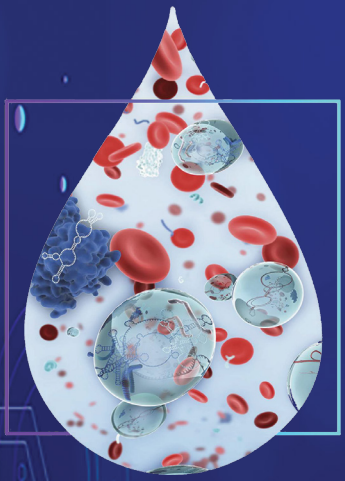


Hellenic Society of Liquid Biopsy Two Day-Conference

"LIQUID BIOPSY: Latest advances and applications in oncology"

20-21 November, 2026

Pasteur Institute, Athens

Free Registration

www.hsliquidbiopsy.com

Organized by:
Evi Lianidou, Professor,
National and Kapodistrian University of Athens
Hellenic Society of Liquid Biopsy

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WELCOME ADDRESS

Dear Friends and Colleagues,

It is a great pleasure to welcome you all to this year's 2 Day-Conference organized by the Hellenic Society of Liquid Biopsy: "Liquid Biopsy: Latest Advances and applications in Oncology".

Liquid biopsy is a minimally invasive approach that is mainly based on the analysis of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). Liquid biopsy is an important tool in the fight against cancer, since it provides the ability to monitor in serial samples a molecular portrait of the tumor in real time. CTC and ctDNA analysis present currently a powerful tool for the management of advanced- and early-stage cancer patients and offers the unique potential to better understand the biology of metastasis and resistance mechanisms to specific treatments. Liquid biopsy has already revealed its tremendous potential to significantly change the therapeutic strategy and management of cancer patients.

This year's 2 Day-Conference of HSLB is focused on the latest advances on liquid biopsy basic research, state of the art technologies, and clinical applications in many types of cancer, from bench to bedside. In this meeting, we bring together Key Opinion Leaders in the field, world-top liquid biopsy clinicians and researchers, that will present state of the art data and technology innovations in the field. During these two days, we will all have again the chance to interact and establish fruitful collaborations in a highly relaxing and stimulating environment.

Warm regards,

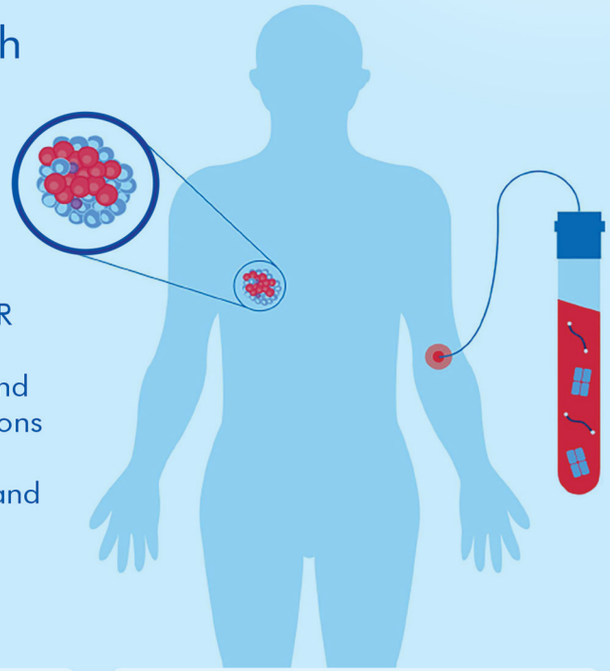


Evi S. Lianidou, PhD
President of the Hellenic Society of Liquid Biopsy
Professor of Analytical Chemistry - Clinical Chemistry
ACTC lab
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Latest advances and applications in oncology

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PROGRAM

Day 1: Friday 20th November 2026

08.30-09.00 **Registration - Coffee**

Session 1: Liquid Biopsy
Chairing: **Evi Lianidou, L. Kaklamanis**

09.00-09.30	Liquid Biopsy: latest advancements	**Evi Lianidou**
09.30-10.00	Extracellular Vesicles as a new Liquid Biopsy component	**An Hendrix**
10.00-10.30	Perspectives in educating molecular pathologists on liquid biopsy: Toward integrative, equitable, and decentralized precision oncology	**Loukas Kaklamanis**
10.30-11.00	Tissue biopsy vs liquid biopsy as complementary tools	**Ioannis Pateras**
11.00-11.30	Pre-analytical conditions and implementation of quality control steps in liquid biopsy analysis	**Aliki Ntzifa**

11.30-12.00 **Coffee break**

Session 2: The potential benefits of the implication of Liquid Biopsy analysis in the Greek Health System
Chairing: **Evi Lianidou** and **Athina Markou**

