healthy individuals in EDTA tubes was significantly higher than in 55 healthy controls with blood samples collected in Streck tubes (p-value < 0.05). The different isolation methods did not result in significantly different plasma cfDNA concentrations. With LIFE-CNA, no major differences regarding the analyzed parameters, such as cfDNA fragmentation and chromatin signatures, were observed to be dependent on the preanalytical workflow in selected healthy controls and CRC patients.
Conclusion: Within our pilot study we showed that neither differences in sample collection tubes nor differences in cfDNA isolation workflows had a significant impact on cfDNA fragmentation analysis. These preliminary results suggest that cfDNA samples shipped across Europe can be used for fragmentation-based ctDNA detection. Thus, enabling us to initiate a larger study. We will investigate the performance of targeted and fragmentation-based ctDNA analysis for the early detection of CRC in a larger cohort of early and late-stage patients in the framework of our collaboration.

PP.101
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100106
NEXT-GENERATION SEQUENCING ANALYSIS OF EXTRACELLULAR VESICLE MICRORNAS FOR BREAST CANCER STAGING

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Introduction: Extracellular Vesicles (EVs) associated miRNA (EVs-miRNAs) profiling has been shown to be particularly altered in breast cancer cells and can serve as a complementary tool for diagnosing and predicting response in localized breast cancer.
Methods: In this prospective longitudinal study, we obtained circulating EVs-miRNAs from serum samples of patients with early-stage breast cancer (n=10), metastatic breast cancer (n=6), and healthy donors (n=9) prior to receiving neoadjuvant chemotherapy, which were subsequently sequenced. EVs were isolated from serum using a serial centrifugation protocol optimized by our laboratory and characterized by western blot and electron microscopy. The isolated EVs were then lysed, and total RNA was extracted using the miRNeasy Kit and sequenced using the TruSeq Small RNA Library Prep Kit. Bioinformatics analysis was performed using the following programs: FastQC for quality control, Cutadapt for adapter removal, Bowtie for sequence mapping, and miRDeep2 for identifying both novel and known miRNAs. The differential expression of genes was analyzed using the EdgeR package in R.
Results: The 25 sequenced samples had an average of 4,883,812 mapped reads, of which a total of 2,656 known miRNAs were successfully mapped. Comparing the expression between each pair of groups (Control vs. Early Stage, Control vs. Metastatic, Early Stage vs. Metastatic) revealed a total of 12 miRNAs with significantly differential expression based on the standard FDR < 0.01. Among them, eight miRNAs showed differential expression in the metastatic stage compared to the early stage: miR-183-5p, miR-200a-3p, miR-100-5p, miR-122-5p, miR-192-5p, miR-500a-3p, miR-141-3p (upregulated), and let-7f-5p (downregulated). Additionally, seven miRNAs were differentially expressed in the metastatic stage compared to healthy controls: let-7a-5p, let-7f-5p, miR-144-5p (downregulated), miR-423-5p, miR-141-3p, miR-100-5p, and miR-320b (upregulated).
Conclusion: While these data need validation in an independent cohort, they suggest that this panel of EVs-miRNAs could be an excellent tool for distinguishing metastatic breast cancer from both healthy and early-stage breast cancer.

Miscellaneous
PP.92
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100107
TECHNICAL ADVANCEMENTS IN CFMEDIP-SEQ LIBRARY PREPARATION

