



ESCUELA DE DOCTORADO
INTERNACIONAL DE LA USC

Elena María
Brozos Vázquez

Tesis doctoral

USO DE LA BIOPSIA LÍQUIDA PARA LA SELECCIÓN DE TRATAMIENTOS EN ONCOLOGÍA

Santiago de Compostela, 2024

Programa de doctorado en Investigación Clínica en Medicina



ESCOLA DE DOUTORAMENTO
INTERNACIONAL DA USC

TESIS DOCTORAL

USO DE LA BIOPSIA LÍQUIDA PARA LA SELECCIÓN DE TRATAMIENTOS EN ONCOLOGÍA

Autora

Elena María Brozos Vázquez

Directores: Rafael López López y Luis Ángel León Mateos

PROGRAMA DE DOCTORADO EN INVESTIGACIÓN CLÍNICA EN MEDICINA

SANTIAGO DE COMPOSTELA

2024

DECLARACIÓN DE CONFLICTOS DE INTERÉS

Dña. Elena María Brozos Vázquez, autora de esta tesis, no tiene conflictos de interés que deba declarar para la realización y presentación de esta tesis doctoral.

A vosotros.

AGRADECIMIENTOS

Este es el capítulo que más me aporta de forma personal, porque es la forma de compartir todos vosotros la alegría que el realizar una tesis doctoral conlleva después del esfuerzo llevado a cabo entre todos. Además, es necesario para mí expresar de manera universal la gratitud a todos aquellos que han sumado tanto con su enorme grano de arena depositado en este trabajo.

Debo comenzar agradeciendo a los directores de tesis, Dr. López y Dr. León, Rafa y Luis, primero al haber aceptado compartir esta aventura conmigo; y después, por su ayuda, ánimo, empuje y soporte continuos a lo largo de la elaboración de esta tesis. Asimismo, a todos aquellos que también me han echado sus dos manos para aspectos formales y normativos, en especial referencia a Pablo Taboada Antelo, por su paciencia, acercamiento y continua disponibilidad para todas mis dudas.

Me dirijo al personal de la Unidad de Análisis de Biopsia Líquida de ONCOMET por haber sido mis faros científicos en materia traslacional. Agradezco de forma especial su implicación a Laura Muínelo, por su enorme capacidad profesional y personal de haber estado a mi lado escuchando, científica y emocionalmente, todas mis inquietudes. Gracias por ser tan buena compañera y amiga.

En el aspecto clínico, he contado con el incondicional apoyo de mis compañeros de la Unidad de Tumores Digestivos, Sonia Candamio, Francis Vázquez, Yolanda Vidal, Vanesa Varela y Juan Ruiz; que de forma totalmente altruista me han ayudado con la selección de pacientes y la recogida de datos clínicos, así como la discusión de resultados y su aplicación a nuestro terreno favorito, la consulta diaria. Es un placer compartir patología con vosotros.

Los autores de los artículos han sido tremendamente generosos por permitir que un trabajo realizado en equipo pueda reflejarse aquí como mi tesis doctoral. Ellos han tirado adelante para que esto hoy vea la luz, con su esfuerzo y conocimientos ha salido mucho más enriquecido. Especialmente, un gracias a Isabel Ferreirós, por su tesón y fuerza a la hora de sacar adelante el estudio de cáncer de colon.

De forma personal, esta tesis supone un crecimiento y aprendizaje inesperados, además de haber estrechado lazos con personas que me han aportado mucho más allá de la ciencia. Los Servicios de Oncología Médica de Santiago de Compostela y de A Coruña son equipos en donde uno crece, se forma como persona, desarrolla habilidades y, sobre todo, disfruta. Gracias por hacer que tantas horas en el hospital sean maravillosas.

Gracias de forma especial a tantos amigos que me rodean, así como sus hombros y oídos, siempre dispuestos y abiertos, ante todo tipo de circunstancias. Merce, María, Lucía, Marta, María, Manuel, Laura, Carmen, Pau, Eva, Manolo...y muchos más que siempre habéis estado en aquellos días que comienzan malos, pero que, tras un ratito con vosotros, mejoran exponencialmente. Es un privilegio teneros cerca.

A mi familia; comenzando por mis padres, por su amor infinito y su fuerza para seguir adelante cuando el cáncer les tocó allí donde más duele. Gracias por transmitirme los valores y principios morales que ahora llevo conmigo, son los bastones que me sostienen en mis debilidades. Además, me enseñasteis la disciplina y fuerza de voluntad necesarias para alcanzar las recompensas. A todos mis abuelos, Miguel y Amable, Manolo y Elena, Adela: gracias por ser la bondad personificada y la sonrisa permanente que transmite paz.

A mis hermanos Miguel, Ana, Rita y Juan, sois los mejores compañeros en cualquier travesía que la vida nos ponga por delante, sean los metros que sean. A nuestros sobrinos, Roi, Erea, Aine, Noela y Anxo: por vosotros haré todo lo posible para que tengáis un mundo mejor. Sois mi motivación para ser feliz cada día.

A Pablo, siempre a mi lado: eres lo mejor que tengo para caminar sin dificultades, salvando los tropiezos y celebrando los triunfos juntos. Contigo no necesito nada más para recorrer esta vida con éxito.

Y por supuesto, gracias a todos los pacientes que, colaborando con la investigación, hacen posible que sueños como este se cumplan. Vosotros sois la energía que nos impulsa hacia adelante. Por vosotros, seguiré soñando.

ÍNDICE

ABREVIATURAS.....	7
RESUMEN.....	8
SUMMARY.....	10
INTRODUCCIÓN.....	12
HIPÓTESIS.....	18
OBJETIVOS.....	19
METODOLOGÍA.....	20
CAPÍTULO 1.....	22
CAPÍTULO 2.....	35
CAPÍTULO 3.....	56
DISCUSIÓN.....	75
CONCLUSIONES.....	85
REFERENCIAS.....	86
ANEXOS.....	92

ABREVIATURAS

ADN	Ácido Desoxirribonucleico
ARN	Ácido Ribonucleico
ALK	Kinasa del linfoma anaplásico
BRAF	<i>v-raf murine sarcoma viral oncogene homolog B1</i>
cEVs	Vesículas Extracelulares circulantes
cfDNA	<i>Cell-free Desoxirribonucleic Acid</i>
ctDNA	<i>Circulating tumour Desoxirribonucleic Acid</i>
CPNM	Carcinoma de Pulmón no Microcítico
CPCP	Carcinoma de Pulmón Célula Pequeña
CPNCP	Carcinoma de Pulmón no Célula Pequeña
CPM	Carcinoma de Pulmón Microcítico
CRC	Carcinoma Colorrectal
CRCm	Carcinoma Colorrectal metastásico
CTCs	Células Tumorales Circulantes
ddPCR	<i>Digital drop Polymerase Chain Reaction</i>
DNA	<i>Desoxirribonucleic Acid</i>
EGFR	<i>Epidermal Growth Factor Receptor</i>
EMT	<i>Epithelial Mesenchymal Transition</i>
GPA	<i>Graded Prognostic Assessment</i>
HER2	<i>Human Epidermal Growth factor Receptor 2</i>
ICIs	<i>Immune Checkpoint Inhibitors</i>
KRAS	<i>Kristen Rat Sarcoma viral oncogene homolog</i>
LCR	Líquido Ceforraquídeo
MAFs	<i>Mutant Allele Fraction</i>
MRD	<i>Minimal Residual Disease</i>
MSI	<i>Microsatellite Instability</i>
NGS	<i>Next Generation Sequencing</i>
NRAS	<i>Neuroblastoma Rat Sarcoma viral oncogene homolog</i>
PCR	<i>Polymerase Chain Reaction</i>
PD-1	<i>Programmed Cell Death Protein 1</i>
PD-L1	<i>Programmed Cell Death Protein 1 Ligand</i>
RAS	<i>Rat Sarcoma viral oncogene homolog</i>
RNA	<i>Ribonucleic Acid</i>
ROS1	<i>ROS Proto-Oncogene 1, receptor tyrosin Kinase</i>
SoC	<i>Standard of Care</i>
SNC	Sistema Nervioso Central
TMB	<i>Tumor Molecular Burden</i>
TCR	<i>T-cell receptor</i>

RESUMEN

El cáncer es una de las enfermedades con mayor mortalidad en situación avanzada, siendo actualmente la segunda causa de muerte a nivel global, lo que pone de manifiesto la necesidad de desarrollar nuevos y mejores enfoques diagnósticos y terapéuticos. La Medicina de Precisión comprende aquellas actuaciones para el tratamiento y prevención de enfermedades que tiene en cuenta la variabilidad individual de los pacientes. De forma más específica, la Oncología de Precisión comprende el uso de terapias dirigidas para el tratamiento de tipos específicos de células cancerosas. Hoy en día el estudio de un paciente oncológico pasa por un diagnóstico molecular, para ofrecer opciones terapéuticas adaptadas a sus características individuales, considerando aquellas alteraciones genómicas para acceder al uso de fármacos específicos para esa diana molecular. La biopsia líquida es una herramienta diagnóstica que está emergiendo como imprescindible para realizar un estudio molecular integral a lo largo de la enfermedad del paciente. Es una técnica no invasiva que permite detectar y analizar células tumorales y material genético en muestras de sangre u otro tejido corporal.

En este sentido, el objetivo principal de esta tesis es presentar el desarrollo de la biopsia líquida en aquellos tumores en situación de pronóstico ensombrecido como herramienta útil para ofrecer a los pacientes una terapia dirigida de forma personalizada en base a la biología molecular de su tumor. El uso de esta técnica diagnóstica ha demostrado utilidad para la personalización de tratamientos en determinados tumores avanzados que presenten siembra al torrente sanguíneo, como son el cáncer de pulmón y colorrectal, donde también está establecida la administración de terapias dirigidas con resultados positivos en cuanto a supervivencia.

En el primer capítulo se presenta en forma de revisión narrativa la utilidad de la biopsia líquida en el uso de inmunoterapia en el de cáncer de pulmón metastásico. Este tipo de tratamiento bloquea la inhibición del sistema inmune favoreciendo así una acción antineoplásica, con beneficios clínicos en determinados tumores. Todavía a día de hoy no disponemos de biomarcadores de respuesta tumoral a estos fármacos, ni de capacidad de predicción de desarrollo de resistencias o toxicidades inmunomediadas. Tras un análisis exhaustivo de numerosos estudios traslacionales, la detección de células tumorales circulantes (CTCs) o ácido dextrorribonucleico libre celular (cfDNA) se muestran como herramientas prometedoras para la selección de pacientes y la monitorización de respuesta a terapias.

En el segundo capítulo, se profundiza en la utilidad de la biopsia líquida en enfermos con aquellos tumores sólidos (pulmón, mama y melanoma, tumores que presentan diseminación sanguínea de material oncológico) que han metastatizado a nivel del sistema nervioso central (SNC). Esta es una de las situaciones clínicas más complejas que afrontamos en nuestro día a día en la clínica, debido a las altas tasas de mortalidad y comorbilidades añadidas que presenta el paciente. Se ha llevado a cabo una revisión de aquellos estudios con inmunoterapia en estos cánceres, y sus resultados en el subgrupo concreto de pacientes que presentan metástasis en SNC. Además, se ha focalizado la revisión en aquellos ensayos que de forma exploratoria emplean la biopsia líquida como técnica predictiva de respuesta a terapia inmune en este contexto de especial mal pronóstico. Varios trabajos presentan la biopsia líquida en fluido cerebral como una herramienta útil para caracterizar el perfil genómico de las metástasis y así poder conocer mejor la biología molecular de estas lesiones.

En el último y tercer capítulo, se presenta un trabajo original llevado a cabo con pacientes en el escenario de cáncer colorrectal metastásico, con la intención de ver la utilidad de la biopsia líquida en nuestro medio, con pacientes con especial mal pronóstico, en situación de enfermedad avanzada irresecable. En él se demuestra la que el uso de esta herramienta como técnica para el estudio del gen *RAS*, necesario para adaptar el tratamiento dirigido en primera línea y sucesivas es válido para cumplir con el objetivo de ofrecer una terapia personalizada. Varios son los factores clínicos que determinan una posible discordancia entre tejido tumoral y plasma, siendo estos la localización derecha y rectal del tumor primario de colon, las metástasis a nivel no hepático y la realización de la cirugía del tumor primario de forma metacrónica al diagnóstico de metástasis. Estas son características que se asocian a disminución de niveles de cfDNA en plasma, por lo que la concordancia con el tejido disminuye.

En general, esta tesis demuestra el potencial de la biopsia líquida como método inocuo, rápido, dinámico y fiable, para el uso de biomarcadores de utilidad pronóstica y predictiva en situaciones oncológicas de evolución especialmente desfavorable y con tumores con capacidad de siembra a sangre, todavía frecuentes en la práctica clínica y de manejo complejo. El uso de este procedimiento puede ayudar a seleccionar de forma personalizada la terapia más adecuada según el momento en el que se encuentren los pacientes y las características clínicas que presenten los tumores.

SUMMARY

Cancer is one of the diseases with the highest mortality in advanced stages, currently the second leading cause of death globally, which highlights the need to develop new and better diagnostic and therapeutic approaches. Precision Medicine comprises those actions for the treatment and prevention of diseases that take into account the individual variability of patients. More specifically, Precision Oncology includes the use of targeted therapies for the treatment of specific types of cancer cells. Nowadays, the study of an oncology patient involves a molecular diagnosis to offer therapeutic options adapted to their individual characteristics, considering those genomic alterations in order to access the use of specific drugs for that molecular target. Liquid biopsy is a diagnostic tool that is emerging as an essential tool for a comprehensive molecular study throughout the patient's disease. It is a non-invasive technique that makes it possible to detect and analyze tumor cells and genetic material in samples of blood or other body tissue.

In this sense, the main objective of this thesis is to present the development of liquid biopsy in those tumors with a poor prognosis as a useful tool to offer patients a personalized targeted therapy based on the molecular biology of their tumor. The use of this diagnostic technique has proven useful for the personalization of treatments in certain advanced tumors that present seeding to the bloodstream, such as lung and colorectal cancer, where the administration of targeted therapies with positive results in terms of survival is also established.

In the first chapter the usefulness of liquid biopsy in the use of immunotherapy in metastatic lung cancer is presented in the form of a narrative review. This type of therapy blocks the inhibition of the immune system, thus favoring an antineoplastic action, with clinical benefits in certain tumors. To date, we still do not have biomarkers of tumor response to these drugs, nor the capacity to predict the development of resistance or immune-mediated toxicities. After an exhaustive analysis of numerous translational studies, the detection of circulating tumor cells (CTCs) or cell-free deoxyribonucleic acid (cfDNA) are promising tools for the selection of patients and the monitoring of response to therapies.

In the second chapter, the usefulness of liquid biopsy in patients with solid tumors (lung, breast and melanoma) that have metastasized to the central nervous system (CNS) is discussed in depth. This is one of the most complex clinical situations that we face in our day-to-day clinical practice, due to the high mortality rates and added comorbidities that the patient presents. We have carried out a review of those studies with immunotherapy in these cancers, and their results in the specific subgroup of patients with CNS metastases. In addition, the review has focused on those trials that exploratory use liquid biopsy as a predictive technique of response to immune therapy in this context of particularly poor prognosis. Several papers present liquid biopsy in cerebral fluid as a useful tool to characterize the genomic profile of metastases and thus to better understand the molecular biology of these lesions.

In the last and third chapter, we present an original work carried out with patients in the setting of metastatic colorectal cancer, with the intention of discovering the usefulness of liquid biopsy in our setting, with patients with a particularly poor prognosis, in a situation of advanced unresectable disease. It demonstrates that the use of this tool as a technique for the study of the

RAS gene, necessary to adapt the targeted treatment in the first line and successive ones, is valid to fulfill the objective to offer a personalized therapy to the patients. There are several clinical factors that determine a possible discordance between tumor tissue and plasma, these being the right and rectal location of the primary colon tumor, non-hepatic metastases and the performance of surgery on the primary tumor metachronously to the diagnosis of metastases. These are characteristics that are associated with decreased plasma cfDNA levels, so that tissue concordance decreases.

In general, this thesis demonstrates the potential of liquid biopsy as a safe, fast, dynamic and reliable method for the use of biomarkers of prognostic and predictive utility in oncological situations of particularly unfavorable evolution and with tumors with blood seeding capacity, still frequent in clinical practice and of complex management. The use of this procedure can help in our daily practice to personalize the selection of the most appropriate therapy according to the stage of the patients and the clinical characteristics of the tumors.

INTRODUCCIÓN

En los últimos años hemos sido testigos de un importante avance en el campo de la Medicina en general, y la Oncología en particular, siendo uno de los principales hitos en los últimos 20 años la llamada Oncología de Precisión. Esta es definida como el estudio del perfil molecular de los tumores para identificar alteraciones genéticas que presenten una terapia dirigida para mejorar el tratamiento y prevención de las enfermedades neoplásicas (1).

El diagnóstico molecular es hoy en día una herramienta integrada en la práctica clínica diaria dirigida a seleccionar pacientes candidatos al uso de tratamientos dirigidos a dianas genéticas alteradas, con la intención de mejorar los resultados en salud. El cáncer de pulmón es el prototipo de neoplasia en la que es obligado un estudio genómico de aquellas alteraciones moleculares accionables para el uso de agentes terapéuticos con beneficio en pacientes con enfermedad avanzada portadores de las mismas. En el cáncer colorrectal también se utiliza el diagnóstico del perfil molecular tumoral desde hace años para la selección de terapias personalizadas, dados los resultados favorables que se obtienen con inhibidores de *EGFR* (Epidermal Growth Factor Receptor) en pacientes *RAS* (rat sarcoma viral oncogene homolog) en estatus nativo. Además, estos dos tipos tumorales, han demostrado presentar en situación avanzada una siembra de material oncológico al torrente sanguíneo que es posible capturar con la técnica de biopsia líquida, al contrario que otros tipos tumorales como los gliomas, con crecimiento en zonas anatómicas estancas con menor repercusión en sangre periférica.

De forma paralela, la inmunoterapia es otro pilar estratégico en el tratamiento de pacientes oncológicos, con resultados realmente impactantes en determinados tumores, tales como el cáncer de pulmón o el melanoma avanzados. Hoy en día no disponemos de alteraciones genéticas predictivas de respuesta en la mayoría de los casos, ni previsoras de toxicidad inmunorrelacionada, por lo que es una necesidad no cubierta el descubrir biomarcadores de respuesta o tolerancia, para ofrecer a los pacientes la terapia más adaptada de forma personalizada.

Debido al desarrollo de resistencias al tratamiento de forma dinámica por la selección clonal tumoral bajo presión de fármacos, la toma de nuevas biopsias en situaciones de progresión tumoral resulta de especial interés a la hora de tomar decisiones clínicas en el día a día asistencial. Es en este contexto donde la biopsia líquida cobra protagonismo como herramienta de utilidad ante aquellos casos en los que se desea evitar un procedimiento cruento a los pacientes.

La biopsia líquida es una herramienta diagnóstica que permite la evaluación de diferentes moléculas obtenidas de un fluido corporal, siendo la sangre el más comúnmente empleado, y monitorizar sus alteraciones a lo largo del tiempo con un método no invasivo, proporcionando información sobre las características moleculares de la enfermedad.

Concretamente en el campo de la Oncología, el uso de biopsia líquida se ha incrementado en las últimas décadas, aportando nuevas oportunidades para mejorar el manejo a diferentes niveles de la enfermedad. Así, la detección temprana de la recurrencia tumoral, el estudio de los mecanismos de resistencia a fármacos y la detección selección clonal tumoral son algunos

de los usos que hoy en día están mostrando mayor interés (2). También su utilización para realizar cribado poblacional de cánceres se postula como un potencial empleo de esta técnica (3). Son varios los estudios y guías clínicas que la posicionan como procedimiento recomendado a la hora de realizar una evaluación personalizada de pacientes y guiar el tratamiento mediante terapia dirigida (4,5).

Asimismo, el análisis mediante biopsia líquida ha emergido recientemente como una alternativa al estudio molecular en tejido tumoral. De hecho, el perfil genético llevado a cabo en tejido tumoral mediante biopsias no siempre es satisfactorio debido a la escasa cantidad y calidad de la muestra, siendo necesario repetir los procedimientos diagnósticos, con la consecuente morbilidad y retraso que esto supone. Otra ventaja es la capacidad de obtener de una forma no invasiva información integral de toda la enfermedad, y no tan solo una muestra que puede no ser representativa de la caracterización molecular del tumor de forma global (6,7). Por otro lado, la monitorización a lo largo del curso de la enfermedad proporciona la posibilidad del estudio dinámico de las alteraciones genéticas que surgen a lo largo del tiempo, sin necesidad de rebiopsiar, ofreciendo así un panorama de la evolución en tiempo real. Además, siendo las metástasis la principal causa de muerte en estos pacientes, puede que presenten rasgos moleculares diferentes al tumor primario, por lo que resulta de interés evaluar la correlación intra- e inter-tumoral dada la heterogeneidad que pueda existir, consiguiendo así proporcionar un manejo más personalizado a cada paciente en cada momento de su enfermedad (8-10).

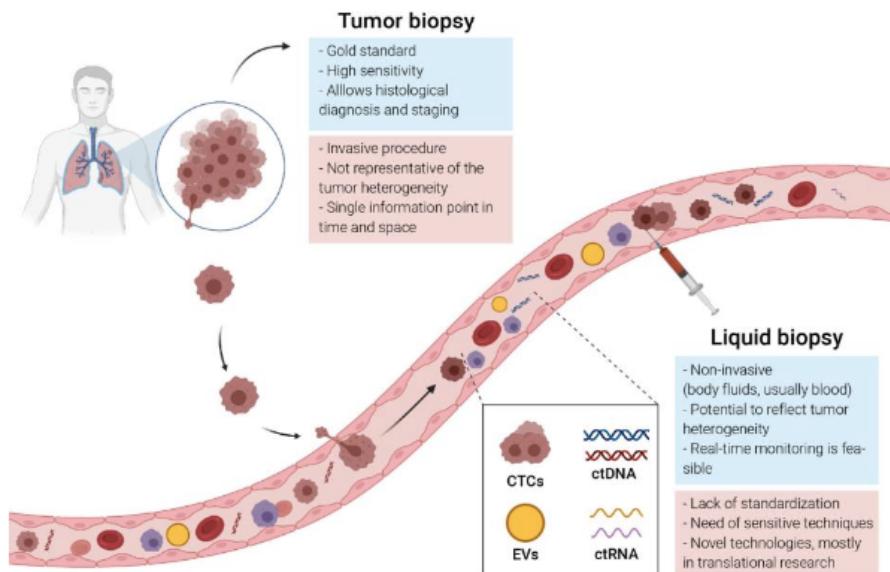


Figura 1: Biopsia tumoral y biopsia líquida: comparación entre ventajas y limitaciones. Reproducida de la referencia (11) con permiso de la editorial, año 2021.

El material tumoral más frecuentemente presente en los biofluidos son las células tumorales circulantes (CTCs), ácidos nucleicos (incluyendo ADN, ácido desoxirribonucleico; y ARN, ácido ribonucleico), y vesículas extracelulares circulantes (cEVs). A través de la caracterización de estos elementos obtenemos información de mecanismos relacionados con diseminación tumoral y progresión, también en el contexto de situaciones complejas como es la metastatización cerebral.

En otros tumores, como el cáncer colorrectal, hay trabajos que demuestran que la caracterización del estatus *RAS* en plasma es factible y presenta una alta sensibilidad, con detección de fracciones alélicas por debajo de 0,01% (12,13). Por lo tanto, este método de diagnóstico molecular en estos tumores ofrece un resultado fiable y con alta concordancia en estudios comparativos en muestra de tejido con biopsia líquida (12,14).

Sin embargo, desde el punto de vista de la asistencia médica habitual, es de especial interés conseguir una mejor caracterización de los factores clínicos que puedan ser la causa de una discordancia entre el estatus del gen *RAS* en muestra de tejido tumoral y plasma, de cara a detectar el perfil de pacientes susceptibles de realizar una técnica u otra. El objetivo final es ofrecer a los mismos la terapia lo más personalizada posible, con el diagnóstico menos cruento y más fiable que nos ayude en la toma de decisiones.

A.1 CÁNCER DE PULMÓN

El cáncer de pulmón, con una incidencia de 480.000 casos nuevos por año a nivel europeo, es el segundo cáncer más diagnosticado, solo por detrás del cáncer de mama en mujeres, causando 380.000 de muertes por año en este continente. Es la primera causa de muerte por cáncer en ambos sexos. Las tasas de incidencia y mortalidad se muestran superiores en hombres que en mujeres, y de 3 a 4 veces más elevada en países industrializados que en aquellos en vías de desarrollo, secundario a los factores de riesgo que lo ocasionan (15).

El cáncer de pulmón es una enfermedad heterogénea, tanto a nivel clínico, histológico y molecular (16,17). Clásicamente hay dos grupos principales de cáncer de pulmón, carcinoma pulmonar no célula pequeña (CPNCP) o carcinoma no microcítico de pulmón (CNMP), y carcinoma pulmonar de célula pequeña (CPCP) o carcinoma microcítico de pulmón (CMP); presentando una distribución de 76% y 13%, respectivamente, siendo el 11% restante asignado a categorías no determinadas. Más del 60% de los casos de nuevo diagnóstico presentan enfermedad locoregional avanzada o enfermedad a distancia ya en el momento de la detección. En los últimos años, el estudio del perfil molecular genómico ha supuesto un avance en la ampliación del arsenal terapéutico disponible, con la introducción de terapias dirigidas contra mutaciones en oncogenes *driver*, y el uso de la inmunoterapia con anticuerpos inhibidores de puntos de control inmunológico (18).

Inicialmente, con la introducción de terapias dirigidas se ha mejorado la supervivencia de los pacientes con cáncer de pulmón avanzado. Sin embargo, tan solo un 15% presentan alteraciones genéticas accionables en genes como *EGFR*, *ALK*, *ROS1*, o *BRAF* (18). Más recientemente, los inhibidores de puntos de control inmunológico (immune checkpoint inhibitor (ICI)) han demostrado beneficio clínico actuando contra la proteína de muerte celular programada (PD-1) o su ligando (PD-L1), siendo establecido su uso con estándar en el escenario de cáncer de

pulmón avanzado, asociado o no al tratamiento con quimioterapia y recomendado por las principales guías clínicas de manejo de esta enfermedad (20-23).

Desafortunadamente, no todos los pacientes responden, siendo tan solo un subgrupo (aproximadamente 20-30%) los que se benefician de la inmunoterapia a largo plazo. Debido a esto, hay una necesidad clara de detectar perfiles de pacientes respondedores a estas terapias antiPD-1/antiPD-L1, a la vez que tratamos de identificar aquellas subpoblaciones en las que son ineficaces para evitar un tratamiento con potenciales eventos adversos graves.

Por otro lado, escenarios de especial mal pronóstico como el diagnóstico de metástasis cerebrales en pacientes con cáncer de pulmón ocurren en un 20-30% de los casos de CPNCP (24,25). En el caso de CPCP la incidencia es de 10-14% al diagnóstico, incrementándose durante el curso de la enfermedad hasta un 50% (26).

Esta situación supone un desafío clínico, ya que su presencia en tumores sólidos se relaciona con un pronóstico infausto y complicaciones graves con un empeoramiento de la calidad de vida de los pacientes oncológicos que las presentan. En global, la metastatización al cerebro se estima en un 20-40% de todos los tumores (27,28). El tratamiento de la enfermedad a nivel cerebral se basa en el uso de cirugía, radioterapia esterotáxica u holocraneal (29,30).

Sin embargo, las indicaciones de estas prácticas están cambiando en los últimos años en el cáncer de pulmón, y en otros tumores como el melanoma, debido al uso de inhibidores tirosina quinasa empleados como terapia dirigida debido a su buena penetrancia en el sistema nervioso central con una buena tasa de respuesta a nivel intracraneal objetivada en determinados ensayos clínicos (31-34).

Sin embargo, en los estudios con inmunoterapia, carecemos de datos sólidos que nos ayuden a identificar qué pacientes se pueden beneficiar del uso de antiPD1/antiPD-L1 en presencia de enfermedad cerebral, ya que clásicamente esta población estaba excluida de la participación en los mismos. La herramienta GPA (Graded Prognostic Assessment) ha tratado de estimar la supervivencia en pacientes con cáncer de pulmón con metástasis cerebrales teniendo en cuenta variables clínicas, pero no moleculares, por lo que hoy en día su uso ha quedado obsoleto al no contemplar factores con implicaciones tanto pronósticas como predictivas ya que influyen en la elección de un tratamiento personalizado con terapia dirigida (35).

Hay, por lo tanto, una necesidad no cubierta de descubrir mediante biomarcadores que puedan ayudar en la práctica clínica habitual a seleccionar pacientes en situación oncológica con pronóstico desfavorable. Además, dado que el perfil molecular puede cambiar a lo largo de la evolución tumoral, por alteraciones genéticas que causen mecanismos de resistencia a fármacos, o conlleven un comportamiento diferente en un momento dado con avidez por metastatizar en determinadas localizaciones, el estudio dinámico de estas situaciones supone un reto atractivo para los investigadores. Al mismo tiempo, la herramienta que cumpla esta misión debe ser no-invasiva para mantener en la medida de lo posible la buena calidad de vida de los pacientes. Además, dada la complejidad que supone obtener tejido tumoral cerebral para su estudio, por la morbilidad para realizar una biopsia en este órgano, el estudio molecular llevado a cabo mediante otras técnicas es de crucial importancia.

A.2 CÁNCER COLORRECTAL

El cáncer colorrectal es uno de los tumores más frecuentes en ambos sexos, con diagnóstico más frecuente en hombres que en mujeres. Es la segunda causa de muerte por cáncer en Europa (14), siendo España uno de los países con una de las tasas de incidencia y mortalidad más elevadas. Su etiología se asocia a factores en relación con estilos de vida, tales como dieta o sedentarismo (36).

La fisiopatología y caracterización molecular son altamente heterogéneas. El adenocarcinoma, subtipo histológico más común, tiene el potencial de originarse en todo el marco colónico y el recto, pero es bien conocido hoy en día que los rasgos genéticos del colon derecho son distintos si los comparamos con el lado izquierdo y recto. Estas diferencias tienen un rol crucial en el escenario metastásico de esta enfermedad, siendo los patrones de diseminación a órganos dependientes de la localización del tumor primario. Como consecuencia de esta heterogeneidad, el tratamiento sistémico de los pacientes con cáncer colorrectal metastásico debe ser personalizado y dependiente de biomarcadores predictivos específicos de esta enfermedad.

El estado mutacional del *RAS* (rat sarcoma viral oncogene homolog) es utilizado hoy en día en la práctica clínica habitual para seleccionar pacientes respondedores a terapia dirigida, y con ello mejorar los resultados oncológicos (37). Cetuximab y panitumumab son anticuerpos monoclonales contra el receptor de factor de crecimiento (*EGFR*) que han demostrado beneficio clínico en pacientes con cáncer colorrectal con estatus nativo de *RAS* administrado con quimioterapia en primera línea de tratamiento (38-40), especialmente en aquellos pacientes cuyo tumor primario se ha originado en el lado izquierdo del colon, incluido el recto (41-46). El 40% de los pacientes presentan mutaciones *missense* en el gen Kristen *RAS* (*KRAS*) y 3-5% en el neuroblastoma *RAS* (*NRAS*), representando un evento precoz en el desarrollo de la carcinogénesis del cáncer colorrectal avanzado (47,48).

