Integrative Multi-Omics Analysis for Biomarker Discovery in Cancer Immunotherapy

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Juan Luis Onieva Zafra

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AUTOR: Juan Luis Onieva Zafra

http://orcid.org/0000-0001-8976-8099

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Integrative Multi-Omics Analysis for Biomarker Discovery in Cancer Immunotherapy

Juan Luis Onieva Zafra

PhD Program in Biomedicine, Translational Research and New Health Technologies

Supervised by: Dra. Isabel Barragán Mallofret
Academic Tutor: Dr. Antonio Rueda Domínguez

Research Group:
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2025

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DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA PARA OBTENER EL TÍTULO DE DOCTOR

D. **Juan Luis Onieva Zafra**

Estudiante del programa de doctorado **Biomedicina, Investigación Traslacional y Nuevas Tecnologías en Salud de la Universidad de Málaga**, autor de la tesis, presentada para la obtención del título de doctor por la Universidad de Málaga, titulada: *Integrative Multi-Omics Analysis for Biomarker Discovery in Cancer Immunotherapy.*

Realizada bajo la tutorización de **Dr. Antonio Rueda Domínguez** y dirección de **Dra. Isabel Barragán Mallofret**.

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En Málaga, a 24 de noviembre de 2025.

Fdo.: Juan Luis Onieva Zafra Doctorando	Fdo.: Antonio Rueda Domínguez Tutor
Fdo.: Isabel Barragán Mallofret Directora de tesis	

Edificio Pabellón de Gobierno. Campus El Ejido. 29071
Tel.: 952 13 10 28 / 952 13 14 61 / 952 13 71 10
E-mail: doctorado@uma.es

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Por la presente los doctores, **D. Antonio Rueda Domínguez**, como tutor académico, Profesor del programa de Doctorado en Biomedicina, Investigación Traslacional, y Nuevas Tecnologías en Salud de la Facultad de Medicina de la Universidad de Málaga y **Dª. Isabel Barragán Mallofret**, como directora de tesis, Investigadora Principal Programa Nicolás Monardes, en el Instituto de Investigación Biomédica de Málaga (IBIMA) Plataforma BIONAND.

CERTIFICAN que **D. Juan Luis Onieva Zafra** ha obtenido y estudiado personalmente bajo nuestra dirección y supervisión los datos necesarios para la realización de su Tesis Doctoral, titulada: *"Integrative Multi-Omics Analysis for Biomarker Discovery in Cancer Immunotherapy"*, que consideramos tiene el contenido y rigor científico necesario para ser sometido al superior juicio de la Comisión que nombre la Universidad de Málaga para optar a grado de Doctor del programa de Doctorado en Biomedicina, Investigación Traslacional y Nuevas Tecnologías en Salud de la Facultad de Medicina. Y que las publicaciones que avalan la presentación de esta tesis en formato de compendio de publicaciones y cuyas referencias son:

1. **Onieva JL**, Xiao Q, Berciano-Guerrero MÁ, et al. High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade. Int J Mol Sci. 2022;23(16):9124. doi:10.3390/ijms23169124
2. **Onieva JL**, Pérez-Ruiz E, Vilkki V, et al. Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma. bioRxiv. 2025:670062. doi:10.1101/2025.08.13.670062
3. Oliver J, **Onieva JL (equally contributed)**, Garrido-Barros M, et al. Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade. Cancers (Basel). 2023;15(13):3357. doi:10.3390/cancers15133357
4. **Onieva JL**, Pérez-Ruiz E, Figueroa-Ortiz L, et al. Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC. medRxiv. 2025:25333961. doi:10.1101/2025.08.19.25333961

no han sido utilizadas en tesis anteriores ni en la Universidad de Málaga ni en otras Universidades. Y para que conste, en cumplimiento de las disposiciones vigentes, expedimos el presente certificado en Málaga el 24 de noviembre de 2025, firman

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Para la realización de esta Tesis Doctoral, el doctorando Juan Luis Onieva Zafra obtuvo la ayuda en régimen de concurrencia competitiva destinada a financiar la contratación predoctoral de personal investigador en formación por los agentes del Sistema Andaluz del Conocimiento (PREDOC_01691), convocadas por la Consejería de Transformación Económica, Industria, Conocimiento y Universidades de la Junta de Andalucía (BOJA NÚM. 111, DE 11 de junio de 2021).

Los proyectos de investigación en los que se enmarca esta tesis son los siguientes:

- Integración de ómicas para la terapia dirigida en inmunoterapia: hacia la biopsia líquida (PI18/01592). Financiado por el Instituto de Salud Carlos III.
- Nuevas estrategias para la identificación de marcadores de respuesta a inmunoterapia en pacientes con cáncer de pulmón no microcítico: carga mutacional y clonalidad de receptores T (C19048). Financiado por Fundación Bancaria Unicaja.
- Farmacogenómica de precisión aplicada al tratamiento de inmunoterapia en cáncer de pulmón y cabeza y cuello (ProyExcel_01002). Financiado por Consejería de Transformación económica, Industria, Conocimiento y Universidades de la Junta de Andalucía.
- Estudio longitudinal y prospectivo de variantes genéticas, epigenéticas y poblaciones celulares como marcadores de respuesta a inhibidores de punto de control inmune en melanoma (PI-0121-2020). Financiado por la Consejería de Salud y Familias de la Junta de Andalucía.

Además, con el objetivo de realizar una tesis internacional, se han realizado dos estancias en el extranjero. La primera tutorizada por el Dr. Nick Tobin en Karolinska Institutet (Estocolmo, Suecia) del 23 de septiembre de 2023 al 23 de diciembre de 2023 gracias a la bolsa de viaje contenida en la concesión PREDOC_01691. Y una segunda estancia dirigida por el Dr. Charles Imbusch en el German Cancer Center – DKFZ (Heidelberg, Alemania) del 1 de agosto de 2024 al 31 de octubre de 2024, gracias a la concesión del Plan Propio de movilidad del Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina con código MOV24-09.

Finalmente, considerando los méritos anteriormente expuestos y el trabajo de investigación desarrollado en los últimos años, esta Tesis Doctoral opta a la Mención de Doctorado Internacional otorgada por la Universidad de Málaga. Por este motivo, se presenta íntegramente redactada en inglés, incluyendo asimismo un resumen en castellano.

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IMMUNOMARK: UNO MÁS.

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La Biología Computacional busca desentrañar el lenguaje de las relaciones entre los componentes de la vida, pero para lograrlo es necesario apreciar plenamente y saber calibrar la magnitud de la complejidad que pretendemos entender.

Ana Conesa

Cada libro, cada tomo que ves, tiene alma. El alma de quien lo escribió, y el alma de quienes lo leyeron y vivieron y soñaron con él.

Carlos Ruíz Zafón

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Agradecimientos

Meraki, es una palabra griega sin traducción directa a otros idiomas. Tiene como significado hacer algo con creatividad, dedicación y pasión; dejando un pedazo de uno mismo. Y eso es lo que he hecho con esta tesis. Pero además, también hay un pedacito de todas aquellas personas que han estado a mi lado, durante todo el proceso o parte de él. Quiero empezar diciendo GRACIAS a todas ellas, aparezca su nombre o no en esta sección.

La primera dedicatoria es para ellos y ellas: los pacientes de IMMUNOMARK. En mis análisis únicamente veía variables de muestras con un código asignado IMK-XXX. Detrás de esa triple X ha habido un paciente que ha aceptado contribuir a la investigación científica. Que le ha tocado una situación complicada teniendo que hacer frente a una enfermedad. Esta tesis es mi granito de arena en su ayuda. Ellos hacen que cada esfuerzo merezca la pena.

Durante estos 4 años de doctorado he podido realizar mi trabajo en unas condiciones inmejorables en la Unidad de Oncología Médica del Hospital Virgen de la Victoria, así como en el Instituto de Investigación Biomédica de Málaga – Plataforma BIONAND. Mi directora de tesis, la Dra. Isabel Barragán, y tutor de tesis, el Dr. Antonio Rueda, han sabido guiarme académica y científicamente, brindándome a lo largo de mi doctorado oportunidades muy enriquecedoras. Han sido mis referentes profesionales mostrándome el camino para tener pasión por la ciencia y luchar ante las adversidades inherentes a una carrera científica en biomedicina. También, al Dr. Emilio Alba, director de la unidad, por confiar en mí y brindarme la oportunidad de pertenecer a una unidad con tanto potencial. Tras ellos, se encuentra el "Despachito". Mi equipo de laboratorio. En 2019, junto a Isabel, conocí al Dr. Javier Oliver, al que puedo denominar sin lugar a dudas mi otro padrino científico. Sus consejos me han hecho llegar a donde estoy hoy. No puedo detenerme en cada miembro de mi equipo, pero tampoco puedo saltarme los abrazos revitalizadores de Laura, la serenidad y consejos musicales de Bea; compañeros que se embarcaron en otras aventuras como Jaime, Patricia, María, Marina y Anabel, o los que se incorporaron durante esta etapa como Andrea, Alberto y Alba, a la que agradezco enormemente su ayuda en lo que ha sido la recta final de esta etapa. También a personas increíbles que he descubierto este año de otros grupos, como Lucía. Además, todo el equipo clínico que se ha involucrado de manera incesante como Miguel Ángel Berciano, Manuel Cobo, Manuel Lozano, Alexandra Cantero y José Carlos Benítez. De forma especial, me gustaría destacar a Elisabeth Pérez por implicarse tanto a

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Con todo mi agradecimiento y emoción, cierro esta etapa con un gracias hacia quienes la hicieron posible.

Juanlu

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Resumen en español

Introducción

1. La inmunoterapia y el reto de predecir respuesta al tratamiento

La inmunoterapia ha transformado el abordaje terapéutico de diversos tipos de cáncer, estableciéndose como un nuevo estándar en el tratamiento de tumores avanzados, especialmente en melanoma y cáncer de pulmón no microcítico (*non-small cell lung cancer*, NSCLC; por sus siglas en inglés). Dentro de esta revolución, los inhibidores de puntos de control inmunitario (*immune checkpoint inhibitors*, ICIs) han demostrado un impacto clínico notable. Sin embargo, a pesar de los resultados prometedores, una proporción considerable de pacientes no responde al tratamiento, aproximadamente entre el 60 % y el 70 %, lo que evidencia la existencia de mecanismos de resistencia a esta terapia. Esta limitación pone de manifiesto la necesidad urgente de identificar biomarcadores predictivos que permitan seleccionar a los pacientes con mayor probabilidad de beneficiarse de los ICIs, optimizando la eficacia y reduciendo tanto los efectos adversos como los costes asociados.

Además de los ICIs, la inmunoterapia abarca otras estrategias terapéuticas: las terapias con virus oncolíticos (virus modificados genéticamente para infectar y lisar selectivamente células tumorales), las vacunas terapéuticas contra el cáncer (que amplifican la respuesta inmune frente a antígenos tumorales específicos), las terapias basadas en citocinas (que promueven la activación y proliferación de células inmunes), y la transferencia adoptiva de células (que consiste en expandir *ex vivo* y reinfundir células inmunes con capacidad antitumoral). No obstante, el presente estudio se centra en el uso de ICIs.

Los tumores desarrollan mecanismos de evasión inmunitaria que incluyen la modulación de vías de señalización, lo que les permite limitar el reconocimiento y la eliminación por parte del sistema inmune. Los ICIs actúan bloqueando estas rutas moleculares que regulan la tolerancia inmunológica, restaurando la capacidad del sistema inmune para reconocer y eliminar células tumorales. Los descubrimientos de James P. Allison y Tasuku Honjo sobre los ejes CTLA-4 y PD-1, que les valieron el Premio Nobel de Medicina en 2018, sentaron las bases del desarrollo de este tipo de fármacos. CTLA-4 es un receptor co-inhibidor expresado

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principalmente en células T activadas y células T reguladoras, mientras que PD-1 es otro receptor inhibidor presente en células T activadas, cuya inhibición permite reactivar células T exhaustas y potenciar su actividad citotóxica frente a las células tumorales. A partir de estos hallazgos, se han desarrollado anticuerpos monoclonales dirigidos contra CTLA-4, PD-1 y su ligando más relevante respecto al mecanismo de acción antitumoral PD-L1.

2. Cáncer: Melanoma y cáncer de pulmón no microcítico

El cáncer comprende un grupo heterogéneo de enfermedades caracterizadas por una proliferación celular descontrolada, con capacidad de invasión local y diseminación a tejidos distantes. Es la principal causa de morbilidad y mortalidad a nivel mundial, lo que lo convierte en una prioridad sanitaria global.

En particular, el melanoma se origina a partir de la proliferación maligna de los melanocitos. Se reconocen distintos subtipos, entre ellos: cutáneo, ocular, de mucosas y de origen desconocido. Por otro lado, el NSCLC representa el subtipo más frecuente dentro del cáncer de pulmón.