12.00-12.30	Ο ρόλος του ΕΔΙΜΟ στην αξιοποίηση των μοριακών αναλύσεων διαχείριση ασθενών με καρκίνο	**K. Stratakis**
12.30-13.00	Εξατομικευμένη Ιατρική και Θεραπευτική Ακριβείας: Η Ιατρική του αύριο, σήμερα	**G. Patrinos**
13.00-13.30	Στόχοι και δράσεις της Ελληνικής Ομοσπονδίας Καρκίνου (ΕΛΛΟΚ) ως προς την εφαρμογή της υγρής βιοψίας στη διαχείριση ασθενών με καρκίνο	**Μαρία Νομικού**
13.30-14.00	Hellenic Society of Medical Oncology (HeSMO): Η συμβολή της ΕΟΠΕ στις εξετάσεις βιοδεικτών για τον καρκίνο	**Manolis Saloustros**

14.00-14.30 **Light Lunch**

Session 3: CTC analysis: clinical applications
Chairing: **Athina Markou**

14.30-15.00	Phenotypic characterization of CTC in pancreatic cancer	**Galatea Kalergi**
15.00-15.30	Detection of MRD in early breast cancer: challenges and opportunities	**Michail Ignatiadis**
15.30-16.00	Prognostic value of liquid biopsy in Waldenström macroglobulinemia	**Tina Bagratuni**
16.00-16.30	Circulating Multiple Myeloma Cells (CMMC) enumeration in patients with plasma cell dyscrasias	**E. Theochari**

16.30 –17.00 **Coffee break**

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Session 5: CTCs: Phenotypic and genotypic characterization
Chairing: **Galatea Kalergi**

17.00-17.30	Phenotypic and genotypic characterization of single circulating tumor cells in the follow-up of high-grade serous ovarian cancer	**Sabine Kasimir-Bauer**
17.30-18.00	Tracking treatment resistance in prostate cancer using ctDNA and CTC in situ expression profiles	**Amin El-Heliebi**
18.00-18.30	Analyzing metastatic phenotypes and CTC clustering mechanisms using microfluidics	**Stuart S. Martin**

Day 2: Saturday 21st November 2026

Session 6: ctDNA analysis: technology advancements and clinical applications
Chairing: **Aliki Ntzifa**

09.00-09.30	The clinical value of ctDNA testing for the management of cancer patients	**Fotini Papageorgiou**
09.30-10.00	The power of ddPCR in liquid biopsy analysis	**Athina Markou**
10.00-10.30	Epigenetics: DNA methylation markers in CTCs and ctDNA	**Ioanna Balgouranidou**
10.30-11.00	PD-L1 in CTCs and immunotherapy response	**Areti Strati**
11.00-11.30	Multimodal liquid biopsy analysis in patients with sporadic Medullary Thyroid Carcinoma (spMTC)	**G. Simeakis**
11.30-12.00	Coffee break	

Session 7: Novel Frontiers in Liquid Biopsy
Chairing: **Evi Lianidou**

12.00-12.30	The potential of FRAGMENTOMICS in liquid biopsy	**Ellen Heitzer**
12.30-13.00	Multimodal liquid biopsy analysis for the detection of homologous recombination deficiency in prostate cancer	**George Vlachos**

Session 8: Young Investigators Forum
Chairing: **Aliki Ntzifa, Ioanna Balgouranidou**

13.00-13.10	Ultra-Sensitive Detection of BRAF V600E in Plasma cfDNA of Early-Stage Melanoma patients based on PD UV-ME ddPCR	**Elina Lagopodi**
13.10-13.20	Targeting MCT1 and MCT4 in Non-Small Cell Lung Cancer: functional and translational studies in cancer cell lines and circulating tumor cells	**Karolina Mangani**
13.20-13.30	Organ-on-a-chip for circulating tumor cell (CTC) culture	**Alexia Tsagkanou**
13.30-13.40	Comparison of PD-L1 expression status between primary tumor tissues and circulating tumor cells (CTCs) of patients with triple-negative breast cancer (TNBC)	**Sofia Chatziavraam**
13.40-13.50	The Added Value of ctDNA ESR1 Testing by NGS vs ddPCR in Women	

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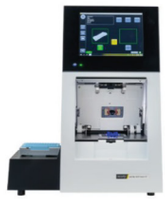


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	with Metastatic Breast Cancer	**Niki Prekete**
13.50-14.00	Longitudinal DNA methylation analysis in plasma cell-free DNA of NSCLC patients monitored during treatment with ICIs	**Elena Themistokli**
14.10-14.20	Tracking FGFR3 and PIK3CA Mutations in Bladder Cancer via Plasma and Urine ddPCR	**Elena Thanou**
14.20-14.30	Proteomic investigation of tumor heterogeneity and therapy resistance in Waldenström macroglobulinemia. Toward proteomics implementation in liquid biopsy approaches	**Ioannis Kollias**
14.30-14.40	Validation of an ultrasensitive ddPCR assay for KRAS G12C genotyping in liquid biopsy samples: a first step to ISO-15189 accreditation	**George Michael**
14.40-15.00	Light Lunch	
	Session 9: CTC analysis: technology advancements and clinical applications Chairing: **Areti Strati**	
15.00-15.30	ESR1 Mutations in Breast Cancer: From Molecular Detection to Clinical Decision-Making - Insights from Greece	**Cleopatra Rapti**
15.30-16.00	ESR1 mutation analysis in CTCs and ctDNA: an update	**Aliki Ntzifa**
16.00-16.30	HER2 analysis in CTCs: an update	**Stavroula Smilkou**
16.30-17.00	Coffee break	
	Session 10: Liquid Biopsy in the clinic: latest update Chairing: **Vasilis Georgoulias, Evi Lianidou**	
17.00-17.30	MCED tests and early detection of cancer	**Nick Papadopoulos**
17.30-18.00	Liquid biopsy approaches for minimal residual disease detection and therapy response monitoring in NSCLC	**Elsa Anagnostou**
18.00-18.30	Novel liquid biopsy technologies for earlier detection of cancer and minimal residual disease	**Mike Makrigiorgos**