Por tanto, aproximadamente un 50% de los pacientes con esta enfermedad presentan resistencia a inhibidores de *EGFR*. Es por ello, que las guías clínicas para el manejo clínico de estos pacientes recomiendan la determinación de la evaluación del estatus mutacional del *RAS* previo a establecer una estrategia de tratamiento (49-52). El estándar para este diagnóstico en práctica clínica habitual es evaluar la presencia de mutaciones *KRAS* y *NRAS* somáticas en tejido tumoral procedente de una biopsia o pieza quirúrgica, mediante el uso de tecnologías basadas en secuenciación específica de mutación, o más recientemente, mediante el uso de plataformas de secuenciación masiva (NGS, next-generation sequencing) (53).

Sin embargo, la evaluación del estatus mutacional del *RAS* utilizando DNA tumoral circulante (ctDNA) mediante la técnica de biopsia líquida se ha establecido como un enfoque alternativo para diagnosticar y monitorizar esta alteración de una forma no invasiva, dinámica y en tiempo real, pudiendo así guiar decisiones terapéuticas de una forma más eficiente (54-57).

Se ha demostrado un nivel de concordancia elevado, con ratios mayores del 85%, entre los procedimientos de secuenciación llevados a cabo en tejido tumoral, y OncoBEAM® *RAS* CRC (cáncer colorrectal), tecnología que usa dPCR (digital polymerase chain reaction) aprobada por la EMA como una herramienta válida para detección de estas mutaciones (58).

En posteriores estudios prospectivos con largas cohortes de pacientes se han determinado algunas de las causas de discordancia entre ambas técnicas de análisis, destacando el impacto de las características clínicas que influyen la detección del ctDNA en plasma. Pequeñas cantidades de ctDNA en sangre de pacientes con previa resección del tumor primario o ausencia de metástasis hepáticas llevan a una disminución en la identificación de mutaciones RAS mediante esta técnica, y a resultados inciertos de concordancia (59-62).

Además, la fracción alélica mutada (MAF, mutant allele fraction) en población con cáncer colorrectal metastásico exclusivamente en pulmón ha sido menor que en pacientes con enfermedad hepática, por lo que este factor supone un aumento en la tasa de falsos negativos (13, 63).

La discordancia en estos resultados puede llevar a comprometer la selección de los mejores candidatos a recibir terapia dirigida con anti-*EGFR*, impidiendo el acceso a una terapia adecuada a nuestros pacientes. Como resultado, se ocasiona un detrimento en la calidad asistencial de nuestra práctica clínica. No cabe duda de que un mejor conocimiento de las características moleculares de los tumores colorrectales es necesario para identificar correctamente subpoblaciones susceptibles de beneficiarse de un tratamiento personalizado. Un estudio estratificado mediante factores clínicos puede ayudar a establecer el correcto uso de biopsia líquida en la rutina clínica.

HIPÓTESIS

En base al conocimiento actual y lo previamente expuesto, planteamos la posibilidad de que la biopsia líquida se establezca como una herramienta de utilidad en pacientes con tumores sólidos en situación avanzada y de pronóstico especialmente desfavorable para ofrecer al paciente alternativas terapéuticas en el marco de la oncología de precisión.

CAPÍTULO 1

Hipótesis 1: La biopsia líquida es una herramienta útil para la selección de pacientes con cáncer de pulmón no célula pequeña respondedores a inmunoterapia.

CAPÍTULO 2

Hipótesis 2: La detección de biomarcadores predictivos mediante biopsia líquida puede ayudar a seleccionar perfiles de pacientes con tumores sólidos y metástasis cerebrales que se beneficien de terapias específicas.

CAPÍTULO 3

Hipótesis 3: El diagnóstico del estatus mutacional de biomarcadores en sangre en cáncer colorrectal metastásico es de utilidad para establecer estrategias de tratamiento personalizada en estos pacientes.

OBJETIVOS

Basándonos en las hipótesis establecidas, el objetivo principal de esta tesis consiste en la demostración de que la búsqueda de biomarcadores mediante biopsia líquida tiene implicaciones pronósticas y predictivas de respuesta a tratamientos en cáncer de pulmón y cáncer colorrectal estadios avanzados.

Este objetivo general puede dividirse en los siguientes objetivos parciales:

CAPÍTULO 1

Objetivo 1: Revisar el potencial del uso de la biopsia líquida en la selección de pacientes con cáncer de pulmón no célula pequeña avanzado respondedores a inmunoterapia.

CAPÍTULO 2

Objetivo 2: Analizar biomarcadores de eficacia mediante biopsia líquida en pacientes con tumores sólidos con enfermedad metastásica cerebral.

CAPÍTULO 3

Objetivo 3: Identificar mediante biopsia líquida perfiles de pacientes con cáncer colorrectal estadio IV que se asocian a respuesta a tratamientos dirigidos.

Objetivo 4: Establecer factores clínicos que condicionan una discordancia entre el análisis mutacional del gen *RAS* realizado en tejido tumoral y biopsia líquida.

Objetivo 5: Determinar el impacto de las características clínico-patológicas en la carga mutacional del gen *RAS* mediante ctDNA.

METODOLOGÍA

Para cumplir el primer y segundo objetivos hemos llevado a cabo dos revisiones narrativas de la literatura existente en el año 2020 la primera y 2022 la segunda. Para ello, el método de proceder fue realizar la búsqueda en Pubmed datados dentro de los últimos 10 años, de artículos científicos correspondientes a trabajos originales con muestras de pacientes que cumplieran las características clínicas de nuestro grupo de interés (cáncer de pulmón no microcítico avanzado en el primer trabajo; y cánceres de pulmón, mama y melanoma con enfermedad metastásica cerebral en el segundo). Los criterios de búsqueda mediante términos MeSH fueron “advanced non-small cell lung cancer AND immunotherapy”, “lung cancer AND liquid biopsy”, “predictive biomarkers AND immunotherapy”, “lung cancer AND brain metastasis AND immunotherapy”, “melanoma AND brain metastasis AND immunotherapy”, “breast cancer AND brain metastasis AND immunotherapy”. Tras una exhaustiva búsqueda, aquellos artículos originales, metaanálisis y revisiones sistemáticas en lengua inglesa fueron seleccionados, siempre y cuando representaran nuestra población diana y el tema de interés, descartando series de casos clínicos y editoriales. Una vez realizado este cribado, se hizo un análisis de todos los resultados con biomarcadores en biopsia líquida que se presentaban y fueron ordenados en texto y tablas para su correcta exposición.

El tercer estudio es un trabajo llevado a cabo en varios centros de Galicia (Complejo Hospitalario Universitario de Santiago (CHUS), Hospital Universitario Lucus Augusti (HULA), Complejo Hospitalario Universitario de Ourense (CHOU), Complejo Hospitalario Universitario de Ferrol (CHUF)), de carácter retrospectivo-prospectivo (ambispectivo) observacional. El estudio comenzó tras el visto bueno del Comité Ético de Investigación Clínica de Galicia). La selección de pacientes se llevó a cabo por parte de los facultativos responsables de los pacientes tratados en los Servicios de Oncología previamente mencionados, diagnosticados tanto histológica como radiológicamente de cáncer colorrectal estadio IV en el momento de inclusión en el estudio y recogida de sangre.

Un total de 366 pacientes fueron seleccionados entre noviembre 2015 y julio 2019. En la mayoría de los casos (79%) la biopsia líquida fue obtenida en los cuatro meses siguientes al diagnóstico de la enfermedad metastásica. El intervalo medio entre la obtención de la biopsia de tejido tumoral sólido y la biopsia de sangre líquida fue inferior a 3 meses (70 días de media) para los pacientes diagnosticados de enfermedad metastásica sincrónica, y de año y medio (535 días de media) para los pacientes diagnosticados de enfermedad metastásica metacrónica recurrente.

La información relativa al estado mutacional de RAS en la muestra de tejido tumoral estaba disponible en el 83% de los pacientes. Por otra parte, el genotipado de *RAS* se consiguió en el 99,5% de las muestras de plasma sanguíneo. La realización del diagnóstico del estatus mutacional *RAS* se realizó mediante BEAMing® ddPCR en el caso del plasma, y mediante la técnica estándar de determinación establecida en cada hospital (Therascreen® *KRAS* RGQ PCR kit Qiagen; COBAS *KRAS* mutation test (Roche Diagnostics, Rotkreuz, Switzerland) or pyrosequencing (PyroMark® Q24, Qiagen).

Los análisis estadísticos se realizaron con subpoblaciones de CCRm predefinidas clínicamente. La concordancia entre los análisis basados en plasma y en tejido tumoral se determinó mediante el estadístico Kappa (κ) con un intervalo de confianza (IC) del 95%. También se calcularon la concordancia global, la sensibilidad (concordancia porcentual positiva) y la especificidad (concordancia porcentual negativa) entre los análisis basados en plasma y tejido y sus IC del 95%.

Se utilizó una regresión logística binaria para modelizar la relación entre las características clínico-patológicas del CCRm y el estado mutacional del *RAS* (discordante frente a concordante) tomado como variable dependiente dicotómica. El resultado principal del análisis fueron las probabilidades de discordancia frente a concordancia entre las pruebas estándar (análisis en tejido) y el análisis de ctADN en plasma. Se utilizó un modelo lineal generalizado y una función logit para estimar las odds ratio (OR) de discordancia y sus intervalos de confianza (IC) del 95%, y para calcular la probabilidad de asociación con la localización del tumor primario, la resección del tumor primario, la localización metastásica y el número de localizaciones metastásicas tomadas como variables independientes. Los pacientes con CCRm originado en el colon izquierdo, sin resección del tumor primario, con metástasis hepáticas, metástasis en ganglios linfáticos, metástasis pulmonares o metástasis peritoneales, y con múltiples localizaciones metastásicas se utilizaron como referencia para las variables independientes.

Se utilizó la regresión logística univariable para estimar el efecto de cada una de las características clínico-patológicas sobre las probabilidades de discordancia, individualmente. También se utilizaron modelos de regresión logística multivariable para estimar el efecto conjunto o la interacción de un grupo de variables clinicopatológicas sobre la probabilidad de que se produjera el resultado.

Por último, se realizaron análisis de regresión logística univariable en subpoblaciones de CCRm estratificadas por la localización del tumor primario (colon izquierdo, recto y colon derecho) para modelizar el efecto de la resección del tumor primario, la localización metastásica y el número de localizaciones metastásicas sobre las probabilidades de que se produjera la discordancia. Se calcularon la mediana y el rango intercuartílico (25-75%; IQR) de los niveles de cfADN y las distribuciones de MAF para las distintas subpoblaciones de mCRC. Se aplicaron los enfoques no paramétricos de Mann-Whitney y Kruskal-Wallis para las comparaciones estadísticas entre dos o más poblaciones, respectivamente. Todas las pruebas estadísticas se realizaron en R versión 3.6.3, GraphPad Prism 6 y VassarStats: Website for Statistical Computation. Las odds ratio ≥ 2 y los valores $p \leq 0,05$ se consideraron estadísticamente significativos.

CAPÍTULO 1

Immunotherapy in non small-cell lung cancer: current status and future prospects for liquid biopsy

Immunotherapy in non small-cell lung cancer: current status and future prospects for liquid biopsy

Elena María Brozos-Vázquez^{1,2} · Roberto Díaz-Peña^{2,3,4} · Jorge García-González^{1,2,3} · Luis León-Mateos^{1,2,3} · Patricia Mondelo-Macia² · María Peña-Chilet^{5,6,7} · Rafael López-López^{1,2,3}

¹ Department of Medical Oncology, Complejo Hospitalario Universitario de Santiago de Compostela, 15706 Santiago de Compostela, Spain.

² Liquid Biopsy Analysis Unit, Translational Medical Oncology (Oncomet), Health Research Institute of Santiago (IDIS), Complejo Hospitalario Universitario de Santiago de Compostela, 15706 Santiago de Compostela, Spain

³ Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), 28029 Madrid, Spain

⁴ Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca 3460000, Chile

⁵ Bioinformatics in Rare Diseases (BiER), Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), 41013 Sevilla, Spain

⁶ Clinical Bioinformatics Area, Fundación Progreso y Salud (FPS), Hospital Virgen del Rocío, 41013 Sevilla, Spain

⁷ Institute of Biomedicine of Sevilla (IBiS, CSIC, Universidad de Sevilla), 41013 Sevilla, Spain

Immunotherapy in non small-cell lung cancer: current status and future prospects for liquid biopsy

Abstract

Immunotherapy has been one of the great advances in the recent years for the treatment of advanced tumors, with non small- cell lung cancer (NSCLC) being one of the cancers that has benefited most from this approach. Currently, the only validated companion diagnostic test for first-line immunotherapy in metastatic NSCLC patients is testing for programmed death ligand 1 (PD-L1) expression in tumor tissues. However, not all patients experience an effective response with the established selection criteria and immune checkpoint inhibitors (ICIs). Liquid biopsy offers a noninvasive opportunity to monitor disease in patients with cancer and identify those who would benefit the most from immunotherapy. This review focuses on the use of liquid biopsy in immunotherapy treatment of NSCLC patients. Circulating tumor cells (CTCs), cell-free DNA (cfDNA) and exosomes are promising tools for developing new biomarkers. We discuss the current application and future implementation of these parameters to improve therapeutic decision-making and identify the patients who will benefit most from immunotherapy.

Keywords Lung cancer · NSCLC · Liquid biopsy · Immunotherapy · CTC · cfDNA

1. Introduction

Lung cancer is currently the most frequently diagnosed malignant tumor, representing the leading cause of cancer death worldwide [1]. Non small-cell lung cancer (NSCLC) accounts for approximately 85% of lung malignancies, and approximately 70% of them are diagnosed with a nonsquamous histology such adenocarcinoma or large cell carcinoma [2]. More than 60% of newly diagnosed patients present locoregional or distant metastases at the time of detection.

Over the past 20 years, the treatment landscape and prognosis of advanced NSCLC patients have changed [3]. First, the introduction of target therapies for oncogene-addicted tumors improved patient survival outcomes. However, only 15% of patients with advanced NSCLC present genetic alterations in EGFR, ALK, ROS1, or BRAF [4]. More recently, immune checkpoint inhibitor (ICI) agents targeting both programmed cell death protein 1 (PD1) or programmed cell death ligand 1 (PD-L1) have been established as a standard treatment in the NSCLC setting. Unfortunately, not all patients respond, and only a subset of them benefit from immunotherapy. Hence, there is a need for new biomarkers. Identifying optimal responsive biomarkers to anti-PD-1/ PD-L1 therapies in NSCLC is essential for selecting patients who will benefit from immunotherapy while limiting ineffective therapy that can produce adverse reactions in patients.

It is within this context that there has been interest in liquid biopsy, a concept that refers to any tumor-derived material circulating through the blood or nonblood body fluids [5]. Liquid biopsy has been developed as an attractive approach to detect biomarkers in a minimally invasive, cost-effective, and rapid manner. Circulating tumor cells (CTCs), cell-free DNA (cfDNA) and extracellular vesicles are the most widely studied components in the field of NSCLC. This review focuses on the use of liquid biopsy in immunotherapy treatment of NSCLC patients, highlighting its potential to be applied in a clinical setting.

2. Immune checkpoint inhibitors in the treatment of NSCLC

The European Medicines Agency (EMA) and the U.S. The Food and Drug Administration (FDA) have approved two classes of ICIs for the treatment of malignancies, including NSCLC [6, 7]: Inhibitors of PD-1 or its ligand PD-L1 and inhibitors of cytotoxic lymphocyte-associated protein 4 (CTLA-4). Table 1 summarizes the different ICIs employed in the treatment of NSCLC.

Drug	Target	Approved use for NSCLC	Line of treatment	Biomarker for patient selection	Comments	References
Nivolumab	PD1	Metastatic	2nd line	None	Monotherapy	[8, 9]
Pembrolizumab	PD-1	Metastatic	2nd line	PD-L1 IC $\geq 1\%$	Monotherapy	[11]
			1st line	PD-L1 IC $\geq 50\%$	Monotherapy	[12]
				None	In combination with platinum-based chemotherapy	[13, 14]
Atezolizumab	PD-L1	Metastatic	2nd line	None	Monotherapy	[15]
			1st line	Nonsquamous NSCLC, independent of the PD-L1 expression level	In combination with bevacizumab, carboplatin, and paclitaxel	[17]
Durvalumab	PD-L1	Unresectable, stage III	Maintenance	None		[20]
Ipilimumab	CTLA-4	Metastatic	1st line	PD-L1 $\geq 1\%$	Nivolumab plus Ipilimumab	[22]
				None	Nivolumab plus Ipilimumab in combination with platinum-based chemotherapy	[23]

TMB tumor mutational burden, *PD-L1 IC* PD-L1 expression in immune cells in the tumor area

Nivolumab plus Ipilimumab for first-line treatment of metastatic NSCLC already has the approval of the European Medicines Agency (EMA)

Table 1. Immune checkpoint inhibitors in the treatment of NSCLC

Nivolumab, a human immunoglobulin G4 (IgG4) monoclonal antibody (mAb) that targets PD-1, has shown improved overall survival (OS), progression-free survival (PFS) and objective response rate in previously treated advanced squamous cell lung carcinoma in comparison with docetaxel according to the results of the CheckMate 017 trial [8]. In nonsquamous NSCLC, the results of the CheckMate 057 trial demonstrated that nivolumab significantly improved OS and objective response rate (ORR) compared to docetaxel [9]. However, as compared to chemotherapy, nivolumab monotherapy has not shown significant effects on improving PFS or OS in previously untreated advanced NSCLC patients with PD-L1 expression on at least 5% of tumor cells [10].

Pembrolizumab, a humanized IgG4 mAb directed against PD-1, was approved for previously treated metastatic NSCLC patients with a PD-L1 tumor expression level of 1% or more, taking into consideration the benefits in the KEYNOTE 10 trial [11]. Recently, the use of pembrolizumab has moved into the first-line setting for recurrent or metastatic NSCLC. The results from the KEYNOTE-024 trial demonstrated that compared with platinum-based chemotherapy, pembrolizumab led to significant improvements in OS, PFS, and ORR in patients with PD-L1 expression on at least 50% of tumor cells [12]. Moreover, the addition of pembrolizumab to platinum-based chemotherapy in previously untreated metastatic NSCLC has recently demonstrated a significant improvement in survival results independent of PD-L1 status [13, 14].

Atezolizumab is a humanized IgG1 mAb that specifically targets PD-L1, blocking its receptors PD-1 and B7.1. In previously treated NSCLC patients, atezolizumab significantly prolonged OS and ORR when compared with docetaxel according to the results of the OAK trial [15]. Consequently, the efficacy of atezolizumab, alone [16] or in combination with chemotherapy [17–19], has been studied in chemotherapy-naïve advanced NSCLC patients in several trials.

Durvalumab, a human IgG1 mAb directed against PD-L1, has been recently approved as consolidation therapy following concurrent chemoradiation in unresectable stage III NSCLC patients [20].

Ipilimumab, a fully human IgG1 mAb directed against CTLA-4, has also been studied in combination with nivolumab in first-line treatment of advanced NSCLC in CheckMate 227, Part 1 [21, 22]. In this trial, in previously untreated NSCLC patients and with high-tumor mutational burden (TMB), PFS was significantly longer with nivolumab plus ipilimumab than with platinum-based chemotherapy, irrespective of PD-L1 expression level [21]. Moreover, in this trial, the combination of nivolumab plus ipilimumab resulted in a longer duration of OS than chemotherapy in patients with NSCLC, independent of the PD-L1 expression level [22]. Finally, the CheckMate 9LA trial has shown that adding 2 cycles of platinum-based chemotherapy to the combination of Nivolumab plus Ipilimumab prolongs the survival of patients with stage IV or recurrent NSCLC [23].

3. Biomarkers in immunotherapy

Intensive work has been carried out in the recent years in the search for biomarkers that identify groups of patients who could benefit most from PD-1/PD-L1 inhibitors. The best-known biomarker in this scenario is PD-L1 expression in tumor or immune cells: a higher probability of response with higher expression of PD-L1 has been described [24, 25]. More than PD-L1 expression on more than 50% of tumor cell NSCLC patients predicts higher effectiveness of pembrolizumab than chemotherapy as a first-line treatment in the metastatic setting [12]. In clinical practice, this marker has limited utility because patients who do not express PD-L1 may respond to PD-1 or PD-L1 checkpoint inhibitors, and in contrast, some patients with elevated expression of PD-L1 do not benefit from the use of these drugs.

High TMB has been linked to the effectiveness of immunotherapy, but given the conflicting results among several studies, this biomarker has not yet been translated into the clinic [21, 22] or authorized by regulatory agencies. The variability among the techniques used for the analysis and interpretation of TMB may influence the value of this biomarker as a predictive factor of response to immunotherapy. As a result, there is an ongoing initiative for increased standardization of TMB interpretation, a subject of active pursuit by the TMB Harmonization Project [26, 27].

In a meta-analysis of 14,395 patients, Yu et al. concluded that the combined use of PD-L1 expression in tissue and TMB is a promising biomarker to evaluate patient survival and response to precision immunotherapy [28]. The further combination of CD8⁺ tumor-infiltrating lymphocytes, PD-L1 expression, and TMB was associated with reliable prognosis, but these results need to be validated in large-scale prospective trials.

4. Liquid biopsy

Tumor diagnosis is made based on tissue biopsy, as well as complementary techniques that can add any genomic information for therapy selection. However, tumor lesions are sometimes difficult to biopsy, and invasive procedures are not the best way to monitor the evolution of the disease. In addition, tumor heterogeneity originates from the appearance of mutations that cause resistance or progression to treatments by selection of more aggressive clones. Considering the

risks and logistical challenges, and even high cost, that limit the application of interventional biopsies, liquid biopsy presents an attractive opportunity for minimally invasive genomic diagnostics.

Tissue provides a snapshot of the tumor at a given time and location, while liquid biopsy has the potential to establish a tumor molecular profile at the beginning of the treatment and during the course of the disease (Fig. 1). In addition, liquid biopsy can capture dynamic intrapatient genomic heterogeneity [5]. These characteristics are especially important in the management of patients with lung cancer due to the great difficulty in obtaining a tissue sample in some cases [29].

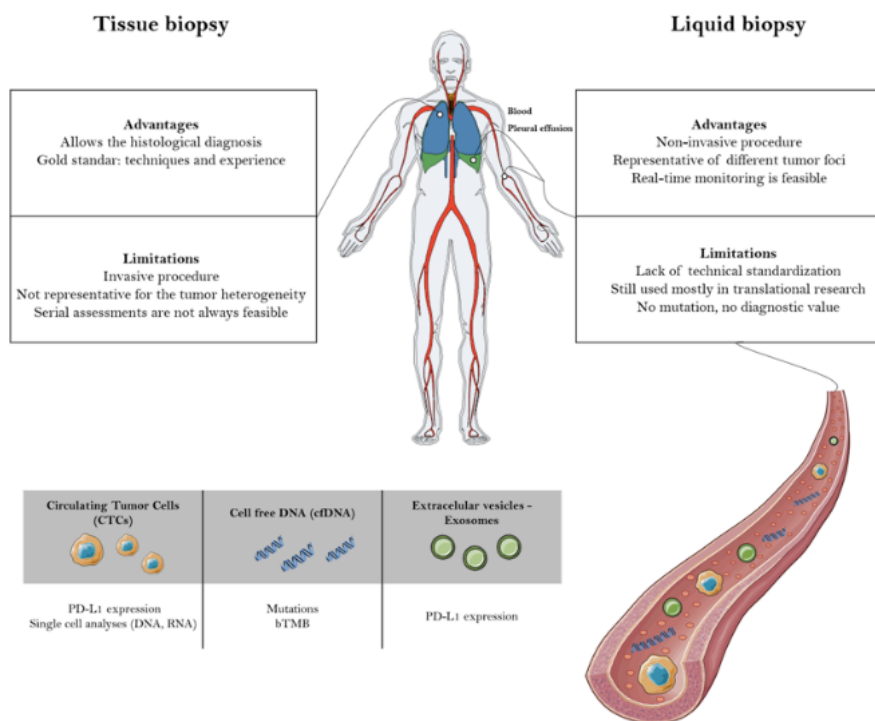


Figure 1. Comparison of the advantages and limitations between tissue and liquid biopsy. Plot generated by the gganatogram R package.

Currently, there are limitations that prevent us from putting into clinical practice the use of the biomarkers used by liquid biopsy. The most relevant is that there is no clear agreement on the usefulness or threshold to be considered for most of the biomarkers studied (notably TMB and PD-L1). Initiatives are underway to harmonize and standardize methods of analysis through universal protocols. An agreement in the scientific community about the ideal biomarker and cut off to define this parameter is needed. The minimally invasive nature of liquid biopsy sampling offers a clear opportunity in immunotherapy. In this work, we will focus on the

potential clinical applications of CTCs and cfDNA, the most widely studied substrates in the field of NSCLC.

4.1 Cell-free DNA

Different types of biological samples are used for liquid biopsies, but cfDNA is currently the material with the greatest potential in clinical practice. The short half-life of cfDNA, approximately 2 h, permits a rapid assessment of tumor-related changes and enables real-time monitoring of molecular biomarkers of response or relapse. Composed of double-stranded fragments of 150–200 base pairs, cfDNA derives from normal physiological tissue remodeling events [30], although a portion of cfDNA results from necrosis and apoptosis of cancer cells, and active cfDNA secretion has also been suspected [31]. The fraction of circulating tumor DNA (ctDNA) in overall cfDNA in patients with cancer varies greatly, from less than 0.1% to more than 90% [30], with ctDNA being distinguished solely through characteristic somatic genomic alterations and its trend to be more fragmented, with sizes ranging from 90 to 150 base pairs.

Therapies for NSCLC patients targeting EGFR, ALK or ROS1 molecular alterations have been proven effective, inhibiting molecules that are pivotal for cell proliferation. Circulating free DNA is now regularly used to manage tyrosine kinase inhibitor treatment in EGFR-mutated NSCLC, and a large number of studies have demonstrated that EGFR gene mutations detected in cfDNA are highly concordant with those detected in tumor tissue of NSCLC patients [32]. What is clear is that cfDNA has been widely studied in the field of targeted therapy. Two FDA- and EMA-approved assay kits to detect somatic mutations in the EGFR oncogene in patients with NSCLC are now available: the Cobas EGFR Mutation Test v2 CE-IVD (Roche, Basel, Switzerland) and theascreen mutation kits (Qiagen, Hilden, Germany). In contrast, the potential of cfDNA to guide the choice of and follow the response to immune therapy is just beginning to be evaluated (Table 2).

4.1.1 Blood tumor mutational burden (bTMB)

One of the biomarkers that could drive the choice of treatment is the blood tumor mutational burden (bTMB) because it can be estimated from cfDNA next-generation sequencing (NGS). Recently, Gandara et al. demonstrated the possibility of using cfDNA to determine TMB [33], also reporting an association with superior results in terms of efficacy in NSCLC treated with immunotherapy. In this regard, a gene panel and algorithm were also optimized for bTMB [34], suggesting that bTMB may serve as a potential biomarker of clinical benefit in patients with NSCLC treated with anti-PD-1 and anti-PD-L1 agents. bTMB may be related to the ability of each tumor to produce antigens that are detected by the immune system, producing an antigenic reaction that triggers an immune response to that tumor. However, studies focused on the relationship between TMB in tissue and the immune response have produced conflicting results [35–37]. In general, further studies are needed to clarify the conditions required to use bTMB in routine clinical practice. The methods for its determination, as well as the threshold to be adopted as a cutoff point for high, intermediate and/or low classification, have yet to be unified and standardized by the scientific community [38]. Moreover, there is a high cost coming from whole-exome sequencing or broad panels, which confers another challenge for clinical implementation. Perhaps it is necessary to move beyond TMB and move to identifying specific somatic alterations detectable in cfDNA that can facilitate selection of patients for

immunotherapy. Not all point mutations participate in the generation of highly immunogenic peptides [39]. Guibert et al. showed that targeted NGS of plasma cfDNA and monitoring its early variations could predict the response to immunotherapy [40]. The study revealed the detrimental effect of STK11 and PTEN mutations in cfDNA on the ICI outcomes reported in tissue. In contrast, mutational transversion (Tv, substitution of a purine by a pyrimidine or vice versa) in KRAS and p53 predicts superior results in terms of PFS. Moreover, the combination of all these specific markers in a predictive model, together with known targetable oncogenic drivers, allows guided patient treatment towards either targeted therapy or ICI.

4.1.2 Minimal residual disease

Detection of minimal residual disease (MRD) following chemoradiation therapy (CRT) predicts the development of progressive disease in NSCLC with high sensitivity and specificity [41]. Immunotherapy is used in the adjuvant setting for patients with unresectable locoregionally advanced NSCLC. The optimal use of immunotherapy requires the identification of patients with MRD to maximize the opportunity for cure, and cfDNA might enable approaches for disease progression monitoring [42]. Antonia SJ et al. showed a survival advantage with durvalumab consolidation therapy after concurrent chemoradiation therapy in patients with stage III [20], unresectable NSCLC. Recently, Moding EJ et al. showed that patients with MRD after CRT receiving ICIs had significantly better outcomes than patients who did not receive consolidation ICI therapy [43]. cfDNA analysis may guide the decision to administer consolidation ICI therapy based on the presence of MRD.

4.1.3 Changes in cfDNA concentration

There are a few studies that propose the cfDNA concentration as a predictive marker of immunotherapy response. In a proof-of-concept study, changes in cfDNA concentration were reported as a valuable tool to assess tumor response in patients treated with anti-PD-1 drugs [44]. Giroux Leprieur et al. reported that low cfDNA concentration at first evaluation (at 2 months) had a relationship with long-term benefit of nivolumab [45]. In addition, a decrease in cfDNA level was correlated with radiological response to immunotherapy [46], suggesting that cfDNA levels may be an early marker of therapeutic efficacy and predict prolonged survival in patients treated with immune checkpoint inhibitors for NSCLC. In another study, NSCLC patients treated with nivolumab and with a cfDNA below their median values survived significantly longer than those with a cfDNA value above their median values [47]. Moreover, the joint analysis of cfDNA with CTCs helped to discriminate a low-risk population that might benefit from continuing nivolumab beyond progression. This suggests that the use of cfDNA and CTCs could help to select those patients who will benefit most from immunotherapy.