Según datos globales, nacionales (España) y regionales (Andalucía) correspondientes a 2022, se registraron aproximadamente 2,5 millones, 34.506 y 5.000 nuevos casos de cáncer de pulmón, respectivamente, mientras que los nuevos diagnósticos de melanoma fueron de 330.000, 10.000 y 1.050. En cuanto a la mortalidad, el cáncer de pulmón causó 1,8 millones de muertes a nivel mundial, 22.500 en España y 3.800 en Andalucía. Para el melanoma, las cifras de mortalidad fueron de 60.000, 1.100 y 200, respectivamente. Cabe destacar que la mayoría de los casos de cáncer de pulmón se diagnosticaron en estadios avanzados, lo que limita considerablemente las opciones terapéuticas y el pronóstico. En contraste, en el melanoma, una proporción importante de los casos se detecta en fases más tempranas, lo que permite aplicar tratamientos con intención curativa en una mayor parte de los pacientes.

En este estudio nos centraremos en melanoma metastásico y NSCLC avanzado. El melanoma metastásico corresponde al estadio IV y se caracteriza por la propagación de las células tumorales desde el tumor primario hacia órganos distantes. Esta diseminación, que ocurre por vías linfática o sanguínea, refleja la pérdida del control local del tumor y un comportamiento más agresivo. Por otro lado, el NSCLC avanzado incluye tanto la enfermedad metastásica (cuando el cáncer se ha diseminado a otros órganos) como la enfermedad localmente avanzada. Esta última corresponde al estadio III e implica que el tumor ha crecido y se ha extendido, afectando ganglios linfáticos cercanos o estructuras adyacentes, sin alcanzar aún otros órganos distantes. Ambos escenarios representan desafíos clínicos importantes y constituyen un marco relevante para el estudio de estrategias terapéuticas sistémicas (tratamientos que alcanzan y actúan sobre células cancerosas en todo el organismo, no solo en el tumor primario) como los ICIs.

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El desarrollo de inmunoterapia basada en ICIs ha supuesto un avance significativo en el tratamiento de ambos tipos de cáncer. Ensayos clínicos como el KEYNOTE-006, KEYNOTE-407 y KEYNOTE-024 han demostrado su eficacia, particularmente en estadios avanzados. No obstante, solo un subconjunto de pacientes experimenta una respuesta duradera, lo que refuerza la necesidad de identificar biomarcadores predictivos que orienten hacia una medicina de precisión en oncología.

3. Biomarcadores en la inmunoterapia del cáncer

Los biomarcadores en cáncer son moléculas biológicas, alteraciones genéticas o características celulares medibles en tejidos o fluidos corporales que se utilizan clínicamente para apoyar el diagnóstico o guiar la toma de decisiones terapéuticas. Según su utilidad clínica, pueden clasificarse en tres categorías: biomarcadores diagnósticos (ayudan a identificar la presencia de células tumorales y facilitar su clasificación); biomarcadores pronósticos (para estratificar a los pacientes según su riesgo de presentar una evolución clínica desfavorable o específica); y biomarcadores predictivos (estiman la probabilidad de respuesta a una intervención terapéutica específica).

Actualmente, existen dos principales fuentes para la obtención de biomarcadores: el tejido tumoral y la biopsia líquida. Mientras que el análisis de tejido se basa en procedimientos invasivos, la biopsia líquida emerge como una alternativa menos invasiva, más escalable y con capacidad de proporcionar información a nivel sistémico. Cada fuente permite extraer distintos tipos de información molecular. A partir del tejido tumoral se pueden obtener ARN, ADN, proteínas y datos histológicos, lo que permite analizar la expresión génica, mutaciones, perfiles epigenéticos y la composición celular del microambiente tumoral. Por otro lado, la biopsia líquida permite analizar componentes como ADN libre circulante (*cell-free DNA*, cfDNA), células mononucleares de sangre periférica (*peripheral blood mononuclear cells*, PBMCs), vesículas extracelulares (*extracellular vesicles*, EVs) y células tumorales circulantes (*circulating tumor cells*, CTCs). Del cfDNA se puede obtener información sobre su cantidad, perfil genómico y epigenómico; de los PBMCs, datos de abundancia celular y perfil transcriptómico y proteómico; de los EVs, el contenido molecular transportado o asociado a su membrana; y de las CTCs, cualquier tipo de perfilado molecular.

Hasta el momento, existen tres biomarcadores predictivos aprobados por la Administración de Alimentos y Medicamentos de Estados Unidos (*Food and Drug Administration*, FDA) en el contexto de inmunoterapia: la expresión de PD-L1, la carga mutacional tumoral (*tumor mutational burden*, TMB) y la inestabilidad de microsatélites (*microsatellite instability*, MSI) junto con la deficiencia en la reparación de errores de apareamiento (*deficient mismatch repair*, dMMR). Por otro lado, la Agencia Europea de Medicamentos (*European Medicines Agency*, EMA) solo ha aprobado hasta la fecha el uso de la expresión de PD-L1 y el estudio de MSI y dMMR. Sin embargo, la capacidad predictiva de estos tres marcadores es limitada

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y, además, se basan exclusivamente en muestras de tejido. Por otro lado, en los últimos años, han surgido biomarcadores experimentales derivados de investigaciones recientes, muchos de ellos centrados en el perfil inmunológico, la expresión génica, y la presencia de marcadores en exosomas o ADN tumoral circulante (*circulating tumor DNA*, ctDNA). No obstante, estos nuevos biomarcadores enfrentan importantes desafíos: la ausencia de estandarización entre estudios, la heterogeneidad intratumoral, los cambios dinámicos inducidos por el tratamiento y la escasa validación prospectiva en cohortes amplias y diversas.

Como resultado, la búsqueda de biomarcadores robustos, multifactoriales y dinámicos sigue siendo un reto clave y un campo de investigación activo en inmuno-oncología.

4. Aplicación de las ómicas para la identificación de biomarcadores en oncología

El término ómica hace referencia a un conjunto de disciplinas científicas orientadas al análisis a gran escala de diversas moléculas biológicas —como el genoma, el transcriptoma, el proteoma, el epigenoma y el metaboloma— en células, tejidos u organismos completos. Estas disciplinas han experimentado un crecimiento exponencial gracias al desarrollo de las tecnologías de secuenciación de nueva generación (*next generation sequencing*, NGS), que han facilitado la generación de datos ómicos de alta resolución y en grandes volúmenes. A su vez, este avance ha impulsado significativamente la identificación de biomarcadores, al permitir la elaboración de perfiles moleculares detallados del estado de la enfermedad. Esta caracterización molecular constituye una base fundamental para descubrir biomarcadores predictivos y pronósticos, comprender los mecanismos de resistencia terapéutica y diseñar estrategias de tratamiento personalizadas.

Nuestro estudio se centra específicamente en cuatro áreas clave: genómica, epigenómica, transcriptómica y proteómica. La genómica tiene como objetivo identificar variaciones genéticas en el genoma, mediante técnicas como la genotipificación de marcadores para detectar diferencias significativas en las frecuencias alélicas entre cohortes de casos y controles. La epigenómica estudia modificaciones heredables y reversibles del ADN y de las proteínas histónicas, como la metilación del ADN, la hidroximetilación y la acetilación de histonas; que regulan la expresión génica sin alterar la secuencia del ADN. La transcriptómica proporciona una visión integral del ARN expresado en una célula o tejido, tanto desde el punto de vista cualitativo como cuantitativo, permitiendo evaluar los patrones de expresión génica. Por su parte, la proteómica se encarga del análisis a gran escala de las proteínas, incluyendo la cuantificación de su expresión, sus modificaciones postraduccionales y las redes funcionales en las que participan.

En la actualidad, la investigación oncológica ha avanzado hacia la aplicación de estos biomarcadores en ensayos clínicos, así como en su incorporación progresiva en la práctica clínica rutinaria. Además, el enfoque multiómico, que integra distintas capas de información molecular, se ha consolidado como una de las líneas más prometedoras y activas en el campo,

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debido a su potencial para capturar la complejidad biológica de los tumores de manera más precisa y holística.

5. El papel de la Inteligencia Artificial

De forma paralela al desarrollo de tecnologías ómicas, la Inteligencia Artificial (*Artificial Intelligence*, AI) se ha consolidado como una herramienta transformadora en múltiples disciplinas científicas, incluida la oncología. Sus aplicaciones son diversas e incluyen la detección precoz del cáncer, la clasificación de subtipos tumorales, la identificación de biomarcadores y la predicción de la respuesta terapéutica.

Dentro del campo de la AI, destaca el aprendizaje automático (*Machine Learning*, ML), que permite a los sistemas aprender de forma autónoma a partir de datos y realizar predicciones al enfrentarse a información nueva. En nuestro estudio, se emplean varios algoritmos representativos de este enfoque, entre ellos: modelos lineales generalizados (*Generalized Linear Model*, GLM), regresión Lasso (técnica de regularización que favorece la selección automática de variables), máquinas de soporte vectorial (*Support Vector Machine*, SVM) (basados en la búsqueda del hiperplano óptimo para separar clases), Random Forest (modelo basado en múltiples árboles de decisión agregados) y XGBoost (*Extreme Gradient Boosting*). Una rama especializada del ML es el aprendizaje profundo (*Deep Learning*, DL), basado en redes neuronales con múltiples capas, capaces de aprender representaciones jerárquicas y complejas de los datos, así como relaciones no lineales. Las principales arquitecturas utilizadas según la tarea son: redes neuronales completamente conectadas (*Feedforward Neural Networks*, FFNNs), redes neuronales recurrentes (*Recurrent Neural Networks*, RNNs), memorias a largo plazo (*Long Short-Term Memory*, LSTMs), redes convolucionales (*Convolutional Neural Networks*, CNNs) y redes generativas adversativas (*Generative Adversarial Networks*, GANs).

En función del tipo de datos y del objetivo, los modelos de aprendizaje se dividen principalmente en dos categorías. Aprendizaje supervisado; donde los modelos se entrenan con datos etiquetados para resolver problemas de clasificación o regresión. Aprendizaje no supervisado; aplicado cuando los datos carecen de etiquetas, y que busca identificar estructuras ocultas, como patrones de agrupamiento (*clustering*) o reducción de la dimensionalidad. También existen otros enfoques menos extendidos, como el aprendizaje por refuerzo, en el que el sistema aprende a través de la interacción con el entorno, o el aprendizaje semi-supervisado, donde solo una parte de los datos está etiquetada.

En el ámbito de la inmunoterapia, se han publicado estudios centrados en el diseño de sistemas de puntuación para cuantificar la inmunogenicidad tumoral o en la mejora de la predicción de la presentación de péptidos por moléculas del complejo mayor de histocompatibilidad (*major histocompatibility complex*, MHC) de clase I. A pesar de su potencial, una de las principales limitaciones comunes a muchos trabajos es la falta de consistencia en la definición de respuesta terapéutica, lo que conduce a variabilidad en la interpretación de los

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resultados y reduce su aplicabilidad clínica. A ello se une la falta de interpretabilidad que ofrecen este tipo de algoritmos de alta complejidad (conocido como el problema de la caja negra), lo que en ocasiones dificulta su adopción en la práctica clínica.

Para abordar estos retos, hemos diseñado un estudio que aplica criterios estandarizados y biológicamente relevantes para la evaluación de la respuesta a inmunoterapia, siguiendo métricas bien definidas de resistencia primaria, según las recomendaciones más recientes de la Sociedad para la Inmunoterapia del Cáncer (*Society for Immunotherapy of Cancer*, SITC). Además, proponemos explorar enfoques aún no aplicados en este contexto, como el uso de técnicas no supervisadas para identificar subtipos moleculares asociados con la respuesta a los ICIs. Con ello, buscamos mejorar la reproducibilidad y la relevancia clínica en el descubrimiento de biomarcadores, contribuyendo al avance real hacia una oncología de precisión.

6. Protocolo clínico y traslacional: un enfoque multiómico

Esta tesis doctoral se enmarca en el proyecto de investigación IMMUNOMARK, centrado en la identificación de biomarcadores predictivos de respuesta y resistencia y pronósticos a ICIs en cáncer. El estudio incluye dos cohortes independientes: melanoma metastásico y NSCLC avanzado, con un total de 180 pacientes reclutados con indicación de tratamiento con ICIs, ya sea en monoterapia o en combinación con otros agentes.

En ambas cohortes, se recogieron muestras de biopsia líquida (n=384 muestras): antes del inicio del tratamiento (T1), tras el segundo ciclo (T2), a los seis meses (T3) y a los doce meses (T4) desde el inicio del tratamiento. Asimismo, se realizaron evaluaciones clínicas y por imagen a los tres, seis y doce meses, con el objetivo de monitorizar la respuesta al tratamiento. De forma complementaria, un subconjunto de pacientes contribuyó con muestras de biopsias tumorales retrospectivas conservadas en bloques de tejido fijado en formalina, embebidos en parafina (*Formalin-Fixed, Paraffin-Embedded*, FFPE). En cuanto a las técnicas ómicas aplicadas, se utilizó secuenciación de ARN (*RNA sequencing*, RNA-seq) en muestras de tejido sólido disponibles. En las biopsias líquidas, se llevó a cabo el fenotipado de PBMCs mediante citometría de flujo y RNA-seq de célula única (*single-cell RNA sequencing*, scRNA-seq), se extrajo cfDNA para el análisis de patrones de metilación, y se realizó secuenciación de ARNs pequeños en EVs.