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FACULTY

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Georgoulias Vasilis Professor Emeritus, University of Crete, Greece

Kaklamanis Loukas MD, PhD, Onassis Hospital, Athens, Greece

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Markou Athina Assoc. Professor, Analysis of Circulating Tumor Cells (ACTC) Lab, Dept of Chemistry, University of Athens (NKUA), Athens, Greece

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Strati Areti Assistant Professor, Medical School, University of Athens (NKUA), Athens, Greece

Theochari Eleni Medical School, University of Athens (NKUA), Athens, Greece

Vlachos George PhD, Medical University of Graz, Austria

INTERNATIONAL SPEAKERS *(online lectures)*

Anagnostou Elsa MD-PhD, Alex Grass Professor of Oncology, Co-Director, Division of Upper Aerodigestive Malignancies Program, Co-Director, Cancer Genetics and Epigenetics Program, Director, Thoracic Oncology Biorepository, Co-Director, Thoracic Oncology Precision Medicine Center of Excellence, Co-Leader, Molecular Tumor Board, Leader, Precision Oncology Analytics, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine,
https://profiles.hopkinsmedicine.org/provider/valsamo-elsa-anagnostou/2701461

El-Heliebi Amin Associate Professor, Gottfried Schatz Research Center, Division of Cell Biology, Histology & Embryology, Medical University of Graz, Graz, Austria,
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Heitzer Ellen Professor, Institute of Human Genetics, Diagnostic and Research Center for Molecular BioMedicine, Medical University of Graz, Graz, Austria,
https://ris.medunigraz.at/?sprache_in=en&ansicht_in=&menue_id_in=101&id_in=2000070

Hendrix An, Professor, Cancer Research Institute Ghent, University of Ghent,
Belgiumhttps://www.crig.ugent.be/en/prof-an-hendrix-phd

Ignatiadis Michail MD, PhD, Director, Breast Medical Oncology Clinic & Program, Medical Oncology Department, Institut Bordet, Associate Professor, Université Libre de Bruxelles, Chair, Breast Cancer Group, EORTC, Belgium,
https://www.esmo.org/about-esmo/biographies/michail-ignatiadis

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Makrigiorgos Mike Professor and Director, Medical Physics and Biophysics, Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA,
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Martin Stuart S Professor, University of Maryland School of Medicine, Baltimore, USA,
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Papadopoulos Nick Professor of Oncology and Pathology, Director of Translational Genetics, Ludwig Center for Cancer Genetics and Therapeutics, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, USA,
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YOUNG INVESTIGATORS FORUM

ABSTRACTS

Ultra-Sensitive Detection of BRAF V600E in Plasma cfDNA of Early-Stage Melanoma patients based on PD UV-ME ddPCR

Elina Lagopodi[1] Stavroula Smilkou[1], Aikaterini Kypraiou[2], Athina Markou[1], Alexander J Stratigos[2], Mike Makrigiorgos[3], Evi Lianidou[1]

[1] ACTC Lab, Lab of Analytical Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, Athens, Greece, [2] First Department of Dermatology, "Andreas Sygros" Hospital for Skin Diseases, National and Kapodistrian University of Athens, Medical School, Athens, Greece, [3] Dana-Farber Cancer Institute and Brigham and Women's Hospital, Department of Radiation Oncology-Harvard Medical School, USA

INTRODUCTION: In melanoma, Liquid Biopsy (LB) plays a critical role, enabling the detection of BRAF mutations in plasma cfDNA, via ddPCR, thus guiding targeted therapy and monitoring treatment response. However, in early-stage melanoma, due to the low tumor burden there is limited analytical sensitivity in the mutant allele detection highlighting the need of wild type (WT) allele masking. We have recently reported that the combination of PD UV-ME and ddPCR can selectively degrade WT alleles through ultraviolet (UV) light, thus enhancing the detection of the BRAF V600E mutation in plasma cfDNA. In this study, using this highly sensitive methodology we report for the first time the detection of BRAF V600E mutations in early-stage melanoma patients at a high percentage.