4.2 Circulating tumor cells

Circulating tumor cells (CTCs) are released from the primary tumor into the blood and have a very short half-life (1–2.4 h). The proportion of CTCs in the bloodstream is very low, approximately 1 CTC per 10^6 – 10^7 leukocytes. Initially, CTCs have an epithelial phenotype; however, the bloodstream is not an ideal place for epithelial tumor cells, and for this reason, they can undergo epithelial-to-mesenchymal transition (EMT). This transition reduces the expression of epithelial markers and increases plasticity and capacity for migration, invasion, and dissemination, being the CTCs isolation a challenge for their total isolation and capture.

cfDNA studies	Sample number	Biomarker	Method	Conclusions	Limitations
Gandara et al. 2018 [33]	259	bTMB	NGS 394 genes	bTMB was validated as a clinical biomarker for PFS in patients under immunotherapy 64% correlation between tTMB and bTMB	The tTMB computational algorithm counted both indels and SNVs, whereas the bTMB counted only SNVs
Wang et al. 2019 [34]	50	bTMB	NGS 150 genes	bTMB was validated as a clinical biomarker for PFS in patients under immunotherapy 62% correlation between tTMB and bTMB	The clinical cohort was obtained from different trials
Chen et al. 2019 [35]	853	MSAF	NGS	MSAF alone or in combination with bTMB can effectively distinguish NSCLC patients with or without OS and PFS benefit from atezolizumab compared with docetaxel	The results require validation from external cohorts
Koeppel et al. 2017 [37]	32	bTMB	WES	cfDNA-WES TML is feasible for patients with good cfDNA fraction There was a weak correlation between variant frequencies of tDNA and cfDNA	The cohort size of the study was small
Guibert et al. 2019 [40]	97	cfDNA	NGS 36 genes	Correlation of cfDNA mutations with survival outcomes: PTEN and STK11 are correlated with poor prognosis TV KRAS and p53 are correlated with better prognosis Early changes in AF can predict response	Patients were not treated with a homogeneous regimen Validation in prospective studies is required
Chaudhuri et al. 2017 [41]	40	cfDNA	CAPP-seq	Molecular residual disease was detected earlier with analyses of cfDNA than imaging (72% patients)	Patients were treated with different therapies, mostly radiotherapy
Cabel et al. 2017 [44]	15	cfDNA	NGS, ddPCR or Bi-PAP	Monitoring of cfDNA levels concluded that undetectable cfDNA at week 8 was associated with superior PFS and OS	The cohort size of the study was small
Giroux Leprieur et al. 2018 [45]	23	cfDNA	NGS 22 genes	cfDNA changes correlated with tumor response, clinical benefit and PFS	Only 15 of 23 patients were eligible for NGS
Goldberg et al. 2018 [46]	28	cfDNA	NGS 24 genes	A cfDNA response (cfDNA decrease <50%) was associated with superior PFS and OS	Patients with undetectable cfDNA levels were excluded
Alama et al. 2019 [47]	89	cfDNA	qPCR	cfDNA is significantly associated with OS in patients under nivolumab treatment	The cohort size of the study was small
Passiglia et al. 2019 [57]	45	cfDNA variations	qPCR	cfDNA > 20% at week 6 correlated with poor outcomes (OS and TTP)	Retrospective design Small number of patients

CTCs studies	Sample	Biomarker	Platform	Conclusions	Limitations
Alama et al. 2019 [47]	89	CTC count	ScreenCell CYTO	≥ 2 CTCs was significantly associated with worse OS in patients under nivolumab treatment	The cohort size of the study was small
Guibert et al. 2018 [50]	96	PD-L1 on CTCs	ISET	No correlation was found between PD-L1 expression in tissue and CTCs	Only 72% of the patients had enough tissue to analyze
Boffa et al. 2017 [51]	112	PD-L1 on CTCs	EPIC science	PD-L1 ⁺ was correlated with better OS (OS-2y: PD-L1 ⁺ 31.2% vs PD-L1 ⁻ 78.8%, (p=0.001599))	Counted nonconventional CTCs (CK negative)
Nicolazzo et al. 2016 [52]	24	PD-L1 on CTCs	CellSearch	PD-L1 ⁺ was correlated with progression disease in 100% of samples	Only 15 patients had available samples after 3 months of treatment
Ilić et al. 2018 [53]	106	PD-L1 on CTCs and white blood cells	ISET	93% concordance between PD-L1 expression in tissue and CTCs	Only 67% of patients presented CTCs in blood
Dhar et al. 2018 [54]	22	PD-L1 on CTCs	Vortex-isolated	100% correlation between PD-L1 expression in tissue and CTCs	Only 4 tissue samples available for correlation analysis
Jainning et al. 2019 [55]	127	PD-L1 on CTCs	CellSearch/Parsortix	No correlation between PD-L1 expression in tissue and CTCs	Not all samples were recruited before the start the treatment
Koh et al. 2019 [56]	67	PD-L1 on CTCs	Microcavity array	No correlation between PD-L1 expression in tissue and CTCs	-
Chen et al. 2019 [58]	51	PD-L1 on CTCs	CMx microfluidic platform	57% concordance between PD-L1 expression in tissue and CTCs	-
Tamminga et al. 2019 [59]	104	CTC count and tDEV	CellSearch	The presence of CTCs was associated with worse PFS and OS in patients under ICI treatment	CTCs detected in only 30% of patients

Table 2. Summary of liquid biopsy studies in NSCLC treated with immunotherapy.

Bi-PAP bidirectional-pyrophosphorolysis-activated polymerization, *bTMB* blood tumor mutational burden, *CAPP-seq* cancer personalized profiling by deep sequencing, *CK* cytokeratin, *ddPCR* droplet digital PCR, *MSAF* maximum somatic allele frequency, *NGS* next-generation sequencing, *OS* overall-survival, *PFS* progression-free survival, *qPCR* quantitative polymerase chain reactions, *SNVs* single-nucleotide variants, *tdEV* tumor derived extracellular vesicles, *TML* tumor mutation load, *tTMB* tissue tumor mutational burden, *TTP* time to progression, *WES* whole exome sequencing.

4.2.1 CTCs isolation platforms

In most of the current assays for the detection of CTCs, the platforms are based on the expression of epithelial markers such as EPCAM and cytokeratins. Both biomarkers are not expressed on mesenchymal cells; therefore, new CTC isolation platforms were developed [48].

Currently, the methods to detect and isolate CTCs are based on different aspects: (1) the surface presence of specific antigens (EpCAM is the most common), such as what is used in the CellSearch[®] system (Menarini), Epic platform (Epic sciences) or GILUPI CellCollector (GILUPI); or (2) size and deformability, such as what is used in Parsortix (Angle), ISET (ISET, Rarecells Diagnostics), Vortex VTX-1 (Vortex Bioscience), and the ClearCell FX device (Biolidics).

4.2.2 CTCs and PD-L1 expression

In the field of immunotherapy, the expression of PD-L1 in tissue samples is the gold-standard biomarker to choose the treatment. Pilot studies in lung cancer have shown PD-L1 as a predictive factor. However, the PD-L1 status can be underestimated in small biopsies, which may not be representative of the entire tumor, causing some to not receive ICI treatment due to tissue sampling bias [49]. PD-L1 analyses in CTCs could be a good option to solve this problem.

The possibility of measuring the expression of PD-L1 in CTCs before and during treatment has been analyzed in several studies (Table 2). Some authors have studied the relationship between baseline CTCs and overall survival, finding an association between a high number of CTCs and an increased risk of death and progression [50]. In addition, the presence of CTCs with positive PD-L1 expression ($\geq 1\%$ of cells) before ICI treatment was correlated with poor prognosis and survival [50, 51], and the persistence of PD-L1 on CTCs after 6 months of ICI therapy may represent the occurrence of tumor escape, which results in disease progression. It may also be a reflection of the aggressiveness of these CTCs, which may show characteristics of the mesenchymal phenotype (via EMT) [52]. In a stage-adjusted Cox model, it was concluded that a high proportion of PD-L1-positive cells was a predictor of mortality [51].

4.2.3 CTCs vs. tissue

The concordance between PD-L1 expression by CTCs and PD-L1 expression in tissue has been evaluated in several studies, showing controversial results [50, 53]. Iliè et al. showed a concordance of 93% between tissue and CTCs regarding PD-L1 expression in a cohort of 106 patients treated with chemotherapy [53], using the platform ISET. Dhar et al. also showed a good correlation between tissue and CTCs using a different isolation platform (Vortex isolation). However, the sample size in this study was only 4 patients [54]. Other studies reveal the opposite situation. Guibert et al., reported no correlation between CTCs and tissue in a cohort of 96 patients treated with nivolumab in 2nd line, isolating the CTCs with the ISET platform [50]. Recently, other two studies reported the same conclusion using two different approaches to isolate the CTCs (Microcavity array and Parsortix system) [55, 56]. It is important to remark that in these studies, the expression of PD-L1 was analyzed using different methods, therefore, different phenotypes of CTCs could be obtained in each study. Also, different antibodies and unbalanced populations were employed, so comparisons between them must be interpreted with caution. More studies are needed to assess a standardized method and PD-L1 antibody to detect and analyze CTCs.

4.3 Other blood biomarkers

There are other markers in the field of liquid biopsy with great potential for future clinical applications. Extracellular vesicles (EVs) are nanosized particles with membranes released by any cell type, including cancer cells. The most common subtypes of EVs are exosomes. Exosomes have a size between 50 and 150 nm, are very abundant, express proteins on their surface and contain several particles, such as proteins, nucleic acids, and lipids [60]. For this reason, exosomes are also a promising biomarker for analyzing the expression of PD-L1. One study in NSCLC patients concluded that PD-L1 expression in exosomes was correlated with disease progression, tumor size, lymph node status, metastasis, and TNM stage. However, no association was demonstrated between PD-L1 expression in exosomes and tissue [61]. In addition, the standardization of methodologies for exosome isolation remains a challenge [49].

Numerous studies have also reported a correlation between immunotherapy and the neutrophil-to-lymphocyte ratio (NLR) [62]. The NLR is defined as the neutrophil count in blood divided by the lymphocyte count in blood and is a marker for the general immune response to distinct stress stimuli. Recently, a meta-analysis concluded that high NLR values were correlated with shorter PFS and OS in NSCLC patients under ICI treatment [63].

Other elements, such as circulating microRNA (miRNA), circulating RNA, platelets, and circulating proteins, have not been included in this review because they are at the early stages of use for tumor genotyping and more specifically for the use in immunotherapy of NSCLC patients [62].

5. Conclusion and future perspectives

Liquid biopsy is being incorporated as a useful procedure in many situations in oncology due to its minimal invasiveness and its great capacity to indicate cancer tumorigenesis. The search for new biomarkers will help us to manage our patients in a personalized way, something that constitutes a clear objective for the implementation of precision oncology.

Over the past few years, several integrated biological strategies have been used to find noninvasive markers to predict response to the administration of immunotherapy in NSCLC. The existence of promising results from observational studies, together with the continued development of analysis platforms, has propelled cfDNA-based liquid biopsy diagnostics into the next developmental phase. Because of its potential to select populations for specific immunotherapy (alone or combined) and predict responders/nonresponders or the appearance of resistance, we foresee that cfDNA analysis will become increasingly important. In the case of lung cancer, cfDNA methods are particularly useful because they minimize the need for tumor tissue biopsy. The capability of cfDNA to guide the choice and follow the response to immune therapy is just beginning to be assessed. Recently, Chabon et al. established the potential of cfDNA for lung cancer screening, underlining the importance of risk-matching cases, and controls in cfDNA-based screening studies [64]. However, we cannot ignore that cfDNA isolation and analysis are still a challenge. Plasma cfDNA is a complex mixture of DNA from many sources, including germline, fetal, infectious, and malignant [31], but there are also mutations in the cfDNA of hematopoietic origin with risk being mis- taken as tumor-derived mutations [65]. Because a large proportion of cfDNA is derived from peripheral blood cells, the impact of clonal haematopoiesis represents a relevant limitation in the implementation of

liquid biopsy for analysing cfDNA. Paired plasma–peripheral blood cells sequencing should be implemented as the standard practice for NGS genomic analysis of cfDNA to prevent misinterpretation of results [66]. Moreover, cfDNA is not tumor-specific and its measurement is prone to false positive results. In fact, the level of cfDNA can increase under some conditions, such as inflammation, infection, and even exercise [30]. The sensitivity and specificity of cfDNA measurement are also affected by the variability of ctDNA, which may vary through tumor evolution, have different origins (primary tumor vs. metastasis; tumor heterogeneity) and be affected by different treatments. We are in a situation in which there are rapidly evolving technologies supporting the use of liquid biopsy, while there is a need for standardization procedures for collecting and processing cfDNA samples. Another aspect to standardize should be the size of the panels for appraising specific cancer mutations and genomic metrics (MRD and TMB), since they vary greatly across assays.

On the other hand, CTCs are intact viable cells with tumor-specific information and can also enable cancer diagnosis and treatment evaluation. Analyses of the presence or absence of proteins, such as PD-L1 or PD-1 on the surface promise to be good options in the future to guide and select treatment, but the strategies will need to be validated in robust studies [49]. In addition, due to methods that allow the preservation and capture of intact CTCs, single-cell analyses or cluster analyses are possible. Single- or cluster-cell characterization of CTCs will allow more precise characterization of the entire cancer in each patient. RNA and DNA analyses are possible and could show the intertumoral and intratumoral heterogeneity in each patient [67]. In contrast, a good and standardized platform to capture CTCs and rigorous validation remain a challenge. Owing to the effects of EMT, a new marker to detect tumor cells undergoing this transition is needed.

Recent studies have focused on the peripheral blood T-cell receptor (TCR) repertoire, as an emergent biomarker to select the most suitable ICI treatment [68]. The analysis of T-cell clonality may reveal the degree of tumor-antigen driven T-cell expansions and help to understand mechanisms underlying T-cell tolerance to cancer antigens. Recently, Reuben et al. showed the heterogeneity of tissue T-cell repertoire in localized NSCLC [69], proposing a positive relation between T-cell density and clonality. They demonstrated an association between higher T-cell density in the blood and improved outcome following surgery. These findings suggest that the peripheral T cell repertoire in NSCLC patients may be reflective of increased systemic immunity. TCR sequencing might be applicable for the treatment selection in patients with EGFR mutations by evaluating the proportions of TCR β clones in the tumor. Miyauchi et al. provided relevant information for understanding the molecular mechanism behind EGFR-mutant patients [70], showing that the low clonal T-cell expansion in NSCLC with EGFR mutations might be a critical factor related to the unfavorable response to ICI. T-cell clonality in the circulation may have predictive value for antitumor responses from checkpoint inhibition. However, since there are very preliminary results, continue this line of research is essential in order to know if it has practical application in the clinic.

In conclusion, liquid biopsy through CTCs and cfDNA analyses could be a promising tool for diagnosis, selection of ICI treatment and monitoring of NSCLC patients under immunotherapy.

CAPÍTULO 2

Immunotherapy in patients with brain metastasis: advances and challenges for the treatment and the application of circulating biomarkers

Immunotherapy in patients with brain metastasis: advances and challenges for the treatment and the application of circulating biomarkers

E. M. Brozos-Vázquez ^{1,2,3,4*}, C. Rodríguez-López ^{1,3,4}, A. Cortegoso-Mosquera ^{1,3,4}, S. López-Landrove ³, L. Muínelo-Romay ^{3,4}, J. García-González ^{1,3,4}, R. López-López ^{1,3,4*} and L. León-Mateos ^{1,3,4}

¹ Medical Oncology Department, Complejo Hospitalario Universitario de Santiago de Compostela, Santiago de Compostela, Spain,

² Medical Oncology Department, Complejo Hospitalario Universitario de A Coruña, Santiago de Compostela, Spain,

³ ONCOMET, Instituto de Investigación Sanitaria de Santiago, Santiago de Compostela, Spain,

⁴ CIBERONC, Madrid, Spain

Immunotherapy in patients with brain metastasis: advances and challenges for the treatment and the application of circulating biomarkers

Abstract

The central nervous system (CNS) is one of the most frequent metastatic sites of various cancers, including lung cancer, breast cancer and melanoma. The development of brain metastases requires a specific therapeutic approach and is associated with high mortality and morbidity in cancer patients. Advances in precision medicine and the introduction in recent years of new drugs, such as immunotherapy, have made it possible to improve the prognosis of these patients by improving survival and quality of life. New diagnostic techniques such as liquid biopsy allow real-time monitoring of tumor evolution, providing molecular information on prognostic and predictive biomarkers of response to treatment in blood or other fluids. In this review, we perform an exhaustive update of the clinical trials that demonstrate the utility of immunotherapy in patients with brain metastases and the potential of circulating biomarkers to improving the results of efficacy and toxicity in this subgroup of patients.

Keywords solid tumors, brain metastasis, biomarkers, immunotherapy, liquid biopsy.

1. Clinical challenge

The brain is one of the most frequent sites of metastases from some types of solid tumors, such as lung, breast cancers and melanoma. The presence of disease in the brain represents a dismal prognosis and devastating complications for the quality of life of oncological patients and can lead to a different evolution in relation to the histological and molecular type, and clinical characteristics of the tumor. Recent advances in Oncology have improved survival rates, with the incidence of brain metastases now estimated at around 20%-40% (1,2). Actually, in recent years overall survival in these cancers has increased significantly due to the introduction of new drugs, including immunotherapy. This treatment has been used in routine clinical practice since phase III clinical trials have demonstrated its efficacy in advanced tumors such as lung cancer (3,4) or melanoma (5,6). However, there is no extensive literature on the subgroup of patients with cerebral or leptomeningeal disease because these patients have traditionally been excluded from the clinical trials.

The mainstays of the treatment in cases with secondary brain involvement are surgery and stereotactic radiosurgery if the patient has a single brain metastasis (BM) or limited number; and radiotherapy and symptomatic management with corticosteroids and/or antiepileptic drugs on patients with extensive number or volume of brain lesions. Classical chemotherapy has played a limited role due to its limited passage through the blood-brain barrier. However, targeted therapy is effective in the presence of onco- addictive molecular alterations, and immunotherapy is nowadays crucial in the landscape treatment of lung cancer and melanoma.

Moreover, we already know the fact that a drug could be effective in controlling metastatic disease at the systemic level, but not necessarily imply that it is active on synchronous disease in the central nervous system (CNS). On the other hand, the genetic characteristics of the primary tumor and different metastatic sites might differ (7,8), and the response to the same treatment may therefore be different. Genetic, epigenetic, and transcriptomic changes in brain metastases lead to changes in the tumor microenvironment, making this disease immunologically more inactive with respect to the primary tumor (9-11).

Besides, there is an unmet clinical need for discovering biomarkers that can help to predict a high risk of brain metastases in certain tumor types, as well as to individualize management once they are present. We have the GPA (Graded Prognostic Assessment) prognostic tool whose objective is to estimate survival in patients with lung cancer based on clinical variables, without considering molecular factors, which nowadays must be considered due to the demonstrated relevance of molecular biology in cancer (12). Given the complexity of obtaining a biopsy of brain tissue, the development of non-invasive biomarkers is of high relevance in the context of secondary brain lesions.

The aim of this review is to analyze published data on the efficacy of immunotherapy as a treatment for patients with metastatic brain disease, focusing on lung, breast, and melanoma tumors. We make an overview of the most relevant clinical trials performed to explore the efficacy of immunotherapy to treat brain metastasis and comment on the current knowledge and the potential of liquid biopsy-based biomarkers to predict the therapy response and monitor the disease evolution.

2. Immunotherapy in lung cancer patients with brain dissemination

Lung cancer (LC) is a heterogeneous disease, histologically and molecularly (13,14). There are two main subtypes of LC, small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC), accounting for 76% and 13%, respectively. LC remains the most common cause of cancer-related death (15). However, in the last decade, mortality associated with NSCLC has decreased due to a reduction in the incidence of LC and an increase in the overall survival of patients with NSCLC (16). Furthermore, the improvement in LC survival is related to a deeper understanding of the genomic profile of NSCLC that has allowed progress in LC treatment in two directions: on the one hand, the introduction of targeted therapies against mutations in driver oncogenes, and, on the other hand, immune checkpoint inhibitors (ICIs) (16).

2.1 Non-small cell lung cancer

BMIs are common in patients with advanced NSCLC and occur in 20-30% of patients (17,18). Currently, treatment of cerebral disease is based on surgery, holocranial radiotherapy (WBRT) or stereotactic radiosurgery (SBRT) (19,20). However, this practice is changing in patients with NSCLC and driver oncogene mutations, as tyrosine kinase inhibitors have shown good CNS penetrance and a high response rate of intracranial disease (21-24).

Immunotherapy with inhibitors against the programmed death 1 (PD-1) receptor, its ligand (PD-L1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has revolutionized the therapeutic scenario for patients with advanced NSCLC (aNSCLC) without driver mutations (25). Today, the current therapeutic approach to an individual patient with aNSCLC is based on tumor expression of PD-L1. Thus, in tumors with 50% PD-L1 expression, the use of ICIs as monotherapy is an effective option (26-28). While the combination of ICI plus platinum-based chemotherapy has been shown to improve efficacy parameters regardless of the percentage of PD-L1 expression (29-32). Although most of the clinical trials in this setting allowed the inclusion of patients with pre-treated and clinically asymptomatic CNS metastases, there are few data about the efficacy of the immunotherapy at the CNS level (Table 1).

Author	Study type	Treatment	No patients with BMs	Outcomes
Ready, et al. (33)	Phase IIIB	Nivolumab plus Ipilimumab	49	mOS: 12.8 m 3-years OS rate: 21.0% mPFS: 2.8 m 3-years PFS rate: 14.2%
Reck, et al. (34)	Phase III	Nivolumab plus Ipilimumab vs chemotherapy	68 66	mOS: 17.4 vs 13.7 m 5-year OS rates: 20% vs 6% 5-year intracranial PFS rates: 16% vs 6%
Carbone, et al. (35)	Phase III	Nivolumab plus ipilimumab and chemotherapy vs chemotherapy	51 50	mOS: 19.3 vs 6.8 m 2-year OS rates: 35% vs 12% mPFS: 10.6 VS 4.1 m
Nadal, et al. (36)	Phase II	Atezolizumab plus chemotherapy, followed by maintenance with pemetrexed plus atezolizumab	40	mOS: 13.6 m 2-year OS rate: 30.5% mPFS: 8.9 m Intracranial mPFS: 6.9 m
Hou, et al. (37)	Phase II	Camrelizumab plus chemotherapy, followed by maintenance with camrelizumab and pemetrexed	45	mOS: 21.0 m mPFS: 7.4 m Intracranial mPFS: 7.6 m
Powell, et al. (38)	Pooled analysis	Pembrolizumab plus chemotherapy vs chemotherapy	171	mOS: 18.8 m vs 7.6 m mPFS: 6.9 m vs 4.1 m
Ozguroglu, et al. (39)	Phase III	Cemiplimab vs chemotherapy	68	mOS: 18.7 vs 11.7 mPFS: 10.4 vs 5.3
Goldberg, et al. (40)	Phase II	Pembrolizumab	42	In patients with NSCLC and PD-L1 $\geq$ 1%: • mPFS 1.9 m • mOS 9.9 m • 2-years OS ratio 34%
Mansfield, et al. (41)	Pooled analysis	Pembrolizumab vs chemotherapy	293	Pts with NSCLC and PD-L1 $\geq$ 1%: • mOS: 13.4 vs 10.3 m Pts with NSCLC and PD-L1 $\geq$ 50%: • mOS: 19.7 vs 9.7 m

BM, brain metastasis; m, months; mOS, median overall survival; mPFS, median progression-free survival; NSCLC, non-small cell lung cancer; Pts, patients.

Table 1. Outcomes of ICI in the treatment of NSCLC with basal BMs.

In the CheckMate 817 clinical trial (33), the efficacy of nivolumab plus ipilimumab was prospectively evaluated in first-line treatment of special populations with NSCLC, including 49 patients with untreated brain metastases. In this subgroup the median OS was 12.8 months, with a 3-year survival rate of 21%. In addition, the median PFS was 2.8 months, with a 3-year PFS rate of 14.2% (42). To reinforce these data, post-hoc analysis data with a minimum five-year follow-up of patients with baseline brain disease included in the CheckMate 227 (Part 1) clinical trial have recently been published (34). In this population, the treatment with the combination of nivolumab plus ipilimumab prolongs OS versus treatment with platinum-based chemotherapy in those patients with CNS metastases at baseline. In addition, less patients with cerebral disease at the inclusion developed new brain metastases with nivolumab and ipilimumab versus chemotherapy (4% and 20%, respectively) (34).

Following this treatment rationale, the nivolumab plus ipilimumab combo was combined with 2 cycles of platinum-based chemotherapy in the CheckMate 9LA clinical trial, which included 101 patients with brain metastases (35). In a post-hoc study of this population, the combination of nivolumab plus ipilimumab chemotherapy showed a hazard ratio for the risk of death of 0.43, with a median OS of 19.3 months versus 6.8 months in patients treated with chemotherapy alone; plus, a 2-year alive rate of 35% (35).

The multicenter phase II Atezo-BRAIN trial evaluated the efficacy and safety of combining atezolizumab with 4 or 6 cycles of carboplatin plus pemetrexed followed by maintenance with atezolizumab plus pemetrexed in patients with non-squamous NSCLC and CNS metastases (36). In this study, 40% of patients had confirmed intracranial response based on RANO-BM criteria (12 partial responses; 4 complete responses) and 19 (47.5%) patients achieved systemic response (all partial responses). No differences in the overall response rate in systemic and intracranial were observed according to PD-L1 expression or corticosteroid use at baseline. With a longer follow-up of 20 months, the median systemic PFS was 8.9 months and the median intracranial PFS was 6.9 months. In addition, the median OS was 13.6 and the estimated 2-year OS rate was 30.5%. OS was explored as a function of PD-L1 expression; thus, the median OS was higher for PD-L1 positive patients at 16.2 months compared to PD-L1 negative patients 10.7 months. However, these differences were not statistically significant due to limited statistical power. There was also no significant difference in OS between those who did and did not receive baseline dexamethasone treatment.

The clinical activity and safety of camrelizumab plus chemotherapy as first-line treatment in patients with advanced NSCLC with brain metastases was evaluated in a study carried out in Chinese population (37). The objective response rate in the intracranial disease was 52.5% in those patients that have at least one post-baseline tumor assessment and 46.7% in the full analysis. On the other hand, the objective response rate in the extracranial tumor burden was 47.5% and 42.2%, respectively.

Strengthening the efficacy of the combined treatment of ICI plus platinum-based chemotherapy are data from a pooled analysis of the clinical trials KEYNOTE-021, -189 and -407 (38). In patients with brain metastases, the median overall survival was 18.8 months with pembrolizumab plus chemotherapy and 7.6 months with chemotherapy with a 52% reduction in the risk of death. Median PFS was longer with pembrolizumab plus chemotherapy versus chemotherapy in patients with cerebral lesions (6.9 months versus 4.1 months). Finally, pembrolizumab with chemotherapy reached a higher objective response rate, regardless of BM status, if we compare it with chemotherapy alone.

The EMPOWER-Lung 1 trial assessed the efficacy and safety of cemiplimab in frontline advanced NSCLC with PD-L1 expression $\geq 50\%$ and included 68 (12.1%) treated brain metastases patients (39). In this subgroup, analysis of PFS and OS showed that patients significantly benefited from cemiplimab compared to platinum-based chemotherapy.

On the other hand, Goldberg et al. (40), analyzed the efficacy of pembrolizumab in two cohorts of patients with NSCLC and CNS metastases: cohort 1 included 37 patients with PD-L1 expression $\geq 1\%$, while cohort 2 included 5 patients with PD-L1 expression $< 1\%$. No responses were observed in patients in cohort 2. In contrast, in cohort 1 the brain response rate was 29.7%. In addition, the overall response rate was 18.9%, considering both systemic and brain disease. A discordant response was observed in 6 patients. In this work the median PFS was 1.9 months and 33% of patients had not progressed to brain level at 12 months. Finally, the median OS was 9.9 months, and the 2-year alive rate was 34%. Importantly, in a pooled analysis of four studies, pembrolizumab was shown to reduce the risk of death by 17% compared to chemotherapy in patients with baseline brain metastases and NSCLC with PD-L1 expression $> 1\%$ (41). Thus, in this population the median OS was 13.4 and 10.3 months, respectively. Furthermore, this benefit appears greater in patients with NSCLC and PD-L1 TPS expression $\geq 50\%$ with brain

metastases at baseline, with a 33% reduction in risk of death; median OS was 19.7 and 9.7 months, respectively. In addition, pembrolizumab provided similar results in patients with brain metastases or without them.

Globally, although limited data are available on the role of immunotherapy in NSCLC and brain metastases, ICIs alone or in combination with chemotherapy have shown promising intracranial clinical activity and safety outcomes (43). Nevertheless, the mechanism of how ICIs act in the brain niche and their interaction with the tumor microenvironment is unknown. Therefore, characterizing the immune phenotype of cerebral disease and better understanding the relation with the immune cells, resident stromal cells along with neoplastic cells are crucial to improve the results of immunotherapy in our patients.

2.2 Small cell lung cancer

SCLC, a highly aggressive type of lung cancer, represents 15% of all lung cancer cases and is strongly linked to tobacco use. Regrettably, patients diagnosed with extensive disease SCLC (ED-SCLC) face a mere 2% 5-year survival rate (44-49). While chemotherapy has long been the established treatment approach for SCLC, recent advancements have introduced alternatives like immunotherapy, which have shown potential in enhancing survival rates (45-48). Nonetheless, a significant portion of ED-SCLC patients do not experience the benefits of this innovative treatment (49).

Brain metastases pose a significant threat to the well-being and survival of individuals with SCLC, leading to notable morbidity and mortality while greatly impacting their quality of life (50). The diagnosis of brain metastases originating from SCLC relies on imaging techniques like CT scans and MRI scans. Research indicates that around 10-14% of SCLC patients already have brain metastases at the time of diagnosis, and during the course of the disease, this number can rise to 50% (50). The incidence of brain metastases is higher in patients with extensive-stage SCLC (ES-SCLC) compared to those with limited stage, LS-SCLC. Typically, the median survival for individuals with brain metastases from SCLC is approximately 6 months, although there have been instances of patients surviving beyond one year. Factors that contribute to improved survival rates include younger age, absence of tumor growth, and a favorable performance status (50).

The standard treatment approach for brain metastases originating from SCLC commonly entails a combination of radiation therapy, chemotherapy, and immunotherapy (50, 51). Due to the aggressive nature of the disease, surgical intervention is seldom employed (51). Historically, radiotherapy has been utilized as a preventive measure to impede the development of metastases in cases of thoracic-confined or metastatic disease (known as prophylactic cranial irradiation) (51). Additionally, radiotherapy is administered as a therapeutic intervention when there is visible macroscopic disease. Chemotherapy, often comprising platinum agents and etoposide, is employed with or without immunotherapy to diminish the risk of recurrence and manage symptoms (51).

Of note, SCLC can be categorized into four subtypes named SCLC-A, SCLC-N, SCLC-P, and SCLC-Y, based on the expression of specific genes, namely achaete-scute homolog 1 (*ASCL1*), neurogenic differentiation factor 1 (*NeuroD1*), POU class 2 homeobox 3 (*POU2F3*), and yes-associated protein-1 (*YAP-1*), respectively (52, 53). Research studies have highlighted the

significance of recruiting and activating T cells at the tumor sites as crucial steps in cancer immunotherapy. A subsequent study indicated that *YAP-1* could serve as a prognostic marker for T cell- induced inflammatory responses (52). The study findings demonstrated an inverse correlation between *YAP-1* and the activation and differentiation of CD4⁺ and CD8⁺ T cells, presenting significant potential for the immunotherapy of LS- SCLC (52). Additionally, the SCLC-Y subtype exhibits a notable inclination towards undergoing epithelial-to-mesenchymal transition (EMT), immune cell infiltration, and heightened antigen presentation capacity (53). This particular SCLC subtype demonstrates relatively higher sensitivity to immunotherapy and combination chemo-immune therapy.

Xiao et al. conducted a meta-analysis aiming to assess the outcomes of lung cancer patients who received immunotherapy alone or in combination with other treatments such as chemotherapy, targeted therapy, or radiotherapy (54). The analysis focused on phase 2 or 3 randomized controlled trials. Additionally, they compared chemotherapy or placebo and examined hazard ratios for overall survival. The meta-analysis revealed a pooled interaction of 0.72 for non-small cell lung cancer (NSCLC) and 1.41 for small cell lung cancer (SCLC) in patients with and without brain metastases. This difference was indicated by a heterogeneity test of interaction between the two subgroups (54).