Los datos moleculares generados fueron correlacionados con distintos resultados clínicos, entre los que se incluyen: respuesta extrema (definida como respuesta sostenida a los 12 meses frente a progresión precoz a los 3 meses), resistencia primaria según los criterios propuestos por SITC (los pacientes no resistentes se caracterizan por una respuesta tumoral calculada según RECIST v1.1, o enfermedad estable prolongada, de duración igual o superior a 6 meses; mientras que los pacientes resistentes experimentaron una estabilización de la enfermedad o una progresión antes de los 6 meses tras recibir al menos dos ciclos completos de ICIs), y

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supervivencia del paciente. En una selección de pacientes, se dispuso de perfiles multiómicos, lo que permitió la realización de análisis integrativos entre distintas capas moleculares.

Este diseño facilitó la identificación de biomarcadores candidatos y proporcionó una comprensión más profunda de los mecanismos subyacentes a la respuesta, resistencia y supervivencia a la inmunoterapia.

7. Hipótesis y objetivos de la tesis

La hipótesis central de esta tesis doctoral es que un enfoque multiómico proporciona un marco integral y robusto para la identificación de biomarcadores predictivos y pronósticos en la inmunoterapia del cáncer. Al integrar información genómica, transcriptómica y epigenética en biopsias sólidas y líquidas, es posible abordar la complejidad biológica de las interacciones tumor- sistema inmunitario y predecir con precisión la respuesta al bloqueo de puntos de control inmunitario.

Objetivos específicos: Esta tesis se estructura como un compendio de cuatro artículos científicos, cada uno alineado con un objetivo de investigación específico derivado de la hipótesis central:

- **Objetivo 1:** Identificar las firmas transcriptómicas asociadas con fenotipos extremos de respuesta al bloqueo de puntos de control inmunitario, mediante la expresión diferencial y el análisis a nivel de señalización celular en biopsias de tumores sólidos.
- **Objetivo 2:** Evaluar la influencia de la distribución de células inmunitarias en la resistencia primaria a la inmunoterapia, estimando la composición celular inmunitaria a partir de datos transcriptómicos y correlacionándola con los resultados clínicos y la biopsia líquida.
- **Objetivo 3:** Evaluar biomarcadores simples en biopsia líquida como predictores no invasivos de la respuesta a la inmunoterapia.
- **Objetivo 4:** Integrar múltiples capas ómicas de la biopsia líquida en un marco analítico unificado, con el objetivo de descubrir subtipos moleculares con distintos mecanismos ocultos de resistencia al bloqueo de puntos de control inmunitario.

8. Estructura y contribución científica de la tesis

Esta tesis doctoral se presenta como un compendio de cuatro artículos de investigación, todos ellos alineados temáticamente con el objetivo central. Las cuatro publicaciones incluidas en este compendio son las siguientes:

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- **Paper #1: Onieva JL**, Xiao Q, Berciano-Guerrero MÁ, et al. High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade. Int J Mol Sci. 2022;23(16):9124. doi:10.3390/ijms23169124
- **Paper #2: Onieva JL**, Pérez-Ruiz E, Vilkki V, et al. Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma. bioRxiv. 2025:670062. doi:10.1101/2025.08.13.670062
- **Paper #3:** Oliver J, **Onieva JL (equally contributed)**, Garrido-Barros M, et al. Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade. Cancers (Basel). 2023;15(13):3357. doi:10.3390/cancers15133357
- **Paper #4: Onieva JL**, Pérez-Ruiz E, Figueroa-Ortiz L, et al. Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC. medRxiv. 2025:25333961. doi:10.1101/2025.08.19.25333961

Resultados

Este trabajo doctoral comprende cuatro estudios interrelacionados orientados a abordar los objetivos planteados en la introducción. Los dos primeros se centran en melanoma metastásico, mientras que los dos últimos investigan NSCLC avanzado. A lo largo de estos estudios se aplicaron diversos enfoques bioinformáticos, según el desenlace clínico analizado—incluyendo respondedores extremos, resistencia primaria y supervivencia global. Un eje central de la tesis es la búsqueda de biomarcadores empleando técnicas de aprendizaje automático y la integración de datos multiómicos. El primer artículo emplea análisis transcriptómicos basados en tejido; el segundo combina tejido y biopsia líquida; y el tercero y cuarto se basan exclusivamente en biomarcadores circulantes obtenidos de sangre.

En el primer estudio, se llevó a cabo un análisis transcriptómico de 16 muestras tumorales basales de pacientes con melanoma cutáneo metastásico tratados con nivolumab, con el objetivo de identificar perfiles moleculares y celulares asociados a una respuesta favorable al tratamiento basándose en la definición de extremos respondedores. El análisis de expresión diferencial resultó en 140 genes diferencialmente expresados, con una firma enriquecida en el linaje de células B en los extremos respondedores. Además, los resultados revelaron una asociación significativa entre la elevada expresión intratumoral del gen *IGKC* y una mayor abundancia de células plasmáticas con una respuesta extrema sostenida a la inmunoterapia. Esta asociación se validó mediante múltiples tecnologías complementarias, incluyendo scRNA-seq en datos de pacientes tratados con ICIs, y análisis de inmunofluorescencia multiplex en tejidos tumorales. Además, se realizaron estudios de repertorio de receptores de

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células B (*B cell receptor*, BCR) y T (*T cell receptor*, TCR), observándose un enriquecimiento de variantes de la cadena IGK dentro de los clonotipos de BCR más relacionados con la respuesta, lo cual refuerza el papel funcional de la inmunidad mediada por células B. En un paso adicional, se construyó un modelo predictivo basado en una firma de 15 genes que, al ser validado en ocho cohortes independientes de pacientes con melanoma tratados con ICIs, mostró un rendimiento superior a las puntuaciones de referencia de la literatura, demostrando su potencial clínico para estratificar pacientes en función de su probabilidad de responder al tratamiento. Este hallazgo sitúa a las células plasmáticas y a la expresión de *IGKC* como nuevos biomarcadores predictivos clave, desafiando el paradigma tradicional centrado en la actividad de las células T.

En un segundo trabajo, se amplió la cohorte de pacientes con melanoma cutáneo metastásico a un total de 46 individuos con el fin de identificar nuevas firmas transcriptómicas capaces de predecir la resistencia primaria a la inmunoterapia, basándose en los criterios SITC. Se identificó una firma de 82 genes diferencialmente expresados entre los grupos de pacientes resistentes y no resistentes, enriquecida en procesos biológicos relacionados con la comunicación entre células inmunes innatas y adaptativas, así como con funciones específicas de las células B. Esta firma permitió clasificar con precisión a los pacientes en función de su respuesta al tratamiento, alcanzando un área bajo la curva (*area under the curve*, AUC) ROC (*receiver operating characteristic*) de 0,814 en una cohorte externa de validación con 54 pacientes. Además, se llevó a cabo una deconvolución inmunocelular de los transcriptomas *bulk*, sin separación de poblaciones, mediante integración de los datos de RNA-seq del tejido tumoral con los datos de scRNA-seq de PBMCs de una fracción de nuestra cohorte de pacientes con melanoma cutáneo metastásico. Este análisis permitió identificar en el microambiente tumoral subpoblaciones inmunitarias específicas con valor pronóstico, incluyendo células plasmáticas, células pre-B, células T $CD4^+$ memoria y células T $CD4^+$ naïve. A partir de estos datos se desarrolló una nueva herramienta de clasificación inmunológica denominada *ImmuneSignature*, que resume el estado del microambiente inmunitario tumoral en relación con la probabilidad de beneficio clínico. Este score fue validado en una cohorte externa de 268 pacientes con melanoma cutáneo metastásico tratados con ICIs. Además, la correlación de estos marcadores con poblaciones celulares en sangre y su dinámica pre-post tratamiento fueron confirmadas mediante citometría de flujo, lo que consolida su papel como posible biomarcador de respuesta clínica y sugiere su potencial como base para el desarrollo de predictores no invasivos.

El tercer estudio se centró en el NSCLC avanzado y exploró el potencial pronóstico de la biopsia líquida a través de la cuantificación de cfDNA antes del inicio del tratamiento con ICIs. Se analizaron dos cohortes independientes: una de descubrimiento (n=61) y otra de validación (n=31), ambas con seguimiento clínico de hasta 40 meses. Se estableció un umbral de concentración en plasma de cfDNA de 0,55 ng/µL, por encima del cual los pacientes presentaban una supervivencia global significativamente más baja. En la cohorte de descu-

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brimiento, este valor de corte permitió diferenciar dos grupos con medianas de supervivencia muy diferentes: 3,8 frente a 17,5 meses (valor p: $3{,}3 \times 10^{-4}$). Resultados similares se observaron en la cohorte de validación (medianas de 5,9 vs. 24,3 meses; valor p: $3{,}8 \times 10^{-2}$). Los análisis de regresión de Cox univariados y multivariados confirmaron que un alto nivel de cfDNA previo al tratamiento era un factor predictivo de mal pronóstico independiente. Estos resultados posicionan la cuantificación fluorométrica del cfDNA como una herramienta sencilla, rápida y escalable para identificar pacientes con NSCLC avanzado que probablemente no se beneficiarán de la inmunoterapia, permitiendo una mejor selección de estrategias terapéuticas alternativas.

El cuarto y último estudio aplicó un enfoque integrador multiómico sobre biopsias líquidas de pacientes con NSCLC avanzado, combinando análisis de metilación del cfDNA y perfiles de microARN (*microRNA*, miRNA) extraídos de EVs. A partir de estos datos se definieron tres subtipos moleculares denominados MDCs (*MOFA-derived clusters*, en inglés) en dos tiempos diferentes: antes del tratamiento; MDC-T1 y tras el segundo ciclo de inmunoterapia; MDC-T2. Ambos mostraron correlaciones claras con resultados clínicos divergentes, particularmente en términos de supervivencia global y supervivencia libre de progresión. Estos subtipos capturaron procesos biológicamente relacionados con la remodelación del citoesqueleto, angiogénesis y señalización inmune, y, concretamente los subtipos de MDC-T2, conservaron su estabilidad a lo largo del tiempo cuando se proyectaron en muestras tomadas a los seis (T3) y doce meses (T4) de tratamiento. Asimismo, los MDCs se correlacionaron con poblaciones inmunitarias específicas detectadas por citometría de flujo en sangre de los mismos pacientes, donde mostraron asociaciones inmunológicas específicas a través de la caracterización de subconjuntos celulares circulantes, como células T $CD8^+PD1^+$, células citotóxicas T $CD8^+$, células T $CD3^+$PD-$L1^+$, células T $CD4^+$ y T reguladoras. De forma destacable, los clasificadores derivados de MDC demostraron una capacidad consistente de estratificación de la supervivencia en cohortes externas, especialmente el subtipo MDC-T2. Este trabajo establece un marco conceptual y técnico basado en biopsia líquida multiómica para la subtipificación molecular de pacientes con NSCLC bajo tratamiento inmunoterapéutico, abriendo nuevas posibilidades para la medicina de precisión.

Discusión

Esta tesis doctoral aborda de forma integrada y multidisciplinar uno de los principales retos actuales en oncología: la identificación de biomarcadores que permitan optimizar la selección de pacientes tratados con ICIs. Se han empleado cohortes de pacientes con melanoma metastásico y NSCLC avanzado, utilizando tanto muestras de tejido tumoral sólido como biopsias líquidas. Se han integrado diferentes capas ómicas (transcriptómica y epigenómica) con metodologías bioinformáticas y enfoques de IA.

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Los dos primeros estudios se centraron en una cohorte de pacientes con melanoma metastásico, donde se disponía de tejido tumoral sólido y biopsia líquida. En el primer estudio, ante la falta de consenso en el campo sobre el momento óptimo para evaluar la respuesta al tratamiento, se diseñó un enfoque basado en fenotipos extremos, comparando pacientes con progresión temprana (en menos de 3 meses) frente a aquellos con respuesta sostenida (de al menos 12 meses). Este diseño permitió una caracterización exhaustiva de la transcriptómica basal del tumor, revelando perfiles moleculares claramente diferenciados entre ambos grupos. En el segundo estudio, tras la definición estandarizada de resistencia primaria por parte de la SITC, se amplió la cohorte de tejido sólido y se incorporó el análisis de células inmunitarias circulantes en sangre mediante el análisis transcriptómico de célula única y mediante citometría de flujo. Esto posibilitó una integración molecular desde distintos compartimentos biológicos y sentó las bases del potencial de una prueba no invasiva implementable en la rutina clínica.