MATERIALS & METHODS: 61 plasma samples, were obtained from 61 early-stage melanoma patients along with 18 plasma (2mL) samples from Healthy Donors (HD) were used as a control group. Plasma cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). All plasma cfDNA samples from both early melanoma patients and HDs were processed by PD-UVME, thus enriching the relative abundance of the BRAF V600E mutant allele. PCR amplification was performed with the objective of the selective amplification of the mutation-enriched DNA. Prior to ddPCR analysis, the amplified UV-ME PCR products were subsequently diluted 1:10 according to their concentration measured using the Quibit fluorometric assay. Mutation Detection was performed by using the ddPCR BRAF V600E Mutation Assay kit by Bio-Rad following the manufacturer's insert instructions.

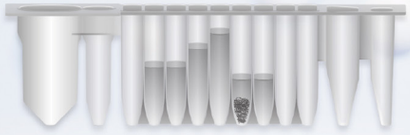
RESULTS: A cutoff threshold of ≥ 4 mutation-positive droplets was established to define positive samples based on the analysis in the HDs group. In plasma cfDNA, 18/61 (29,5%) samples were found positive for the *BRAF V600E* mutation. Of note, all samples which were identified as positive via PD-UVME, were negative through direct ddPCR. All HD plasma cfDNA samples (0/18, 0%) were negative.

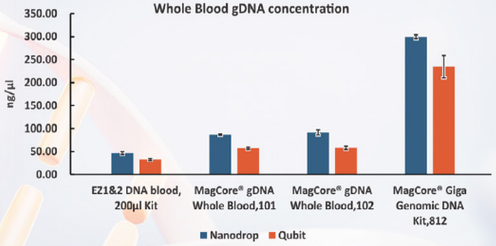
CONCLUSIONS: Combination of UV-ME and ddPCR appears to be very effective since it enables the selective masking of WT alleles, thereby improving the detection of the druggable *BRAF V600E* mutant alleles in early-stage melanoma patients. We strongly believe that the implications of this finding in the therapeutic management of these patients should be further explored.

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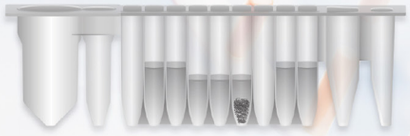


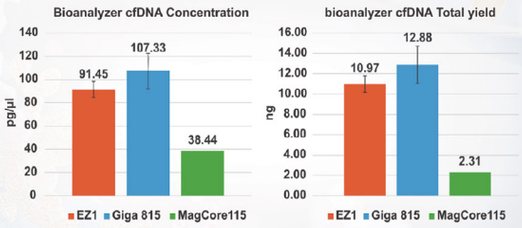


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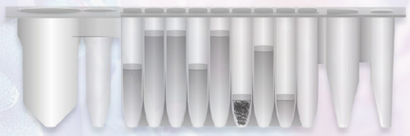


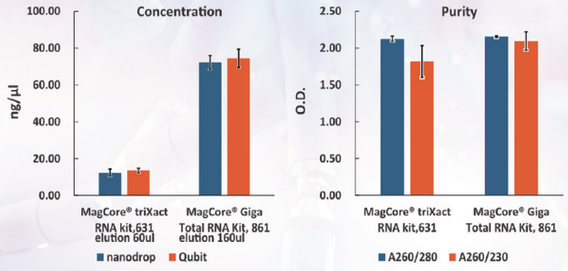


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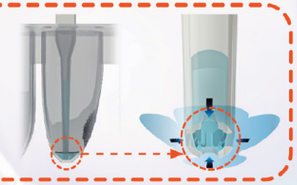
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CE IVD

Targeting MCT1 and MCT4 in Non-Small Cell Lung Cancer: functional and translational studies in cancer cell lines and circulating tumor cells

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Background: Monocarboxylate transporters MCT1 and MCT4 have been reported to be overexpressed in circulating tumor cells (CTCs) derived from non-small cell lung cancer (NSCLC) patients, with their expression showing significant correlations with clinical outcome. This could be attributed to their role in supporting tumor growth and metastasis by mediating lactate transport within the tumor microenvironment.

Methods: The present study investigated the effects of the dual MCT1/MCT4 inhibitor syrosingopine in H1975 and CTC-MCC-41 cells, as well as in CTCs and circulating tumor-associated cells (CTACs) from NSCLC patients. Upon treatment, apoptosis was evaluated using M30 antibody, mitochondrial ROS (mtROS) levels were measured using MitoSOX™ Red, while cell migration was assessed by transwell assays. Furthermore, MCT1 and MCT4 expression was analyzed by qPCR and Western blot. Blood samples from 12 NSCLC patients were also cultured, treated with syrosingopine, and stained for CK, CD45, M30, MCT1 and MCT4. Samples were analyzed using the VyCAP platform, followed by statistical analysis. An IC_{50} of 13μM was established after 48h of syrosingopine treatment and used for subsequent experiments.