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Introduction: Cell-free methylated immunoprecipitation sequencing (cfMeDIP-Seq) is a promising approach to assess non-invasive methylomics from plasma samples. Despite its cost-efficiency and accuracy, cfMeDIP-Seq setup and processing are technically challenging. In 2019, Shen et al. published a detailed protocol for cfMeDIP-Seq, which can now be partially automated, potentially improving reproducibility and yield.
Methods: We designed a pilot study to assess the performance of cfMeDIP-Seq under four library preparation conditions. From the same healthy donor, we collected four K2EDTA and four PAXgene ccfDNA tubes (BD Vacutainer™), the latter ones specifically designed to avoid cfDNA degradation. K2EDTA and PAXgene tubes were processed respectively within 2 and 6 hours of blood draw, as previously described (Dameri et al., 2023). Ten nanograms of cfDNA were used as input material for library preparation. Each condition underwent two parallel preparation procedures: one following the manual protocol described by Shen et al., and the other following a semi-automated version of the original protocol using the IP-Star® Compact instrument (Diagenode). A total of 16 libraries were generated, four for each condition, and sequenced on an Illumina NovaSeq 6000 device.
Results: No significant differences were observed across the four conditions in terms of GC content (47%, sd = 0.49). The number of uniquely mapped reads was comparable (p-value = 0.8) between semi-automated and manually processed libraries, with an average of 19.9 MReads per sample. The percentage of duplicates was reduced (p-value = 0.0012) in semi-automated libraries (median = 32.88; IQR = 31.94-34.06) compared to manually prepared ones (median = 41.48; IQR = 35.61-42.53). Semi-automated libraries showed a longer mean insert size (255 bp) compared to manually processed libraries (243 bp) with a p-value < 0.01. Principal component analysis revealed no major differences between K2EDTA and PAXgene samples processed with the semi-automated

protocol.
Conclusions: In this pilot study, we demonstrated that semi-automated library preparation outperformed the fully manual protocol, regardless of tube type. Potential differences between K2EDTA and PAXgene were marginal compared to the library preparation procedure, provided that K2EDTA were processed shortly after collection. Partial automation of fluidic handling seems extremely relevant to increase yield and quality of cfMeDIP-Seq experiments.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100108
SOMATIC MUTATION CALLING FROM CFDNA USING MACHINE LEARNING

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Introduction: Sequencing cell-free DNA (cfDNA) fragments from plasma samples to analyze tumor mutations is a non-invasive method for tumor profiling and monitoring. However, given that sequencers are erroneous and vast majority of these cfDNA are derived from healthy cells, it is challenging to computationally differentiate between errors and ctDNA mutations. To improve the sensitivity, some assays sequence only small genomic regions (panel) with expected mutations deeply and computational methods learn the error function in a position-specific manner. Therefore, existing methods make a trade-off between detection sensitivity and applicability to general genomic positions. We aim to use machine learning to learn the complex function of errors to tackle both generalizability and sensitivity.
Methods: Here, we implemented a computational analysis pipeline to detect somatic mutations from a single deep-sequenced plasma sample. We used unique molecular index (UMI) barcoding to label errors during PCR amplification. For the remaining errors, we trained a random forest classifier on multiple features, including the read sequence, the base quality and diverse read mapping properties, to distinguish errors from true mutations. To train and test our model, we used diluted cell line data from 60 samples as reliable ground truth. To analyze generalizability, we excluded mutations on chromosome 1 and 17 during model training and used them only for performance testing.
Results: To balance the training data, we down-sampled 195,535 labeled candidate mutations and used 16,128 for training and 105,804 data points as test data. The model achieved a recall of 0.85, accuracy of 0.90, and false positive rate of 0.12 on the test set.
Conclusion: As a proof-of-concept, we showed that using machine learning to statistically model error has the potential to be both generalizable and sensitive. However, in our pipeline we still have a relatively high false positive rate, which could be resolved by incorporating more features and acquiring more independent training data. Increased accuracy of ctDNA mutation calling might enable a more complete molecular characterization of tumors and facilitate broader clinical applications for liquid biopsies.