Uno de los hallazgos más relevantes en estos estudios fue la identificación consistente de las células B y, especialmente, de las células plasmáticas como elementos asociados a una mejor respuesta clínica. Las células B y las células plasmáticas no solo producen anticuerpos específicos frente a antígenos tumorales, sino que también participan en la presentación antigénica a células T y en la modulación del microambiente tumoral mediante la secreción de citocinas proinflamatorias. Aunque no se pudo demostrar directamente la presencia de estructuras linfoides terciarias (*tertiary lymphoid structures*, TLS), la evidencia bibliográfica respalda su asociación con una mayor supervivencia. Además, nuestros resultados coinciden con estas observaciones, especialmente con la sobreexpresión del gen *CXCL13* en los pacientes no resistentes primarios y en el modelado del score pronóstico *InmuneSignature*. El *InmuneSignature*, compuesto por células plasmáticas, células pre-B, células T $CD4^+$ memoria y células T $CD4^+$ naïve; presenta un valor alto en aquellos pacientes con mejor supervivencia global. En conjunto, nuestros datos, junto con la literatura previamente publicada, destacan el papel complejo y clínicamente relevante de las poblaciones derivadas predominantemente de la línea B, así como de las células T $CD4^+$, en la configuración del microambiente inmunológico tumoral y en la modulación de la eficacia de los ICIs.

Los dos primeros estudios abordan importantes desafíos inherentes al ML, como la maldición de la dimensionalidad (espacios de alta dimensión, donde el aumento de variables compromete la generalización de los modelos) y la fuga de datos (*data leakage*, en inglés), que ocurre cuando información del conjunto de validación se filtra al de entrenamiento, sesgando el rendimiento del modelo. Para hacer frente a estos problemas, se implementaron estrategias rigurosas, tales como la validación cruzada y la incorporación de cohortes de validación externa, prácticas que no se han adoptado de manera sistemática en otros trabajos que desarrollan modelos predictivos de respuesta a ICIs en melanoma. Asimismo, nuestro modelo supera a algoritmos establecidos, como TIDE, mostrando una capacidad predictiva superior.

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En la segunda parte de la tesis, se abordaron dos estudios centrados en pacientes con NSCLC avanzado, en los que la obtención de tejido sólido representaba un reto clínico. En este contexto, la biopsia líquida emergió como la única vía para el análisis molecular. En el tercer estudio de esta tesis doctoral, se evaluó el valor pronóstico de los niveles de cfDNA desde una perspectiva cuantitativa, identificando subgrupos con diferente pronóstico. El análisis basal de la concentración de cfDNA permitió identificar pacientes con NSCLC avanzado con baja probabilidad de respuesta favorable a ICI. Esta medida refleja procesos biológicos complejos asociados a la agresividad tumoral en un pool de cfDNA —no exclusivamente ctDNA— que incluye fragmentos de ADN procedentes no solo de células tumorales, sino también del microambiente tumoral y de células inmunes implicadas en la respuesta antitumoral, liberados mediante mecanismos como necrosis, secreción, apoptosis y NETosis. Sin embargo, para profundizar en la comprensión de los mecanismos moleculares que subyacen a la respuesta al tratamiento, resulta imprescindible la aplicación de enfoques multiómicos, que constituyen la base de nuestro cuarto estudio.

En el último análisis, se desarrolló un enfoque no supervisado basado en la integración de datos multiómicos (EV-miRNAs y cfDNA metilado), lo cual permitió definir subtipos moleculares diferenciados en cuanto a supervivencia proyectables en distintos momentos del tratamiento. La integración de perfiles circulantes de miRNA y metilación de cfDNA reveló patrones diferenciados y complementarios en la estructura molecular latente para las muestras previas al tratamiento ICI (T1), MDC-T1, y las muestras obtenidas tras el segundo ciclo de tratamiento (T2), MDC-T2. En el modelo T1 (MDC-T1), la varianza equilibrada entre ambas capas sugiere una contribución conjunta en etapas previas al inicio del tratamiento con ICIs. En contraste, en T2 (MDC-T2) se evidenció una mayor dominancia de las señales epigenéticas, lo que podría reflejar una transición hacia procesos regulados por metilación durante las fases iniciales del tratamiento. Esta dimensión temporal resulta especialmente relevante en contextos de monitoreo longitudinal, donde la metilación de cfDNA podría actuar como un marcador más sensible de reprogramación epigenética, mientras que los perfiles de miRNA reflejarían respuestas regulatorias más rápidas y transitorias. Además, los subtipos obtenidos en cada tiempo mostraron una sólida asociación con señales inmunológicas relevantes, como la presencia de células $CD8^+PD1^+$ y el incremento de células $CD4^+$ en los grupos con mejor pronóstico. De forma destacada, el marcador sanguíneo de célula T $CD4^+$ se ha observado de forma consistente en los estudios #2 y #4, actuando como componente clave en la firma inmune (*ImmuneSignature*), y asociándose con el subtipo MDC caracterizado por un mejor pronóstico. Un análisis más exhaustivo y optimizado permitirá determinar si otras poblaciones inmunes relevantes identificadas comparten patrones entre ambos tipos de tumor tratados con ICIs, lo que podría reforzar su valor como biomarcadores transversales y profundizar en la comprensión de los mecanismos inmunológicos comunes subyacentes a la respuesta terapéutica.

Nuestros análisis presentan una serie de limitaciones que deben ser consideradas. En primer

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lugar, el reducido tamaño muestral en la cohorte de scRNA-seq de PBMCs limita la generalización de los hallazgos de marcadores predictivos a nivel de célula única. Asimismo, la ausencia de marcadores en la citometría de flujo más optimizados dificultó la identificación completa de las subpoblaciones celulares incluidas en la *ImmuneSignature*. Por otra parte, se requieren estudios adicionales que permitan profundizar en los mecanismos funcionales subyacentes, ya sea mediante ensayos funcionales o transcriptómica espacial. Otra limitación importante es la falta de cohortes de validación amplias que integren ambas capas ómicas en el último estudio. En este contexto, las líneas futuras del proyecto se orientan a solventar estas limitaciones, incluyendo la ampliación de cohortes, un análisis más exhaustivo de los datos longitudinales y la optimización de los modelos predictivos mediante la reducción del panel de biomarcadores. Estos avances permitirán avanzar hacia una aplicación clínica más costo-efectiva, con el objetivo final de estandarizar paneles predictivos y plantear ensayos clínicos que validen los biomarcadores propuestos en esta tesis.

En conjunto, este trabajo destaca el potencial de los biomarcadores circulantes en sangre y de los enfoques computacionales avanzados, incluyendo técnicas de AI y bioinformática, en el campo de la inmunoterapia. Además, propone una estrategia integradora que combina datos clínicos, moleculares y longitudinales para abordar la complejidad biológica inherente a la respuesta a los ICIs. De este modo, esta tesis establece un marco sólido para futuras investigaciones traslacionales orientadas a la validación clínica de los biomarcadores identificados, y abre nuevas oportunidades para el diseño de ensayos clínicos que impulsen el avance hacia una medicina personalizada.

Conclusiones

Esta tesis doctoral ha explorado la aplicación de análisis bioinformáticos, técnicas de aprendizaje automático e integración de datos multiómicos para identificar biomarcadores predictivos y pronósticos de la respuesta a la inmunoterapia contra el cáncer. A continuación se resumen las principales conclusiones derivadas de la investigación presentada:

1. Se desarrollaron y validaron dos firmas genéticas —un panel de 15 genes que predice los pacientes con respuesta extrema y un panel de 82 genes que predice la resistencia primaria a los ICIs en el melanoma cutáneo metastásico— con modelos de aprendizaje automático supervisado, logrando un AUC superior a 0,8 en cohortes de validación externas.

2. Las subpoblaciones de células B con alta expresión de *IGKC* y expansión clonal de IGK BCR se enriquecieron significativamente en el microambiente tumoral en los pacientes con respuesta extrema.

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3. Se estableció y validó una puntuación *ImmuneSignature* basada en cuatro poblaciones de células inmunitarias (células plasmáticas, células pre-B, células T $CD4^+$ naïve y células T $CD4^+$ de memoria) en tejido de melanoma cutáneo metastásico, lo que permitió identificar un subgrupo con una mejora significativa de la supervivencia global en pacientes tratados con ICIs.

4. Una puntuación subrogada del *ImmuneSignature* empleando la biopsia líquida de la firma inmunológica (células T $CD4^+$ y células plasmáticas —$CD20^-$, $CD27^+$, $CD138^+$) medida en sangre tras el segundo ciclo de inmunoterapia demostró su potencial como biomarcador mínimamente invasivo para la monitorización del tratamiento.

5. La cuantificación del cfDNA se validó como biomarcador pronóstico no invasivo en el NSCLC avanzado, con un umbral de <0,55 ng/µL que estratificó eficazmente a los pacientes con mejor supervivencia libre de progresión y supervivencia global, superando a los marcadores clínicos tradicionales.

6. La integración multiómica de miRNA derivados de EVs y la metilación del cfDNA identificó tres subtipos moleculares en el NSCLC avanzado con resultados de supervivencia distintos, donde los subtipos de alta supervivencia mostraron un enriquecimiento de células circulantes T $CD8^+PD\text{-}1^+$ y T $CD4^+$.

7. La alta expresión de los miRNA *hsa-miR-451a* y *hsa-miR-4732-5p*, junto con la hipermetilación en regiones específicas de los genes *BLK* y *ADARB2*, se observó de forma consistente en los subtipos moleculares asociados con una mejora de la supervivencia global antes del inicio de la inmunoterapia y después del segundo ciclo de tratamiento (semanas 2-3), lo que sugiere su potencial como biomarcadores pronósticos tempranos.

Palabras clave: Cáncer, Inmunoterapia, Multiómica, Biomarcador, Aprendizaje atomático.

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Abstract

Introduction: Immunotherapy, particularly immune checkpoint inhibitors (ICIs), has transformed the treatment landscape for advanced cancers such as metastatic melanoma and advanced non-small cell lung cancer (NSCLC), providing durable responses in a subset of patients. Nevertheless, treatment resistance remains major clinical barriers, with up to 70 % of patients failing to derive long-term benefit. Existing biomarkers, including PD-L1 expression and tumor mutational burden, offer limited predictive value and are often inconsistent or technically demanding. Furthermore, intratumoral heterogeneity and the lack of assay standardization hinder their broad clinical application. A comprehensive understanding of the molecular biology of tumors and their interaction with the immune system is essential to elucidate variability in treatment response. In this context, multi-omic integration has emerged as a powerful strategy to capture the complexity underlying therapeutic outcomes. Furthermore, the application of machine learning approaches enables the identification of predictive patterns across high-dimensional datasets, facilitating the development of robust biomarker signatures. In addition, liquid biopsy has emerged as a promising strategy, as it provides a non-invasive and scalable platform for biomarker discovery and longitudinal monitoring. Together, these innovations hold potential to improve patient stratification and optimize the clinical efficacy of immunotherapy.

Methods: This study integrates data from two patient cohorts: one with metastatic melanoma and the other with advanced NSCLC, comprising a total of 180 individuals treated with ICIs, either as monotherapy or in combination with other agents, and followed longitudinally. Liquid biopsy samples (n=348) were collected prior to treatment initiation (T1), after the second cycle (T2, approximately 3–4 weeks), and at six (T3) and twelve months (T4) post-initiation. Clinical and radiographic evaluations were conducted at three, six, and twelve months to assess therapeutic response. A subset of patients also contributed retrospective formalin-fixed paraffin-embedded tumor biopsies, enabling parallel molecular characterization of solid tissues. Molecular analyses were performed on both tumor biopsies and liquid biopsy components, including peripheral blood mononuclear cells (PBMCs), cell-free DNA

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(cfDNA), and extracellular vesicles (EVs). These molecular data were systematically correlated with clinical outcomes, particularly focusing on extreme response phenotypes (defined as sustained responders at 12 months and early progressors at 3 months), primary resistance based on Society for Immunotherapy of Cancer (SITC) criteria, and overall survival. In a selected subset of patients, matched multi-omic analyses across multiple molecular layers were performed from the same individuals, allowing for an integrative profiling. This design facilitated the identification of candidate biomarkers and mechanistic insights into response and resistance to immunotherapy.