Results: Results showed altered gene and protein expression of MCTs, and reduced cell viability by 46% (p=0.0029) in H1975 and 21% (p=0.0052) in CTC-MCC-41 cells. Significantly, M30-positive cells were increased by 370% (p=0.0004) in H1975, and 45% (p<0.0001) in CTC-MCC-41. Furthermore, increased mtROS levels by 140% (p=0.0001) in H1975 and 90% (p=0.0022) in CTC-MCC-41 cells were observed, while cell migration was reduced to 78% in H1975 cells (p=0.0001). In patients' samples, total CTAC numbers and MCT1-positive CTACs were both decreased after treatment (p=0.055) and (p=0.041), respectively.

Conclusions: These findings highlight the potential of MCT1/MCT4 inhibition to disrupt tumor metabolic adaptation and metastasis. Further investigation in CTCs from NSCLC patients is warranted to validate these results.

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Organ-on-a-chip for circulating tumor cell (CTC) culture

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Background: Circulating tumor cells (CTCs) are the primary mediators of metastatic dissemination, detaching from the primary tumor and entering the bloodstream. CTC detection remains a challenging process in the liquid biopsy field due to their low abundance in circulation. Microfluidic technologies have emerged as an interdisciplinary approach for CTC isolation, using microscale devices that enable fluid control. These platforms offer portability, rapid sample processing, accurate experimental control, and high analytical sensitivity. "Organ-on-a-chip" technology represents an advanced tool for investigating metastasis by creating controlled microenvironments that closely mimic human organ function in cancer research. These systems enable real-time investigation of the biomechanical cues that regulate cancer cell morphology, aggressiveness, migration, and invasion.

Methods: The current study aimed to determine the optimal substrate using different resins for 3D printing of an "Organ-on-a-chip" device for CTC isolation. Deliberately, in this project, the only available CTC cell line (CTC-MCC-41) was used, derived from a patient with metastatic colon cancer. Two different resins were evaluated, each prepared with three different surface roughness conditions: Formlabs Clear Resin V4.1 and Formlabs BioMed Clear Resin. CTCs were cultured on both resin substrates, and their viability was subsequently assessed in comparison with control samples and across the two substrate types.

Results: The results revealed a statistically significant difference in the biocompatibility of the two resin materials. Notably, Clear Resin V4.1 exhibited higher cytotoxicity, with cell viability remaining below 15%. In contrast, BioMed Clear Resin demonstrated significantly greater biocompatibility, with cell survival rates in the range of 50%-65%. Statistical analysis of three independent biological replicates demonstrated higher biocompatibility of BioMed Clear Resin compared to Clear Resin V4.1 (R1: p<0.0001, R2: p=0.001, R3: p=0.0399). These results confirmed that iBioMed Clear Resin is a suitable substrate for the 3D printing of the "Organ-on-a-chip" device. Based on these findings, BioMed Clear Resin was selected for the fabrication of the Organ-on-a-chip device, with all device components successfully printed.

Conclusions: Overall, the results highlight the importance of proper resin selection in the development of biocompatible 3D-printed platforms and support the use of BioMed Clear Resin for further development of CTC-based Organ-on-a-chip applications.

Comparison of PD-L1 expression status between primary tumor tissues and circulating tumor cells (CTCs) of patients with triple-negative breast cancer (TNBC)

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Background: PD-L1 is an established biomarker and therapeutic target in triple-negative breast cancer (TNBC). However, its expression may dynamically evolve in response to therapeutic pressure and metastatic progression. Liquid biopsy offers a minimally invasive approach for real-time assessment of tumor-associated biomarkers. Here, we evaluated PD-L1 status in parallel in primary tumor tissue and circulating tumor cells (CTCs) from patients with TNBC.

Methods: Primary tumor tissues and matched peripheral blood (PB) samples from 92 patients with TNBC were analyzed. Tissue analysis included assessment of tumor-infiltrating lymphocytes (TILs) and determination of the PD-L1 combined positive score (CPS). PD-L1 positivity was additionally assessed separately in cancer cells and mononuclear inflammatory cells (MICs). CTCs were enriched from PB using Ficoll density gradient centrifugation and microfluidic cell separation (Parsortix®), and identified by triple immunofluorescence staining for cytokeratins/PD-L1 (E1L3N)/DAPI.

Results: High TIL density (≥50%) was observed in 19.6% of primary tumors. A positive CPS (≥1) was detected in 56.5% of tumors, with PD-L1 expression predominantly observed in MICs rather than cancer cells (59.8% vs. 18.5% of tumors, respectively). CTCs were detected in 23.9% of patients (total CTCs: n=185). Among CTC-positive patients, PD-L1-positive CTCs were identified in 59.1%, accounting for 49.2% of all detected CTCs. PD-L1-positive CTCs were less frequently detected in patients with high TIL density in the primary tumor compared with those with low TIL density (20% vs. 70.6%, respectively; p=0.116). PD-L1 expression was more frequently observed on CTCs than on primary tumor cancer cells (59.1% vs. 13.6% of patients, respectively). Notably, only one patient exhibited PD-L1 positivity in both compartments, whereas in 54.5% of patients, PD-L1 was exclusively expressed by CTCs (positivity discordance: 93.3%, p=0.013; McNemar test).