PP.94
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100109
IMPACT OF CFDNA INPUT ON ENZYMATIC METHYLATION CONVERSION AND LIBRARY PREPARATION ROBUSTNESS

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Introduction: Liquid biopsies and cell-free DNA (cfDNA) analysis have emerged as novel non-invasive diagnostic tools in oncology. Analysis of cfDNA methylation status has been proposed as promising approach for several diagnostic purposes, and robust methylation-detecting technologies maintaining their performance when used to analyze challenging DNA samples as cfDNA are of primary importance for the clinical implementation of novel diagnostic assays. Gold standard method to detect methylation is bisulfite sequencing, but its chemical aggressiveness towards DNA prompted the development of novel technologies that would better preserve DNA integrity. One of such methods substitutes chemical conversion with less aggressive enzymatic conversion and is commercialized by New England Biolabs under the Enzymatic Methyl-Seq (EM-Seq™) name. In this study we assessed the influence of different amounts of cfDNA used as EM-seq input on methylation conversion and library preparation robustness.
Methods: >2000 cfDNA samples were extracted from 3-4mL of plasma. 10-20ng cfDNA were spiked-in with RRBS unmethylated/methylated controls and used as input for EM-seq™, which was run according to manufacturer's instructions. After enzymatic conversion, adaptor-ligated samples were quantified by qPCR, while converted libraries by using Varioskan microplate. Libraries were subject to hybrid-capture with probes against the spike-in controls and sequenced on Novaseq 6000. The analysis was performed in R environment.
Results: We have analyzed the influence of different cfDNA inputs (less than 20ng or 20ng) on the robustness of EM-seq™ workflow by evaluating the impact of cfDNA input on key EM-seq™ parameters. As expected, we've identified differences between cfDNA inputs in the yield of obtained adaptor-ligated DNA fragments ($p<0.05$) and of library DNA ($p<0.05$). Differences were identified also in the PCR duplicates proportions, with libraries prepared from 20ng cfDNA input having lower PCR duplicates proportions compared to libraries prepared from less than 20ng ($p<0.01$). cfDNA input had instead no impact on the conversion efficiency of EM-seq™ kit ($p=0.49$).
Conclusion: Analytical validation of novel methods is crucial for their implementation in clinical practice. With this study, we've verified the robustness of EM-seq™ method relative to changes in cfDNA inputs and provide an initial assessment of its potential use within novel liquid biopsy NGS diagnostic applications.

PP.95
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100110
THE DIRECT INTERACTION BETWEEN LUNG CANCER CELLS AND PLATELETS PROMOTES RESISTANCE TO CHEMOTHERAPY

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Introduction: Platelets are active players in tumorigenesis, although their direct impact on tumor cells remains largely unknown.
Preliminary studies in our laboratory showed that coculturing lung and other cancer cell lines with platelets enhances the proliferation of the former and induces phenotype changes.
One of the many functions proposed for this interaction between platelets and tumor cells is that platelets protect tumor cells against cell death and apoptosis and they could be protecting against treatments. In order to demonstrate that, in vitro tests were carried out with three different lung cancer cell lines (A549, H1975 and H1299) and platelets in coculture treated or not with CIS-platin, Docetaxel or Erlotinib.
Methods: Platelets were obtained from peripheral blood samples from a healthy donor. Platelets were added at a concentration of 100,000 platelets/µL.
The tests were carried out with different treatments at calculated IC50 concentrations with or without platelets.
The effect on cell death and apoptosis was evaluated at 24 h after treatment using annexin-V/7AAD kit. We also carried out xCelligence assays analyzing cell proliferation and cytotoxicity. We also evaluated the capability of migration.
Results: The IC50 concentrations of all drugs increased in all tumor cell lines when cells had 24h of contact with platelets.
Apoptosis in the A549 cell line co-cultured with platelets and treated with CIS-platin showed a statistically significant downward at 24h versus cells without platelets ($P<0.05$). However, cell death was decreased but not significant. In the coculture of H1975 cell line or H1299 cell lines with platelets treated with CIS-platin similar results were found: apoptosis decreased statistically significant ($P<0.05$) and cell death was decreased but not significant.
Regarding the xCelligence proliferation assays, significant differences were found between the condition of treated co-culture and treated cells without platelets for both cell lines at 24 and 48 h after treatment ($P<0.05$).
No significant differences were found in the migration assay.
Conclusion: These results suggest that tumor cells might be protected by platelets. Platelets are able to interact with tumor cells and prevent apoptosis. Furthermore, platelets promote the growth of tumor cells even if these cells are treated with different drugs.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100111
A NEW PLATFORM FOR THE DIRECT PROFILING OF NUCLEIC ACIDS IN BIOFLUIDS