Results: Firstly, transcriptomic profiling of tumor samples from 16 metastatic cutaneous melanoma patients revealed that high intratumoral expression of *IGKC* and increased abundance of plasma cells were strongly associated with favorable extreme response to nivolumab. These findings were validated through single-cell RNA sequencing and multiplex immunofluorescence. B-cell receptor (BCR) and T-cell receptor (TCR) analyses showed an enrichment of IGK chain sequences among extreme responders. Additionally, a 15-gene predictive model outperformed established reference scores across eight independent ICIs-treated melanoma cohorts, underscoring the predictive relevance of B cell–mediated immunity. In a second study expanding the cohort to 46 patients, an 82-gene signature enriched in tumor–immune interaction pathways and B cell-related processes was identified, enabling accurate classification of metastatic cutaneous melanoma patients as resistant or non-resistant based on primary resistance to ICIs. The predictive model achieved an AUC of 0.814 in an external validation cohort. Integration with single-cell RNA-seq of PBMCs and validation by flow cytometry confirmed the prognostic value of specific immune populations, including plasma cells, pre-B cells, and $CD4^+$ T cell subsets, whose combined contribution forms the basis of the Immune-Signature score. In the third study, focused on advanced NSCLC, pre-treatment quantification of cfDNA levels using liquid biopsy in discovery (n=61) and validation (n=31) cohorts demonstrated that concentrations above 0,55 ng/µl were significantly associated with poorer overall survival. This cut-off effectively stratified patients into high- and low-risk groups, with median survival differences exceeding one year. cfDNA concentration was confirmed as an independent prognostic factor, supporting its use as a simple and scalable tool to inform clinical decision-making in ICIs-treated NSCLC advanced. Finally, an integrative multi-omic analysis of cfDNA methylation and extracellular vesicle–derived miRNAs in NSCLC advanced defined molecularly distinct subtypes (Multi-Omics Factor Analysis (MOFA)-derived clusters, MDCs) associated with divergent survival outcomes and immune-related features affecting the clinical benefit of ICI. These MDCs captured molecular signatures and were validated in independent external cohorts. This liquid biopsy–based framework provides a novel strategy for molecular subtyping and therapeutic stratification in patients undergoing ICIs treatment.

Conclusions: In summary, these studies provide new insights into the molecular drivers of response and resistance to ICIs in metastatic melanoma and advanced NSCLC. The iden-

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tification of B cell–related signatures—particularly plasma cell activity and *IGKC* expression—establishes a novel prognostic framework that expands beyond the conventional focus on T cells. Moreover, the integration of tumor transcriptomic data with PBMC profiles enabled the development of an ImmuneSignature linked to improved survival, shaped by distinct immune cell phenotypes. Notably, through the NSCLC studies, where tumor tissue unavailability hinders precision medicine, liquid biopsy emerges as a non-invasive, scalable, and clinically informative platform, with cfDNA levels and multi-omic subtypes. These findings support the adoption of liquid biopsy–based, multi-omic strategies to personalize immunotherapy and guide clinical decision.

Keywords: Cancer, Immunotherapy, Multi-omics, Biomarker, Machine Learning.

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Glossary of Terms and Abbreviations

Abbreviations

AI	Artificial Intelligence
APCs	Antigen-Presenting Cells
ATAC-seq	Assay for Transposase-Accessible Chromatin using Sequencing
AUC	Area Under the Curve
BCR	B-cell Receptor
BS-seq	Bisulfite Sequencing
CAR	Chimeric Antigen Receptor
cfDNA	Cell-Free DNA
CNSs	Convolutional Neural Networks
CNVs	Copy Number Variations
CPS	Combined Positive Score
CTCs	Circulating Tumor Cells
CTLA-4	Cytotoxic T-lymphocyte-Associated Antigen 4
ctDNA	Circulating Tumor DNA
DL	Deep Learning
dMMR	Deficient Mismatch Repair
DMRs	Differentially Methylated Regions
DNA	Deoxyribonucleic Acid
EMA	European Medicines Agency
EM-seq	Enzymatic Methyl-seq
EV-miRNA	Extracellular Vesicle-Associated microRNA
EVs	Extracellular Vesicles
FDA	U.S. Food and Drug Administration
FFNNs	Feedforward Neural Networks
FFPE	Formalin-Fixed Paraffin-Embedded
GANs	Generative Adversarial Networks
GCO	Global Cancer Observatory
GLM	Generalized Linear Models
HLA	Human Leukocyte Antigen
IARC	International Agency for Research on Cancer

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ICIs	Immune Checkpoint Inhibitors
IFN-α	Interferon-Alpha
IGK	Immunoglobulin Kappa
IL-2	Interleukin-2
Indels	Insertions/Deletions
IPS	Immunophenoscore
IT	Immunotherapy
lncRNAs	Long Non-Coding RNAs
LSTMs	Long Short-Term Memory Networks
MDCs	MOFA-Derived Clusters
ML	Machine Learning
mRNA	Messenger RNA
MS	Mass Spectrometry
MSI	Microsatellite Instability
miRNAs	microRNAs
MOFA2	Multi-Omics Factor Analysis v2
NEXT	Neural-Based Models for Integrating Gene Expression and Visual Texture Features
NGS	Next-Generation Sequencing
NK	Natural Killer
NSCLC	Non-Small Cell Lung Cancer
ORR	Objective Response Rates
OS	Overall Survival
PD-1	Programmed Cell Death-1
PD-L1	Programmed Cell Death Ligand-1
PFS	Progression-Free Survival
PTMs	Post-Translational Modifications
qRT-PCR	Quantitative Real-Time PCR
RF	Random Forest
RNA	Ribonucleic Acid
RNA-seq	RNA Sequencing
RNNs	Recurrent Neural Networks
ROC	Receiver Operating Characteristic
scRNA-seq	Single-Cell RNA-seq
SCLC	Small Cell Lung Cancer
SITC	Society for Immunotherapy of Cancer
SNVs	Single Nucleotide Variants
SVM	Support Vector Machines
TCR	T-cell Receptor
TIDE	Tumor Immune Dysfunction and Exclusion

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TILs	Tumor-Infiltrating Lymphocytes
TMB	Tumor Mutational Burden
TME	Tumor Microenvironment
TLSs	Tertiary Lymphoid Structures
Tregs	Regulatory T Cells
T-VEC	Talimogene Laherparepvec
TPS	Tumor Proportion Score
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
XGBoost	Extreme Gradient Boosting

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Chapter 1: Introduction

In the evolving landscape of cancer treatment, immunotherapy offers transformative clinical benefits. In melanoma and non-small-cell lung cancer, subsets of patients achieve remarkable and durable survival gains, while others exhibit resistance that limits the overall efficacy of this approach. This outcome disparity reflects a complex molecular architecture whose complexity poses significant challenges to interpretation but is increasingly accessible through applied bioinformatics and machine learning. This thesis explores this molecular landscape by leveraging multi-omics and artificial intelligence techniques to identify biomarkers capable of transforming response prediction from an empirical art into a data-driven science.

1.1 Immunotherapy and the Challenge of Response Prediction

Throughout the last decade, immunotherapy has redefined the landscape of the treatment of various cancer types, particularly with immune checkpoint inhibitors (ICIs) targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) or its ligand PD-L1 [1]. These agents have led to durable responses in previously hard-to-treat patient subsets with advanced malignancies—including melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma—tumors historically associated with poor prognosis and limited therapeutic options [2, 3]. Their impact has changed the clinical outlook and established new standards of care [4]. However, despite this therapeutic potential, response rates remain highly variable. While a fraction of patients achieve sustained tumor control, up to 60–70 % experience resistance to therapy [5, 6]. This heterogeneity underscores an urgent need for robust predictive biomarkers to guide patient selection, improve clinical outcomes, and enhance the cost-effectiveness of immunotherapy.

The conceptual definition of cancer immunotherapy lies in the recognition of the immune system's capacity to identify and eliminate malignant cells. The first significant advances on the field dates back to the late 19th century when William B. Coley observed tumor regression following bacterial infections [7]—an insight that led to the development of "Coley's toxins," considered the first immunotherapeutic approach [8]. Nonetheless, the field remained empirical until the early 21st century, when a breakthrough occurred with the

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discovery and functional characterization of immune checkpoints such as CTLA-4 and PD-1. These molecules were found to be pivotal in regulating immune tolerance and preventing autoimmunity [9]. The significance of these findings was recognized with the 2018 Nobel Prize in Physiology or Medicine, awarded to James P. Allison and Tasuku Honjo for their respective contributions to the understanding of CTLA-4 and PD-1 [10, 11]. Their work laid the foundation for the development of ICIs, which have become central to modern cancer therapy. This milestone firmly established immunotherapy as a standard modality within contemporary oncology.

ICIs constitute one of the five major categories of state-of-the-art immunotherapy of cancer [12]. The other four include oncolytic virus therapies, cancer vaccines, cytokine therapies, and adoptive cell transfer (Figure 1).

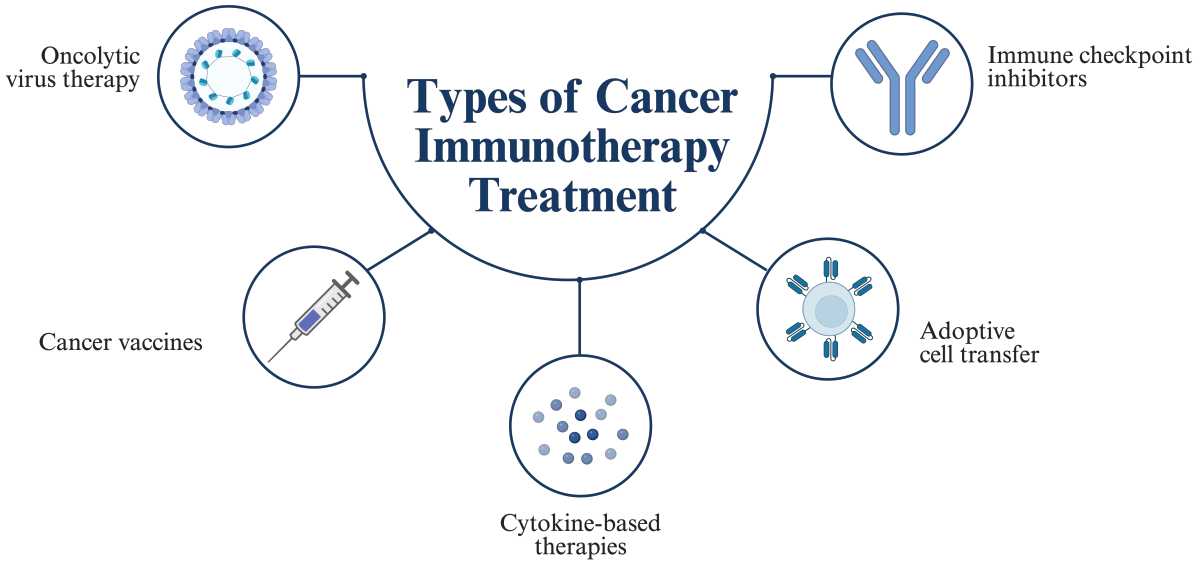

Figure 1. Schematic representation of the five main categories currently encompassed within immunotherapy. Created with BioRender.com.

- **Oncolytic virus therapies** employ genetically modified viruses capable of selectively infecting and lysing malignant cells. Beyond direct oncolysis, this process facilitates the release of tumor-associated antigens, thereby enhancing antigen presentation and promoting systemic anti-tumor immune responses [13]. Talimogene laherparepvec (T-VEC), a modified herpes simplex virus type 1, was the first oncolytic virus therapy approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of subsets of patients with melanoma [14].
- **Cancer vaccines** are designed to elicit or amplify immune responses directed against tumor-specific or tumor-associated antigens, thereby facilitating the recognition and

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elimination of malignant cells [15]. Unlike oncolytic viruses, which induce immunogenic cell death and secondary antigen release, cancer vaccines engage the immune system in a more targeted manner by priming it against selected immunogenic markers. A clinically relevant example is sipuleucel-T, a Dendritic Cell-based immunotherapy that has been approved for the treatment of advanced prostate cancer [16] by FDA and EMA.

- **Cytokine-based therapies** leverage immune-modulating proteins—such as interleukins and interferons—to promote the activation, proliferation, and effector functions of immune cells [17]. Cytokines function as key signaling molecules that coordinate immune cell communication and response. Their rapid and context-specific release facilitates robust and integrated immune signaling cascades directed against antigenic targets. Clinically, cytokines such as interleukin-2 (IL-2) and interferon-alpha (IFN-α) have demonstrated therapeutic benefit in malignancies like melanoma and chronic myeloid leukemia [18, 19]. Nonetheless, their clinical utility has been constrained by dose-limiting systemic toxicities. Current strategies are focused on the development of engineered cytokine variants with enhanced selectivity and reduced toxicity.

- **Adoptive cell transfer** encompasses the ex vivo expansion and reinfusion of autologous or allogeneic immune cells to target and eradicate cancer cells [20]. This approach includes therapies such as tumor-infiltrating lymphocytes (TILs), T-cell receptor (TCR) engineered T cells, and chimeric antigen receptor (CAR) T cells. CAR T-cell therapy, in particular, has shown remarkable success in hematologic malignancies [21].

- **ICIs** are a class of monoclonal antibodies that has emerged as a cornerstone of modern cancer immunotherapy. These agents function by blocking inhibitory pathways that restrain T-cell activation, thereby restoring and enhancing anti-tumor immunity. The most extensively studied and clinically utilized targets include CTLA-4, PD-1, and its ligand PD-L1. Collectively, these immunotherapeutic strategies have revolutionized cancer treatment paradigms, offering durable responses and improved survival outcomes across various malignancies [4].