Conclusions: PD-L1 expression demonstrates substantial discordance between primary tumors and CTCs in patients with TNBC, with a clear predominance of PD-L1 expression at the CTC level. These findings support further investigation of liquid biopsy as a complementary, minimally invasive approach for real-time assessment of PD-L1 status in TNBC.

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The Added Value of ctDNA ESR1 Testing by NGS vs ddPCR in Women with Metastatic Breast Cancer

Niki Prekete, Aikaterini Oikonomaki, Andromachi Vagena, Stavroula Samara, Stamatina Roussou, Irini Tsiasioti, Ioanna Georgousi, Leman Karantai, Despoina Avramopoulou, Stavros Bournazos, Georgia Christopoulou, Pantelis Constantoulakis

Genotypos MSA, Athens, Greece

Introduction: ESR1 mutations (estrogen receptor 1) are well-known drivers of resistance to endocrine therapy (i.e. aromatase inhibitors) in estrogen receptor-positive (ER+) metastatic breast cancer (mBC). They serve as predictive biomarkers for FDA- and EMA-approved therapies. ESR1 activating variants are mainly detected by NGS or ddPCR on liquid biopsies (LB) to identify mBC patients who could benefit from i.e. Elacestrant (Orserdu), Imlunestrant (Inluriyo), specifically approved for ER-positive, HER2-negative mBC. The method of choice is debatable. We aim to evaluate the utility of particular ddPCR and NGS methods in this context.

Patients and methods: In a total of 140 women with mBC, eligible for ESR1 LB testing, who were referred to our lab, 66 were tested by ddPCR (ddPlex ESR1 mutation detection kit, QX Manager Software 2.3.1, BioRad). These were highly selected patients, with HR+/HER2- mBC who had disease progression after at least one line of therapy. The remaining 74 were a more heterogeneous mBC cohort, tested by a 34-gene targeted NGS panel (oncoReveal™ Essential LBx-NGS Panel Kit, PiVAT, Pillar Biosciences).

Results: 25/66 (37.87%) patients were ESR1+ by ddPCR and 12/74 (16.21%) by NGS. The mutation detection rate by both approaches was within the range reported by the scientific community (10-50% in mBC). Overall, ddPCR detected 28 and NGS 29 ESR1 Tier1 variants, with variant frequencies ranging from 0.06-23.48% and 0.06-57.14%, respectively. Notably, some patients had >1 ESR1 variants. Most frequently mutated codons were Tyr537 and Asp538 in both approaches, as expected. ddPCR detected twice as many variants as NGS, at least partially due to its higher sensitivity; LOD 0.01-0.025% vs 0.1% for NGS. In addition, the “NGS cohort” included mBC patients with less stringent recruiting criteria. In the NGS cohort, 54 patients had 97 non-ESR1 Tier1/2 variants. Besides TP53, Tier 1 variants were most frequent in AKT-pathway genes (42/97; 43.3%); PIK3CA (30/97; 30,9%), PTEN (11/97; 11,3%), and 1 AKT1.

Conclusions: Both ddPCR and NGS methods applied were efficient in selecting ESR1+ mBC patients, eligible for FDA-approved Selective Estrogen Receptor Degrader (SERD) therapy. However, NGS detected additional patients with AKT-pathway mutations, thereby granting eligibility for approved AKT inhibitor therapy. Although the ddPCR method applied has higher sensitivity, it targets only ESR1 hotspots, limiting therapeutic options. By applying the liquid biopsy NGS approach, more patients may benefit from the detection of a broader spectrum of predictive biomarkers.

Longitudinal DNA methylation analysis in plasma cell-free DNA of NSCLC patients monitored during treatment with ICIs

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Introduction: Resistance to immune checkpoint inhibitors (ICI) in non-small cell lung cancer (NSCLC) remains a clinical challenge. Epigenetic changes such as DNA methylation could serve as potential biomarkers for the early detection of resistance to immunotherapy. In this study, we studied DNA promoter methylation of five genes implicated in tumor progression and tumor immunity (NALCN, KEAP1, RASSF1A, SOX17, SHOX2) in plasma cell-free DNA (cfDNA) of NSCLC patients longitudinally monitored during treatment with ICIs.

Patients and methods: Peripheral blood (PB) samples were collected from 79 metastatic NSCLC patients at three time points during treatment with ICIs: a) Vo, before treatment (n=79), b) V1, two months after ICIs (n=46) and c) V2 at progression of disease (PD) (n=7). Plasma samples (2mL) from 10 healthy donors (HD) were used as a control group and cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). The EZ DNA Methylation Gold Kit (ZYMO Research, Irvine, CA, USA) based on sodium bisulfite (SB) was used for DNA methylation analysis. Previously developed and analytically validated real time MSP assays were used for DNA methylation analysis.