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Introduction: circulating nucleic acids such as miRNAs, cDNAs, lncRNAs and circRNAs have been identified as potential biomarkers for early detection, prognosis and drug efficacy prediction of several diseases. However, their use in clinical settings has been hindered by the absence of appropriate detection techniques. Most of the current technologies require complex protocols, not yet able to deliver robust and cost-effective assays in the field of clinical diagnostics.
Methods: we describe the development of an innovative platform for profiling circulating nucleic acids. We designed and synthesized a set of reagents that combines a new chemical-based method with a chemiluminescent reaction, which is detected using a novel, compact silicon photomultiplier-based reader. Semi-automated protocol has been implemented for outstanding reproducibility.
Results: the combination of a unique chemical-based method for nucleic acid detection with a novel silicon photomultiplier-based reader created an innovative product (ODG platform) with diagnostic utility, for the direct qualitative and quantitative analysis of nucleic acids in biological fluid. The unique assay allows single base specificity, capturing the target directly in the matrix of interest, with no false positives. First, we explored and analysed the performances of the platform regarding LoD, LLoQ, precision and accuracy in relevant biological matrices, such as plasma and serum, with synthetic miRNA spike-ins. Later, we successfully detected miRNA-21 from plasma of lung cancer patients as a proof of concept.
Conclusions: nucleic acids profiling without extraction, pre-amplification or pre-labeling of the target is now possible. Avoiding such limitations, ODG platform will allow scientists and clinicians to rely on reproducible and certain results from precious liquid biopsies. Our proof of concept was performed with miRNAs, however, the platform can be tuned for other nucleic acids of interest.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100112
CAF-DERIVED EXOSOMES AS POTENTIAL CHROMATIN REGULATORS

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Introduction: Advances in tumor biology emphasize the importance of tumor microenvironment, where Carcinoma-Associated Fibroblasts (CAFs) are one of the most abundant populations with protumorigenic effects such as proliferation and drug resistance. Within of tumor microenvironment, cell-to-cell communication through exosomes plays an important role modulating surrounding cells and components. In a previous study, we identified by RNA-Seq an enrichment of specific non-coding RNA in exosome cargo released by primary cultures of CAFs from colon cancer (CR) patients, suggesting a contribution in paracrine communication. Based on these results, we stablished a CAF-derived exosomes signature contained 50 predicted target genes of exosomal ncRNA released by CAFs, which would indicate a greater active stromal component and strongly associated with shorter overall survival of CR patients. The aim of our study is identify potential mechanisms of chromatin regulation by CAF-derived exosomes in tumors with higher active stromal component. Methods. A series of 243 colon adenocarcinoma tumors from TCGA (Firehose) with methylation data and mRNA gene expression (RNA-Seq) were evaluated. Tumors were classified as high or low active exoCAF-component using a risk score based on the CAF-derived exosomes signature. A panel of 124 epigenetic-related genes and genome DNA methylation patterns were interrogated. Results. Eleven epigenetic-related genes were significantly associated with exoCAF-component (FDR<0.05; FC 1.5), 8 of them showing overexpression and 3 underexpresion in tumors with high exoCAF-component. Thus, deregulation of histone methyltransferases and deacetylases was found in tumors with active exoCAF-compoennt. In addition, using data about methylation across the genome, we found that these tumors showed 888 hypermethylated genes and 220 hypomethylated (p-adj<0.05; Δβ-value 0.10). For 77 of them, mRNA expression were strongly associated with methylation status (p<0.05; rho<-0.40). Enrichment pathways analysis of these 77 genes showed a relation with inflammatory response, epithelial to mesenchymal transition and proteoglycans in cancer. Conclusions. This in silico study suggests that effects carried out by exosomes may be mediated by epigenetic regulation, modifying DNA methylation and histone marks and support the idea that exosome released by CAFs can affect important pathways in cancer and are attractive targets to normalize an active stromal component in the tumors.