Given their clinical and biological relevance, we have focused specifically on ICIs in our study. To understand their mechanism of action, it is essential to examine the key components of the immune checkpoint pathway, as illustrated in Figure 2. Immune checkpoints are coinhibitory molecules that play a fundamental role in maintaining peripheral tolerance and preventing autoimmune responses. However, numerous tumor types exploit these regulatory mechanisms to evade immune surveillance. ICIs act by blocking these inhibitory signals, thereby restoring T-cell activity and facilitating the immune-mediated elimination of malignant cells. Two major immune checkpoint axes have been extensively explored in the context of ICIs: the CTLA-4 axis and the PD-1/PD-L1 axis [9].

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- **CTLA-4 axis:** CTLA-4 is a coinhibitory receptor expressed primarily on activated T cells and regulatory T cells (Tregs). It competes with the costimulatory molecule CD28 for binding to B7 ligands (CD80/CD86) on antigen-presenting cells (APCs). Tumors may exploit this checkpoint indirectly by promoting a tolerogenic environment that enhances CTLA-4–mediated suppression. Blocking CTLA-4 with monoclonal antibodies (e.g., ipilimumab) releases this brake on the immune system, leading to enhanced T-cell activation and expansion (Figure 2).

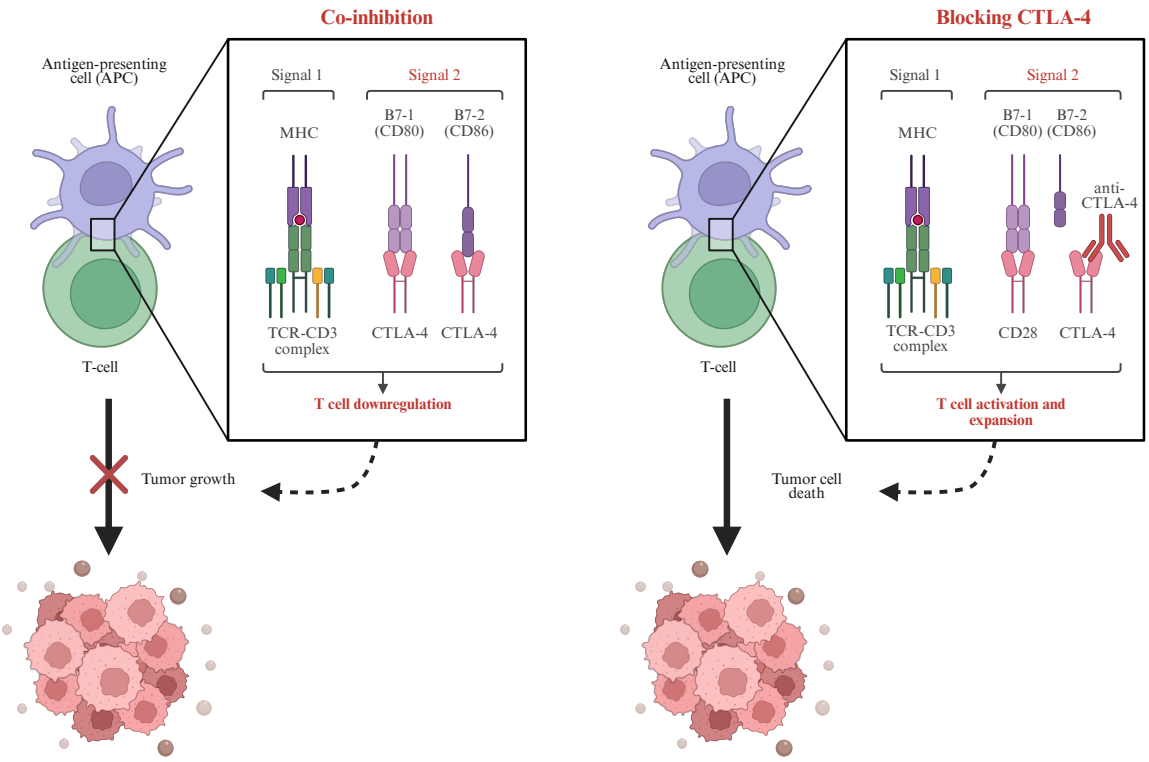

Figure 2. Diagram illustrating the role of CTLA-4 in immune checkpoint inhibition. The figure shows the co-inhibitory process following the blockade of signal 1 (via MHC) and signal 2 (via CD80 and CD86 binding to CTLA-4) contributing to T cell downregulation and tumor growth. In contrast, anti-CTLA-4 antibodies block this inhibitory interaction, thereby enabling T cell activation and expansion aimed at eliminating tumor cells. MHC: Major Histocompatibility Complex. Created with BioRender.com.

- **PD-1/PD-L1 axis:** PD-1 is another inhibitory receptor expressed on the surface of activated immune cells, mainly in T cells, but also in B cells and natural killer (NK) cells. Its ligands, PD-L1 and PD-L2, are expressed on tumor cells, stromal cells, and immune cells within the tumor microenvironment (TME). When PD-1 binds to PD-L1, it

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delivers an inhibitory signal that reduces TCR signaling, limits cytokine secretion, and promotes T-cell exhaustion. Many tumors upregulate PD-L1 as an adaptive resistance mechanism. Therapeutic antibodies targeting PD-1 (e.g., nivolumab, pembrolizumab) or PD-L1 (e.g., atezolizumab, durvalumab) disrupt this axis and reinvigorate exhausted T cells, enhancing their cytotoxic activity against malignant cells (Figure 3).

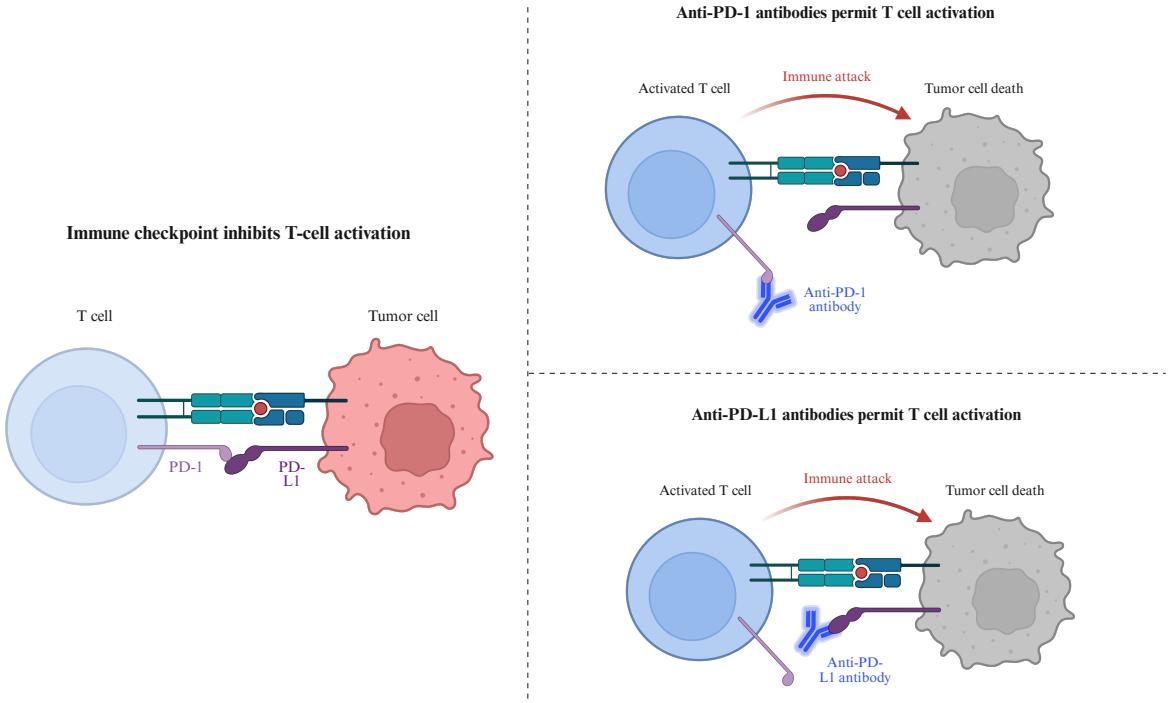

Figure 3. Diagram illustrating the role of PD-1/PD-L1 in immune checkpoint inhibition. The left panel shows how PD-1/PD-L1 interaction suppresses T cell activation. The right panels depict how blockade with anti-PD-1 or anti-PD-L1 antibodies restores T cell activation, promoting an antitumor immune response. Created with BioRender.com.

In metastatic melanoma, combination therapy with CTLA-4 and PD-1 inhibitors achieves objective response rates (ORR) of up to 60 % [22], whereas monotherapy with PD-1 inhibitors alone results in lower response rates around 40 % [2]. Similarly, in advanced NSCLC, response rates to anti-PD-1/PD-L1 therapies vary significantly, ranging from approximately 20 % to 45 % [3, 23], depending mainly on tumor PD-L1 expression levels and individual clinical contexts. These relatively low response rates can be attributed primarily to the complex interplay between tumor heterogeneity, the immunosuppressive TME, intrinsic or adaptive resistance mechanisms, and variability in host immune responses. Hence, inadequate patient

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stratification due to the current limitations of available predictive biomarkers contributes substantially to these inconsistent clinical outcomes.

Several tumor-intrinsic mechanisms have been identified as contributors to resistance in melanoma [24] and NSCLC [25]. In melanoma, one of the most relevant is the loss of antigen presentation, frequently driven by reduced HLA class I expression, which compromises cytotoxic T-cell recognition [26]. Additionally, defects in interferon-γ signaling, particularly mutations in *JAK1* or *JAK2* [27], limit the tumor's ability to respond to immune pressure and reduce inducible PD-L1 expression. In NSCLC, mutations affecting antigen expression pathways decrease antigen availability and hinder T-cell recognition [28]. Exogenous tumor-microenvironmental factors, including increased M2 macrophages, regulatory T cells, and hypoxia-driven *VEGF* expression, further reinforce immunosuppression. Acquired resistance may emerge through epigenetic remodeling, altered protein translation, or clonal evolution [29].

In summary, while immunotherapy has revolutionized the therapeutic landscape of oncology, the field continues to face substantial obstacles in accurately predicting which patients will derive benefit. The development of robust, reproducible, and clinically applicable biomarkers remains a critical priority. A shift toward multi-omic integration and longitudinal, patient-specific analyses is increasingly seen as the most promising path forward in overcoming these limitations.

1.2. Cancer: Melanoma and Non-Small Cell Lung Cancer

Cancer encompasses a heterogeneous group of diseases characterized by the uncontrolled proliferation of abnormal cells, which may lead to the development of malignant tumors with the capacity to invade surrounding tissues and metastasize to distant organs. It stands as one of the leading causes of morbidity and mortality worldwide, carrying significant implications for public health. Our focus, due to their clinical relevance in the context of immunotherapy, is on melanoma and NSCLC [4].

Melanoma originates from the uncontrolled proliferation of melanocytes, the pigment producing cells responsible for melanin synthesis and keratinization. The main subtypes of melanoma include cutaneous melanoma, ocular melanoma, mucosal melanoma and melanoma with unknown primary. These subtypes differ based on clinical outlook, histopathological growth patterns, anatomical location, and associated risk factors. In contrast, NSCLC is the most common histological subtype of lung cancer, accounting for approximately 85 % of all cases [30]. It comprises a group of epithelial lung malignancies that can be distinguished from small cell lung cancer (SCLC) by their slower proliferation rate, distinct cellular morphology, and differential therapeutic responses. The principal NSCLC subtypes include adenocarcinoma,

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squamous cell carcinoma, and large cell carcinoma. These are classified based on the type of cells involved and their appearance under histological examination. Adenocarcinoma arises from glandular epithelial cells and is often located in the lung periphery; squamous cell carcinoma originates from bronchial epithelial cells and is typically centrally located; and large cell carcinoma represents an undifferentiated form that lacks the specific features of the other two and is diagnosed by exclusion. Both melanoma and NSCLC are examples of highly immunogenic tumors, characterized by elevated tumor mutational burden (TMB)—which will be defined and discussed in more detail in a later section—substantial infiltration of effector T cells, and increased expression of immune checkpoint molecules such as PD-L1. These features enhance the immune system's ability to recognize and target tumor cells, contributing to the favorable clinical responses observed with ICIs [31].

According to the Global Cancer Observatory (GCO) of the International Agency for Research on Cancer (IARC) [32], approximately 20 million new cancer cases and 9.7 million cancer-related deaths were recorded worldwide in 2022. Among these, melanoma accounted for an estimated 330,000 new cases and nearly 60,000 deaths. Cancers of the trachea, bronchus, and lung (classified by IARC as a single group for statistical reporting purposes) represented one of the most prevalent and lethal groups, with approximately 2.5 million new cases and 1.8 million deaths globally underscoring their aggressive clinical behavior and the significant challenges associated with their management.

In Spain [33], the estimated number of new cancer cases in 2022 was approximately 265,000. Lung cancer ranked as the third most frequently diagnosed malignancy, with 34,506 new cases, while melanoma accounted for around 10,000 new cases. Cancer remains the leading cause of death among men and the second among women in the country. Lung cancer, in particular, stands out due to its high mortality burden, with around 22,500. In contrast, melanoma was associated with an estimated 1,100 deaths. Focusing on Andalusia [33], cancer incidence in 2022 was estimated at approximately 47,500 new cases, including 5,100 lung cancer diagnoses and 1,050 cases of melanoma. Lung cancer accounted for 3,800 deaths in the region, with a marked gender disparity in mortality rates—69 per 100,000 among men compared to 20 per 100,000 among women. In contrast, melanoma was responsible for around 200 deaths, with mortality distributed relatively equally between males and females.