Results: At V0, before treatment initiation, RASSF1A promoter methylation was detected in 10/79 (12.7%) patients, SOX17 in 32/79 (40.5%), SHOX2 in 8/79 (10.1%), KEAP1 in 4/79 (5.1%) and NALCN in 6/79 (7.6%). At V1, after two months, RASSF1A promoter methylation was detected in 5/46 (10.9%) patients, SOX17 in 8/46 (17.4%), SHOX2 in 3/46 (6.5%) KEAP1 in 2/46 (4.3%) and NALCN in 1/46 (2.2%). At V2, PD, DNA methylation analysis was performed for only 7 NSCLC patients; RASSF1A promoter methylation was detected in 14.3%, SOX17 in 42.9% and SHOX2 in 14.3%. Direct comparison of 46 matched cfDNA samples before and after immunotherapy revealed that 21/46 (45.6%) patients had at least one methylated gene before immunotherapy whereas 16/46 (34.8%) were positive for at least one of these DNA methylation markers after immunotherapy. DNA methylation profile for all these gene promoters varied between V0 and V1 whereas six patients remained positive for SOX17 methylation before and after ICI treatment.

Conclusions: Longitudinal DNA methylation analysis in plasma cell-free DNA of NSCLC patients monitored during treatment with ICIs could be a promising tool for the early detection of resistance. These findings should be further validated in larger cohort studies to investigate the clinical significance of these DNA methylation biomarkers and their association with response to immunotherapy.

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Tracking FGFR3 and PIK3CA Mutations in Bladder Cancer via Plasma and Urine ddPCR

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Introduction: Bladder cancer (BC) is a common malignancy with a high local and systemic recurrence rate, necessitating reliable surveillance tools. Hotspot mutations have been identified in the TERT promoter as well as in the FGFR3 and PIK3CA genes. Importantly, these alterations are not only implicated in tumorigenesis but also have therapeutic relevance, as targeted therapies against FGFR3 alterations and PI3K pathway inhibitors for PIK3CA-mutant tumors have received FDA approval, underscoring their clinical significance. Currently, molecular testing to guide therapeutic decision-making relies primarily on tumor tissue analysis, which requires invasive sampling and may not adequately capture tumor heterogeneity or enable longitudinal monitoring. cfDNA from liquid biopsies, such as urine supernatant and plasma, holds significant promise as a source of non-invasive biomarkers. In this study, we developed drop-off droplet digital PCR (dddPCR) assays to detect hotspot FGFR3 and PIK3CA mutations in liquid biopsy samples.

Patients and methods: A highly sensitive dddPCR assay was developed, optimized, and analytically validated for the simultaneous detection of four hotspot FGFR3 mutations (S249C, R248C, Y373C, and G370C). The assay was specifically designed to enable multiplex mutation screening in low-abundance cfDNA and demonstrated robust analytical performance. It was subsequently applied to matched plasma and urine cfDNA samples from 33 individuals (8 healthy donors and 25 BC patients). In parallel, a previously established dual dddPCR assay for PIK3CA hotspot mutations was implemented on the same sample set, enabling comprehensive mutation profiling.

Results: Analytical validation demonstrated that the assay achieves a sensitivity down to 0.1% mutant allele fraction, enabling reliable detection in low tumor burden samples such as urine and plasma cfDNA. Among the 25 BC patients, FGFR3 mutations were identified in 1/25 patients (4%) in both urine and plasma cfDNA, consistent with NGS analysis of the corresponding tumor tissue. In this case, the mutation score was higher in plasma than in urine. Two additional patients (8%) were positive for FGFR3 mutations exclusively in plasma cfDNA, while their matched urine samples were negative. Using the PIK3CA assay, 3/25 patients (12%) were positive for mutations in plasma cfDNA, whereas only 1/25 (4%) also showed detectable mutations in the matched urine sample.

Conclusions: The developed multiplex dddPCR assays enable highly sensitive detection of actionable FGFR3 and PIK3CA mutations in urine and plasma cfDNA. Detection of mutations exclusively in plasma in several cases underscores the complementary value of liquid biopsy over tissue-based testing. This minimally invasive approach may facilitate real-time molecular profiling and support targeted therapeutic decision-making in bladder cancer.

Proteomic investigation of tumor heterogeneity and therapy resistance in Waldenström macroglobulinemia. Toward proteomics implementation in liquid biopsy approaches

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Introduction: Waldenström macroglobulinemia (WM) is a rare indolent B-lymphoproliferative neoplasm characterized by bone-marrow infiltration by lymphoplasmacytic cells that are producing IgM paraprotein. The MYD88 L265P point mutation and the mutations of CXCR4 are the major genetic features of the disease affecting 90-95% and 30-40% respectively and contributing to disease development, clinical presentation and patient outcomes. Although Bruton's tyrosine kinase inhibitors (BTKis), with ibrutinib as their first approved agent, have substantially improved patient outcomes, intra-tumor and inter-patient heterogeneity lead to some patients failing to respond or eventually relapse. The mechanisms underlying response and resistance as well as tumor heterogeneity remain insufficiently understood. Bone marrow aspiration remains, to this day, the gold standard for the diagnosis of the disease. However, it is a painful and invasive procedure that is difficult to perform at regular intervals to monitor the development of the disease. Liquid biopsy refers to the set of methods that utilize information derived from peripheral blood and thus serves as a minimally invasive complementary approach to bone marrow biopsy. This study suggests the use of proteomic methods to elucidate tumor heterogeneity and to provide information at the liquid biopsy level that may be of prognostic significance.