PP.98
THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100113
COMPUTATIONAL PROFILING OF ABERRANT DNA METHYLATION IN CANCER: A PROMISING SOURCE OF BIOMARKERS FOR LIQUID BIOPSIES

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Introduction: DNA methylation alterations are known to accompany and contribute to carcinogenesis, making them candidate biomarkers for cancer, particularly due to their stability, frequency, and accessibility in bodily fluids. The advent of newer minimally-invasive methods such as liquid biopsies provide the perfect setting for methylation-based biomarker development. Despite their huge potential, accurate and robust biomarkers for the conclusive diagnosis of most cancer types are still lacking, hence sustained research in this field is still needed. Moreover, studies have focused on identifying cancer types individually, but common and multi-cancer markers are underexplored.
Methods: We used The Cancer Genome Atlas (TCGA) to investigate genome-wide methylation profiles of 14 different cancer types and identify biomarkers for cancer diagnosis (N=6502). After differential methylation analysis through linear mixed models, we selected pan-cancer and type-specific differentially methylated probes (DMPs). We then developed a three-step computational approach, using dimensionality reduction, clustering, and discriminant analysis, to select candidate biomarker CpG sites. We also validated the identified pan-cancer markers in a novel in-house methylation assay, in 8 of the most lethal cancer types worldwide, and tested their viability in cell-free DNA.
Results: In total, we discovered 1,991 pan-cancer and between 75 and 1,803 cancer-specific differentially methylated CpG sites. We also cataloged differentially methylated blocks and regions that were conserved between cancer types on a large scale. Through our computational approach, a combination of four pan-cancer CpG markers was identified from these sites and externally validated (AUC = 0.90), maintaining comparable performance across tumor stages. Additionally, 20 tumor-specific CpG markers were identified and made up the final type-specific prediction model, which could accurately differentiate tumor types (AUC = 0.87-0.99). In our experimental validation, the pan-cancer markers could discriminate tumor from normal samples with a sensitivity of 0.95 and a specificity of 0.91.
Conclusion: Our study highlights the power of the methylome as a rich source of cancer biomarkers, and the computational method we developed provides a new resource for identifying highly performing markers, genome-wide, for use in liquid biopsies. Moreover, the markers we identified, combined with our novel DNA methylation assay, have strong applicability in liquid biopsies for cancer detection.

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SIMULATING CELL-FREE CHROMATIN USING BREAST AND COLORECTAL CANCER PRECLINICAL MODELS FOR CANCER-SPECIFIC BIOMARKER DISCOVERY

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Introduction: Cell-free chromatin (cf-chromatin) is a valuable source of biomarkers in many conditions, including cancer. Tumor-derived cf-chromatin can

be accessed noninvasively from the circulation for epigenetic profiling, such as through whole genome sequencing (WGS) to analyze nucleosome positioning. However, the low fractional abundance of tumor-derived cf-chromatin in blood plasma and limited blood sample availability poses challenges for efficient and reproducible biomarker discovery. To overcome this, we developed a novel framework to simulate cf-chromatin from conditioned media of preclinical cancer models, namely breast and colorectal cancer.