In the CASPIAN trial, the combination of durvalumab with the etoposide-platinum (EP) regimen was investigated for the treatment of patients with extensive-stage SCLC (55). The trial demonstrated a median overall survival (OS) of 13 months, which was 2.7 months longer than the control group. The study included patients with brain metastases, either receiving treatment or without symptoms in the central nervous system (CNS) (10% in the control group and 10.4% in the monoclonal antibody group). The results indicated that the combination of monoclonal antibody and chemotherapy regimen delayed intracranial progression, reduced the need for brain radiotherapy, and provided certain benefits in terms of improving progression-free survival (PFS) and OS in patients with brain metastases.

The IMpower 133 trial, a large global phase III clinical trial, aimed to evaluate the effectiveness of atezolizumab in combination with chemotherapy as a first-line treatment for metastatic SCLC. The trial results demonstrated a statistically significant 2-month improvement in overall survival ($p=0.015$) for this subgroup of patients (47). However, only 8.7% of patients with CNS metastasis were included in this trial, making it inconclusive regarding the benefits of atezolizumab for patients with BM (56). On the other hand, the KEYNOTE-604 trial, a double-blind, placebo-controlled phase III trial, enrolled previously untreated patients with extensive-stage SCLC (ES-SCLC) (49). These participants were randomly assigned to receive EP (etoposide-platinum) chemotherapy with or without pembrolizumab. The median overall survival (OS) for the experimental group was 10.8 months, while the control group had a median OS of 9.7 months. The difference between the two groups was statistically significant ($P = 0.0164$). However, the confidence interval (CI) values for subgroups with fewer than three metastatic sites and brain metastases were wide and overlapped with the total population. Consequently, more patients with BM need to be included in studies to accurately assess the efficacy of pembrolizumab in this particular scenario.

The genomic profile of SCLC is characterized by extensive chromosomal rearrangements and a high mutational burden, including the inactivation of tumor suppressor genes *TP53* and *RBI* (57). However, there are currently no validated predictive biomarkers available to guide

treatment decisions or stratify patients (58). Liquid biopsies, such as circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs), show promise as alternative methods to guide the therapy selection and the follow-up (58–60).

3. Immunotherapy in breast cancer patients with brain dissemination

Breast cancer is one of the most prevalent tumors in the world and the one that most frequently metastasizes to the central nervous system (CNS), being the second most common cause of brain metastases after lung carcinoma and above melanoma. Between 15- 30% of patients with advanced breast carcinoma will develop CNS disease, which will have prognostic implications, limiting survival to around 15 months, as well as a major impact on their quality of life due to the neurological complications that these patients develop (61,62).

There are some histological subtypes with molecular features that determine a different biological behavior and natural history of disease. HER2⁺ and triple negative (TNBC) are the ones that most frequently cause BM. For the appropriate management of this disease, we have the added difficulty that patients with CNS disease are often excluded from clinical trials, so there is less evidence available. Currently, the recommended treatment of brain metastatic breast cancer disease (BCBM) includes locoregional therapy and systemic therapy, or a combination of both. In the case of HER2⁺ BC, different drugs directed against the HER2 receptor have proven to be effective in the treatment of advanced disease (63), including cerebral disease, sometimes avoiding aggressive surgery or brain irradiation with medium and long-term consequences.

In recent years, systemic therapies for the treatment of advanced TNBC have included ICIs such as atezolizumab and pembrolizumab, in combination with chemotherapy, for patients whose tumor expresses PD-L1, quantified by IHC with non- homogeneous methods in different studies. In these studies, exclusion criteria have prevented recruitment of patients with BM. Therefore, we do not have useful biomarkers of response to immunotherapy in liquid biopsy in metastatic brain disease due to breast carcinoma which are valid for use in routine clinical practice. From retrospective literature studies that obtain information from different genetic databases to perform differential expression analysis between primary breast tumors and BM, some genes emerge as possible subjects of future research to test their use as potential biomarkers predictive of drug response or therapeutic targets (64,65).

A study published by Lu et al. found increased plasma cell infiltration in BM and decreased infiltration by M2 macrophages, as well as overexpression of the immuno-related genes *THY1* and *NEU2*, proposing them as possible therapeutic targets (7). The *ARG2* expression, which has an immunosuppressive role, appears to be higher in BCBM, indicative of a lower T-cell mediated response (10). In terms of epigenetic alterations, HLA-A methylation has typically been identified in metastatic TNBC disease, resulting in decreased expression of HLA-A and associated immune cells, which may be a biomarker of unresponsiveness to ICIs as well as a therapeutic target, using DNA demethylating drugs in combination (11). The signature Macrophage receptor with collagenous structure (MARCO) has been linked to prognosis, immune cell infiltration and ICI treatment in different tumors, and is a potential biomarker that still needs to be further explored (66).

There are some approaches about molecular development in this subpopulation of patients (67). An ongoing phase II investigates the efficacy of different combinations depending on molecular subtype; the cohort of TN patients receive bevacizumab, SHR1316 (a novel anti-PD-L1) and platinum (NCT04303988). Two studies explore the efficacy of immunotherapy in combination with radiosurgery (SRS), a phase I/II study with pembrolizumab in BCBM (NCT03449238), and a phase II study with atezolizumab in TNBM (NCT03483012). The use of other ICIs, such as ipilimumab (anti-CTLA4), are being tested in advanced TNBC, so far without high efficacy data and without specific development in cases of brain disease. Other newer approaches emerging in the field of immunotherapy that are being explored in the HER2⁺ subtype, such as the use of CAR-T, are also lacking research in this setting of BM disease by triple-negative subtype.

Some ongoing clinical trials are evaluating the efficacy of immunotherapy in combination with other drugs or radiotherapy in patients with BCBM depending on molecular subtype. The use of other ICIs, such as ipilimumab (anti-CTLA4), are being tested in advanced TNBC, so far without high efficacy data and without specific development in cases of brain disease. Other newer approaches emerging in the field of immunotherapy that are being explored in the HER2⁺ subtype, such as the use of CAR-T, are also lacking research in this setting of metastatic brain disease by triple-negative subtype.

4. Immunotherapy in melanoma patients with brain dissemination

Melanoma is the third most common type of cancer, after breast and lung cancer, to metastasize to CNS. Between 40% and 50% of patients diagnosed with melanoma will generate clinically detectable CNS metastases during the course of the disease (68). Prior to the development of new targeted therapies and immunotherapy, the presence of BM in melanoma was associated with poor prognosis, with a median overall survival of 4 months (68, 69). Leptomeningeal involvement, the size and number of BM, the presence or absence of neurological symptoms and mutational status are different prognostic factors that affect the survival of these patients (69).

Historically, treatments for melanoma with CNS metastases consisted of surgery and radiotherapy, with poor results and short survival. In the last 10 years, with the emergence of targeted therapies (*BRAF* inhibitors and *MEK* inhibitors) and the efficacy of immunotherapy in this context, the prognosis of these patients has changed. The *BRAF V600* mutation is present in 40-50% of metastatic melanomas and leads to activation of MAPK and *ERK* pathways. In a study of matched samples of primary melanoma tumors and BM, a mutation concordance of 80% was observed (70). The presence of *NRAS* and C-KIT mutation in patients with melanoma and BM was 22% and 11% respectively (71). Loss of *PTEN* function has been detected in about 10-30% of melanomas, most frequently in *BRAF*-mutated tumors (71). Activating mutations in *AKT1* and *AKT3* are rare (1-2%) (71). In studies of paired biopsy samples from extracranial and CNS metastases, a similar mutation profile tends to be observed. As an exception, BM express specific molecular features in the *PI3K/AKT* pathway (72). Melanoma CNS metastases showed increased expression of various protein markers that activate the *PI3K/AKT* pathway compared to extracranial disease, being a potential therapeutic target (72).

In the melanoma population with BM and *BRAF* mutated tumors, the combination of immunotherapy with *BRAF* and *MEK* inhibitors has been tested in recent years, as well as the sequencing of these treatments. Data have recently been published from the TRICOTEL clinical trial, a phase II study with 2 cohorts patients diagnosed with melanoma and BM: one cohort without *BRAF V600* mutated tumors, and another one with *BRAF V600* mutated ones (73). The cohort *BRAF* negative was treated with atezolizumab and cobimetinib. The other group was treated with atezolizumab, cobimetinib and vemurafenib. Sixty-five patients were enrolled in the study. The intracranial objective response rate was 42% in the *BRAF V600* mutation-positive cohort and 27% in the non-mutated population. Another study also analyzed the time to develop central nervous system metastases in patients treated with atezolizumab or placebo plus vemurafenib and cobimetinib (74). In the 514 patients included in the immunotherapy and targeted therapy cohort, 25% of patients developed brain metastases with a follow-up of 29.8 months, and 28% in the placebo group with a follow-up of 22.8 months. The time to onset of first CNS metastases was later in the atezolizumab, vemurafenib and cobimetinib group, which is interesting because represents some kind of activity in patients that are kind to generate brain metastases.

Several clinical trials assessed the efficacy of immunotherapy specifically in patients with melanoma and intracranial metastases. One of the first studies published in this context evaluates the role of Ipilimumab (Anti-CTLA-4) (75). This phase II study included patients in cohort A, neurologically asymptomatic and without corticosteroid therapy; and cohort B, symptomatic and on stable doses of corticosteroids. They received 4 doses of ipilimumab every 3 weeks, followed by 1 dose every 12 weeks if clinically stable. Disease control measuring the intracranial metastases was achieved in 24% of patients in cohort A and in 10% of cohort B patients. Extracranial disease control was 27% and 5% respectively. The different outcomes of the cohorts were related to patient characteristics, with cohort B being a poorer prognostic group and to the negative effects of corticosteroid therapy on immunotherapy. In the NIBIT-M1 phase II clinical trial, they combined treatment with ipilimumab and fotemustine, since chemotherapy-induced release of tumor antigens could amplify the anti-tumor activity of immunotherapy (76). Of the 20 patients included in the study, 50% had disease control, showing benefit if compared with historical cohort of patients with these features.

The efficacy of treatment with pembrolizumab and nivolumab monotherapy was also studied in the context of patients with BM. In a phase II study with patients affected by melanoma or untreated NSCLC adenocarcinoma with CNS metastases the activity of pembrolizumab was interrogated (77). Of the 18 melanoma patients included, response of BM was achieved in four patients. In another phase II clinical trial, 23 patients with untreated asymptomatic melanoma and BM were included without the need for corticosteroids. Seventy percent of the patients had received prior systemic therapy (78). Twenty-six percent of patients had a brain response, which was concordant with the systemic response. Median PFS was 2 months and OS was 17 months.

In clinical trials using immunotherapy in monotherapy, intracranial objective response rates were 16% with ipilimumab, 26% with Pembrolizumab and 20% with nivolumab (75-80). The combination of nivolumab and ipilimumab was investigated in two separate studies for patients with melanoma BM (79,80). In the Australian study (ABC), asymptomatic patients without prior local treatment were assigned to nivolumab plus ipilimumab 4 doses every 3 weeks, followed by nivolumab every 2 weeks (Cohort A), or nivolumab every 2 weeks (Cohort B and C) (80). Cohort C included patients with failure of local therapy, with neurological symptoms

or leptomeningeal disease, to receive nivolumab monotherapy. The best intracranial response at 12 weeks or later was 51%, 20% and 6% respectively in each cohort. PFS at 24 months was 49%, 15% and 6%, respectively. OS at 24 months was 63%, 51% and 19% respectively. The phase II CheckMate 204 study enrolled asymptomatic patients to receive nivolumab and ipilimumab every 3 weeks, 4 doses, followed by nivolumab every 2 weeks (79). The intracranial clinical benefit rate (defined as the percentage of patients had stable disease at 6 months, complete or partial response) was 57%, with 26% complete responses. The combination seemed to prevent intracranial progression for more than 6 months in 64% of patients (150). The treatment with ipilimumab and nivolumab achieved higher intracranial response rates in the different clinical trials compared to anti-PDL1 monotherapy (nivolumab or pembrolizumab) at the cost of higher toxicity (Table 2).

Author	Study type	Treatment	No patients with BMs	Outcomes
Margolin, et al. (75)	Phase II	Ipilimumab	Cohort A 51	IDCR: 25% mOS: 7.0 m mPFS: 2.7 m Intracranial mPFS: 1.9 m
			Cohort B ^a 21	IDCR: 10% mOS: 3.7 m mPFS: 1.3 m Intracranial mPFS: 1.2 m
Di Giacomo, et al. (76)	Phase II	Ipilimumab and Fotemustine	20	IDCR: 50.0% mOS: 13.4 m mPFS: 4.5 m Intracranial mPFS: 3 m
Goldberg, et al. (77)	Phase II	Pembrolizumab	18	IRR: 22%
Kluger, et al. (78)	Phase II	Pembrolizumab	23	IRR: 26% mOS: 17 m mPFS: 2 m Intracranial mPFS: 3 m
Long, et al. (80)	Phase II	Nivolumab plus Ipilimumab, followed by Nivolumab	Cohort A 35	IRR 46% mOS: NR mPFS: 13.8 m Intracranial mPFS: NR
		Nivolumab	Cohort B 25	IRR 20% mOS: 13.8 m mPFS: 2.6 m Intracranial mPFS: 2.6 m
		Nivolumab	Cohort C ^d 16	IRR 6% mOS: 5.1 m mPFS: 2.6 m Intracranial mPFS: 2.3 m
Tawbi, et al. (79)	Phase II	Nivolumab plus Ipilimumab, followed by Nivolumab	94	IDCR: 57% 1-year OS rate: 81.5% 1-year PFS rate: 56.6% 1-year intracranial PFS rate: 59.5%

IDCR, intracranial disease control rate; CR, complete response; PR, partial response; SD, stable disease. NR, Not reached; PFS, Progression Free Survival; OS, Overall Survival.
^aPatients neurologically symptomatic.

Table 2. Clinical Trials with immunotherapy in patients with melanoma and BM.

5. Liquid biopsy to characterize brain metastases and guide immunotherapy.

There is a clear interest in addressing the study of liquid biopsy in tumors that have metastasized to the brain. Surgery, in patients with BM located in eloquent or deep brain area, could be difficult and may cause neurological sequelae. Therefore, the possibility of characterizing the disease non-invasively represents a window of opportunity to guide the diagnosis, treatment, and monitoring of the therapeutic response when there is brain dissemination. Likewise, the focal study of a brain lesion by traditional biopsy may not fully capture intertumoral heterogeneity (81). In addition, it is known that BM may have different genomic alterations than primary extracranial tumors, and it is important to analyze genomic alterations specific to brain lesions in order to select the optimal therapeutic approach (72).

To overcome these limitations, the study of circulating markers provides us with a more global vision of the molecular features of different regions within the same lesion and the possibility of approaching the study of BM in a non-invasive way (82).

In the era of precision oncology, the ability to analyze liquid biopsies represents an important element to guide the selection of the treatment and supervise tumor evolution in real time. Any biofluid containing tumor material that can be molecularly characterized, represents a liquid biopsy. This term therefore includes blood, but also other corporal fluids like saliva, urine, bile, pleural effusion, or cerebrospinal fluid, among others. The tumor material present in these biofluids consists mainly of circulating tumor cells (CTCs), nucleic acids (cNAs) including DNA and RNA, and circulating extracellular vesicles (cEVs). Through the characterization of these circulating elements, we can obtain valuable information on mechanisms that favor tumors dissemination and progression, also in the context of brain lesions (82) (Figure 1). Knowledge about the application of each of these tumor elements in the context of BM is described in more detail below.

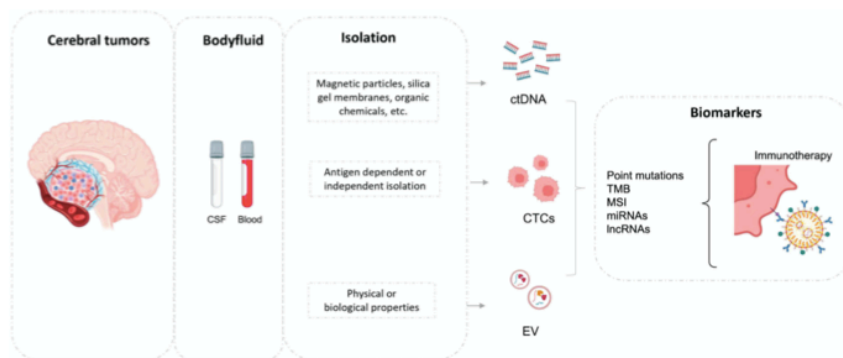


Figure 1. Brain metastases shed to blood or CSF, and biomarkers could be studied (ctDNA, CTCs or EV).

CSF, cerebrospinal fluid; ctDNA, circulating tumoral DNA; EV, extracellular vesicles.

5.1 Circulating tumor DNA

Free tumor DNA can be released from primary tumor cells, CTCs, micrometastases, or macrometastases. Most of this DNA comes from tumor cells that enter apoptosis or necrosis and release their DNA with varying degrees of fragmentation into the bloodstream. The fraction of ctDNA in the total free circulating DNA (cfDNA) released by healthy cells is usually low, less than 1%, but can vary from less than 0.1% to more than 90% (83). ctDNA is characterized by the presence of somatic genetic alterations such as SNVs (single-nucleotide variants), CNVs (copy number variants) or Indels, as well as a tendency to be more fragmented, showing sizes that range from 90 to 170 base pairs (84). ctDNA is also known to reflect the epigenetic signatures of nuclear DNA (85, 86).

In order to detect ctDNA, it is necessary to apply highly sensitive techniques that can target a small panel of mutations such as digital PCR or BEAMing or cover a large panel of genes and even the whole exome/genome (NGS, Next-generation Sequencing; WGS, Whole-genome sequencing, or WES, Whole-exome sequencing). Generally, higher genome coverage is associated with lower sensitivity of the analysis (87).

There is already a great deal of evidence on the value of cfDNA analysis to genotype advanced stage tumors for personalized therapy selection, but also as a tool to monitor response to treatment or detect the presence of minimal residual disease (MRD) post-surgery and guide adjuvant treatment. In fact, the FDA (Food and Drug Administration) has approved several diagnostic tests to detect EGFR or PIK3CA mutations in lung and breast tumors. The FoundationOne Liquid CDx and Guardant360 liquid CDx panels (using free circulating DNA, cfDNA) have also recently been approved as companion diagnostic tests for several targeted therapies and immunotherapy (88). In the context of immunotherapy treatment, numerous studies have demonstrated the possibility of analyzing both tumor mutational rate and microsatellite instability (MSI) status in circulating free DNA samples, two biomarkers that predict response to checkpoint inhibitors (89). On the other hand, TMB has traditionally been tested in tumor tissue samples. However, Gandara et al. reported an analysis of TMB by NGS in a subgroup of patients from the POPLAR and OAK studies, comparing tumor sample with pre-treatment plasma in the same patient cohort (90). A positive correlation (0.64 Spearman rank) was found between tissue and blood sample, so that plasma TMB (bTMB) by liquid biopsy is considered a predictive biomarker of progression-free survival in patients treated with Atezolizumab monotherapy, when the value is higher than 16 mutations per megabase, as a clinically meaningful and technically robust threshold (90). A biomarker analysis with bTMB was also conducted in the phase III MYSTIC study, demonstrating its association with OS, PFS and ORR in patients treated with ICIs (91).

Although blood is the most used fluid for the study of cfDNA, several studies have shown that the concentration of tumor free DNA in brain and central nervous system lesions is higher in cerebrospinal fluid (CSF), which could be the preferred biofluid for the molecular characterization of these lesions, regardless of their origin (42,92). This is because CSF is in more direct contact with brain lesions, so shedding may be more evident in this fluid by a contiguity mechanism (93). For example, Pan et al. analyzed cfDNA samples from plasma and CSF of 7 patients with brain tumors, including metastatic lesions, using a combined digital PCR (polymerase chain reaction) and sequencing strategy and found a higher concentration of somatic mutations characteristic of primary tumors such as *EGFR* or *PI3KCA* in the CSF (94).

Of note, a postmortem analysis of patients with BCBM in which ctDNA was evaluated in CSF identified truncal mutations from the tumors of origin but also variants unique to the brain lesions, highlighting the potential for diagnosing this type of lesion in breast tumors (92). Using targeted sequencing, Liang et al. analyzed the somatic variants present in CSF from 21 patients with glioma and 7 patients with cerebral disease with primaries located mainly in the lung and digestive tract (95). The results of the study showed that the genes altered in primary and metastatic brain tumors are different. Specifically, the ctDNA of metastatic brain lesions was characterized by the presence of alterations in ALK and MDM2. The authors suggest that this differential profile could be of interest for the diagnosis and therapy of BM (95).

Rubio-Pérez et al. characterized the immune composition of brain lesions and matched CSF by means of single-cell RNA sequencing and also genotyped the T cell receptors+ (96). The results obtained showed that tumor immune infiltration and specifically CD8⁺ T cell infiltration can be assessed in CSF samples (96). Consistently, the same T cell receptor clonotypes were present in brain lesions and CSF, demonstrating immune cell exchange between the tissue and the CSF (96). Overall, these data support the use of CSF to analyze the immune composition in a minimally invasive sample and represent a promising tool to anticipate the response to ICI (96). There is another in which single cell RNA sequencing (scRNA-Seq) was performed to characterize cell types in CSF from 19 patients with leptomeningeal metastases from different solid tumors, mostly breast cancer, who received treatment with ICIs, found a significant increase in CD8⁺ T lymphocyte population and higher levels of IFN- γ after treatment in the patients who obtained the greatest clinical benefit (97).

As it was previously commented, there are currently active treatments to treat metastatic brain lesions such as immunotherapy or targeted therapies, such as *ALK*, *MEK*, *BRAF* or *HER2* inhibitors. Studies have shown that clinically relevant variants in these genes can be detected by studying ctDNA in CSF from lung, melanoma, or breast tumors, among others (92,94,98). As with these targeted therapies, the study of ctDNA in CSF samples has shown value in determining the tumor mutational burden (TMB) which, as mentioned above, is a predictive biomarker of response to immunotherapy (89). Thus, Guao et al. described a high correlation between TMB measured in tumor DNA present in CSF and tumor tissue of patients with gliomas, and the result may be transferable to the characterization of BM (99).

Despite the lower concentration of ctDNA in the blood released by cerebral metastatic lesions, several studies have demonstrated the possibility of genotyping these lesions using this strategy. For example, in patients diagnosed with advanced lung cancer with BM, EGFR status has been successfully characterized in plasma ctDNA, showing good levels of concordance with the original primary tumor (100). Also, in plasma DNA from BC patients with brain lesions, Pangen et al. have recently demonstrated the presence of differentially methylated markers (CTD-2028 M8, CCDC8, miR3193 and miR124-2) already present in primary tumors, showcasing that plasma can provide valuable information also in this patient profile (101). On the other hand, in the BREAK2 clinical trial, patients with BRAF-mutated metastatic melanoma were evaluated for response to oral dabrafenib. This study included a comparative analysis of BRAF status in ctDNA in plasma and tissue. Data obtained from plasma analysis showed good concordance with tissue and predictive value for response, including patients with brain lesions (102).

In addition to point mutations, numerous studies have demonstrated the possibility to analyze both TMB and MSI in plasma cfDNA and select immunotherapy in patients with advanced tumors regardless of metastatic location, although it is important to determine the possibility of false negatives (90, 103). ctDNA monitoring may be helpful in distinguishing progression during immunotherapy treatment, although no studies have addressed this utility particularly in patients with BM (104, 105). Exceptionally, in a study published by Lee et al. the role of ctDNA in monitoring melanoma patients with BM treated with anti-PD1 immunotherapy was examined (106). They analyzed *BRAF*, *NRAS* mutations and *c-KIT* in cfDNA in serial plasma samples during the first 12 weeks of treatment and assessed both intracranial and extracranial disease using the RECIST criteria. Of the 72 patients included, 13 had intracranial metastases exclusively and 59 had intra and extracranial metastases. Detection of ctDNA was 0% and 64%, respectively (106). Detection of ctDNA in plasma was associated with disease volume, while absence of ctDNA detection during treatment was associated with extracranial but not with intracranial response (106). The median OS in those patients with undetectable ctDNA versus detectable ctDNA at baseline was 39.2 and 10.6 months, respectively (106). These results suggest that ctDNA could be considered a strong prognostic biomarker in patients with melanoma and cerebral disease, especially in the subgroup with extracranial disease. In addition, our research group demonstrated the value of monitoring total cfDNA levels in 46 patients with non-small cell lung cancer who received anti- PD1 treatment but only 3 patients in this cohort had cerebral metastatic lesions (107).

5.2 Circulating tumor cells

The development of metastasis is a multistep procedure during which tumor cells must gradually detach from the primary tumoral lesion and locally occupy the stroma and surrounding tissues to reach the circulatory or lymphatic system. At this point, intravasated tumor cells or CTCs represent a valuable element in understanding the process of malignant dissemination but also as a reflection of tumor burden and the dynamic evolution of tumors (108,109). The low proportion of CTCs in the bloodstream together with the molecular heterogeneity that characterizes these cells is the principal challenge for CTCs isolation and detection. All technologies isolate these cells focusing on differential features between CTCs and blood cells, such as protein expression, morphology, and biophysical properties. These technologies can be categorized based on the method of isolation as antigen- dependent or antigen-independent (110). The most employed strategy is usually carried out using antibodies that recognize epithelial cell adhesion molecule (EpCAM) conjugated with magnetic nanoparticles (111). Among the current EpCAM-based technologies, CellSearch[®] system is the “gold standard” for the CTC detection methods (112). Antigen-independent methods are based on the physical properties of CTCs such as density, electric charges (DEP, dielectrophoresis), size and deformability, among others. The principal advantage in comparison with the antigen-dependent methods is the isolation of CTCs with a low epithelial phenotype. Size-based methods are the most common antigen independent strategies. They are since tumor cells are larger than blood cells and they can be isolated using filter-based strategies such as ISET assay microfluidic chips, Parsortix system and methods based on centrifugal forces (56,113,114).

Independently of the isolation strategy, CTCs main clinical utility has been demonstrated in patients with advanced neoplasia where its validity as a prognostic biomarker has been demonstrated in numerous studies with different solid tumors, such as breast, colon, prostate,

or lung cancer (115). In addition to its value as a prognostic and monitoring biomarker, the molecular characterization of the population of CTCs present in different biofluids is a very useful tool when tumor lesions are not accessible, as in the case of BM. This molecular characterization has provided a better comprehension of the mechanisms that facilitate the appearance of BM. Thus, for example, analyzing CTCs from lung cancer patients in comparison with the original tumor and metastatic disease describes the appearance of mutations in genes involved in the response to cellular stress, such as *Keap-1*, *Nrf2* and *P300*, which are key players in the *Keap1-Nrf2-ARE* signaling pathway and which could provide a survival mechanism for CTCs when they are in the process of distant tumor dissemination (116).

On the other hand, Boral et al. characterized the population of CTCs associated with the presence of BM in patients with advanced breast cancer (117). This study identified the activation of an important Notch signaling pathway, which is associated with the appearance of brain lesions, as well as the identification of new inflammatory and immunomodulatory networks, whose role in immune evasion could favor the appearance of brain metastases (117). Prior to this work, the same research group associated the existence of a *HER2*⁺/*EGFR*⁺/*HPSE*⁺/*Notch1*⁺ population in tumor lines generated from patient CTCs with the generation of BM (118).

Another relevant study characterizing CTCs from patients with advanced breast cancer identified cyclooxygenase COX2 (*PTGS2*), the epidermal growth factor receptor (*EGFR*) ligand *HBEGF* and the alpha2,6-sialyltransferase *ST6GALNAC5* as key players for tumor cells to cross the blood-brain barrier (119). Finally, also in breast cancer, the characterization of CTCs in the subtype named triple negative breast cancer patients, which frequently metastasize to the brain, identified HER2 positivity as a hallmark of tumor progression (120).

In addition to the study in blood, the presence of CTCs has been described in CSF in patients with brain dissemination. For example, Ruan et al. established an efficient procedure for collecting CTCs from CSF in five patients with leptomeningeal metastases of lung adenocarcinoma and characterized the CTCs at the transcriptomic level (121). These cells showed increased expression of genes regulating cell adhesion mechanisms such as *MMP7*, *CLDN7* and *ICAM1*, as well as genes associated with a primarily epithelial phenotype (121). The detection of CTCs in CSF also represents a diagnostic tool of great interest to confirm the presence of leptomeningeal disease in tumors of different origin (122). Also, Darlix et al. analyzed the presence of CTCs in 40 breast cancer patients with suspected leptomeningeal metastases obtaining a good diagnostic sensitivity (100%) and specificity (78%) (123).

Despite all these studies demonstrating the value of CTCs analysis to characterize primary tumors located at central nervous system or BM, their utility to predict or monitor the activity of immunotherapy in this tumor context is still unexplored.

5.3 Extracellular vesicles

Extracellular vesicles (EVs) are known to play a relevant role as communicative mediators favoring the preparation of the pre-metastatic niche, including BM (124, 125). In addition to this communicative role, the analysis of Evs levels and their molecular cargo in different body fluids represents a promising strategy to obtain a liquid biopsy from the tumor (126). Besides,

tumor- secreted Evs have the intrinsic ability to breach the brain barrier and therefore can participate in the brain colonization by cells originated from other tumor location (127).

Evs isolation can be addressed with different strategies based on their biochemical composition, size, and density, among other properties (128). The most employed strategies are ultracentrifugation, ultrafiltration, and immunoaffinity capture, size exclusion chromatography and density gradient separation. Ultracentrifugation employs high speed centrifugation to separate the Evs while size exclusion chromatography and density gradient isolation separate Evs from other debris. Affinity-based methods for EV isolation are known to produce highly specific isolation results. Immunoaffinity strategies are based on the use of antibody-coated beads to specifically recognize surface antigens (129,130). The selection of the isolation method should be mainly made based on the sample requirements for downstream analyses.

Taking this into account the study of Evs in plasma or CSF from patients with BM has gained interest in the last years. Thus, a recent work by Carretero-González et al. compared the levels of plasmatic Evs in patients with different solid tumors with or without BM and described lower number of cEVs levels while higher protein concentration in patients with brain affection (131). In particular, melanoma patients with cerebral disease have decreased STAT3 activation and increased PD-L1 content in plasma Evs. In this sense, release of Evs PD-L1⁺ has been described as a resistance mechanism in patient treated with immune checkpoint inhibitors, thus the presence of Evs PD-L1⁺ in patients with BM may potentially have an impact on the response to immunotherapy (132).

In addition to the cEV levels or the total Evs protein content different molecules have been described in plasma cEVs as potential biomarkers or biological mediators in tumors with brain dissemination. For instance, different miRNAs contained in cEVs have shown to be specifically increased in tumors with BM. Thus, Wei and collaborators described the presence of MicroRNA-550a- 3-5p in plasma Evs from lung cancer patients as an important factor promoting BM thanks to the direct regulation of *YAP1* (133). Also in lung cancer patients, the characterization of Evs associated miRNAs present in CSF samples served to identify miR-34b-3p and miR-335-5p as specially expressed in lung cancer patient with brain dissemination (134). Also, in CSF high levels of miR-200 family members were found in BC patients with brain secondary disease (135). In a recent study, Catelan et al. compared a panel of genes in serum exosomes isolated in tumor with brain dissemination and high-grade gliomas, finding a common increase of miR-21 levels in both cancer groups in comparison with healthy controls and a specific increment of miR-124-3p in the tumors with brain dissemination (136). Another non-coding RNAs such as *LncRNA XR_429159.1* or *LncRNA GSI-600G8.5* expressed in Evs were reported as relevant for the brain barrier disruption in the context of SCLC and BC (137, 138). Also, *XIST LncRNA* has been described associated with brain dissemination in BC patients. It was found to be downregulated in BM and this downregulation was associated with increased exosomal miR-503 secretion, which regulates microglia polarization and suppresses T cell growth (139).