Patients and methods: In an effort to assess the WM proteome using a liquid biopsy approach, we performed plasma proteomics in a cohort of 35 patients using the proximity extension assay (PEA) developed by Olink Proteomics. The panel (Olink Explore HT) is targeted to detect more than 5,400 proteins. The heterogeneity among patients and the temporal heterogeneity within the same patients before and after treatment were highlighted using this technique, offering a potential future tool for prognosis and patient monitoring.

Results: Specifically, the two distinct time points are effectively distinguished by 248 differentially expressed proteins (DEPs) associated with known pathways involved in the disease, such as the JAK/STAT pathway, TNF-α, cytokine secretion, and inflammatory response. Furthermore, 64 proteins successfully distinguished the R group from the NR group at baseline, enabling the development of a tool with potential prognostic significance using Elastic Net regression. Our complimentary results performing liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics in immunomagnetically enriched CD19+ bone marrow mononuclear cells (BMMCs) reveals noteworthy differences in cell biology at baseline of 25 patients, of whom 17 responders (R) and 8 non-responders (NR). The complexity and temporal tumor heterogeneity are also evident by the shifts in proteomic signatures before and after 6 months of treatment. Specifically, baseline CD19+ cells showed enrichment of mitochondrial, oxidative phosphorylation and respiratory chain assembly proteins (COA3/5/7, COX7C, NDUFB6, BCL2L1), consistent with metabolically active, apoptosis-resistant cells. Following BTKi treatment, N-linked glycosylation and ER-Golgi protein trafficking pathways became significantly enriched, driven by proteins including DPM3, RPN2, TMED2 and ARF1. Baseline comparison between responders and non-responders identified 92 proteins detected in ≥25% of responder samples but absent from all non-responder samples. These proteins suggest that responding clones exhibit increased cell-cycle activity (CDK4, CKS1B, DLGAP5, RACGAP1, CKAP2L, ANAPC5), apoptosis priming with stress resistance (CRADD, PARP16, TRIM24, AKT3, SMAD5), and potential dependence on tumor microenvironment signaling (CD200, BTLA).

Conclusions: Future goals include assessing the patient proteome of plasma and CTCs, using highly sensitive mass spectrometry-based assays that combine the best of both worlds: the untargeted capabilities of LC-MS/MS and the minimally invasive liquid biopsy methodology.

Validation of an ultrasensitive ddPCR assay for KRAS G12C genotyping in liquid biopsy samples: a first step to ISO-15189 accreditation

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Introduction: Implementation of circulating tumor DNA (ctDNA) analysis into routine clinical practice requires strict compliance with international quality standards. A rigorous validation framework is necessary to use molecular assays for routine clinical purposes with diagnostic reliability. The aim of this study was to perform analytical and clinical validation of a droplet digital PCR (ddPCR) assay for *KRAS p.G12C* genotyping in cfDNA samples, based on the performance criteria required for clinical laboratory accreditation under the ISO15189 standard.

Patients and methods: The commercially available PrimePCR™ ddPCR™ Mutation Detection Assay Kit (Bio-Rad) for the simultaneous detection of *KRAS WT* and *KRAS p.G12C* was used. gBlocks carrying the KRAS G12C mutation and WT were used as positive controls and to obtain a series of standard samples with the desired MAF range. Validation parameters included Limit of Blank (LoB), Limit of Detection (LoD), repeatability, reproducibility (cross-platform agreement), and linearity were performed in QX200™ and QX600™ (Bio-Rad) platforms. Plasma cfDNA was isolated from 28 colorectal cancer patients (41 longitudinal samples) and 10 healthy donors. All experimental workflows were implemented within an ISO 15189-compliant environment.

Results: The LoD was established at a concentration of 0.28 copies/μL (corresponding to an average of 5.6 mutant copies in a background of 818.2 wild-type copies per reaction) ensuring stable repeatability despite inherent sampling variability (CV = 33.8%). WT gBlocks were analyzed to establish a LoB of 0.06 copies/μL (3 droplets). Cross-platform comparison revealed negligible instrumentation bias. The validated ddPCR assay demonstrated 100% concordance with the reference genotyping method in 10 pre-characterized clinical samples (7 cfDNA and 3 FFPE tissue samples). All healthy donors cfDNA samples analyzed for *KRAS G12C* mutation were negative whereas only one clinical sample was found positive for *KRAS G12C* mutation.

Conclusions: Following these validation steps based on ISO 15189 requirements, the ddPCR assay for ultrasensitive KRAS G12C genotyping is ready for immediate routine diagnostic implementation.

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