Methods: We optimized nuclease treatment of conditioned media cf-chromatin across various 2D and 3D models to generate nucleosomal distributions that resemble the mono- and oligo-nucleosomal topology of cf-chromatin present in human plasma. We conducted low-pass WGS of nuclease-treated cf-chromatin from breast (CAMA-1) and colorectal (HCT116) cancer cell lines (1.1X median coverage) and assessed nucleosome positioning associations with gene expression and chromatin accessibility. Furthermore, we compared media cf-chromatin profiles to previously published plasma WGS samples (n=54 for breast cancer and n=27 for colorectal cancer).

Results: Nuclease-treated media cf-chromatin exhibited nucleosome positioning patterns correlated with gene expression (amplitude: r=0.87, p=0.0010; mean coverage: r=-0.86, p=0.0016; central coverage: r=-0.89, p=0.00060), with highly expressed genes showing a nucleosome-depleted region and strong positioning of surrounding nucleosomes. When assessing chromatin variants with enriched accessibility in CAMA-1 relative to HCT116, we observed distinct cell-specific nucleosome profiles (Welch Two Sample t-test, amplitude: p= 0.00079, mean coverage: p=0.093; Wilcoxon rank-sum test, central coverage: p=0.022). Moreover, compared with plasma cell-free nucleosome positioning, cf-chromatin generated from conditioned media displayed stronger nucleosome positioning patterns at genomic locations of accessible chromatin from patient tissue (Wilcoxon rank-sum test, amplitude: p= 2.6e-08, mean coverage: p = 0.35; central coverage: p= 2.6e-08).

Conclusion: Our results demonstrate that simulated cf-chromatin reflects the chromatin landscape of the cell of origin, revealing cellular phenotypes more clearly than plasma WGS, in which non-tumour-derived cf-chromatin dramatically dilutes tumour-derived cf-chromatin nucleosome positioning features. Altogether, we show the broad applicability of preclinical simulation of cf-chromatin using breast and colorectal cancer models, providing an opportunity to accelerate cancer-specific epigenetic liquid biopsy biomarker discovery.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100115

EXTRACELLULAR VESICLES AS MEDIATORS OF RESISTANCE TO EGFR-TYROSINE KINASE INHIBITORS IN NON-SMALL CELL LUNG CANCER PRECLINICAL MODELS

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Introduction: Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases and the leading cause of cancer-related mortality globally. Thirty-two percent of patients with NSCLC harbor activating mutations in EGFR and benefit from EGFR-tyrosine kinase inhibitors (TKIs). Although EGFR-TKIs demonstrated higher efficacy than standard chemotherapy in phase-III clinical trials, resistance is inevitable and leads to disease progression. Acquired resistance to TKIs is a complex process and the biology is still poorly understood. Here, we aim to study the role of extracellular vesicles (EVs) in the resistance to EGFR-TKIs.

Methods: EVs were isolated by differential ultracentrifugation from conditioned medium (CM) of two NSCLC cell lines harboring EGFR mutations (PC-9 and H1975). We further developed osimertinib- and/or afatinib-resistant versions of these cell lines. EVs were characterized using transmission-electron microscopy and EVs markers protein expression. Cell viability was assessed using CellTiter-Glo® in the EVs and CM transfer experiments between EGFR TKIs-sensitive and -resistant cells (Fig.1A).

Results: We established the IC_{50} values of EGFR-TKIs for sensitive and resistant cell lines. As expected, the acquired resistant cells had a 25±6.7- and 71±53.7-fold higher IC_{50} for osimertinib and afatinib compared to sensitive cell lines. Incubation of the two sensitive cell lines with either CM (Fig.1B) or isolated EVs (Fig.1C) from the matched resistant cell lines led to a significant increase in the resistance to osimertinib and afatinib, compared to sensitive cells incubated with their own EVs or CM. Moreover, sensitive cells showed increased resistance to TKIs after incubation with CM depleted from EVs, particularly higher in PC-9 cells treated with osimertinib (Fig.1C).