All these data exemplify the interest of cEVs in the context of BM to better understand the mechanisms favoring brain colonization but also in the modulation of the response to therapy, especially in the context of immunotherapy.

6. Conclusions and future directions

In recent decades, solid tumors have made significant advances in the treatment of advanced disease, increasing survival and quality of life. New treatments such as immunotherapy and targeted therapy are the cause of this improvement. However, there are still scenarios, such as metastatic disease at the brain level, which remain a challenge due to high morbidity and mortality. Traditionally, antineoplastic drugs do not penetrate brain tissue because of the blood-brain barrier. In this review, we summarize results obtained in patients with brain disease recruited in the principal studies evaluating immunotherapy strategies, who represent a small cohort of patients due to the low representation of these patients in phase III clinical trials and the limited number of specific studies in this population. Derived from this factor, the present review has the limitation of the small number of patients, the heterogeneity of the population which includes limited and extensive cerebral disease and diverse types of primary tumors. In addition, we noticed that this group of patients is not always described in the articles, which makes it difficult to extrapolate to clinical practice. Besides is important to mention the fact that our work is not a systematic review, but a narrative comprehensive qualitative study. Although these limitations the review highlights the benefit of using immunotherapy to treat breast, lung, and melanoma tumor with BM, opening new therapeutic options to manage disseminated disease. However, immunotherapy has some serious toxicity that must be monitored and treated. Therefore, it is crucial to be able to accurately select patients who will benefit most of the immunotherapy at brain level and avoid endangering vital organs.

In this clinical context, the application of liquid biopsy may have special relevance, as a tool that avoids invasive procedures and provides molecular information in real time for the monitoring of the disease throughout its evolution. As it was detailed in this review, there is evidence supporting the utility of liquid biopsy analyses for immunotherapy selection and monitoring in lung, breast, and melanoma cancers. Combined use of immunotherapy and liquid biopsy have a proven benefit, making them important achievements in the therapeutic and diagnosis arsenal of the mentioned tumors. Several works have demonstrated that liquid biopsy analysis (mainly CSF and blood markers) in the context of BM from breast, lung, and melanoma tumors, allow to molecularly characterize the disease taking into account the tumor evolution and heterogeneity and determine the status of well established immune biomarkers such as PD-L1, TMB or MSI condition. In this sense, cfDNA analyses in CSF have demonstrated special value to interrogate the tumors located in CNS, including BM. However, our review showed the general lack of information about the specific utility of the cfDNA assessment and other markers such as CTCs and EVs in patients with BM receiving immunotherapy. In this regard, it is important to mention that all the phase III trials described in advanced tumors (NSCLC, SCLC, breast, melanoma) were not associated with translational studies, so there is no analysis of liquid biopsy that could serve as secondary endpoints. This makes it difficult to draw conclusions about the behavior of circulating biomarkers during immunotherapy treatment, but at the same time it creates the need and challenge to improve the design in future clinical trials focused on a subgroup of patients of particular concern due to their poor prognosis. Of note, the present review englobes for the first time the results regarding the combined value of immunotherapy and liquid biopsy in the context of BM and provides comprehensive knowledge that might open the door to the creation of specific clinical trials to interrogate value of circulating biomarkers for the diagnosis, prognosis, and monitoring of the response to immunotherapy in patients with brain lesions. Fortunately, there are ongoing studies and preliminary results in lung, breast, and melanoma cancer, which aim to provide more molecular

information in this setting to better meet the needs of this subgroup of patients. Further research is warranted to make progress in the application of immunotherapy to treat solid tumors with BM and liquid biopsy to guide the treatment in a cohort of patients with poor therapeutic alternatives.

CAPÍTULO 3

Clinicopathological Profiles Associated with Discordant *RAS* Mutational Status between Liquid and Tissue Biopsies in a Real-World Cohort of Metastatic Colorectal Cancer

Clinicopathological Profiles Associated with Discordant *RAS* Mutational Status between Liquid and Tissue Biopsies in a Real-World Cohort of Metastatic Colorectal Cancer

Elena Brozos-Vázquez ^{1,†}, Ramón Manuel Lago-Lestón ^{1,2,†}, Marta Covela ³, Juan de la Cámara Gómez ⁴, Ana Fernández-Montes ⁵, Sonia Candamio ¹, Yolanda Vidal ¹, Francisca Vázquez ¹, Alicia Abalo ^{1,2}, Rosa López ¹, Cristina Blanco ¹, Laura Muínelo-Romay ^{1,2,6}, Isabel Ferreirós-Vidal ^{1,2,*,‡} and Rafael López-López ^{1,2,6,*,‡}

¹ Translational Medical Oncology Group, Oncomet, University Hospital of Santiago de Compostela (CHUS), 15706 Santiago de Compostela, Spain;

² Liquid Biopsy Unit, Health Research Institute of Santiago de Compostela (IDIS), 15706 Santiago de Compostela, Spain

³ Department of Medical Oncology, Lucus Augusti University Hospital of Lugo (CHULA), 27003 Lugo, Spain;

⁴ Department of Medical Oncology, University Hospital of Ferrol (CHUF), 15405 Ferrol, Spain;

⁵ Department of Medical Oncology, University Hospital Complex of Ourense (CHUO), 32005 Ourense, Spain; afm1003@hotmail.com

* Correspondence: isabel.ferreiros.vidal@sergas.es (I.F.-V.); rafael.lopez.lopez@sergas.es (R.L.-L.)

† These authors contributed equally to this work.

‡ These authors jointly supervised this work.

Clinicopathological Profiles Associated with Discordant *RAS* Mutational Status between Liquid and Tissue Biopsies in a Real-World Cohort of Metastatic Colorectal Cancer

Simple Summary: The evaluation of *RAS* mutations from plasma circulating tumour DNA (ctDNA) has emerged as an efficient approach to guide therapeutic decisions in the treatment of metastatic colorectal cancer (mCRC). However, disagreements about the *RAS* mutational status in tests using liquid and tumour tissue biopsies can lead to misinterpretation of the tumour genotype and compromise effective therapy. We used digital droplet PCR (ddPCR) technology and logistic regression models to decipher the clinicopathological profiles that commonly influence the levels and detection of plasmatic *RAS* mutations and are associated with discordant assays. The absence of liver metastases and the resection of the primary tumour were associated with reduced ctDNA levels and low percentages of positive agreement between tissue and ctDNA tests, predominantly when the mCRC originated in the right colon and rectum. Thus, ctDNA assays reporting undetected *RAS* mutations in these patients should be taken prudently, and further investigations should be considered before any decision about treatment.

Abstract: We aimed to identify common mCRC profiles associated with a discordant mutational status of *RAS* between the standard of care (SoC) tumour tissue tests and ctDNA tests to understand ctDNA detection and improve treatment responses. This was a multicentre, retrospective and prospective study. A total of 366 Spanish mCRC patients were independently recruited. BEAMing ddPCR technology was employed to detect ctDNA *RAS* mutations, and logistic regression analyses were performed to investigate clinicopathological factors associated with discordance. The highest concordance ratios were observed in profiles with multiple metastatic sites when the liver was present (89.7%; 95% CI 84.8–93.2), profiles with synchronous disease without primary tumour resection (90.2%; 95% CI 83.6–94.3) and profiles with mCRC originating in the left colon (91.3%; 95% CI 85.0–95.0). Metachronous disease originating in the right colon (OR = 6.1; 95% CI 1.7–26.5; p -value = 0.006) or rectum (OR = 5.0; 95% CI 1.5–17.8; p -value = 0.009) showed the highest probability of discrepancies. Primary tumour resection and a higher frequency of single metastases in the peritoneum or lungs in these patients were associated with reduced plasmatic mutation allele fractions (MAFs) and an increased probability of showing false-negative genotypes. Additional testing of patients with mCRC originating in the right colon or rectum with a single non-mutated ctDNA test is advised before the choice of therapy.

Keywords: liquid biopsy; colorectal cancer; metastatic colorectal cancer; mCRC; *RAS*; ctDNA; anti-EGFR therapy

1. Introduction

Colorectal cancer (CRC) is one of the most frequently occurring solid cancers world- wide, with a higher incidence in developed countries associated with lifestyle risk factors, such as diet and lack of exercise [1]. The prevalence patterns are generally similar in men and women, but an alarming increased rate has lately been observed in adults younger than 55 years old [2–4]. Notably, it is the second leading cause of cancer-related death in Europe [5], and Spain has been determined to be one of the European countries with the highest incidence and mortality rates [1].

The aetiology, physiopathology and molecular characteristics of CRC are highly heterogeneous. Adenocarcinoma has the potential to originate in any location in the colon or rectum, and the molecular features of right-sided colon cancers are different compared with left-sided colon and rectal cancers [6]. These differences in CRC aetiology have a key role in the metastatic settings of the disease. The burden and dissemination patterns [7,8] of mCRC have been shown to significantly depend on the location of the primary tumour. As a consequence of the great heterogeneity in mCRC molecular and clinicopathological characteristics, systemic therapy for mCRC patients must be personalised and dependent on the disease-specific predictive markers.

The mutational status of the rat sarcoma viral oncogene homolog (*RAS*) family of genes—and, more recently, the tumour sidedness—are the current mCRC biomarkers used to predict the targeted therapy response in clinical practice [6]. Cetuximab and panitumumab are monoclonal antibodies against the epidermal growth factor receptor (*EGFR*) that have been demonstrated to improve outcomes in mCRC patients with wild-type (WT) status compared to the anti-VEGF used as the first line of treatment [9–11], particularly in those patients whose primary tumour originated in the left side of the colon [12–17]. However, approximately 40% of CRCs show activating missense mutations in the Kirsten *RAS* (*KRAS*) gene and 3–5% in the neuroblastoma *RAS* (*NRAS*) [18], representing an early event in the carcinogenesis of mCRC [19]. Therefore, around half of mCRC cases show resistance to *EGFR*-targeted therapy. Hence, current guidelines for the clinical management of mCRC recommend the evaluation of the *RAS* mutational status, especially exons 2, 3 and 4 of the *KRAS* and *NRAS* genes, before the administration of *EGFR* inhibitors [20–22].

The standard of care (SoC) procedure in clinical practice is to evaluate the presence of somatic *KRAS* and *NRAS* mutations with a solid tumour specimen from surgical or biopsy samples at the diagnosis of the metastatic disease [23], commonly by using mutation- specific sequencing-based technologies or, more recently, next-generation sequencing (NGS) approaches [24]. However, the evaluation of *RAS* mutations using circulating tumour DNA (ctDNA) from minimal-invasive blood-based liquid biopsies has been established as a valuable approach to stratify and monitor mCRC patients in real time throughout the course of the disease [25], guiding clinical decisions more efficiently [26–29]. Also, in other tumour types, such as NSCLC, *EGFR* genotyping through ctDNA assessment is well- accepted, especially when invasive procedures may be risky or contraindicated [30,31].

Previous studies [32–34] have shown high correlations, with concordance ratios above 85%, between routine sequencing procedures performed with solid tumour tissue and the OncoBEAM[®] *RAS* CRC, a technology that uses BEAMing digital droplet PCR and has been

approved by the European Commission as a tool for the in vitro diagnosis of *RAS* mutations [35]. Prospective studies including larger cohorts of patients have already elucidated some of the frequent causes of discordance between tumour tissue and plasma ctDNA analysis, highlighting the impact of clinical features on the detection of plasmatic ctDNA. Small amounts of plasmatic ctDNA in mCRC patients with resection of the primary tumour or absence of liver metastases led to undetectable *RAS* mutations in plasma and inconclusive results concerning concordance [36–39]. In addition, ctDNA *RAS* mutant allele fraction (MAF) in the mCRC population with exclusive lung metastatic disease was frequently found to be lower than in patients with at least one liver metastasis, which led to false-negative results in the ctDNA-based tests [40,41].

Liquid biopsy tests reporting false-negative results are expected to have an impact on the choice of therapy. Despite broad scientific knowledge about the disease conditions potentially leading to inconclusive results, difficulties exist in the day-to-day medical care to interpret discordances between liquid biopsy tests and SoC procedures performed in tumour tissue, thereby compromising the selection of the best candidates to receive anti-*EGFR* therapy.

A better understanding of the clinicopathological characteristics that predominantly impact the molecular detection of *RAS* mutations in mCRC is still needed to identify properly the mCRC subpopulations susceptible to presenting misleading ctDNA results. Enhanced patient stratification will improve tailored therapy and the definitive establishment of ctDNA testing in the clinical routine.

2. Materials and Methods

2.1. Study Design and Patients

This is a multicentre, retrospective and prospective observational study. A total of 366 unrelated mCRC patients were independently recruited from November 2015 to July 2019 in four hospitals in Galicia, northwest Spain: 233 patients recruited from the University Hospital of Santiago de Compostela, 67 from the Lucus Augusti University Hospital of Lugo, 38 from the University Hospital of Ferrol and 28 patients from the University Hospital of Ourense.

Patients had been previously diagnosed with Stage I, Stage II (IIA, IIB, IIC), Stage III (IIIA, IIIB, IIIC) or Stage IV (IVA, IVB and IVC) CRC following the recommendations of the American Joint Committee on Cancer (AJCC) TNM Staging System for Colon Cancer [42] published at the NCCN Guidelines for Colon Cancer [43]. All patients were selected to have been histologically and radiologically diagnosed with Stage IV metastatic colorectal cancer at the time of enrolment in this study.

All patients had at least one blood draw taken at the metastatic stage. In most cases (79%), blood biopsies were obtained within four months of the diagnosis of metastatic disease. The average time interval from the collection of the solid tumour tissue biopsy to the liquid blood biopsy was less than 3 months (70 days on average) for patients diagnosed with synchronous metastatic disease and a year and a half (535 days on average) for patients diagnosed with recurrent metachronous metastatic disease.

Information regarding the mutational status of *RAS* in the tissue tumour sample was available in 83% of the patients. On the other hand, *RAS* genotyping by BEAMing ddPCR was achieved in 99.5% of the plasma blood samples. The regional ethics committee (Comité Ético de Investigación Clínica de Galicia) approved the study (ref 2015/746), and written informed consent was obtained from all patients.

2.2. Procedures

Peripheral blood samples were collected in 10 mL Cell-Free DNA STRECK BCT[®] tubes (Streck, Nebraska, United States). Two-step centrifugation was carried out to isolate the plasma from the whole blood. Blood samples were centrifuged for 10 min at 1600× *g* at room temperature. The supernatant was collected and centrifuged for 15 min at 6000× *g* to remove the remaining cells. Plasma samples were stored in aliquots at −80 °C until further analyses. Plasma cell-free DNA (cfDNA) was isolated from 5 mL of peripheral blood using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and quantified with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, United States). *RAS* genotyping was performed using BEAMing Digital Droplet PCR (ddPCR) Technology (OncoBEAM[™] *RAS* CRC CEIVD kit, Sysmex Inostics GmbH, Hamburg, Germany), which detected 34 mutations in the codons 12, 13, 59, 61, 117, and 146 of the *KRAS* and *NRAS* genes, and compared the *RAS* mutational status in formalin-fixed and paraffin-embedded (FFPE) tissue sections derived from primary tumours or from metastasized tumour tissue, which had been previously genotyped according to the procedures validated in each of the hospitals of the patients recruited: Therascreen *KRAS* RGQ PCR kit (Qiagen); COBAS *KRAS* mutation test (Roche Diagnostics, Rotkreuz, Switzerland) or pyrosequencing (PyroMark Q24, Qiagen) testing.

2.3. Statistical Analysis

Statistical analyses were performed with clinically predefined mCRC subpopulations. Concordance between plasma- and tumour-tissue-based analyses were determined using a Kappa statistic (κ) with a 95% confidence interval (CI). Overall concordance, sensitivity (positive percent agreement), and specificity (negative percent agreement) between plasma- and tissue-based analyses and their 95% CIs were also calculated.

Binary logistic regression was used to model the relationship between the mCRC clinicopathological characteristics and the *RAS* mutational status (discordant vs. concordant) taken as the dichotomous dependent variable. The main outcome of the analysis was the odds of discordance versus concordance between SoC and ctDNA tests. We used a generalized linear model and a logit function to estimate the odds ratios (ORs) of discordance and their 95% confidence intervals (CIs), and to calculate the probability of association with the site of the primary tumour, the resection of the primary tumour, the metastatic site and the number of metastatic sites taken as the independent variables. Patients with mCRC originating in the left colon, without primary tumour resection, with liver metastases, lymph node metastases, lung metastases or peritoneal metastases, and with multiple metastatic sites were used as the reference for the independent variables.

We used univariable logistic regression to estimate the effect of each of the clinico-pathological characteristics on the odds of discordance, individually. Multivariable logistic regression

models were also used to estimate the joint effect or interaction of a set of clinicopathological variables on the probability that the outcome happened. Finally, univariable logistic regression analyses in mCRC subpopulations stratified by the location of the primary tumour (left colon, rectum and right colon) were undertaken to model the effect of the resection of the primary tumour, the metastatic site and the number of metastatic sites on the odds of discordance occurring.

The median and interquartile range (25–75%; IQR) of the cfDNA levels and MAFs distributions were calculated for the different mCRC subpopulations. The Mann–Whitney and the Kruskal–Wallis non-parametric approaches were applied for the statistical comparisons between two or more populations, respectively. All statistical tests were performed in R version 3.6.3, GraphPad Prism 6 and the VassarStats: Website for Statistical Computation. Odds ratios ≥ 2 and p -values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Cohort Characteristics

A total of 366 patients were recruited from four hospitals in Galicia, northwest Spain. At the time of the inclusion in the study, all patients had been diagnosed with mCRC. Disease staging was based on the histopathology of biopsies or surgical specimens and on imaging diagnoses. The baseline characteristics of patients are shown in Table 1. The majority (71.6%) of mCRC patients were male and the average age was 66 ± 11 years old. The primary tumour was located in the left side of the colon in 42% of patients, in the right side of the colon in 22% of patients, in the rectum in 31% of patients, and was of mixed origin in 5% of patients. Considering the timing of the diagnosis of metastatic disease, 70% of patients had been diagnosed with synchronous primary and metastatic tumours while 29% of patients presented a metachronous onset of the metastatic disease. About half of the patients showed only a single location of metastases at the diagnosis of mCRC. In these cases, the liver was the most frequent metastatic location (61.8%), followed by the lung (14.2%) and the peritoneum (13.2%). In addition, the liver was also the most common metastatic site among patients presenting metastases at more than one anatomical location. Almost 70% of these patients had been diagnosed with metastases in the liver and in other anatomical locations.

Characteristics	Number	Percentage
Gender		
Female	104	28.4%
Male	262	71.6%
Age (years)		
Median (range)	67 (22–88)	
<50	29	7.9%
50–80	305	83.4%
>80	32	8.7%
Histology		
Adenocarcinoma	338	92.3%
Mucinous adenocarcinoma	14	3.8%
Others	4	1.1%
Unknown	10	2.7%
Primary tumour site		
Right (cecum, ascending and transverse colon)	79	21.6%
Left (descending and sigmoid colon)	154	42.1%
Rectum	112	30.6%
Mix	19	5.2%
Unknown	2	0.6%
Stage at diagnosis ^a		
I	5	1.4%
II	32	8.7%
III	70	19.1%
IV	256	70.0%
Unknown	3	0.8%
Metastases presentation		
Metachronous	107	29.2%
Synchronous	256	70.0%
Unknown	3	0.8%
Number Metastatic sites		
1	204	55.7%
2	103	28.1%
≥3	50	13.7%
Unknown	9	2.5%

Characteristics	Number	Percentage
Metastases site		
Liver (only liver)	250 (125)	68.3% (34.2%)
Lung (only lung)	129 (29)	35.3% (7.9%)
Peritoneal (only peritoneal)	75 (27)	20.5% (7.4%)
Lymph node (only lymph nodes)	78 (15)	21.3% (4.1%)
Others (only others)	21 (7)	5.7% (1.9%)
Unknown	9	2.5%
Resection of primary tumour ^b		
Yes	220	60.1%
No	144	39.3%
Unknown	2	0.6%
Resection metastases ^c		
Yes	44	12.0%
No	320	87.4%
Unknown	2	0.6%
Systemic chemotherapy ^d		
Yes	106	29.0%
No	254	69.4%
Unknown	6	1.6%
Anti-EGFR therapy ^e		
Yes	122	33.3%
No	231	63.1%
Unknown	13	3.6%
Tissue biopsy location		
Primary tumour	256	69.9%
Metastasis	28	7.7%
Both	12	3.3%
Unknown	70	19.1%
Plasma ctDNA/BEAMing ^f		
Mutated	180	49.2%
Non mutated	184	50.3%
Unknown	2	0.5%
Tumour Tissue/SoC ^g		
Mutated	173	47.3%
Wild type	130	35.5%
Unknown	63	17.2%

Table 1. Clinicopathological characteristics of the mCRC patients included in the study.

Table 1 contains the clinical and pathological information of the 366 patients and samples recruited for the study.

^a Stage at the first diagnosis of CRC disease. ^{b,c} Surgery performed previous to plasma collection. ^d Systemic treatment received previous to plasma collection. ^e Anti-EGFR therapy received after plasma collection at any line of treatment. ^f A total of 55% (166/303) of the *RAS* mutations of the 303 tissue samples successfully genotyped with the SoC techniques were found in *KRAS* gene, and 3% (7/303) were found in *NRAS* gene. ^g BEAMing analysis showed *KRAS* mutations in 45% (165/366) of plasma samples and *NRAS* mutations in 5% (18/366).

All mCRC patients had at least one blood liquid biopsy taken in the metastatic stage. At blood biopsy collection, 60% of patients had undergone surgical resection of the primary tumour and 12% had undergone resection of metastasized tumour lesion due to any local complication. A total of 69% of patients were chemotherapy naïve, 22% had received adjuvant chemotherapy after primary tumour resection, and 15% had received systemic chemotherapy (+anti-VEGF in three of the cases) for palliative or life-extending care. Thirty- three percent of patients received anti-EGFR therapy after blood sample collection.

3.2. Concordance of RAS Mutational Status between Analyses Based on Solid Tumour Tissue and Analysis Based on Plasmatic ctDNA

RAS mutations were found in 47% of solid tumour tissue samples genotyped with SoC techniques and in 49% of plasma ctDNA samples analysed by BEAMing ddPCR (Table 1). The overall concordance of the RAS mutational status between the tests in 301 mCRC

samples with genotyping information in both solid tumour tissue and plasma liquid biopsies was 84.4%, CI 79.9–88.1 (Cohen's Kappa = 0.69; 95% CI 0.61–0.77), with a sensitivity of 81.3% and a specificity of 88.5% (Table 2). The highest overall concordance ratios of the study were observed in the mCRC population with multiple metastatic sites when the liver was present (89.7%, CI 84.8–93.2), in patients with synchronous mCRC that was not previously treated with resection of the primary tumour (newly diagnosed synchronous mCRC) (90.2%, CI 83.6–94.3) and in patients with mCRC originating in the left colon (91.3%, CI 85.0–95) (Table 2).

	N ^o	Concordance ¹	Sensitivity ²	Specificity ³	K (95% CI) ⁴	OR (95% CI) ⁵	p-Value ⁶
All samples ^a	301	84.4	81.3	88.5	0.69 (0.61–0.77)		
Primary tumour site ^b							
Left Colon	126	91.3	87.1	95.3	0.83 (0.73–0.92)		
Rectum	97	83.5	82.5	85.0	0.66 (0.51–0.82)	2.1 (0.9–4.8)	0.083
Right Colon	65	76.9	73.3	85.0	0.52 (0.31–0.73)	3.1 (1.4–7.5)	0.008
Primary tumour resection ^c							
Not	122	90.2	90.9	89.3	0.80 (0.70–0.91)		
Yes	177	81.4	75.2	90.3	0.63 (0.52–0.74)	1.9 (1.0–3.9)	0.073
Synchronous	94	86.2	81.0	94.4	0.72 (0.58–0.86)	1.5 (0.6–3.4)	n.s.
Metachronous	83	75.9	68.1	86.1	0.53 (0.34–0.71)	2.9 (0.4–6.5)	0.007
Metastatic site ^d							
Liver							
Yes	204	89.7	90.2	89.0	0.79 (0.71–0.88)		
Not	88	76.1	65.5	93.9	0.53 (0.34–0.71)	2.7 (1.4–5.3)	0.003
L. nodes							
Yes	65	86.2	86.8	85.2	0.72 (0.54–0.89)		
Not	227	85.5	80.8	91.8	0.71 (0.62–0.80)	1.1 (0.5–2.5)	n.s.
Lung							
Yes	110	85.5	82.9	90.0	0.70 (0.56–0.84)		
Not	182	85.7	81.6	90.5	0.72 (0.61–0.82)	1.0 (0.5–2.0)	n.s.
Peritoneum							
Yes	61	82.0	71.9	93.1	0.64 (0.45–0.84)		
Not	231	86.6	84.6	89.5	0.72 (0.63–0.81)	0.7 (0.3–1.6)	n.s.
N ^o metastatic sites ^e							
Multiple	128	89.1	89.0	89.1	0.78 (0.67–0.89)		
Single	164	82.9	76.8	91.3	0.66 (0.55–0.78)	1.7 (0.9–3.4)	0.141
Liver	98	88.8	88.5	89.1	0.78 (0.65–0.90)	1.0 (0.4–2.4)	n.s.
L. nodes	12	83.3	85.7	80.0	0.67 (0.22–1.00)	1.6 (0.2–7.0)	n.s.
Lung	25	72.0	63.2	100.0	0.45 (0.11–0.80)	3.2 (1.1–8.8)	0.029
Peritoneum	21	71.4	53.8	100.0	0.47 (0.11–0.83)	3.3 (1.0–9.5)	0.035
Others	7	71.4	50.0	100.0	0.46 (0.00–1.00)	3.3 (0.4–16.8)	0.181

Table 2. Concordance of *RAS* mutational status between SoC analysis in tumour tissue samples and ctDNA analysis in tumour tissue samples and ctDNA analysis using BEAMing technology.

Table 2 includes information on 301 patients with available *RAS* status in solid tumour tissue and plasmatic ctDNA samples. ¹ Overall percentage agreement; ² positive percentage agreement; ³ negative percentage agreement; ⁴ Cohen's kappa coefficient (95% confidence interval); ⁵ odds ratio (95% confidence interval); ⁶ n.s. = *p*-value > 0.05. ^a Discordant outcomes between SoC and BEAMing analyses were observed in 47 (15.6%) of 301 patients with *RAS* status available in tumour tissue and in plasma ctDNA samples: 10.6% of cases showed exclusive *RAS* mutations in the tumour tissue sample, and in 5% of cases, *RAS* was found to be mutated in the ctDNA sample of a patient with a wild-type *RAS* genotype in the tumour tissue sample. ^b Information about the location of the primary tumour was available in 288 patients. Patients with mixed locations were excluded from this analysis. Left Colon refers to primary tumours located at the descending and sigmoid colon. Right Colon refers to primary tumours located at the cecum or the ascending and transverse colon. All ORs were calculated in reference to the left colon location. ^c Information about surgical resection of tumour tissue was available in 299 cases. The time of presentation of the metastatic disease was known in 299 patients. Not, patients diagnosed with synchronous mCRC who had not undergone surgery or any treatment before the collection of the liquid biopsy sample. Yes, patients who had been treated with resection of the primary tumour before the collection of the liquid biopsy sample (33 patients had also been treated with resection of metastatic tissue). Synchronous refers to cases diagnosed with synchronous mCRC who had undergone resection of the primary tumour before the collection of the liquid biopsy sample (17 patients had also been treated with resection of metastatic tissue). All patients with metachronous metastases had undergone resection of the primary tumour (16 patients had also been treated with resection of metastatic tissue). ORs were calculated in reference to cases with no resection. ^d Site of metastases. Numbers included mCRC cases with (Yes) or without (Not) the metastases at the indicated site. ORs were referred to cases with the specified metastatic site. ^e Number of metastatic sites was obtained in 292 cases. Multiple sites include all cases showing metastases in more than one anatomical location. All ORs were calculated in reference to cases with multiple metastatic sites. n.s. indicates not significant *p*-values.

3.3. Association of the mCRC Clinicopathological Features with Discordance of RAS Mutational Status between Analysis Based on Tumour Tissue and Analysis Based on Plasmatic ctDNA

We used logistic regression analyses to decipher the clinicopathological characteristics with the most significant association with discordance in our mCRC cohort (Table 2). The metastatic pattern was a main factor predictor of the discordance between assays. In patients with metastases at more than one anatomical location, the absence of liver metastases significantly increased the probability of discordance (OR = 2.7, 95% CI [1.4–5.3]; *p*-value = 0.003). We observed that the sensitivity rates were significantly decreased among tests of patients with only one metastatic site (76.84% vs. 89.09%; *p*-value = 0.044), mainly when this site was located at the lung (63.2%) or the peritoneum (53.8%). Consistent with this observation, patients with only metastases in the lung (OR = 3.2, 95% CI [1.1–8.8]; *p*-value = 0.029) or the peritoneum (OR = 3.3, 95% CI [1.1–9.5]; *p*-value = 0.035) showed the highest probability of having a mutational status of *RAS* discordant between analyses based on tissue and on plasma.

In addition to the absence of liver metastases, the resection of tumour tissue was also significantly associated with a lack of concordance. The probability of having discordant results between assays increased when patients had undergone the resection of tumour tissue before the collection of the blood liquid biopsy (OR = 1.9, 95% CI [1.0–3.9]; *p*-value = 0.073), mostly noticeable in profiles with metachronous mCRC (OR = 2.9, 95% CI [0.4–2.5]; *p*-value = 0.007) than in profiles with synchronous mCRC (OR = 1.5, 95% CI [0.6–3.4]; *p*-value = 0.365) (Tables 2 and S1). Discordance associated with metachronous mCRC populations was also dependent

on the metastatic locations since multivariable logistic regression analyses showed that the probability of showing discordance was reduced in the metachronous mCRC population with liver metastases (OR = 1.9, 95% CI [0.8–4.6]; p -value = 0.127).

3.4. Association of the mCRC Origin Site with Discordance of RAS Mutational Status between Analysis Based on Tumour Tissue and Analysis Based on Plasmatic ctDNA

The probability of discrepancies between both assays was strongly associated with the anatomical location of the primary tumour. Patients with mCRC originating in the right colon had a likelihood of presenting discordant genotypes three times higher than patients with primary tumours located in the left colon (OR = 3.1, 95% CI [1.4–7.5]; p -value = 0.008). Moreover, patients with primary tumours originating in the rectum showed discordant assays twice as frequently as patients with primary tumours in the left colon (OR = 2.1, 95% CI [0.9–4.8]; p -value = 0.083) (Table 2).

The probability of discrepancies between assays in both populations, the mCRC originated at the right colon (OR = 6.1, 95% CI [1.7–26.5]; p -value = 0.006) and the mCRC originated at the rectum (OR = 5.0, 95% CI [1.5–17.8]; p -value = 0.009), increased significantly among patients without liver metastases (Table 3). More specifically, the highest frequencies of discordant RAS status in the mCRC originating in the right colon were observed in patients with only peritoneal metastases (OR = 7.7, 95% CI [1.3–54.5]; p -value = 0.030), and in cases with mCRC originated in the rectum, discordant results increased in patients with only lung metastases (OR = 5.8, 95% CI [1.3–2.6]; p -value = 0.018).