This study focuses on metastatic melanoma and advanced NSCLC. Metastatic melanoma corresponds to stage IV and is characterized by the spread of tumor cells from the primary lesion to distant organs. This dissemination occurs via lymphatic or hematogenous routes and reflects a loss of local tumor control as well as a more aggressive clinical behavior. Advanced NSCLC includes both metastatic disease, in which cancer has spread to distant organs, and locally advanced disease. The latter corresponds to stage III and involves tumor growth and extension affecting nearby lymph nodes or adjacent structures, without distant organ involvement. Both conditions represent significant clinical challenges and provide a relevant framework for studying systemic therapeutic strategies—treatments designed to

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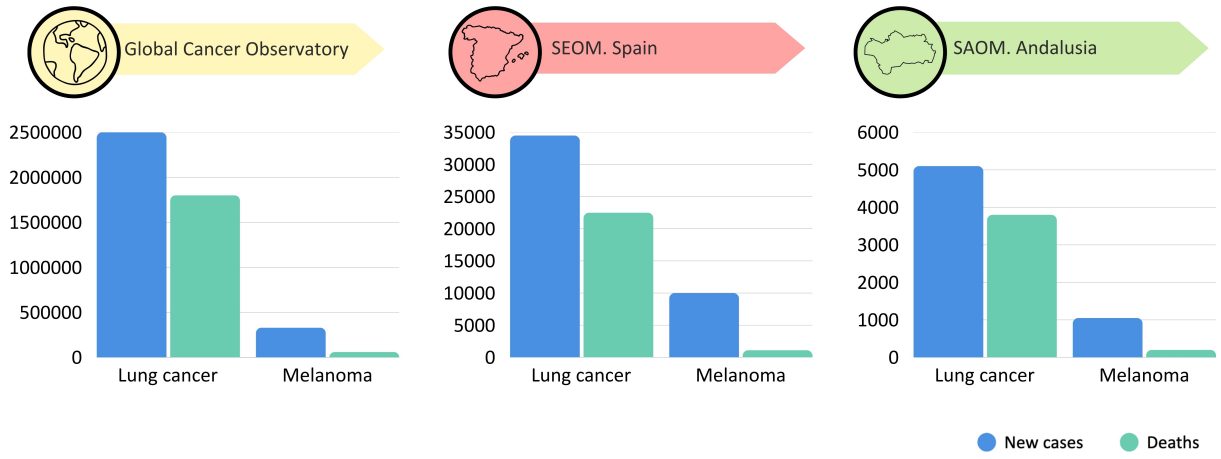

Figure 4. Bar plot showing the number of new cancer cases and cancer-related deaths globally in 2022, in Spain, and in Andalusia. Data sources: Global Cancer Observatory (GCO, IARC) and AECC Cancer Observatory. Created with Canvas.com.

target cancer cells throughout the body rather than solely at the primary tumor site—such as ICIs.

Traditionally, treatment guidelines for NSCLC recommended surgical resection for early-stage tumors and platinum-based chemotherapy, often in combination with radiotherapy, as the standard of care for advanced-stage disease [34]. However, as discussed, ICIs have revolutionized the therapeutic landscape of NSCLC, particularly in the first-line setting for advanced disease [35]. For instance, the KEYNOTE-407 trial [36], which included patients regardless of PD-L1 expression, demonstrated that the combination of pembrolizumab and chemotherapy achieved a median overall survival (OS) of 15.9 months and a median progression-free survival (PFS) of 6.4 months. In contrast, the chemotherapy with placebo arm reported an OS of 11.3 months and a PFS of 4.8 months. The benefit of ICIs becomes even more pronounced in patients with high PD-L1 expression. In the KEYNOTE-024 trial [23], patients with PD-L1 expression $\geq$50 % treated with pembrolizumab monotherapy achieved a median OS of 30.0 months and a median PFS of 10.0 months, compared to 14.2 months OS and 6.0 months PFS in the chemotherapy group. Similarly, the therapeutic landscape of advanced melanoma was historically limited to surgical resection for localized disease, chemotherapy, high-dose IL-2, or radiotherapy, none of which provided consistent or substantial long-term benefit [37]. Only targeted therapies, particularly for patients with *BRAF* mutations, led to measurable improvements in PFS and OS. The introduction of ICIs, however, dramatically disrupted this paradigm. A decade ago, in the pre-ICI era, the 5-year survival rate for metastatic melanoma was approximately 10 %. Today, with the implementation of ICI-based therapies, 5-year survival exceeds 40 % and 20-30 % of patient could be

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cure, marking a transformative shift in outcomes for this historically aggressive malignancy [38].

Despite the success of ICIs in melanoma and NSCLC, only a subset of patients achieves durable clinical benefit. The reasons behind this selective response remain largely unclear. Understanding the mechanisms of resistance and identifying non-responders early—either before or during treatment—would allow for better therapeutic decisions, exploration of alternative strategies, and a reduction in unnecessary toxicity and healthcare costs. Progress toward precision oncology depends on the development of reliable biomarkers that can predict response to ICIs, monitor treatment efficacy in real time, and guide personalized therapeutic approaches. In this context, biomarker discovery is essential to improve patient selection and optimize the clinical use of immunotherapy.

1.3. Biomarkers in Cancer Immunotherapy

Cancer biomarkers are biological molecules, genetic alterations, or cellular features measurable in tissues or bodily fluids that are utilized clinically to guide diagnosis, prognosis, and therapeutic decision-making. Biomarkers are typically categorized based on their clinical application: diagnostic biomarkers, prognostic biomarkers and predictive biomarkers.

- **Diagnostic biomarkers** facilitate the identification of malignancy and enable the classification of cancer into distinct histological or molecular subtypes.
- **Prognostic biomarkers** are often identified from observational data and are regularly used to identify patients more likely to have a particular outcome.
- **Predictive biomarkers** estimate the likelihood of response to specific therapeutic strategies, thus enabling personalized oncology.

Predictive biomarkers in immunotherapy have emerged as critical tools to select patients most likely to benefit from treatment with ICIs. Robust biomarkers predicting therapeutic efficacy are urgently required to optimize clinical outcomes.

In the context of biomarker discovery and clinical application, tissue-based analysis and liquid biopsy constitute the two primary sources of molecular information with diagnostic, prognostic, and predictive relevance. Traditionally, tumor tissue has been considered the gold standard for biomarker assessment, offering comprehensive insights into histopathological architecture, genomic alterations, gene and protein expression profiles, DNA methylation status, and immune cell infiltration [39]. However, tissue sampling is invasive, constrained by both spatial and temporal heterogeneity, and often fails to reflect the dynamic molecular changes that occur during disease progression and treatment. To overcome these limitations,

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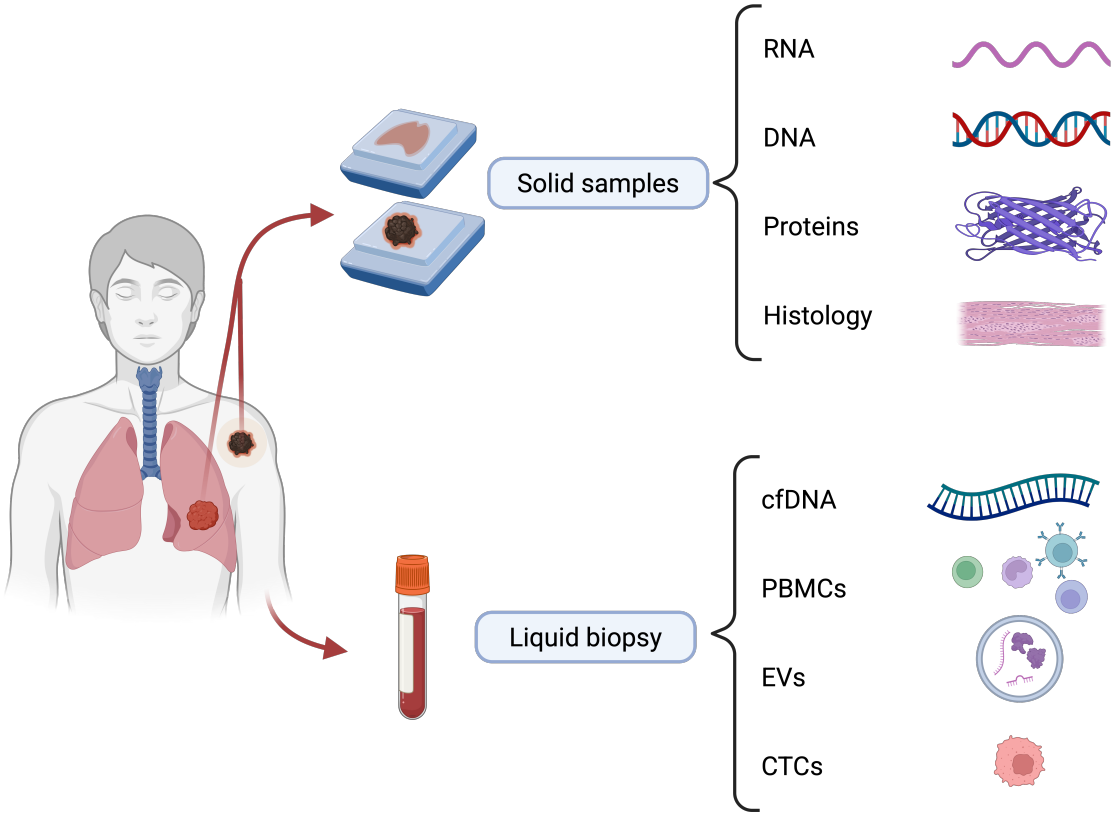

Figure 5. Overview of molecular data accessible from different sources. Solid biopsies can provide DNA, RNA, and protein and histological information, while liquid biopsies (e.g., blood) allow the extraction of circulating free DNA (cfDNA), peripheral blood mononuclear cells (PBMCs), extracellular vesicles (EVs) and circulating tumor cells (CTCs). Created with BioRender.com

liquid biopsy has emerged as a minimally invasive approach, enabling the analysis of circulating biomarkers—including the quantification of cell-free DNA (cfDNA), as well as its genomic and epigenetic landscape, circulating tumor cells (CTCs), RNA, proteins, extracellular vesicles (EVs), and immune-related components—from blood or other body fluids [40]. Liquid biopsy facilitates real-time monitoring of tumor evolution, therapeutic response, and the development of resistance mechanisms, offering a dynamic view of disease progression that tissue sampling alone cannot achieve. Together, tissue-based and liquid biopsy analyses yield complementary information, and their integration is increasingly critical for the advancement of personalized immuno-oncology (Table 1, Figure 5).

A wide range of biomarkers have been identified to guide immunotherapy decisions in cancer, particularly in melanoma and NSCLC. Among them, several have been validated and approved by the U.S. FDA due to their predictive or prognostic value in specific clinical contexts.

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Table 1. Summary of molecular materials and corresponding analytical methods. The table outlines molecular extractable from clinical samples and their applicable analysis techniques. Key limitations and challenges for each material are also highlighted. FFPE: Formalin-Fixed Paraffin-Embedded. Seq: Sequencing. TCR: T-cell receptor. BCR: B-cell receptor. HLA: Human Leukocyte Antigen

Molecular material	**Analytical methods**	**Challenges / Limitations**
DNA	Whole Genome Sequencing Whole Exome Sequencing Methylation profiling	Sample degradation, contamination, and limited yield from formalin-fixed tissues
RNA	Bulk RNA-Seq Single-cell RNA-Seq Immune repertoire profiling (TCR/BCR/HLA-seq)	RNA is inherently unstable and highly susceptible to degradation, particularly in FFPE samples
cfDNA	Quantification Fragment size analysis Methylation assays Mutation detection	Low abundance, fragmentation, potential contamination with genomic DNA
PBMCs	Single-cell RNA-Seq Bulk RNA-Seq Immune repertoire profiling (TCR/BCR/HLA-seq) Flow cytometry	Concerns regarding cell viability and low RNA yield per cell. In liquid biopsies, genomic DNA contamination, while in FFPE samples, single-cell RNA extraction remains technically difficult
EVs	Small RNA-Seq	Low yield, isolation complexity
Proteins	Mass spectrometry Western blot ELISA	Complex extraction, high dynamic range
CTCs	Quantification Whole Genome Sequencing Whole Exome Sequencing Methylation profiling bulk RNA-Seq single-cell RNA-Seq	Extremely low frequency

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These biomarkers are currently used to select patients most likely to benefit from ICIs and to tailor therapeutic strategies accordingly. In addition to these established markers, numerous experimental biomarkers have been reported across a growing body of literature.

FDA-Approved Biomarkers

There are three FDA-approved biomarkers: PD-L1 expression, TMB and MSI/dMMR [41].