Conclusion: We show that EGFR TKIs-resistant cells transfer the resistance to neighboring cells directly via EVs and other secreted factors. Obtained results will be validated using EVs from patients with NSCLC in order to elucidate driver resistance mechanisms.

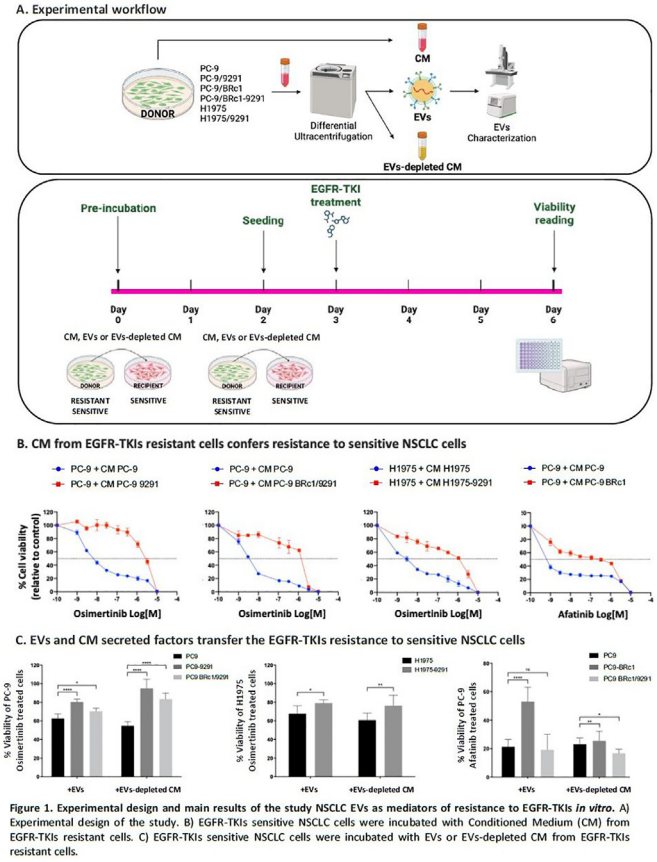

Figure 1. Experimental design and main results of the study NSCLC EVs as mediators of resistance to EGFR-TKIs *in vitro*. A) Experimental design of the study. B) EGFR-TKIs sensitive NSCLC cells were incubated with Conditioned Medium (CM) from EGFR-TKIs resistant cells. C) EGFR-TKIs sensitive NSCLC cells were incubated with EVs or EVs-depleted CM from EGFR-TKIs resistant cells.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100124

PIK3CA TESTING IN HR+/HER2− METASTATIC BREAST CANCER: REAL-WORLD DATA FROM REFERRAL MOLECULAR PATHOLOGY LABS

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Introduction: Activating mutations in *PIK3CA* occur in ~40% of hormone receptor (HR)+/HER2- breast cancers (BC). However, *PIK3CA* testing in BC is systemically complex, involving the selection of the proper biological specimen to test (i.e., primary tumor, metastasis, ctDNA) and the most appropriate testing method (i.e., RT-PCR, NGS). This work aims to provide real-life experience on *PIK3CA* testing in BC.

Methods: Clinical data on *PIK3CA* mutations in HR+/HER2- BC patients (tissue or liquid biopsy samples) were collected from two Italian referral molecular pathology laboratories between 2019 and 2023. *PIK3CA* mutational analyses were carried out using NGS and/or RT-PCR.