In addition, the resection of the tumour before the collection of the blood liquid biopsy significantly increased the chances of obtaining discordant results between assays, particularly among patients with metachronous mCRC originated in the rectum (OR = 8.8, 95% CI [2.6–36]; p -value = 0.001) and in the right colon (OR = 2.9, 95% CI [0.6–16.2]; p -value = 0.187) (Tables 3 and S2).

	N ^a	Left Colon OR (95% CI)	p -Value	N ^a	Rectum OR (95% CI)	p -Value	N ^a	Right Colon OR (95% CI)	p -Value
Primary tumour resection ^a									
Not	40			57			19		
Yes	86	1.3 (0.3–6.0)	n.s.	40	5.7 (1.8–21.8)	0.005	46	1.9 (0.5–9.1)	n.s.
Synchronous	47	1.5 (0.3–7.5)	n.s.	15	2.0 (0.3–11.7)	n.s.	29	1.4 (0.3–7.4)	n.s.
Metachronous	39	1.0 (0.2–5.9)	n.s.	25	8.8 (2.6–36.1)	0.001	17	2.9 (0.6–16.2)	0.187
Metastatic site ^b									
Liver									
Yes	90			63			42		
Not	36	0.9 (0.2–3.5)	n.s.	30	5.0 (1.5–17.8)	0.009	20	6.1 (1.7–26.5)	0.006
L. Nodes									
Yes	21			23			18		
Not	105	0.9 (0.2–6.2)	n.s.	70	2.2 (0.5–14.7)	n.s.	44	1.0 (0.3–4.3)	n.s.
Lung									
Yes	41			53			14		
Not	85	1.3 (0.4–6.3)	n.s.	40	0.7 (0.2–2.2)	n.s.	48	1.1 (0.3–5.4)	0.046
Peritoneum									
Yes	32			6			20		
Not	94	1.6 (0.4–10.8)	n.s.	87	0.2 (0.0–0.9)	0.027	42	0.8 (0.2–3.0)	n.s.
N° metastatic sites ^c									
Multiple	47			51			26		
Single	79	1.1 (0.3–4.2)	n.s.	42	2.5 (0.8–8.8)	0.127	36	3.4 (0.9–16.3)	0.088
Liver	49	1.0 (0.2–4.3)	n.s.	22	0.9 (0.1–4.6)	n.s.	23	1.6 (0.3–9.1)	n.s.
L. nodes	6	2.2 (0.1–18.7)	n.s.	3	n.a.	n.a.	2	7.7 (0.3–234)	0.186
Lung	10	n.a.	n.a.	13	5.8 (1.3–2.6)	0.018	2	n.a.	n.a.
Peritoneum	11	1.1 (0.1–8.3)	n.s.	1	n.a.	n.a.	8	7.7 (1.3–54.5)	0.030
Others	3	5.4 (0.2–70.4)	n.s.	3	4.6 (0.2–57.5)	n.s.	1	n.a.	n.s.

Table 3. Discordance of RAS mutational status between SoC and ctDNA analyses associated with the mCRC origin site.

Table 3 includes information from 288 patients with known clinicopathological data. The location of the primary tumour was unknown in 13 of the 301 patients with available *RAS* status in solid tumour tissue and plasmatic ctDNA samples. ^a Information on the location of the primary tumour was available in 288 patients. Patients with mixed locations were excluded from this analysis. ORs were calculated in reference to cases with no resection. ^b The site of metastases was available in 281 patients. The numbers included mCRC cases with (Yes) or without (Not) metastases at the indicated anatomical site. ORs were referred to cases with the specified metastatic site. ^c The number of metastatic sites was obtained in 281 cases. Multiple sites included all cases with metastases in more than one anatomical location. All ORs were calculated in reference to cases with multiple metastatic sites. n.a., statistical power was not enough for calculation. n.s., *not significant* *p*-values.

These two mCRC populations, patients with metachronous mCRC originated in the rectum (56.3%) or in the right colon (54.6%), showed the lowest sensitivity ratios as well as the lowest confidence ratios to predict true plasmatic WT *RAS* genotypes, as reflected by their negative predicted value (NPV) (Table 4). Thus, compared to a 13.6% (95% CI 3.6–36.0) probability of showing a false-negative plasmatic WT *RAS* genotype in the population with metachronous mCRC originating in the left colon, this probability was 50.0% (95% CI 20.1–79.9) and 53.8% (95% CI 26.1–79.6) in cases with right-sided and rectal metachronous mCRC, respectively (Table 4).

	N ²	Concordance ¹	Sensitivity ²	Specificity ³	K ⁴ (95% CI)	PPV ⁵ (95% CI)	NPV ⁶ (95% CI)
Synchronous not resection							
Left	40	92.5	90.0	95.0	0.85 (0.69–1.00)	97.7 (71.9–99.7)	90.5 (68.2–98.3)
Rectum	57	93.0	96.7	88.9	0.86 (0.73–0.99)	90.6 (73.8–97.5)	96.0 (77.7–99.8)
Right	19	84.2	90.9	75.0	0.67 (0.33–1.00)	83.3 (50.8–97.1)	75.0 (35.6–95.5)
Synchronous resection ^a							
Left	47	89.4	86.4	92.0	0.79 (0.61–0.96)	90.5 (68.2–98.3)	88.5 (68.7–97.0)
Rectum	15	86.7	81.8	100.0	0.71 (0.33–1.00)	100.0 (62.9–100.0)	66.7 (24.1–94.0)
Right	29	79.3	73.9	100.0	0.54 (0.21–0.87)	100.0 (77.1–100.0)	50.0 (22.3–77.7)
Metachronous ^b							
Left	39	92.3	85.0	100.0	0.85 (0.68–1.0)	100.0 (77.1–100.0)	86.4 (64.0–96.4)
Rectum	25	60.0	56.3	66.7	0.21 (0.0–0.59)	75.0 (42.8–93.3)	46.2 (20.4–73.9)
Right	17	64.7	54.6	83.3	0.33 (0.0–0.76)	85.7 (42.0–99.2)	50.0 (20.1–79.9)

Table 4. Concordance of *RAS* mutational status between SoC and ctDNA analyses related to tumour surgery.

Table 4 includes information from 288 patients with known clinicopathological data. The location of the primary tumour was unknown in 13 of the total 301 patients with available *RAS* status in solid tumour tissue and plasmatic ctDNA samples. Discordant outcomes between SoC and BEAMing analyses were observed in 23 cases (12.1%) with synchronous metastases and 19 cases (24.7%) with metachronous metastases. ¹ Overall percentage agreement; ² positive percentage agreement; ³ negative percentage agreement; ⁴ Cohen's kappa coefficient (95% confidence interval); ⁵ positive predictive value (95% confidence interval); ⁶ negative predictive value (95% confidence interval). ^a Primary tumour resection was performed in 43.5% of patients with synchronous metastases. ^b Primary tumour resection was performed in 100% of patients with metachronous metastases.

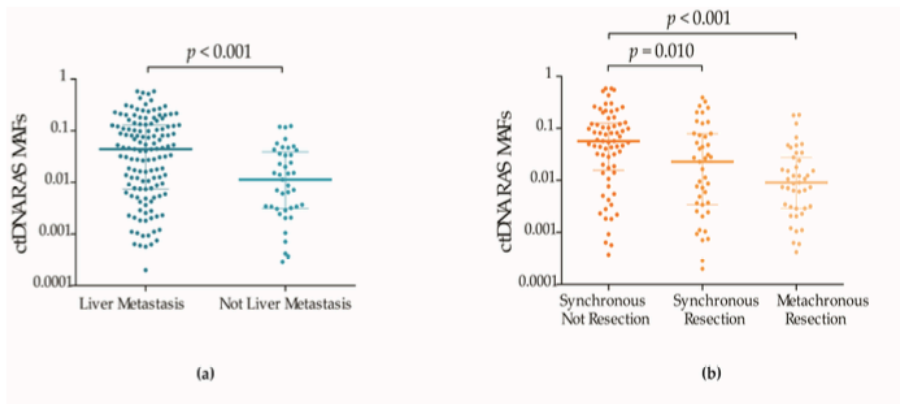
Additionally, 12% of cases with metachronous mCRC originating in the rectum showed *RAS* mutations in plasma, despite *RAS* mutations not being observed in their tumour tissue assays, demonstrating that the plasma assays from patients with metachronous mCRC originating in the rectum showed the lowest specificity of the study as well (Table 4).

3.5. Impact of the mCRC Clinicopathological Characteristics on the ctDNA RAS Mutational Load

The plasmatic *RAS* mutational load has been shown to be lower in patients with- out liver metastases [32,37,41] and to decrease upon primary tumour resection [34,40] or systemic chemotherapy [32]. The median of *RAS* MAFs in plasmatic ctDNA from 165 patients with *RAS* mutated genotypes was 0.029, IQR [0.005–0.104], ranging from a minimum level of 0.0002 to a maximum of 0.58. This wide dispersion suggested that the *RAS* MAF levels were highly variable among the mCRC patients.

According to the above-cited studies, we observed that the ctDNA *RAS* MAFs were significantly higher in patients with at least one liver metastasis than in patients without liver metastases but with metastases at other anatomical locations (median = 0.044, IQR [0.007–0.129] vs. median = 0.011, IQR [0.003–0.039]; p -value < 0.001) (Figure 1a). In line with these observed ctDNA levels, fluorometric quantification of plasmatic cfDNA estimated that the mCRC subpopulation with at least one liver metastasis showed significantly higher levels of cfDNA in plasma than patients who did not have any liver metastases but had metastases at other sites (median = 36.7 ng/mL, IQR [17.7–87.3] vs. median = 12.9 ng/mL, IQR [9.8–18.9]; p -value < 0.001). cfDNA levels were also higher in patients who had metas- tases only at the liver than in patients with lung metastases alone (median = 37.1 ng/mL, IQR [20.4–80.4] vs. 13.2 ng/mL, IQR [12.5–17.2], p -value = 0.004) or exclusive peritoneal metastatic disease (median = 37.1 ng/mL, IQR [20.4–80.4] vs. 12.4 ng/mL, IQR [9.8–20.5], p -value = 0.031).

Furthermore, we observed that patients diagnosed with synchronous mCRC, who had not been treated either with tumour resection or with systemic chemotherapy at the moment of the blood biopsy extraction, had higher ctDNA *RAS* MAFs than patients with synchronous mCRC who were naïve to systemic chemotherapy but treated with resection of the primary tumour (8.9% had also been subjected to resection of metastasized tissue), (median = 0.057, IQR [0.016–0.125] vs. median = 0.025, IQR [0.003–0.079]; p -value = 0.010) and also significantly higher than the plasmatic *RAS* MAFs that were observed in patients with a metachronous presentation of the metastases (median = 0.009, IQR [0.003–0.028]; p -value < 0.001) (Figure 1b).



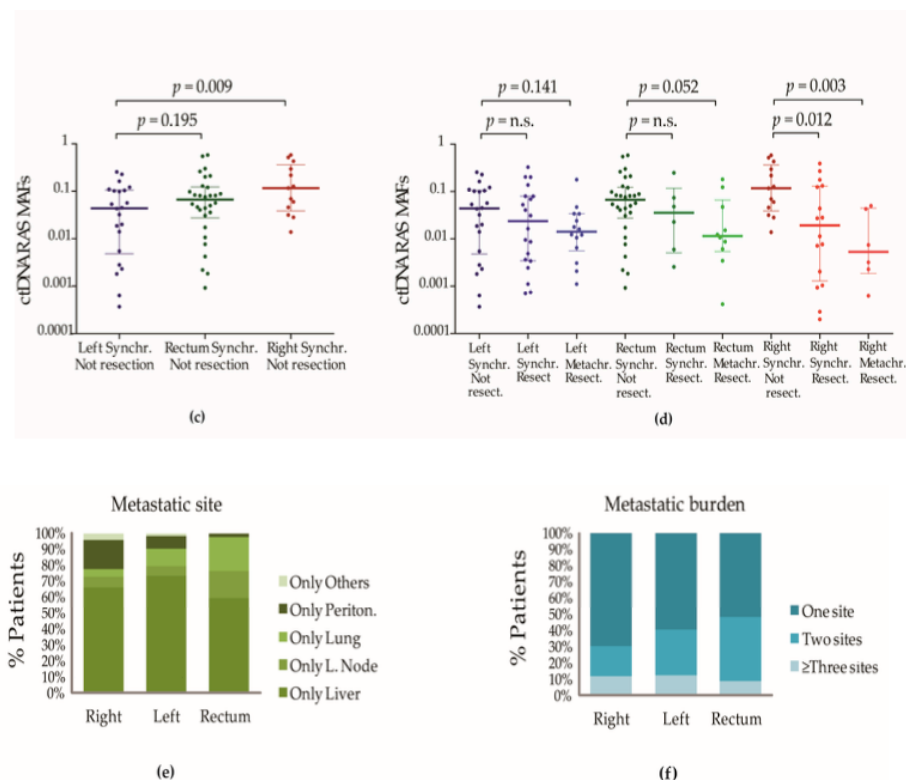


Figure 1. Impact of the mCRC clinicopathological characteristics on the detection of plasmatic *RAS* mutations.

(a) Impact of the metastatic site on the plasmatic *RAS* MAFs. Dark blue dots (column 1) represent ctDNA *RAS* MAFs of patients with at least one liver metastasis. Light blue dots (column 2) represent ctDNA *RAS* MAFs of patients with at least one metastatic site at other anatomical locations than the liver. Lines represent the median and the 25–75% IQR.

(b) Impact of surgical treatment and palliative/life-extending systemic chemotherapy on the ctDNA *RAS* MAFs. Plasmatic *RAS* mutations were detected in 113 patients with synchronous metastases and chemotherapy-naïve and in 43 patients with metachronous metastases who had been at least previously treated by tumour surgery. Dark orange dots (column 1) represent ctDNA *RAS* MAFs of 69 patients with newly diagnosed synchronous mCRC who had not received surgical treatment. Light orange dots (column 2) represent ctDNA *RAS* MAFs of patients with synchronous metastases treated with resection of the primary tumour (4 had also been subjected to the removal of metastatic tissue). Yellow dots represent (column 3) ctDNA *RAS* MAFs of 43 patients with metachronous metastases; 34 had only been treated with resection of the primary tumour and 9 had been treated with resection of primary and metastatic tumour tissue. Adjuvant systemic chemotherapy had been administered to 24 of the cases before plasma collection. Lines represent the median and the 25–75% IQR.

(c) Plasmatic *RAS* MAFs in patients with synchronous mCRC and different origins of the primary tumour who were not subjected to previous tumour surgery or systemic chemotherapy. Blue, green and red dots represent ctDNA *RAS* MAFs of patients with mCRC that originated in the left colon, rectum and right colon, respectively. Lines represent the median and the 25–75% IQR.

(d) Plasmatic *RAS* MAFs from patients with primary tumours in different locations and treated with surgical resection. Blue, green and red dots represent ctDNA *RAS* MAFs of patients with mCRC that originated in the left colon, rectum and right colon, respectively. Colour gradients were used to show the decrease in the *RAS* MAFs in the mCRC subpopulations treated with resection of the primary tumour. Lines represent the median and the 25–75% IQR.

(e) Correlation of primary tumour location with the metastatic pattern of mCRC. Plots showed the number of metastatic sites (e) and the anatomical location of the metastases.

(f) in 242 patients with synchronous tumours. All patients were naive to surgery or chemotherapy. The frequency of only one single site of metastasis was higher in patients with primary tumours located in the right colon. The liver was the predominant site of metastasis in mCRC with left colon origin, whereas lung metastases were more frequent in mCRC originating at the rectum. Peritoneal metastases and metastases to other sites were frequent in right-sided mCRC. Non-parametric Mann–Whitney U and Kruskal–Wallis tests were used to calculate *p*-values when two or more groups were compared.

Lower ctDNA *RAS* MAF medians were observed in all mCRC populations treated with surgery independently of the location of the primary tumour (Figure 1d). However, the population with mCRC originating in the right colon showed the most significant differences in the fractions of circulating *RAS* mutations between patients treated and patients not treated with tumour resection (Figure 1d). We observed that those patients diagnosed with synchronous mCRC originating in the right colon who had not yet received any treatment showed the highest ctDNA *RAS* MAF medians (Figure 1c). Conversely, the population with mCRC originating in the right colon treated with resection of the tumour contained the highest number of patients carrying plasmatic *RAS* MAFs closer to the 0.01% limit of detection of the BEAMing technology (Figure S1a,b). Among them, patients with metachronous mCRC originating in the right side of the colon showed the lowest *RAS* MAF medians (Figure 1d), coinciding with the lowest sensitivity ratios and NPVs (Table 4).

4. Discussion

The mutational status of *RAS* genes is a well-known prognostic and predictive mCRC biomarker conferring poor prognosis and a lack of response to anti-*EGFR* antibodies. Therefore, guidelines strongly recommend the evaluation of the *RAS* mutational status before the choice of the first line of therapy [44–47]. Alternatively to the invasive SoC analyses carried out in locally restricted tumour tissue specimens obtained by surgery or throughout a biopsy at the time of the diagnosis of the disease, the evaluation of the *RAS* mutational status using plasmatic ctDNA isolated from peripheral blood allows a spatial-temporal view of the tumour [32]. Therefore, ctDNA assays have emerged as a promising minimally invasive tool with the potential to improve the management and therapy outcomes of the CRC patient by guiding clinical decisions [40,48].

Actually, ctDNA genotyping technologies and, more in particular, the OncoBEAM *RAS* CRC test, have demonstrated to be highly accurate tools, detecting down to 0.01% *RAS* allelic fractions in plasma samples from mCRC patients [32,40]. Hence, the detection of somatic *RAS* mutations through the analysis of plasmatic ctDNA in mCRC has been shown to be reliable and highly concordant with that observed in tumour tissue samples analysed with the gold standard tests [32,33]. In our study, using the mentioned technology to genotype plasmatic ctDNA from a real-world and heterogenous cohort of 366 mCRC patients, representative of the day-to-day care at four different hospitals in Galicia, we observed a concordance of 84.4% with the gold

standard approach (SoC analysis in tumour tissue specimens). Similar to previous studies [32,49,50], the concordance ratios in our study were significantly higher in patients with liver metastases (89.7%) than in patients with metastases at other sites (76.1%), and in patients who had not been treated by resection of the primary tumour, as those with newly diagnosed synchronous tumours (90.2%), compared to those patients with synchronous (86.2%) and metachronous metastases (75.9%) who had already been treated by resection of the primary tumour.

In addition, binary logistic regression models, used to explore the odds of presenting discordant versus concordant assays depending on the mCRC clinicopathological characteristics, showed that the origin of the primary tumour was also an important predictor of discordant outcomes between the SoC and ctDNA tests. In fact, we observed that more than 91% of ctDNA genotypes from patients with mCRC originated in the left colon concurred with SoC tests. However, ctDNA tests carried out in samples from patients with mCRC originated in the rectum or right colon assigned the same genotype as SoC analysis in 84% and 77% of the cases, respectively. Up to 80% of the genotyping discrepancies observed in mCRC originated in the right colon and 63% of the discrepancies observed in mCRC originated in the rectum resulted from the failure of the ctDNA assay to detect any mutated *RAS* allele in the plasma of mCRC patients.

The accuracy of the liquid biopsy tests to detect ctDNA isolated from peripheral blood depends on the fraction of mutated DNA within the whole cell-free DNA released to the bloodstream, mainly through cell death processes [51,52]. Although the characteristics of tumours known to be highly cfDNA shedders are still poorly understood, the anatomical location of the tumour lesions has been demonstrated to be an important factor determining the presence and, therefore, the detection of ctDNA in the plasma of patients with mCRC and other tumours [50,53,54]. In line with these observations, it has been proposed that the good vascularisation [40] and usually high volume of liver metastatic lesions [41,49] would contribute to increased cfDNA shedding into the bloodstream and, therefore, explain why mCRC patients diagnosed with liver metastases are frequently associated with higher plasmatic *RAS* MAFs, as we have also reported here.

In this study, the mCRC subpopulation with at least one metastatic lesion in the liver showed significantly higher levels of cfDNA in plasma than patients without liver metastases but with metastases located at other anatomical sites. Consistent with this observation, the OncoBEAM assay detected higher fractions of mutated *RAS* alleles in plasma samples of patients with at least one liver metastasis than in patients without liver metastases. In addition, our results showed that the accuracy of the ctDNA assays was dramatically reduced to almost 50% when plasma samples belonged to mCRC cases with only metastases at the peritoneum, and to approximately 60% if samples had been obtained from cases with only lung metastases.

These new data obtained in a real-world cohort are in line with previous works addressed by ddPCR assays and also NGS and represent new evidence about the already known importance of the presence of liver metastases [32,37,41] to assess the plasmatic fractions of *RAS* mutations with enough sensitivity in mCRC patients.

Importantly, the plasmatic fraction of *RAS* mutations has also been shown to decrease after surgical resection of the primary tumour [34,36,40]. In agreement with these previous findings,

patients from our cohort treated with resection of the primary tumour tissue showed significantly lower *RAS* MAFs in plasma than non-operated patients, with the lowest plasmatic ctDNA content observed in metachronous mCRC originating in the right colon and in the rectum. Consistent with the results, these populations showed negative predicted values below 50% and, thus, the highest probabilities of showing false-negative *RAS* genotypes in our study.

Hence, our study also highlighted the relevance of the primary tumour location to understanding our capacity to detect plasmatic ctDNA in mCRC. It is well-accepted among the clinical and scientific community that the anatomical location of the primary tumour precedes the anatomical spreading of the metastatic lesions, with cancers originating in different bowel sites predisposing to different metastatic patterns [8]. Thus, left-sided mCRC, known to spread through the hepatic portal system to distal sites, is more prone to metastasis first to the liver and then spreading to the lungs. However, metastases in thoracic organs such as the lungs have more commonly been observed in rectal cancers with only a single metastatic site than in left colon cancers with single metastases [55]. On the other hand, patients with right-sided mCRC show peritoneal metastases with a higher frequency than patients with left-sided or rectal mCRC [8,56].

Therefore, in relation to the association of concordance ratios with the origin of the primary tumour in mCRC, we summarized that the higher ratios of concordance observed among assays from patients with primary tumours located at the left colon could be linked to the fact that these patients present clinicopathological features that have been significantly associated with higher fractions of mutated *RAS* in plasmatic ctDNA. Thus, more than 40% of these patients had more than one metastatic site when they were diagnosed with mCRC (Figure 1f), and as we have mentioned above, patients with mCRC that originated in the left colon have a higher preference to develop liver metastases [8]. Moreover, while the lung and the peritoneum were also organs that commonly showed metastases in patients with rectal and right-sided mCRC with only one metastatic site, the liver was the most common site for metastases in patients with mCRC originating in the left colon with only one metastatic site (Figure 1e).

Based on these observations, left-sided mCRC has to be expected as a disease with increased ctDNA shedding and in general, presenting highly concordant *RAS* genotypes between tissue and plasma assays.

The association of the mCRC origin with the sensitivity to detect plasmatic fractions of mutated *RAS* alleles is of enormous relevance in the context of the selection of therapy for mCRC [16]. In a recent clinical trial, the evaluation of *RAS* mutations in plasma has already been shown to be relevant to monitor minimal residual disease after surgery in patients with non-metastatic CRC stages to decide the administration of adjuvant therapy [57]. Moreover, recent results obtained as part of the CIRCULATE study have shown that the detection of ctDNA after surgical treatment is more frequent in subjects with tumours located on the left side of the colon [58]. Therefore, based on our results, we advise that particular attention should be paid to mCRC originated in the right colon and rectum treated by resection, particularly in the metachronous setting, since the significantly reduced sensitivity observed in ctDNA assays from these patients could lead to inconclusive ctDNA results. In addition, the fact that 12% of the patients with WT *RAS* primary tumours originated in the rectum showed plasmatic *RAS* mutations when diagnosed with metachronous metastases later on is suggestive of the existence of molecular mechanisms of tumour evolution through the emergence of *RAS* mutations in these

patients, which, if they are left inconclusively detected, could potentially reduce therapy responses.

Our study has some limitations, such as the different timing of the liquid biopsy collection, hindering the homogeneity of the population and making decisions regarding the data obtained. However, the observed post hoc statistical power based on the large sample size was high, and the BEAMing ddPCR genotyping was carried out in the same laboratory and by the same personnel, which makes the results reliable and reproducible.

5. Conclusions

In conclusion, our study confirmed that fractions of circulating *RAS* mutations and concordance between SoC and ctDNA assays were higher in mCRC with liver metastases and when the primary tumour was not resected.

On the contrary, patients with metachronous mCRC that originated in the right colon or rectum, usually treated with resection of the primary tumour and presenting higher frequencies of single metastases at the peritoneum or the lung, showed the lowest fractions of circulating *RAS* mutations. Despite the high sensitivity of the BEAMing ddPCR technology, the evaluation of plasma circulating *RAS* mutations in patients with mCRC originating in the right colon or rectum, when operated on, showed a high probability of reporting false-negative outcomes and discordances with the SoC procedures. Approximately only 50% of the negative ctDNA tests in this mCRC population were predicted to be true negative and therefore showed a conclusive non-mutated *RAS* result.

DISCUSIÓN

La biopsia líquida se está incorporando en la práctica clínica como una herramienta útil en muchas situaciones en Oncología, debido a ser un método no invasivo y con gran capacidad de mostrar en tiempo real y de forma personalizada la evolución de la tumorigénesis del cáncer. La búsqueda de biomarcadores que nos ayuden a seleccionar pacientes para ofrecer un tratamiento individualizado es uno de los objetivos para la implantación de la Medicina de Precisión en nuestra práctica clínica asistencial.

En los últimos años se han utilizado varias estrategias biológicas integradas para encontrar marcadores moleculares no invasivos que permitan predecir la respuesta a la administración de inmunoterapia en diferentes tumores, entre ellos el CPNM. Se trata además de uno de los tumores más frecuentes en nuestra población, con capacidad de diseminación a sangre (lo que permite estudiar sus características moleculares en este medio), y con diagnóstico avanzado en un alto porcentaje de pacientes, con pronóstico ensombrecido en estos casos. La existencia de resultados prometedores en estudios observacionales y retrospectivos, junto con el desarrollo continuado de plataformas de análisis molecular, ha impulsado el diagnóstico por biopsia líquida basado en cfDNA a la siguiente fase de estudio. Debido a su potencial para seleccionar poblaciones de pacientes candidatos a inmunoterapia específica (sola o combinada) con ICIs, y predecir respondedores o no respondedores, así como la aparición de resistencias primarias o secundarias, prevemos que el análisis de cfDNA será cada vez más importante en la rutina asistencial.

En el caso del cáncer de pulmón avanzado, los métodos de análisis de cfDNA son especialmente útiles porque minimizan la necesidad de realizar o repetir la biopsia del tejido tumoral, evitando así en nuestros pacientes complicaciones derivadas de la realización de un procedimiento agresivo. La capacidad del cfDNA para guiar la elección del tratamiento ideal y seguir la respuesta tumoral a la inmunoterapia apenas estaba empezando a evaluarse en el año 2020. Uno de los trabajos pioneros en este campo llevado a cabo por Chabon et al, estableció el potencial del análisis de cfDNA en plasma mediante biopsia líquida para realizar el cribado de cáncer de pulmón, subrayando la importancia de emparejar casos y controles de riesgo en los estudios de cribado basados en este biomarcador (64) en sangre periférica.

Sin embargo, no podemos ignorar que el aislamiento y el análisis del cfDNA siguen siendo un reto hoy en día. El cfDNA en plasma es una mezcla compleja de ADN procedente de muchas fuentes, como son las células de línea germinal, origen fetal, causa infecciosa o maligna (65), pero también hay mutaciones en el cfDNA de origen hematopoyético con riesgo de ser tomadas erróneamente como mutaciones derivadas del tumor (66). Dado que una gran proporción del cfDNA plasmático procede de células sanguíneas periféricas, el impacto de la hematopoyesis clonal representa una limitación relevante en la aplicación de la biopsia líquida para analizar el cfDNA como parámetro, y dificulta su interpretación en pacientes oncológicos. La secuenciación emparejada de plasma y células sanguíneas periféricas debería implementarse como práctica estándar para el análisis genómico mediante NGS del cfDNA para evitar la interpretación errónea de los resultados (67). Además, el cfDNA no es específico de un tumor concreto y su medición es propensa a resultados que terminan siendo falsos positivos. De hecho, el nivel de cfDNA puede aumentar en algunas condiciones no malignas, como son la inflamación, la infección e incluso en situaciones fisiológicas como es el ejercicio físico (68).

La sensibilidad y especificidad de la medición del cfDNA en plasma también se ven afectadas por la variabilidad del ctDNA (cuyo origen es tumoral), que puede variar a lo largo de la evolución del cáncer, así como tener distintos orígenes (tumor primario frente a metástasis; mostrando así la heterogeneidad tumoral) y estar afectado por distintos tratamientos aplicados a lo largo de la evolución oncológica. Actualmente nos encontramos en una situación en la investigación en la que las tecnologías que apoyan el uso de la biopsia líquida evolucionan de forma rápida y exponencial, al mismo tiempo que nos percatamos de que se necesitan procedimientos estandarizados para la recogida y procesamiento de muestras de cfDNA con el objetivo de homogenizar estos estudios a nivel internacional. Otro aspecto a concretar en la comunidad científica debería ser el tamaño de los paneles moleculares para evaluar aquellas mutaciones específicas del cáncer y métricas genómicas (MRD, *minimal residual disease*; y TMB, *tumor mutational burden*), ya que varían mucho de un ensayo a otro. Esto dificulta la interpretación de los resultados y la posibilidad de establecer redes de trabajo comunes dada la dificultad de comunicación si no hablamos un lenguaje común.

Por otro lado, las CTCs son células viables intactas con información específica del tumor y también pueden permitir el diagnóstico del cáncer y la evaluación del tratamiento con una monitorización en tiempo real. Los análisis de la presencia o ausencia de proteínas, como son PD-L1 o PD-1 en la superficie de estas células, prometen ser buenas opciones en el futuro para guiar y seleccionar el tratamiento de forma personalizada a nuestros pacientes. Pero estas estrategias deberán ser validadas en estudios robustos con un buen diseño para demostrar esta hipótesis (69). Además, gracias a los métodos que hoy en día permiten la conservación y captura de CTCs intactas, es posible realizar análisis unicelulares o de clusters celulares. La caracterización de células individuales o de grupos de CTCs permitirá un estudio molecular más preciso de todo el cáncer en cada uno de los pacientes. Los análisis de ARN y ADN son factibles y podrían mostrar la heterogeneidad inter- e intratumoral en cada enfermo (70), pudiendo emplear esta información para el uso de terapia dirigida o personalizada de forma individual. En cambio, encontrar una plataforma buena y estandarizada para capturar las CTCs y una validación rigurosa para el empleo diario siguen siendo un reto clínico. Debido a los efectos de la TME (transición epitelio mesenquimal), se necesitan nuevos marcadores biológicos para detectar las células tumorales que experimentan esta transformación y así poder adaptarnos al momento concreto de la evolución de ese cáncer en particular.

Estudios recientes se han centrado en el repertorio de receptores de células T (TCR) de sangre periférica como biomarcador emergente para seleccionar el tratamiento inmunoterápico más adecuado (71). El análisis de la clonalidad de las células T puede revelar el grado de expansión de estas células T impulsada por el antígeno tumoral y ayudar a comprender los mecanismos subyacentes a la tolerancia de las células T a los antígenos del cáncer. En un trabajo llevado a cabo por Reuben et al. se demostró la heterogeneidad del repertorio tisular de células T en el CPNM localizado (72) proponiendo una relación positiva entre la densidad de células T y la clonalidad. Demostraron en este estudio una asociación entre una mayor densidad de células T en la sangre y un mejor resultado tras la cirugía realizada en estos pacientes. Estos resultados sugieren que el repertorio periférico de células T en pacientes con CPNM puede reflejar un aumento de la inmunidad sistémica. Con esta idea en mente, se sugiere que la secuenciación del TCR podría ser aplicable para la selección del tratamiento de una forma personalizada en pacientes con mutaciones del gen *EGFR* mediante la evaluación de las proporciones de clones TCR β en el tumor.

En otro trabajo de investigación, Miyauchi et al. proporcionaron información relevante para comprender el mecanismo molecular que subyace en los pacientes con mutaciones del gen *EGFR* (73), mostrando que la baja expansión clonal de células T en CPNM con esta mutación podría ser un factor crítico relacionado con la respuesta desfavorable a ICIs. La clonalidad de las células T en la circulación del torrente sanguíneo podría tener un valor predictivo para las respuestas antitumorales de la inmunoterapia con fármacos con mecanismo de inhibición de puntos de control inmunológico. Sin embargo, dado que se trata de resultados muy preliminares todavía, es esencial continuar esta línea de investigación para saber si tiene aplicación práctica en la vida real.

En conclusión al primer capítulo presentado en esta tesis, mostramos que en el cáncer de pulmón no microcítico avanzado hay una necesidad no cubierta a la hora de identificar con antelación los pacientes que se podrían beneficiar de la administración de inmunoterapia, pudiendo predecir así la respuesta tumoral; o por el contrario, averiguar cuáles son aquellos que progresan durante el curso de esta terapia debido a resistencias primarias o secundarias. La biopsia líquida mediante análisis de CTCs y cfDNA se presenta como una herramienta prometedora para el diagnóstico, la selección del tratamiento con ICIs y el seguimiento de pacientes con CPNM tratados con esta terapia.

En las últimas décadas, los tumores sólidos han experimentado avances significativos en el tratamiento de la enfermedad avanzada, aumentando la supervivencia y la calidad de vida. Los nuevos tratamientos, como la inmunoterapia y la terapia dirigida, son la causa de esta mejoría. Sin embargo, todavía existen escenarios, como la enfermedad metastásica a nivel del sistema nervioso central (SNC), que siguen siendo un reto debido a la elevada morbilidad que ocasiona en nuestros pacientes. Tradicionalmente, los fármacos antineoplásicos no penetran en el tejido cerebral debido a la barrera hematoencefálica, por lo que estos pacientes presentan un pronóstico infausto sin opciones terapéuticas sistémicas a este nivel.

En la revisión que presentamos como segundo capítulo de esta tesis, resumimos los resultados obtenidos en pacientes con enfermedad cerebral reclutados en los principales estudios que evalúan estrategias de inmunoterapia en determinados tumores en los que esta terapia está aprobada. Representan una cohorte pequeña de pacientes debido a la escasa representación de estos pacientes en los ensayos clínicos de fase III y al limitado número de estudios específicos en esta población. Derivado de este factor, el presente trabajo tiene la limitación del reducido número de pacientes incluidos, la heterogeneidad de la población que incluye enfermedad cerebral limitada o extensa, así como pacientes con diversos tipos de tumores primarios. Además, observamos que este grupo de pacientes no siempre está descrito en los artículos, lo que dificulta su extrapolación a la práctica clínica con la consiguiente incertidumbre a la hora de tratar a estos pacientes con los datos de la medicina basada en la evidencia.

Además, es importante mencionar el hecho de que nuestro trabajo no es una revisión sistemática, sino un análisis cualitativo narrativo exhaustivo a raíz de estudios publicados. A pesar de estas limitaciones, la revisión destaca el beneficio del uso de la inmunoterapia en el tratamiento de tumores de pulmón, mama y melanoma con metástasis a nivel cerebral abriendo nuevas opciones terapéuticas para el manejo de la enfermedad. Sin embargo, la inmunoterapia tiene cierta toxicidad grave que debe vigilarse y tratarse de una forma concreta consecuente al mecanismo de acción de estos fármacos. Por lo tanto, es crucial poder seleccionar con precisión

a aquellos pacientes que más se beneficiarán de la inmunoterapia presentando enfermedad cerebral para así evitar poner en peligro órganos vitales expuestos a una toxicidad potencialmente grave.

A nivel global, aunque se dispone de pocos datos sobre el papel de la inmunoterapia en el caso del cáncer no microcítico de pulmón con metástasis cerebrales, la inmunoterapia sola o en combinación con quimioterapia ha mostrado una actividad clínica intracraneal y unos resultados de seguridad prometedores mejorando el pronóstico de estos pacientes. No obstante, se desconoce el mecanismo de actuación de los ICI en el nicho cerebral y su interacción con el microambiente tumoral. Por lo tanto, caracterizar el fenotipo inmunitario de la enfermedad cerebral y comprender mejor la relación de las células inmunitarias y las células estromales residentes junto con las células neoplásicas es crucial para mejorar los resultados de la inmunoterapia en nuestros pacientes.

En el caso del cáncer microcítico de pulmón el perfil genómico se caracteriza por extensos reordenamientos cromosómicos y una elevada carga mutacional, incluida la inactivación de los genes supresores tumorales. Sin embargo, actualmente para su uso en la práctica clínica no se dispone de biomarcadores predictivos validados para guiar las decisiones de tratamiento o estratificar a los pacientes. Las biopsias líquidas con análisis de ctDNA y CTCs, resultan prometedoras como métodos alternativos para la selección del tratamiento y el seguimiento.

Considerando el cáncer de mama como uno de los tumores más prevalentes en el mundo y el que con más frecuencia metastatiza al SNC, es el segundo tumor que genera metástasis cerebrales después del carcinoma de pulmón y por encima del melanoma. Algunos ensayos clínicos en curso están evaluando la eficacia de la inmunoterapia en combinación con otros fármacos o radioterapia en pacientes con cáncer de mama avanzado en función del subtipo molecular. El uso de inmunoterapia se está testando hasta ahora sin datos de alta eficacia y sin desarrollo específico en casos de enfermedad cerebral. Otros abordajes más novedosos en el campo de la inmunoterapia se están explorando en subtipos concretos, como el *cluster* HER2+. La investigación en este escenario de enfermedad cerebral metastásica es necesaria para aportar luz a un subgrupo de pacientes con mal pronóstico vital y con la intención de mejorar la adaptación de la terapia personalizada para la mejora de resultados.

En el caso del melanoma hemos revisado la tasa de beneficio clínico intracraneal reportada en los ensayos clínicos pivotaes de inmunoterapia con un alto porcentaje de respuestas completas o parciales, evidenciando así un nicho de pacientes en los que mejoran de forma significativa su pronóstico y calidad de vida. El tratamiento con fármacos ICI tales como ipilimumab y nivolumab alcanzan altas tasas de respuesta intracraneal en los distintos trabajos a costa de una mayor toxicidad. Evidenciamos, por tanto, una oportunidad para continuar la investigación en esta subpoblación de pacientes debiendo hacer un balance entre eficacia y seguridad en el uso de este tipo de terapias, y optimizando el diseño de ensayos clínicos para poder trasladar los resultados a nuestra práctica clínica asistencial.

En este contexto clínico, la aplicación de la biopsia líquida puede tener especial relevancia como herramienta que evita procedimientos invasivos, y proporciona información molecular en tiempo real para la monitorización y seguimiento de la enfermedad oncológica a lo largo de su evolución. Como se detalla en esta revisión, existen evidencias que apoyan la utilidad de los análisis de biomarcadores mediante esta técnica para la selección y monitorización de la

inmunoterapia en cánceres de pulmón, mama y melanoma. El uso combinado de inmunoterapia y biopsia líquida tiene un beneficio claro, lo que los convierte en importantes logros en el arsenal terapéutico y diagnóstico de los tumores mencionados. Varios trabajos han demostrado que el análisis de biopsia líquida (principalmente marcadores de fluidos como líquido cefalorraquídeo, LCR; y sangre) en el contexto de enfermedad a nivel cerebral derivado de tumores con origen en pulmón, mama y melanoma, permitiendo así la caracterización molecular de la enfermedad teniendo en cuenta la evolución y heterogeneidad tumoral. Esto se llevaría a cabo determinando en sangre el estado de biomarcadores inmunológicos bien establecidos como PD-L1, TMB o la condición MSI (inestabilidad de microsatélites).

Sin embargo, nuestra revisión evidenció la escasa información sobre la utilidad específica de la evaluación del cfDNA y otros marcadores como las CTCs y las vesículas extracelulares circulantes (cEVs) en pacientes con metástasis cerebrales que reciben inmunoterapia. En este sentido, es importante mencionar que todos los ensayos de fase III descritos en tumores avanzados (CPNM, CPM, mama o melanoma) no estaban asociados a estudios traslacionales con toma de biopsia líquida, por lo que no hay análisis de plasma ni otros fluidos que puedan servir como criterios de valoración secundarios. Esto dificulta extraer conclusiones sobre el comportamiento de los biomarcadores circulantes durante el tratamiento con inmunoterapia, pero al mismo pone de relieve la necesidad y el reto de mejorar el diseño en futuros ensayos clínicos centrados en este subgrupo de pacientes de especial interés por su mal pronóstico vital. Cabe destacar que la presente revisión engloba por primera vez los resultados relativos al valor combinado del uso de la inmunoterapia como tratamiento y la biopsia líquida como técnica diagnóstica en el contexto de enfermedad cerebral, proporcionando así un conocimiento exhaustivo que podría abrir la puerta a la creación de ensayos clínicos específicos para estudiar el valor de los marcadores biológicos en plasma u otros fluidos con utilidad para establecer el diagnóstico, pronóstico y monitorización de respuesta a la inmunoterapia en pacientes con lesiones cerebrales.

Afortunadamente, existen estos estudios en curso y con los resultados preliminares en cáncer de pulmón, mama y melanoma obtendremos más información. El objetivo es proporcionar más conocimiento a nivel molecular en este contexto para satisfacer mejor las necesidades de este subgrupo de pacientes. Es de interés seguir investigando en la línea de la aplicación de la inmunoterapia al tratamiento de tumores sólidos con metástasis cerebrales y el uso de biopsia líquida podría guiar el tratamiento en una subpoblación de pacientes con escasas alternativas terapéuticas.

Uno de los tumores en los que se ha implementado de una forma más temprana el uso de tratamiento personalizado mediante el diagnóstico molecular es el cáncer colorrectal metastásico, ya que al momento del diagnóstico está recomendado realizar el estatus del gen *RAS* para el diseño de la mejor estrategia terapéutica en primera y sucesivas líneas de tratamiento desde hace ya unos años. El estado mutacional de los genes *RAS* es un biomarcador pronóstico y predictivo bien establecido en nuestra práctica clínica asistencial, sabiendo que confiere un mal pronóstico y una falta de respuesta a los anticuerpos anti-*EGFR*. Por lo tanto, las directrices internacionales recomiendan encarecidamente la evaluación del estado mutacional *RAS* antes de la elección de la primera línea de tratamiento (49-52). Este estudio se obtiene con procedimientos invasivos para la toma de biopsia para el estudio del perfil molecular realizado en muestras de tejido tumoral conseguidas mediante la cirugía de resección del tumor primario, o a lo largo de una biopsia mediante colonoscopia en el momento del

diagnóstico. La evaluación del estado mutacional del gen *RAS* utilizando ctDNA plasmático aislado en sangre periférica permite una visión tanto espacial como temporal del tumor (74) y su evolución a lo largo del tiempo. Por lo tanto, los ensayos de ctDNA han surgido como una prometedora herramienta mínimamente invasiva con el potencial de mejorar la gestión y los resultados de la terapia del paciente con CCRm mediante la orientación de las decisiones clínicas (13,75) en base a estos resultados moleculares para poder ofrecer al paciente la mejor alternativa terapéutica posible.

De hecho, las tecnologías de genotipado de ctDNA y, en particular, el test OncoBEAM® *RAS* CRC, han demostrado ser herramientas muy precisas y eficaces, detectando hasta un 0,01% de fracciones alélicas del gen *RAS* en muestras de plasma de pacientes con CRCm (74,13). Por lo tanto, la detección de mutaciones somáticas de *RAS* mediante el análisis de ctDNA plasmático en CRCm ha demostrado ser fiable y altamente concordante con la observada en muestras de tejido tumoral analizadas con las pruebas en tejido tumoral, todavía hoy en día considerado el *gold standard* (74, 76) para el análisis mutacional en esta población de pacientes.

En nuestro estudio presentado como tercer capítulo de esta tesis hemos querido trasladar el uso de la biopsia líquida en un tumor frecuente en la población, con alta capacidad de siembra a torrente sanguíneo y con implicaciones en el diseño de la terapia personalizada para alcanzar mejores resultados en la evolución tumoral. En nuestro trabajo de campo hemos utilizado la tecnología mencionada para genotipar el ctDNA plasmático de una cohorte real y heterogénea de 366 pacientes con CRCm, representativa de la asistencia diaria en cuatro hospitales diferentes de Galicia, nuestro medio. Se observa una concordancia del 84,4% del análisis mutacional del *RAS* con el enfoque de referencia (análisis de *standard of care*, SoC, en muestras de tejido tumoral). Al igual que en estudios previos (74,77,78) los ratios de concordancia en nuestro trabajo fueron significativamente más elevados en los pacientes con metástasis a nivel hepático (89,7%) que en los pacientes con lesiones metastásicas de otras localizaciones (76,1%). También en los pacientes que no han sido tratados mediante cirugía de resección del tumor primario, así como aquellos con tumores metastásicos al debut (90,2%), en comparación con aquellos pacientes con metástasis sincrónicas (86,2%) y metacrónicas (75,9%) a los que se les ha realizado resección del tumor primario.

Además, los modelos de regresión logística binaria llevados a cabo en este estudio para explorar las probabilidades de presentar resultados discordantes frente a concordantes en función de las características clínico-patológicas del CCRm, mostraron que el origen del tumor primario también es un factor predictivo importante de los resultados discordantes entre las pruebas de análisis del gen *RAS* mediante SoC y de ctDNA. De hecho, observamos que más del 91% de los genotipos de ctDNA de pacientes con CCRm originados en el colon izquierdo concordaban con las pruebas realizadas en tejido. Sin embargo, las pruebas de ctDNA realizadas en muestras de plasma de pacientes con CCRm de origen primario en el recto o el colon derecho asignaron el mismo genotipo que el análisis de tejido en el 84% y el 77% de los casos, respectivamente. Hasta el 80% de las discrepancias de genotipado observadas en el CRCm con tumor primario en el colon derecho y el 63% de las discrepancias observadas en el CRCm originado en el recto se debieron a que el estudio de ctDNA no detectó ningún alelo *RAS* mutado en el plasma de los pacientes con CRCm.

La precisión de las pruebas de biopsia líquida realizadas para detectar ctDNA aislado de sangre periférica depende de la fracción de DNA mutado dentro del DNA libre de células enteras

liberado al torrente sanguíneo, principalmente a través de procesos de muerte celular (79,80); bien sea por apoptosis, necrosis u otros mecanismos. Aunque todavía no se conocen bien las características que presentan los tumores altamente excretores de cfDNA, se ha demostrado que la localización anatómica de las lesiones tumorales es un factor importante que determina la presencia y, por tanto, la detección de cfDNA en el plasma de pacientes con CCRm y otros tumores (78, 81,82). En consonancia con estas observaciones, se ha propuesto que la buena vascularización (58) y el volumen generalmente elevado de las lesiones metastásicas hepáticas (63,77) contribuirían a un mayor vertido de cfDNA al torrente sanguíneo y, por tanto, explicarían por qué los pacientes con CCRm diagnosticados con lesiones hepáticas se asocian con frecuencia a MAFs *RAS* plasmáticos más elevados, como también hemos demostrado en nuestro estudio.

Además, en nuestro trabajo, destacamos que la subpoblación de CCRm con al menos una lesión metastásica en el hígado mostró niveles significativamente más altos de cfDNA en plasma que los pacientes sin metástasis hepáticas, pero con lesiones presentes en otras localizaciones anatómicas. En línea con esta idea, el ensayo OncoBEAM detectó fracciones más elevadas de alelos *RAS* mutados en muestras de plasma de pacientes con al menos una metástasis hepática que en pacientes sin ninguna metástasis a nivel de hígado. Además, nuestros resultados mostraron que la precisión de los ensayos de ctDNA se reducía drásticamente a casi el 50% cuando las muestras de plasma pertenecían a casos de CRCm con enfermedad únicamente presente en el peritoneo, y a aproximadamente el 60% si las muestras se habían obtenido de casos con metástasis únicamente a nivel pulmonar.

Estos nuevos datos obtenidos en nuestra cohorte están en consonancia con trabajos anteriores abordados mediante ensayos de ddPCR (digital drop polymerase chain reaction) y también mediante secuenciación masiva con la tecnología NGS (*next generation sequencing*). Representan nuevas pruebas sobre la importancia ya conocida previamente de la presencia de metástasis hepáticas (60,63,74) para evaluar las fracciones plasmáticas de mutaciones del gen *RAS* con suficiente sensibilidad en pacientes con CRCm.

Es importante destacar que también se ha demostrado que la fracción plasmática de mutaciones (MAF) del gen *RAS* disminuye tras la resección quirúrgica del tumor primario (58,59,83). En consonancia con estos hallazgos previos, los pacientes de nuestra cohorte tratados con resección quirúrgica del primario mostraron MAFs *RAS* en plasma significativamente más bajas que los pacientes no operados, observándose el contenido más bajo de ctDNA a nivel plasmático en el caso de CRCm metacrónico con origen tumoral en el colon derecho y en recto. En concordancia con los resultados, estas poblaciones mostraron valores negativos inferiores al 50% y, por tanto, las mayores probabilidades de mostrar genotipos *RAS* considerados como falsos negativos en nuestro estudio. Por lo tanto, nuestro trabajo también ha puesto de relieve la importancia de la localización del tumor primario para comprender nuestra capacidad de detectar ctDNA en el plasma de los pacientes con CRCm.

Está bien aceptado entre la comunidad clínica y científica que la localización anatómica del tumor primario precede a la diseminación anatómica de las lesiones metastásicas, y que los cánceres originados en diferentes localizaciones intestinales predisponen a distintos patrones metastásicos (84). Así, el CCRm del lado izquierdo, conocido por propagarse a través del sistema portal hepático a sitios distales por la circulación sanguínea, es más propenso a las metástasis primero en el hígado, y luego en los pulmones. Sin embargo, estas metástasis en

órganos torácicos se han observado con más frecuencia en cánceres de recto como un único foco metastásico que en cáncer de colon izquierdo con metástasis únicas (85). Por otra parte, los pacientes con CCRm derecho presentan metástasis peritoneales con mayor frecuencia que los pacientes con origen izquierdo o rectal (84,86), siguiendo así un patrón de diseminación tumoral más concordante con mecanismo de propagación local que a nivel del torrente sanguíneo.

Comparando estos resultados con otros similares, son varios los grupos de investigación que han realizado comparativas entre su determinación en tejido y plasma, apreciando en general un alto nivel de concordancia entre los dos métodos. Nuestro estudio multicéntrico con 366 pacientes con características clínicas heterogéneas muestra una concordancia global del 84,4%, presentando variaciones según factores clínicos, como son la presencia de metástasis hepáticas (con una concordancia del 89,7%) o el diagnóstico metastásico de forma sincrónica (con una concordancia del 90,2%), siendo estas situaciones dos condiciones frecuentes en nuestro día a día.

Por lo tanto, en relación con la asociación de las ratios de concordancia con el origen del tumor primario en el cáncer colorrectal avanzado, sugerimos que las mayores ratios de concordancia observadas entre los ensayos de pacientes con tumores primarios localizados en el colon izquierdo podrían estar relacionados con el hecho de que estos pacientes presentan características clinicopatológicas que se han asociado significativamente con mayores fracciones de *RAS* mutado en el ctDNA plasmático. Así, más del 40% de estos pacientes presentaban más de una localización metastásica cuando se les diagnosticó la enfermedad en situación avanzada y, como hemos mencionado anteriormente, los pacientes con CCRm originado en el colon izquierdo tienen una mayor preferencia a desarrollar metástasis a nivel hepático (84).

Además, mientras que el pulmón y el peritoneo también fueron localizaciones que presentaron metástasis con frecuencia en pacientes con CCRm rectal y colon derecho con un solo foco metastásico, el hígado es el lugar más frecuente de metástasis en pacientes con CCRm originado en el colon izquierdo con un único lugar de metástasis. Basándonos en estas observaciones, cabe esperar que el CCRm izquierdo sea una enfermedad con una mayor diseminación de ctDNA y que, en general, presente genotipos del gen *RAS* altamente concordantes entre los análisis de tejido y plasma.

Analizando las características que podrían influenciar una discordancia entre ambos tests, la localización tumoral primaria se postula como un predictor de la misma. El cáncer originado en colon derecho o recto muestran una concordancia del 77% y del 84%, respectivamente. Uno de los motivos de discrepancia es derivado de la no detección de alelos *RAS* en ctDNA en el plasma de los pacientes. Se sugiere que la localización tumoral y la presencia de metástasis en órganos muy vascularizados son condiciones que mejoran la detección de ctDNA (debido a un mayor *shedding*), de ahí que el lado izquierdo y las lesiones hepáticas sean las que mayor descarga de alelos *RAS* al torrente sanguíneo presenten. Otro de los factores que condicionan menores niveles de ctDNA en sangre es la resección del tumor primario, especialmente si es realizada con anterioridad a la aparición de enfermedad metastásica (situación metacrónica).

Por el contrario, los pacientes con CCRm metacrónico originado en el colon derecho o en el recto, normalmente tratados con resección del tumor primario y que presentaban mayores

frecuencias de metástasis únicas en el peritoneo o en el pulmón, mostraron las fracciones más bajas de mutaciones *RAS* a nivel circulante. A pesar de la alta sensibilidad de la tecnología BEAMing® ddPCR, la evaluación de las mutaciones del *RAS* en plasma de pacientes con CRCm originado en el colon derecho o el recto, cuando fueron operados, mostró una alta probabilidad de reportar resultados falsos negativos y discordancias con los procedimientos realizados en tejido tumoral mediante la técnica estándar. Aproximadamente sólo el 50% de las pruebas de ctDNA negativas en esta población de CRCm se predicen como verdaderas negativas y, por lo tanto, muestran un resultado concluyente de estatus mutacional del gen *RAS* como no mutado.

Así, teniendo en cuenta estos factores, debemos presentar especial atención a aquellos pacientes con origen del tumor en colon derecho y recto operados, particularmente en la aparición de metástasis de forma metacrónica y en otros órganos diferentes al hígado. Estos son las situaciones que suponen un reto a la hora de establecer el método de diagnóstico para el análisis del estado mutacional del gen *RAS*, que debe ser lo más adecuado posible para ofrecer a los pacientes terapias dirigidas de forma apropiada.

Así las cosas y acorde con nuestros resultados, ofrecemos la recomendación a la comunidad científica y clínica de que se preste especial atención a los CCRm originados en el colon derecho y el recto, y que han sido tratados mediante resección del tumor primario, en particular en el contexto de situación metacrónica, ya que la sensibilidad significativamente reducida observada en los ensayos de ctDNA de estos pacientes podría dar lugar a resultados del estatus mutacional del gen *RAS* no concluyentes. Además, el hecho de que el 12% de los pacientes con tumores primarios con perfil molecular *RAS* nativo y originados en el recto hubiesen presentado mutaciones plasmáticas *RAS* cuando se diagnosticaron de metástasis en momento metacrónico es sugestivo de la existencia de mecanismos moleculares de evolución tumoral a través de la aparición de mutaciones en el gen *RAS* en este subgrupo de pacientes, que, si no se seleccionan de forma adecuada, podrían reducir potencialmente las respuestas terapéuticas a fármacos de terapias dirigidas que aportan toxicidad a nuestros pacientes.

Nuestro estudio tiene algunas limitaciones, como por ejemplo, el diferente momento de la recogida del plasma para realizar la biopsia líquida en nuestra cohorte de pacientes, cuestión que dificulta la interpretación del análisis dada la escasa homogeneidad de la población y consecuentemente, el traslado de resultados para la toma de decisiones clínicas respecto a los datos obtenidos. Sin embargo, la potencia estadística *post hoc* observada basada en el gran tamaño de la muestra fue alta, y el genotipado mediante BEAMing® ddPCR se llevó a cabo en el mismo laboratorio y por el mismo personal, lo que hace que los resultados sean fiables y reproducibles.

En conclusión, nuestro estudio confirmó que las fracciones de mutaciones del gen *RAS* a nivel de plasma circulante y la concordancia entre los ensayos de estudio de tejido y ctDNA en plasma fueron mayores en el CRCm con metástasis hepáticas y cuando no se reseca el tumor primario.

En base al conocimiento actual creemos que la biopsia líquida podrá establecerse como una herramienta de utilidad en pacientes con tumores sólidos en situación metastásica y con pronóstico desfavorable, para ofrecer al paciente alternativas terapéuticas dentro de la

Oncología de Precisión. Además, el trabajo de campo realizado en pacientes de nuestro medio demuestra de forma empírica la utilidad de esta técnica para una situación habitual en nuestro día a día como oncólogos.

CONCLUSIONES

En esta tesis, la utilidad de la biopsia líquida queda demostrada para la búsqueda de biomarcadores circulantes con capacidad para el uso de fármacos basados en terapia dirigida, con beneficio clínico en pacientes con cáncer de pulmón, mama y melanoma con metástasis cerebrales, así como en cáncer colorrectal metastásico, situaciones especialmente preocupantes en nuestro día a día.

Los datos obtenidos de nuestro estudio experimental nos llevan a presentar las siguientes conclusiones:

CAPÍTULO 1

1. La biopsia líquida se postula como una herramienta para la selección de pacientes con cáncer de pulmón no célula pequeña respondedores a inmunoterapia.

CAPÍTULO 2

2. La detección de biomarcadores predictivos mediante biopsia líquida selecciona perfiles de pacientes con tumores sólidos estadio IV cerebral candidatos a tratamientos dirigidos.

CAPÍTULO 3

3. La determinación de biomarcadores circulantes es de utilidad para establecer estrategias de tratamientos personalizados en cáncer colorrectal metastásico.
4. La ausencia de metástasis hepáticas y la presencia de enfermedad avanzada metacrónica con tumor primario localizado en colon derecho y recto condicionan mayor discordancia entre el estado mutacional del gen *RAS* realizado en tejido tumoral y biopsia líquida.
5. La enfermedad metastásica a nivel hepático y el tumor primario no resecado son características clínico-patológicas que influyen en mayor carga mutacional del gen *RAS* detectado mediante ctDNA.

REFERENCIAS

1. Schwartzberg L, Kim ES, Liu D, Schrag D. Precision Oncology: Who, How, What, When, and When Not? *Am Soc Clin Oncol Educ Book*. 2017;37:160-169.
2. Heidrich I, Ačkar L, Mossahebi Mohammadi P, Pantel K. Liquid biopsies: Potential and challenges. *Int J Cancer*. 2020;(May):1–18.
3. Bradley SH, Barclay ME. “Liquid biopsy” for cancer screening. Vol. 372, *BMJ* (Clinical research ed.). England; 2021. p. m 4933.
4. Planchard D, Popat S, Kerr K, Novello S, Smit E.F, Faivre-Finn C, et al. Metastatic Non-Small Cell Lung Cancer: ESMO Clinical Practice Guidelines for Diagnosis, Treatment and Follow-up. *Ann. Oncol.* **2018**, 29, IV192–IV237.
5. Pascual J, Attard G, Bidard FC, Curigliano G, De Mattos-Arruda L, Diehn M, et al. ESMO Recommendations on the Use of Circulating Tumour DNA Assays for Patients with Cancer: A Report from the ESMO Precision Medicine Working Group. *Ann. Oncol.* **2022**, 33, 750–768.
6. Chouaid C, Dujon C, Do P, Monnet I, Madroszyk A, Le Caer H, et al. Feasibility and clinical impact of re-biopsy in advanced non small-cell lung cancer: a prospective multicenter study in a real-world setting (GFPC study 12-01). *Lung Cancer*. 2014 Nov;86(2):170–3.
7. Vanderlaan PA, Yamaguchi N, Folch E, Boucher DH, Kent MS, Gangadharan SP, et al. Success and failure rates of tumor genotyping techniques in routine pathological samples with non-small-cell lung cancer. *Lung Cancer*. 2014 Apr;84(1):39–44.
8. Ganesh K, Massagué J. Targeting metastatic cancer. *Nat Med*. 2021;27(1):34–44.
9. Hudson TJ, Anderson W, Aretz A, Barker AD, Bell C, Bernabé RR, et al. International network of cancer genome projects. *Nature*. 2010;464(7291):993–8.
10. Campbell PJ, Getz G, Korbel JO, Stuart JM, Jennings JL, Stein LD, et al. Pan- cancer analysis of whole genomes. *Nature*. 2020;578(7793):82–93).
11. Mondelo-Macia P, García-González J, León-Mateos L, Castillo-García A, López-López R, Muinelo-Romay L, et al. Current Status and Future Perspectives of Liquid Biopsy in Small Cell Lung Cancer. *Biomedicines*. 2021 Jan 7;9(1):48.
12. Vidal J, Muinelo L, Dalmases A, Jones F, Edelstein D, Iglesias M, et al. Plasma CtDNA RAS Mutation Analysis for the Diagnosis and Treatment Monitoring of Metastatic Colorectal Cancer Patients. *Ann. Oncol.* **2017**, 28, 1325–1332.
13. García-Foncillas J, Tabernero J, Élez E, Aranda E, Benavides M, Camps, C, et al. Prospective Multicenter Real-World RAS Mutation Comparison between OncoBEAM-Based Liquid Biopsy and Tissue Analysis in Metastatic Colorectal Cancer. *Br. J. Cancer* **2018**, 119, 1464–1470.
14. Grasselli J, Elez E, Caratù G, Matito J, Santos C, Macarulla T, et al. Concordance of Blood- and Tumor-Based Detection of RAS Mutations to Guide Anti-EGFR Therapy in Metastatic Colorectal Cancer. *Ann. Oncol.* **2017**, 28, 1294–1301.
15. Dyba T, Randi G, Bray F, Martos C, Giusti F, Nicholson N, et al. The European cancer burden in 2020: Incidence and mortality estimates for 40 countries and 25 major cancers. *Eur J Cancer*. 2021 Nov; 157:308-347.
16. Lewis DR, Check DP, Caporaso NE, Travis WD, Devesa SS. US lung cancer trends by histologic type. *Cancer*. (2014) 120(18):2883–92. doi: 10.1002/cncr. 28749.