Results: Among the total 120 patients with HR+/HER2- BC tested for *PIK3CA*, n=32 (26.7%) were from external consultations. Most of the analyses (n=108; 90.0%) were conducted on tissue samples only, whereas plasma samples were

profiled in n=12 (10.0%) cases, including n=4 cases with both type of samples analyzed. In terms of the diagnostic methods employed, NGS targeted panels were at the forefront of *PIK3CA* testing since they were used in n=114 (95.0%) of cases. Overall, n=35/120 (29.2%) cases were *PIK3CA*-mutated. Considering the specific *PIK3CA* mutations detected, most of them occurred in the exons 9 (p.E542K/x, p.E545K/x, and p.Q546K/R/x) and 20 (p.T1025A, p.D1029N, p.H1047L/R, p.G1049R, and p.N1068Lfs*5). In one case, while the druggable mutation p.H1047R was detected through tissue testing, the corresponding plasma analysis showed a wild-type *PIK3CA* status.

Conclusion: In metastatic HR+/HER2- breast cancer, *PIK3CA* testing is more frequently performed on tissue samples. Liquid biopsies provide a valuable alternative, particularly in cases with insufficient material. The presence of *PIK3CA* mutations in plasma samples highlights their clinical importance, warranting further research for treatment decisions. However, techniques with high sensitivity are needed to detect low-frequency alterations from ctDNA routine samples.

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THE JOURNAL OF LIQUID BIOPSY 1 (2023) 100010 100125

BLU-945 MONOTHERAPY AND COMBINATION WITH OSIMERTINIB IN PREVIOUSLY TREATED PATIENTS WITH ADVANCED EGFR-MUTANT (EGFRM) NSCLC IN PHASE 1/2 SYMPHONY STUDY

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Background: BLU-945, an investigational, oral tyrosine kinase inhibitor (TKI), targets common EGFR-activating and T790M and C797X resistance mutations and is selective against EGFR wild type (WT). SYMPHONY (NCT04862780) is an ongoing phase 1/2 study of BLU-945 monotherapy and in combination with osimertinib (updated from Elamin YY, et al. J Clin Oncol. 2023;41(16_suppl):9011).

Methods: For phase 1 dose escalation, patients (≥18 years; metastatic EGFRm NSCLC; ECOG PS 0–1; treated with ≥1 EGFR TKI) received BLU-945 monotherapy and patients progressing on osimertinib as the last therapy line received BLU-945 with 80 mg osimertinib. Dose escalation followed a Bayesian Optimal Interval design. Safety, pharmacokinetics, preliminary efficacy, and circulating tumor DNA (ctDNA) dynamics were assessed.

Results: By April 28, 2023, 112 patients received BLU-945 monotherapy (25–600mg QD; 100–300mg BID). Patients had a median of 3.5 lines of prior therapy and were genomically complex with ctDNA-detectable EGFR on-target and/or off-target resistance alterations at baseline. DLTs occurred in 12 patients at doses of ≥400 mg/day; most common was Grade 3 elevated alanine transaminase and aspartate transaminase. EGFR-WT–associated AEs were low grade and infrequent (<10%). Monotherapy yielded dose-dependent reductions in EGFRm ctDNA levels at Day 15; however, efficacy was limited with 2 confirmed partial responses (PR) observed at ≥400 mg/day.

Fifty-five patients with a median of 2 lines of prior therapy received BLU-945 (200–400 mg QD; 100–200 mg BID) in combination with osimertinib. EGFR-WT–associated AEs were infrequent and the majority were Grade 1. DLTs occurred in 3 patients at ≥200 mg/day (Grade 3 acute respiratory failure, Grade 4 pneumonitis, Grade 3 dermatitis acneiform). Dose-dependent reductions in EGFRm ctDNA levels were observed at Day 15. Tumor reductions, including confirmed PRs in 4 patients progressing on osimertinib, were observed at ≥300 mg/day. Early robust reductions in EGFR on-target resistance mutant allele levels correlated with patients who demonstrated target lesion reduction. Dose escalation is ongoing.

Conclusion: Profiling of early ctDNA dynamics as pharmacodynamic readout offers potentially promising correlative insights in better understanding clinical response. Phase 1 data support BLU-945 plus osimertinib as a differentiated, fully oral, novel combination for treatment of EGFRm NSCLC, warranting further clinical development.