17. Ding L, Getz G, Wheeler DA, Mardis ER, McLellan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature*. (2008) 455 (7216):1069–75.
18. Howlader N, Forjaz G, Mooradian MJ, Meza R, Kong CY, Cronin KA, et al. The effect of advances in lung-cancer treatment on population mortality. *New Engl J Med* (2020) 383(7):640–9.
19. Barlesi F, Mazieres J, Merlio JP, Debieuvre D, Mosser J, Lena H, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French Cooperative Thoracic Intergroup (IFCT). *Lancet* 2016; 387:1415– 1426.
20. Ettinger DS, Wood DE, Aisner DL, Akerley W, Bauman JR, Bharat A, et al. NCCN Guidelines® Insights: Non-Small Cell Lung Cancer, Version 2.2023. *J Natl Compr Canc Netw*. 2023 Apr;21(4):340-350.
21. Hendriks LE, Kerr KM, Menis J, Mok TS, Nestle U, Passaro A, et al. ESMO Guidelines Committee. Electronic address: clinicalguidelines@esmo.org. Non-oncogene-addicted metastatic non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2023 Apr;34(4):358-376.
22. Singh N, Temin S, Baker S Jr, Blanchard E, Brahmer JR, Celano P, et al. Therapy for Stage IV Non-Small-Cell Lung Cancer Without Driver Alterations: ASCO Living Guideline. *J Clin Oncol*. 2022 Oct 1;40(28):3323-3343.
23. García-Campelo R, Sullivan I, Arriola E, Insa A, Juan Vidal O, Cruz-Castellanos P, et al. SEOM-GECP Clinical guidelines for diagnosis, treatment and follow-up of small-cell lung cancer (SCLC) (2022). *Clin Transl Oncol*. 2023 Sep;25(9):2679-2691.
24. Waqar SN, Samson PP, Robinson CG, Bradley J, Devarakonda S, Du L, et al. Non-small-cell lung cancer with brain metastasis at presentation. *Clin Lung Cancer*. (2018) 19(4):e373–9.
25. Barnholtz-Stoan JS, Sloan AE, Davis FG, Vigneau FD, Lai P, Sawaya RE. Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the metropolitan detroit cancer surveillance system. *J Clin Oncol* (2004) 22(14):2865– 72.
26. Dumoulin DW, Dingemans AC, Aerts JGJV, Remon J, DeRuyscher DKM, Hendriks LEL. Immunotherapy in small cell lung cancer: One step at a time: A narrative review. *Trans Lung Cancer Res* (2021) 10(6):2970–87.
27. Sul J, Posner JB. Brain metastases: Epidemiology and pathophysiology. *Cancer Treat Res* (2007) 136:1–21.
28. Riihimäki M, Hemminki A, Fallah M, Thomsen H, Sundquist K, Sundquist J, et al. Metastatic sites and survival in lungcancer. *Lung Cancer (Amsterdam Netherlands)*. (2014) 86(1):78–84.
29. Ernani V, Stinchcombe TE. Management of brain metastases in Non-Small-cell lung cancer. *J Oncol Practice*. (2019) 15(11):563–70.
30. Suh JH, Kotecha R, Chao ST, Ahluwalia MS, Sahgal A, Chang EL. Current approaches to the management of brain metastases. *Nat Rev Clin Oncol* (2020) 17 (5):279–99.
31. Ramalingam SS, Vansteenkiste J, Planchard D, Cho BC, Gray JE, Ohe Y, et al. Overall survival with osimertinib in untreated, EGFR-mutated advanced NSCLC. *New Engl J Med* (2020) 382(1):41–50.
32. Peters S, Camidge DR, Shaw AT, Gadgeel S, Ahn JS, Kim DW, et al. Alectinib versus crizotinib in untreated ALK-positive Non-Small-cell lung cancer. *New Engl J Med* (2017) 377(9):829–38.

33. Camidge DR, Kim HR, Ahn M, Yang JC, Han JY, Lee JS, et al. Brigatinib versus crizotinib in ALK-positive Non-Small-cell lung cancer. *New Engl J Med* (2018) 379 (21):2027–39.
34. Solomon BJ, Bauer TM, Mok TSK, Liu G, Mazieres J, de Marinis F, et al. Efficacy and safety of first-line lorlatinib versus crizotinib in patients with advanced, ALK- positive non-small-cell lung cancer: Updated analysis of data from the phase 3, randomised, open-label CROWN study. *Lancet Respir Med* (2023) 11(4):354–66.
35. Sperduto PW, Kased N, Roberge D, Xu Z, Shanley R, Luo X, et al. Summary report on the graded prognostic assessment: An accurate and facile diagnosis-specific tool to estimate survival for patients with brain metastases. *J Clin Oncol* (2012) 30 (4):419–25
36. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Patterns and Trends in Colorectal Cancer Incidence and Mortality. *Gut* **2017**, 66, 683–691)
37. Dekker E, Tanis PJ, Vleugels JLA, Kasi PM, Wallace MB. Colorectal Cancer. *Lancet* **2019**, 394, 1467–1480)
38. Van Cutsem E, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus Irinotecan, Fluorouracil, and Leucovorin as First-Line Treatment for Metastatic Colorectal Cancer: Updated Analysis of Overall Survival according to Tumor KRAS and BRAF Mutation Status. *J. Clin. Oncol.* **2011**, 29, 2011–2019.
39. Bokemeyer C, Bondarenko I, Hartmann JT, De Braud F, Schuch G, Zube A, et al. Efficacy According to Biomarker Status of Cetuximab plus FOLFOX-4 as First-Line Treatment for Metastatic Colorectal Cancer: The OPUS Study. *Ann. Oncol.* **2011**, 22, 1535–1546.
40. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Final Results from PRIME: Randomized Phase III Study of Panitumumab with FOLFOX4 for First-Line Treatment of Metastatic Colorectal Cancer. *Ann. Oncol.* **2014**, 25, 1346–1355.
41. Venook AP, Niedzwiecki D, Innocenti F, Fruth B, Green C, O’Neil BH, et al. Impact of Primary (1o) Tumor Location on Overall Survival (OS) and Progression-Free Survival (PFS) in Patients (Pts) with Metastatic Colorectal Cancer (MCR): Analysis of CALGB/SWOG 80405 (Alliance). *J. Clin. Oncol.* **2016**, 34, 3504.
42. Venook AP. Right-Sided vs Left-Sided Colorectal Cancer. *Clin. Adv. Hematol. Oncol.* **2017**, 15, 22–24.
43. Tejpar S, Stintzing S, Ciardiello F, Tabernero J, Van Cutsem E, Beier F, et al. Prognostic and Predictive Relevance of Primary Tumor Location in Patients with RAS Wild-Type Metastatic Colorectal Cancer: Retrospective Analyses of the CRYSTAL and FIRE-3 Trials. *JAMA Oncol.* **2017**, 3, 194–201.
44. Bahl A, Talwar V, Sirohi B, Mehta P, Arya D, Shrivastava G, et al. Primary Tumor Location as a Prognostic and Predictive Marker in Metastatic Colorectal Cancer (MCR). *Front. Oncol.* **2020**, 10, 964.
45. Benavides M, Díaz-Rubio E, Carrato A, Abad A, Guillén C, García-Alfonso P, et al. Tumour Location and Efficacy of First-Line EGFR Inhibitors in KRAS/RAS Wild-Type Metastatic Colorectal Cancer: Retrospective Analyses of Two Phase II Randomised Spanish TTD Trials. *ESMO Open.* **2019**, 4, e000599.
46. Arnold D, Lueza B, Douillard JY, Peeters M, Lenz HJ, Venook A, et al. Prognostic and Predictive Value of Primary Tumour Side in Patients with RAS Wild-Type Metastatic Colorectal Cancer Treated with Chemotherapy and EGFR Directed Antibodies in Six Randomized Trials. *Ann. Oncol.* **2017**, 28, 1713–1729).

47. Serebriiskii IG, Connelly C, Frampton G, Newberg J, Cooke M, Miller V, et al. Comprehensive Characterization of RAS Mutations in Colon and Rectal Cancers in Old and Young Patients. *Nat. Commun.* **2019**, 10, 3722.
48. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of KRAS Mutations and Acquired Resistance to Anti-EGFR Therapy in Colorectal Cancer. *Nature* **2012**, 486, 532–536.
49. Benson AB, Venook AP, Al-Hawary MM, Arain MA, Chen YJ, Ciombor KK, et al. Colon Cancer, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw.* 2021 Mar 2;19(3):329-359.
50. Cervantes A, Adam R, Roselló S, Arnold D, Normanno N, Taïeb J, et al. ESMO Guidelines Committee. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol.* 2023 Jan;34(1):10-32.
51. Morris VK, Kennedy EB, Baxter NN, Benson AB 3rd, Cercek A, Cho M, et al. Treatment of Metastatic Colorectal Cancer: ASCO Guideline. *J Clin Oncol.* 2023 Jan 20;41(3):678-700.
52. Fernández Montes A, Alonso V, Aranda E, Élez E, García Alfonso P, Grávalos C, et al. SEOM-GEMCAD-TTD clinical guidelines for the systemic treatment of metastatic colorectal cancer (2022). *Clin Transl Oncol.* 2023 Sep;25(9):2718-2731.
53. Bayle A, Basile D, Garinet S, Rance B, Laurent-Puig P, Blons H, et al. Next-Generation Sequencing Targeted Panel in Routine Care for Metastatic Colon Cancers. *Cancers* **2021**, 13, 5750.
54. Wang F, Huang YS, Wu HX, Wang ZX, Jin Y, Yao YC, et al. Genomic Temporal Heterogeneity of Circulating Tumour DNA in Unresectable Metastatic Colorectal Cancer under First-Line Treatment. *Gut* **2022**, 71, 1340–1349.
55. Thierry AR, El Messaoudi S, Mollevi C, Raoul JL, Guimbaud R, Pezet D, et al. Clinical Utility of Circulating DNA Analysis for Rapid Detection of Actionable Mutations to Select Metastatic Colorectal Patients for Anti-EGFR Treatment. *Ann. Oncol.* **2017**, 28, 2149–2159.
56. Garlan F, Laurent-Puig P, Sefrioui D, Siauve N, Didelot A, Sarafan-Vasseur N, et al. Early Evaluation of Circulating Tumor DNA as Marker of Therapeutic Efficacy in Metastatic Colorectal Cancer Patients (PLACOL Study). *Clin. Cancer Res.* **2017**, 23, 5416–5425.
57. García-Alfonso P, García-Carbonero R, García-Foncillas J, Pérez-Segura P, Salazar R, Vera R, et al. Update of the Recommendations for the Determination of Biomarkers in Colorectal Carcinoma: National Consensus of the Spanish Society of Medical Oncology and the Spanish Society of Pathology. *Clin. Transl. Oncol.* **2020**, 22, 1976–1991.
58. García-Foncillas J, Alba E, Aranda E, Diaz-Rubio E, López-López R, Tabernero J, et al. Incorporating BEAMing Technology as a Liquid Biopsy into Clinical Practice for the Management of Colorectal Cancer Patients: An Expert Taskforce Review. *Ann. Oncol.* **2017**, 28, 2943–2949.
59. Bachet JB, Bouché O, Taieb J, Dubreuil O, Garcia ML, Meurisse A, et al. RAS Mutation Analysis in Circulating Tumor DNA from Patients with Metastatic Colorectal Cancer: The AGE0 RASANC Prospective Multicenter Study. *Ann. Oncol.* **2018**, 29, 1211–1219.
60. Vitiello PP, De Falco V, Giunta EF, Ciardiello D, Cardone C, Vitale P, et al. Clinical Practice Use of Liquid Biopsy to Identify RAS/BRAF Mutations in Patients with Metastatic Colorectal Cancer (MCRC): A Single Institution Experience. *Cancers* **2019**, 11, 1504.

61. Hamfjord J, Guren TK, Glimelius B, Sorbye H, Pfeiffer P, Dajani O, et al. Clinicopathological Factors Associated with Tumour-Specific Mutation Detection in Plasma of Patients with RAS-Mutated or BRAF-Mutated Metastatic Colorectal Cancer. *Int. J. Cancer* **2021**, 149, 1385–1397.
62. Manca P, Corallo S, Busico A, Lonardi S, Corti F, Antoniotti C, et al. The Added Value of Baseline Circulating Tumor DNA Profiling in Patients with Molecularly Hyperselected, Left-Sided Metastatic Colorectal Cancer. *Clin. Cancer Res.* **2021**, 27, 2505–2514)
63. Bando H, Kagawa Y, Kato T, Akagi K, Denda T, Nishina T, et al. A Multicentre, Prospective Study of Plasma Circulating Tumour DNA Test for Detecting RAS Mutation in Patients with Metastatic Colorectal Cancer. *Br. J. Cancer* **2019**, 120,982–986.
64. Chabon JJ, Hamilton EG, Kurtz DM, Esfahani MS, Moding EJ, Stehr H et al. Integrating genomic features for non-invasive early lung cancer detection. *Nature.* **2020**, 580:245–251
65. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer.* **2017**, 17:223–238.
66. Hu Y, Ulrich BC, Supplee J, Kuang Y, Lizotte PH, Feeney NB, et al. False-positive plasma genotyping due to clonal hematopoiesis. *Clin Cancer Res.* **2018**, 24:4437–4443.
67. Chan HT, Nagayama S, Chin YM, Otaki M, Hayashi R, Kiyotani K, et al. Clinical significance of clonal hematopoiesis in the interpretation of blood liquid biopsy. *Mol Oncol.* **2020**, 14:1719–1730.
68. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. *N Engl J Med.* **2020**, 379:1754–1765.
69. Hofman P, Heeke S, Alix-Panabières C, Pantel K. Liquid biopsy in the era of immuno-oncology: is it ready for prime- time use for cancer patients? *Ann Oncol.* **2019**, 30:1448–1459.
70. Cortés-Hernández LE, Eslami-S Z, Pantel K, Alix-Panabières C. Molecular and functional characterization of circulating tumor cells: from discovery to clinical application. *Clin Chem.* **2019**, 66:97–104.
71. Rossi G, Russo A, Tagliamento M, Tuzi A, Nigro O, Vallome G, et al. Precision medicine for NSCLC in the era of immunotherapy: new biomarkers to select the most suitable treatment or the most suitable patient. *Cancers.* **2020**, 12:1125.
72. Reuben A, Zhang J, Chiou SH, Gittelman RM, Li J, Lee WC, et al. Comprehensive T cell repertoire characterization of non-small cell lung cancer. *Nat Commun.* **2020**, 11:603.
73. Miyauchi E, Matsuda T, Kiyotani K, Low SK, Hsu YW, Tsukita Y, et al. Significant differences in T cell receptor repertoires in lung adenocarcinomas with and without epidermal growth factor receptor mutations. *Cancer Sci.* **2019**, 110:867–874.
74. Vidal J, Muinelo L, Dalmases A, Jones F, Edelstein D, Iglesias M, et al. Plasma CtDNA RAS Mutation Analysis for the Diagnosis and Treatment Monitoring of Metastatic Colorectal Cancer Patients. *Ann. Oncol.* **2017**, 28, 1325–1332.
75. Montagut C, Vidal J. Liquid Biopsy for Precision Adjuvant Chemotherapy in Colon Cancer. *N. Engl. J. Med.* **2022**, 386, 2330–2331.
76. Grasselli J, Elez E, Caratù G, Matito J, Santos C, Macarulla T, et al. Concordance of Blood- and Tumor-Based Detection of RAS Mutations to Guide Anti-EGFR Therapy in Metastatic Colorectal Cancer. *Ann. Oncol.* **2017**, 28, 1294–1301.

77. Kagawa Y, Elez E, García-Foncillas J, Bando H, Taniguchi H, Vivancos A, et al. Combined Analysis of Concordance between Liquid and Tumor Tissue Biopsies for RAS Mutations in Colorectal Cancer with a Single Metastasis Site: The METABEAM Study. *Clin. Cancer Res.* **2021**, 27, 2515–2522.
78. Bando H, Nakamura Y, Taniguchi H, Shiozawa M, Yasui H, Esaki T, et al. Effects of Metastatic Sites on Circulating Tumor DNA in Patients with Metastatic Colorectal Cancer. *JCO Precis. Oncol.* **2022**, 6, e2100535.
79. Heitzer E, Auinger L, Speicher MR. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends Mol. Med.* **2020**, 26, 519–528.
80. Lo YMD, Han DSC, Jiang P, Chiu RWK. Epigenetics, Fragmentomics, and Topology of Cell-Free DNA in Liquid Biopsies. *Science* **2021**, 372, eaaw3616.
81. Uesato Y, Sasahira N, Ozaka M, Sasaki T, Takatsuki M, Zembutsu H. Evaluation of Circulating Tumor DNA as a Biomarker in Pancreatic Cancer with Liver Metastasis. *PLoS ONE* **2020**, 15, e0235623.
82. Reichert ZR, Morgan TM, Li G, Castellanos E, Snow T, Dall’Olio FG, et al. Prognostic Value of Plasma Circulating Tumor DNA Fraction across Four Common Cancer Types: A Real-World Outcomes Study. *Ann. Oncol.* **2023**, 34, 111–120.
83. Schmiegel W, Scott RJ, Dooley S, Lewis W, Meldrum CJ, Pockney P, et al. Blood-Based Detection of RAS Mutations to Guide Anti-EGFR Therapy in Colorectal Cancer Patients: Concordance of Results from Circulating Tumor DNA and Tissue-Based RAS Testing. *Mol. Oncol.* **2017**, 11, 208–219.
84. Hugen N, Nagtegaal ID. Distinct Metastatic Patterns in Colorectal Cancer Patients Based on Primary Tumour Location. *Eur. J. Cancer* **2017**, 75, 3–4.
85. Riihimäki M, Hemminki A, Sundquist J, Hemminki K. Patterns of Metastasis in Colon and Rectal Cancer. *Sci. Rep.* **2016**, 6, 29765.
86. Weiss JM, Pfau PR, O’Connor ES, King J, LoConte N, Kennedy G, et al. Mortality by Stage for Right- versus Left-Sided Colon Cancer: Analysis of Surveillance, Epidemiology, and End Results-Medicare Data. *J. Clin. Oncol.* **2011**, 29, 4401–4409.
87. Tie J, Wang Y, Cohen J, Li L, Hong W, Christie M, et al. Circulating Tumor DNA Dynamics and Recurrence Risk in Patients Undergoing Curative Intent Resection of Colorectal Cancer Liver Metastases: A Prospective Cohort Study. *PLoS Med.* **2021**, 18, e1003620.
88. Kotani D, Oki E, Nakamura Y, Yukami H, Mishima S, Bando H, et al. Molecular Residual Disease and Efficacy of Adjuvant Chemotherapy in Patients with Colorectal Cancer. *Nat. Med.* **2023**, 29, 127–134.

ANEXOS

ANEXO 1: ASPECTOS ÉTICOS

DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Carlos Rodríguez Moreno, Presidente/a del Comité de Ética de la Investigación de Santiago-Lugo

CERTIFICA:

Que este Comité evaluó en su reunión del día 16/12/2015 el estudio:

Título: Valor de la Tecnología BEAMing para el análisis de mutaciones del gen RAS en biopsia líquida Promotor: Rafael López López.

Tipo de estudio: Otros

Versión: Versión 4 de diciembre 2015.

Código del Promotor: RAS-Bliq-001 Código de Registro: 2015/746

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos

legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y el la Circular no 07 / 2004, investigaciones clínicas con productos sanitarios.

- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del Comité.

Emite un INFORME FAVORABLE para la realización del estudio por el/la investigador/a del centro:

Centros Investigadores Principales:

- C.H. Universitario de Santiago. Laura Muinelo Romy, Rafael López López

Sin perjuicio al dictamen procedente emitido, en el apartado ¿cómo se protegerá la confidencialidad de mis datos? Debe corregirse la siguiente errata: en la última frase de la penúltima página de la hoja de información al paciente, se indica que “el lugar de realización de los análisis será la Unidad de Análisis de Biopsia Líquida del Complejo Hospitalario de Santiago de Vigo”. Debe indicarse correctamente el centro.

INFORMACIÓN Y CONSENTIMIENTO INFORMADO - PACIENTES

TÍTULO DEL ESTUDIO: Identificación de marcadores de diagnóstico, pronóstico y seguimiento mediante el análisis de biopsia líquida en pacientes con cáncer

PROMOTOR: Rafael López López

NOMBRE DE INVESTIGADORES PRINCIPALES: Rafael López López, Roberto Díaz Peña, Laura Muinelo Romay

CENTRO: Complejo Hospitalario Universitario de Santiago de Compostela **VERSIÓN UNO**
(31 de octubre de 2017)

Este documento tiene por objeto informarle y solicitar su consentimiento escrito antes de comenzar un estudio de investigación en el que se le invita a participar; y que se llevará a cabo por los profesionales del Complejo Hospitalario Universitario de Santiago de Compostela (CHUS). Este estudio ha sido aprobado por el Comité de Ética de la Investigación con medicamentos de Galicia.

Si decide participar en el mismo, recibirá información personalizada del investigador. Antes, debe Usted leer este documento y hacer todas las preguntas que precise para comprender los detalles del mismo. Si así lo desea, puede llevarse el documento, consultarlo con otras personas y tomarse el tiempo necesario para decidir si se participa o no.

La participación en este estudio es completamente voluntaria. Usted puede decidir no participar o, de hacerlo, cambiar de idea retirando su consentimiento en cualquier momento sin obligación de dar explicaciones. Esta decisión no afectará a la relación con su médico ni a la asistencia sanitaria a la que usted tiene derecho.

¿Cuál es el objetivo del estudio?

El objetivo general del estudio es encontrar nuevas formas de analizar tumores como el suyo a través de una muestra de sangre, de obtención menos invasiva que la de tejido. De esta forma esperamos comprender mejor los cambios que puedan presentarse en el tumor en respuesta al tratamiento, y que esto finalmente pueda ayudar a los oncólogos a proponer un tratamiento más dirigido o personalizado. Además, de este conocimiento pueden derivarse biomarcadores tanto con utilidad diagnóstica y pronóstica, como para monitorizar la evolución de la enfermedad.

¿Por qué me ofrecen participar?

Se le ofrece participar en el estudio porque ha sido diagnosticado/a de un cáncer, con el objeto de mejorar la identificación de moléculas (biomarcadores) que sean de utilidad a la hora de diagnosticar y escoger el mejor tratamiento en tumores como el suyo. En concreto, en este estudio en el que va a participar se pretende caracterizar el tumor durante el tratamiento para reconocer de forma temprana si los pacientes responden al mismo y también para entender mejor por qué dejan de responder a él.

¿En qué consiste mi participación?

Coincidiendo con una analítica de rutina asistencial se le extraerán tres tubos adicionales de sangre con los que analizar biomarcadores en biopsia líquida. A mayores, si por rutina hospitalaria el facultativo realiza una biopsia tumoral del tejido de interés, esa muestra podrá ser utilizada con el objetivo de valorar la concordancia con las diferentes alteraciones genómicas descritas mediante biopsia líquida. Su tratamiento será el mismo que se le administre de forma estándar al resto de pacientes que tienen su mismo tumor. No se le hará ningún seguimiento especial ni se le pedirán más datos de los necesarios para hacer el seguimiento habitual de su enfermedad, aunque sí se consultarán los datos de su historia médica. El investigador puede decidir finalizar el estudio antes de lo previsto o interrumpir su participación. En todo caso se le informará de los motivos de su retirada.

¿Qué molestias o inconvenientes puede generarle la participación en el estudio?

Su participación en el estudio no implicará ninguna molestia adicional a mayores de la derivada de la propia extracción de sangre y de las de la práctica asistencial habitual.

¿Cuáles son los beneficios esperables y los riesgos potenciales de este estudio?

Usted no obtendrá ningún beneficio por permitir el análisis de sus muestras biológicas. La cesión de muestras para investigación es voluntaria y altruista. Su único beneficio es el que corresponde al avance de la medicina en beneficio de la sociedad, y el saber que ha colaborado en este proceso. Las muestras no podrán ser objeto directo de actividades con ánimo de lucro. No obstante, la información generada a partir de los estudios realizados sobre su muestra podría ser fuente de beneficios comerciales. En tal caso, están previstos mecanismos para que estos beneficios reviertan en la salud de la población, aunque no de forma individual en el donante.

¿Recibiré la información que se obtenga del estudio?

Si usted lo desea, se le facilitará un resumen de los resultados del estudio. También podrá recibir los resultados de las pruebas que se realicen con sus muestras si así lo solicita dirigiéndose al investigador. Estos resultados pueden no tener aplicación clínica ni una interpretación clara, por lo que, si quiere disponer de ellos, debería ser comentado previamente con el médico del estudio.

¿Se publicarán los resultados del estudio?

Los resultados de este estudio serán remitidos a publicaciones científicas para su difusión, pero no se transmitirá ningún dato que pueda llevar la identificación de los pacientes.

¿Como se protegerá la confidencialidad de mis datos y mis muestras?

Los datos personales que se recojan sobre Usted, únicamente serán tratados y accedidos por el personal médico y sanitario que realizará el estudio. Dicha información será confidencial y procesada de acuerdo con la Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal y la legislación vigente. En todo momento, usted podrá acceder a sus datos, corregirlos o cancelarlos, solicitándoselo al investigador del estudio.

El equipo investigador y las autoridades sanitarias, que tienen el deber de guardar la confidencialidad, tendrán acceso a los datos recogidos en el estudio. Se podrá transmitir la información a terceros siempre que no pueda ser identificado/a el/la paciente. En el caso de que alguna información sea transmitida a otros países, se hará con el nivel de protección de datos equivalente, como mínimo, a lo exigido por la normativa de nuestro país. Sus datos y muestras biológicas serán recogidos y conservados hasta acabar el estudio de modo codificado, que quiere decir que poseen un código con el cual sólo el equipo investigador podrá conocer a quien corresponden.

Los responsables de la custodia de los datos y las muestras serán los investigadores principales del estudio Rafael López López, Roberto Díaz Peña y Laura Muínelo, y el lugar de realización de los análisis será la Unidad de Análisis de Biopsia Líquida del Complejo Hospitalario de Santiago de Compostela.

Hasta la realización de los análisis correspondientes, las muestras serán custodiadas en la Unidad de Análisis de Biopsia Líquida del CHUS. Al terminar el estudio sus muestras se guardarán codificadas en la colección de muestras con fines de investigación registrada en el registro de biobancos con el número C0003811 y cuyo titular es el Dr. Rafael López López.

Usted podrá solicitar la destrucción o anonimización de sus muestras en cualquier momento dirigiéndose al investigador principal. Tiene que saber que esto no será de aplicación a los datos resultantes de los análisis que ya fueran efectuados

¿Existen intereses económicos en este estudio?

Usted no será retribuido por participar. Es posible que de los resultados del estudio se deriven productos comerciales o patentes. En este caso, usted no podrá participar en los beneficios económicos originados. El investigador no recibirá retribución específica por su dedicación al estudio.

¿Cómo contactar con el equipo investigador del estudio?

Usted puede contactar con el equipo investigador en el teléfono 981955451 o dirección de correo electrónico roberto.diaz.pena@sergas.es, laura.muínelo.romay@sergas.es y rafael.lopez.lopez@sergas.es.

Muchas gracias por su colaboración.

CONSENTIMIENTO INFORMADO

Yo, _____

He leído la hoja de información al paciente del estudio arriba mencionado que se me entregó, pudiendo hablar con el investigador..... y hacer todas las preguntas sobre el estudio necesarias.

Comprendo que mi participación es voluntaria, y que puedo retirarme del estudio cuando quiera, sin tener que dar explicaciones y sin que esto repercuta en mis cuidados médicos.

Accedo a que se utilicen mis datos en las condiciones detalladas en el presente documento. Presto libremente mi conformidad para participar en este estudio.

A continuación indique si da su consentimiento o no:

___ El participante autoriza que se guarden sus muestras en la colección.

___ El participante no lo autoriza y desea que se destruyan.

___ El participante autoriza a que se guarden las muestras anonimizadas.

Fdo.: la/el participante,

Fdo.: El investigador/a que solicita el consentimiento

Nombre y apellidos: _____ Nombre y apellidos: _____

Fecha:

Fecha:

ANEXO 2: LISTA DE PUBLICACIONES PRESENTADAS EN ESTA TESIS Y PERMISOS DE PUBLICACIÓN

A. Immunotherapy in nonsmall-cell lung cancer: current status and future prospects for liquid biopsy.

Brozos-Vázquez EM*, Díaz-Peña R*, García-González J, León-Mateos L, Mondelo-Macia P, Peña-Chilet M, López-López R.

*Corresponding authors.

Cancer Immunol Immunother. 2021 May;70(5):1177-1188.

Epub 2020 Oct 28.

PMID: 33113004.

Texto completo disponible: <https://doi.org/10.1007/s00262-020-02752-z>

Permission: Open access Creative Commons CC BY license; no special permission is required to reuse all or part of article published under that license, including figures and tables.

Journal Citation Reports (JCR) category rank (2020): Q1 (46/242): Oncology field.

Journal Impact factor (2020) – 6.968

Contribución de Brozos-Vázquez EM a este trabajo: La autora ha realizado la conceptualización de este artículo, metodología, investigación, redacción y borrador original, así como su revisión y edición para su visualización en la publicación final.

B. Immunotherapy in patients with brain metastasis: advances and challenges for the treatment and the application of circulating biomarkers.

Brozos-Vázquez EM*, Rodríguez-López C, Cortegoso-Mosquera A, López-Landrove S, Muínelo-Romay L, García-González J, López-López R*, León-Mateos L.

*Corresponding authors.

Front Immunol. 2023 Nov 3;14:1221113.

PMID: 38022574

PMCID: PMC10654987.

Texto completo disponible: doi: 10.3389/fimmu.2023.1221113.

Permission: Frontiers is fully compliant with open access mandates, by publishing its articles under the Creative Commons Attribution license (CC-BY); no special permission is required to reuse all or part of article published under that license, including figures and tables.

Journal Citation Reports (JCR) category rank (2022): Q1 (44/177): Immunology field.

Journal Impact factor (2020) – 7.3

Contribución de Brozos-Vázquez EM a este trabajo: La autora ha realizado la conceptualización de este artículo, metodología, investigación, redacción y borrador original, así como su revisión y edición para su visualización en la publicación final.

C. Clinicopathological Profiles Associated with Discordant *RAS* Mutational Status between Liquid and Tissue Biopsies in a Real-World Cohort of Metastatic Colorectal Cancer.

Brozos-Vázquez E, Lago-Lestón RM, Covela M, de la Cámara Gómez J, Fernández-Montes A, Candamio S, Vidal Y, Vázquez F, Abalo A, López R, Blanco C, Muínelo-Romay L, Ferreirós-Vidal I*, López-López R*.

*Corresponding authors.

Cancers (Basel). 2023 Jul 12;15(14):3578.

doi: 10.3390/cancers15143578.

PMID: 37509239

PMCID: PMC10377339.

Permission: Open access Creative Commons CC BY license; no special permission is required to reuse all or part of article published under that license, including figures and tables.

Journal Citation Reports (JCR) category rank (2022): Q1 (44/177): Oncology field.

Journal Impact factor (2022) – 5.2

Contribución de Brozos-Vázquez EM a este trabajo: La autora ha realizado la conceptualización de este artículo, recogida de datos clínicos, análisis formal, investigación, redacción y preparación del borrador original, así como su revisión y edición para su visualización en la publicación final.

D. Permiso de reproducción de imagen 1.

Referencia: Mondelo-Macía P, García-González J, León-Mateos L, Castillo-García A, López-López R, Muínelo-Romay L, et al. Current Status and Future Perspectives of Liquid Biopsy in Small Cell Lung Cancer. *Biomedicines*. 2021 Jan 7;9(1):48.

Permission: Open access Creative Commons CC BY license; no special permission is required to reuse all or part of article published under that license, including figures and tables.

La terapia dirigida y la inmunoterapia han revolucionado el tratamiento de los pacientes oncológicos en los últimos años. En tumores como el cáncer de pulmón y cáncer de colon cobra especial importancia el estudio molecular para adaptar el tratamiento en el marco de la Oncología de Precisión. La biopsia líquida se ha posicionado como una herramienta útil para el diagnóstico de alteraciones moleculares de una forma no invasiva, dinámica, rápida y fiable. Esta tesis investiga el valor de los biomarcadores en sangre para ofrecer a los pacientes tratamientos personalizados en situaciones clínicas complejas, con una mejor caracterización molecular y así mejorar su supervivencia y calidad de vida.