- The most widely used and clinically validated FDA-approved biomarker is **PD-L1 expression**. PD-L1 is assessed by immunohistochemistry in tumor tissue and serves as a predictive biomarker for response to anti-PD-1/PD-L1 therapies in both melanoma and NSCLC [42]. PD-L1 levels are commonly reported using two scoring systems: the tumor proportion score (TPS), which represents the percentage of PD-L1-expressing tumor cells, and the combined positive score (CPS), which incorporates PD-L1 expression in tumor and mononuclear inflammatory cells.
- **TMB** is another predictive and prognostic biomarker, measured from tumor tissue via whole-exome sequencing (WES) [43]. TMB is defined as the number of somatic mutations per megabase of the genome and is associated with the likelihood of generating neoantigens, which may enhance immune recognition.
- **Microsatellite Instability (MSI) and Mismatch Repair Deficiency (dMMR)** detected via immunohistochemistry, PCR, or next-generation sequencing (NGS). MSI and dMMR are used as predictive biomarkers across solid tumors [44]. These alterations impair DNA damage response mechanisms, increasing the mutation likelihood and therefore the probability of response to ICIs due to enhanced tumor immunogenicity.

To date, the EMA has approved only PD-L1 expression, MSI and dMMR [45].

Emerging and Experimental Biomarkers

- The presence of TILs and the immunoscore, which quantifies TILs, have been associated with better prognosis and immunotherapy responsiveness in both melanoma and NSCLC, although the phenotypic and functional diversity of infiltrating immune cells limits standardization. In addition, tertiary lymphoid structures (TLS) have also been linked to improved clinical outcomes [46].
- Specific gene expression signatures, have been linked to better responses to ICIs. For instance, a 10-gene IFN-γ signature has demonstrated predictive value in metastatic melanoma patients treated with anti-PD-1 therapies [47].
- Human Leukocyte Antigen (HLA) class I expression on tumor cells is crucial for antigen presentation and subsequent T-cell-mediated immune responses. Studies have shown that HLA class I expression correlates with better outcomes in melanoma patients

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receiving anti-CTLA-4 therapy, though its predictive value for anti-PD-1 therapies remains less clear [48].

- The distribution and phenotype of peripheral immune cell subsets also correlate with treatment outcomes. High circulating $CD4^+$ and $CD8^+$ effector-memory T cell levels have been associated with improved survival in melanoma and NSCLC [49, 50]. Additionally, neoantigen-specific T cell responses have been documented in responders to ICIs in both cancer types [51, 52].

- Soluble immune checkpoint molecules, such as PD-L1 and CTLA-4, especially when carried by exosomes, may reflect dynamic tumor immune adaptation. Elevated circulating PD-L1 on exosomes released from melanoma tumors has been shown to stratify clinical responders from non-responders during early treatment [53].

- Circulating tumor DNA (ctDNA) is an emerging non-invasive biomarker. In NSCLC, a reduction of >50 % in ctDNA levels after treatment with anti-PD-1/PD-L1 agents has been correlated with better radiographic response, improved PFS, and longer OS [54-56]. In this context, the use of TMB measured via ctDNA, namely bTMB (blood TMB), as a noninvasive biomarker to guide ICI therapies has garnered considerable interest [57]. However, this approach still presents limitations, notably the lack of concordance between bTMB and tissue-based TMB measurements [58].

- Inflammatory cytokines such as VEGF, angiopoietin-2, and IL-8 also have prognostic relevance. Elevated baseline VEGF and angiopoietin-2 levels are associated with poorer outcomes in melanoma patients treated with ipilimumab, while on-treatment increasing IL-8 levels have been linked to disease progression in both melanoma and NSCLC [59] .

Nonetheless, many of these biomarkers lack the robustness, reproducibility, and clinical generalizability needed for widespread implementation. Limitations include variability in detection platforms and assay protocols, lack of standardization across studies, tumor heterogeneity, dynamic changes during treatment, and a general limited availability of prospective validation in large cohorts. Moreover, most current biomarkers capture only a fragment of the complex and evolving tumor-immune interaction, which varies between cancer types, anatomical sites, and treatment contexts. As a result, the search for reliable, multifactorial, and dynamic biomarkers remains a key challenge and an active field of research in immuno-oncology. Current efforts are increasingly focused on integrating multi-omic data, longitudinal sampling, and advanced computational modeling to better predict therapeutic response, monitor treatment efficacy in real time, and understand mechanisms of resistance. These approaches aim to move the field closer to truly personalized immunotherapy guided by robust, clinically actionable biomarker profiles.

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1.4. Omics Applications for Biomarker Discovery in Oncology

The term "omics" refers to a collection of scientific disciplines aimed at the large-scale, comprehensive analysis of biological molecules—such as the genome, transcriptome, proteome, epigenome, and metabolome—within a cell, tissue, or organism. The ongoing expansion of omics research has been driven by significant advancements in high-throughput platforms, most notably NGS, which has revolutionized biomedical research and cancer biology [60]. NGS enables the systematic molecular profiling of the state of the disease, providing critical insights for identifying predictive and prognostic biomarkers, understanding mechanisms of drug resistance, and designing personalized treatment strategies. This integrative approach aligns with the core objectives of precision oncology: delivering the right therapy to the right patient at the right time, with maximal efficacy and minimal toxicity.

Among the different omics approaches, this section will focus on genomics, epigenomics, transcriptomics, and proteomics [61] (Figure 6).

Genomics aims to identify genetic variations across the genome. These studies typically involve the genotyping of millions of markers to detect statistically significant differences in allele frequencies between case and control cohorts.

- Input material: DNA from tumor tissue or matched normal tissue, circulating cfDNA in liquid biopsy or DNA from PBMCs.
- Typical output: Variant Call Format (VCF) files containing single nucleotide variants (SNVs), insertions/deletions (indels), copy number variations (CNVs), and structural variants (translocations).
- Downstream analysis: Variant annotation, pathogenicity prediction, association with clinical phenotypes, mutational burden estimation, and identification of driver mutations.
- NGS options: Whole Genome Sequencing (WGS) for comprehensive coverage, WES for protein-coding regions, and targeted panels for specific cancer or resistance-associated genes.

Epigenomics investigates reversible and heritable modifications of DNA and histone proteins—such as DNA methylation, hydroxymethylation, and histone acetylation—that influence gene expression without altering the underlying DNA sequence. These epigenetic changes, shaped by both genetic and environmental factors, play a crucial role in regulating cell fate and contributing to disease progression.

- Input material: DNA from tumor tissue or matched normal tissue and cfDNA.
- Typical output: DNA methylation landscapes, histone modification profiles, and chromatin accessibility data.

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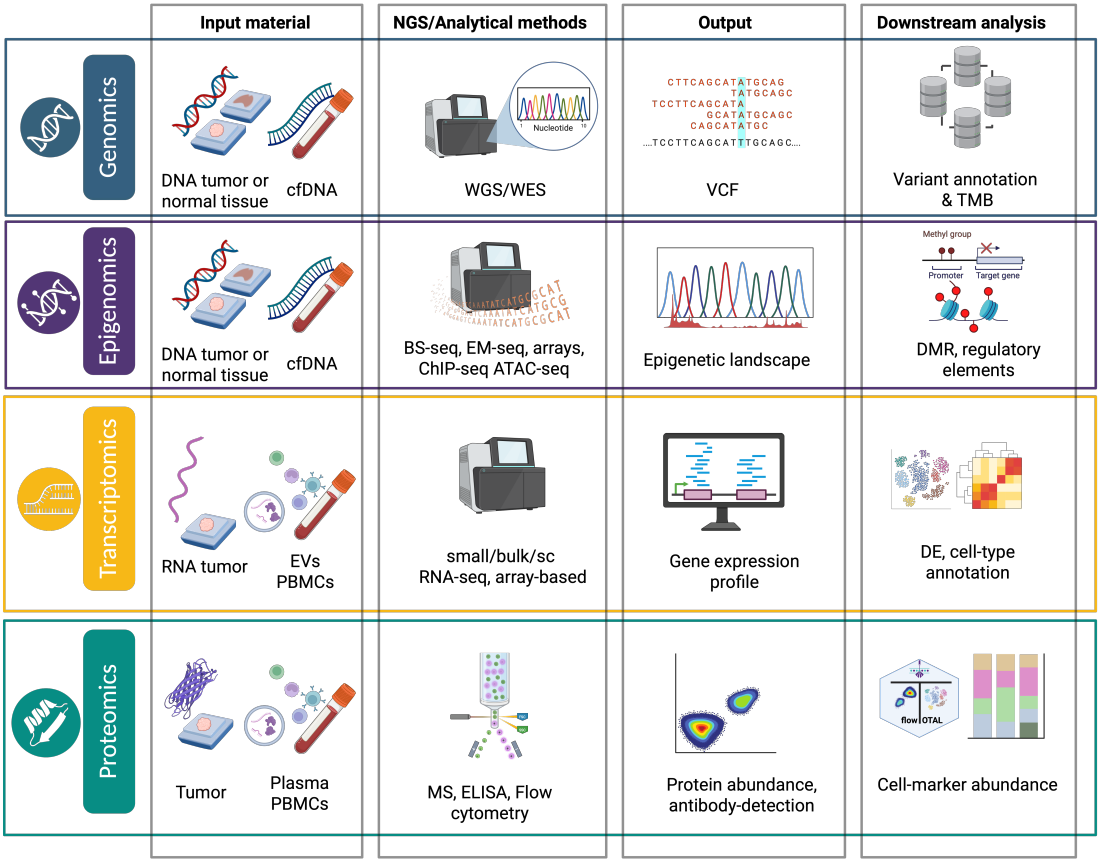

Figure 6. Overview of omics workflows, from input material to downstream analysis. This figure summarizes the key components of genomics, epigenomics, transcriptomics, and proteomics pipelines, including the input biomaterials, analytical methods, outputs generated, and typical downstream analyses. VCF: Variant Call Format. WGS: Whole Genome Sequencing. WES: Whole Exome Sequencing. DMR: Differentially Methylated Region. DE: Differential Expression. MS: Mass Spectrometry. Created with BioRender.com.

- Downstream analysis: Identification of differentially methylated regions (DMRs), mapping of regulatory elements, and integration with transcriptomic data.
- NGS options: Bisulfite sequencing (BS-seq), Enzymatic Methyl-seq (EM-seq), arrays (e.g. EPIC), ChIP-seq, and ATAC-seq.

Transcriptomics provides a comprehensive view of RNA expression at the genome-wide level, both qualitatively and quantitatively. Depending on the experimental design, transcriptomic analyses may target the complete transcriptome, messenger RNA (mRNA), or various classes of non-coding RNAs, including long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNAs.

- Input material: Tumor tissue, liquid biopsy,e.g., plasma RNA, PBMCs or EVs.

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- Typical output: Gene expression profiles, alternative splicing patterns, RNA editing events, and non-coding RNA expression data.
- Downstream analysis: Differential expression analysis, functional enrichment of pathways, co-expression network construction, and cell-type identification and quantification.
- NGS options: Bulk RNA sequencing (RNA-seq), single-cell RNA-seq (scRNA-seq), small RNA-seq, total RNA-seq and array-based technologies (e.g., NanoString nCounter).

Proteomics is dedicated to the large-scale analysis of proteins, including their expression levels, post-translational modifications (PTMs), and interaction networks.

- Input material: Tumor tissue, liquid biopsy (e.g., serum, plasma), PBMCs.
- Typical output: Quantification of protein abundance, protein-protein interaction maps, and profiles of PTMs .
- Downstream analysis: Functional annotation, identification of protein complexes, and reconstruction of signaling pathways.
- NGS options: While not directly reliant on NGS, high-throughput proteomic profiling is commonly performed using mass spectrometry (MS), ELISA and flow cytometry-based platforms.

The advancement of multi-layered molecular knowledge in cancer research has enabled the development of biomarker-stratified and genome-informed clinical trial designs, in which patient enrollment and treatment allocation are guided by individual molecular profiles. As a result of these efforts, several NGS-based panels have been introduced into clinical practice [62]. For instance, the Oncomine Focus Panel [63] includes 52 genes primarily associated with targets of currently available therapies, supported by published clinical evidence. Larger panels have also emerged, such as the FDA-approved FoundationOne CDx [64] and MSK-IMPACT panel [65]. While these broad molecular panels significantly contribute to the understanding of cancer genomics, they also encourage further research aimed at refining panel composition [66]. Such efforts may ultimately support the development of practical and cost-effective panels for routine clinical application in oncology (Figure 7).

Despite considerable progress, immuno-oncology still faces significant challenges in fully capturing the complexity of tumor heterogeneity and its dynamic interactions with the immune system, as well as in translating these insights into clinical practice [67]. Current efforts are focused on overcoming these limitations through the development of robust analytical frameworks and the validation of biomarkers in large, heterogeneous patient cohorts. In this context, we advocate for a comprehensive approach to cancer biology through the integration of multi-omics strategies in a longitudinal prospective cohort [68]:

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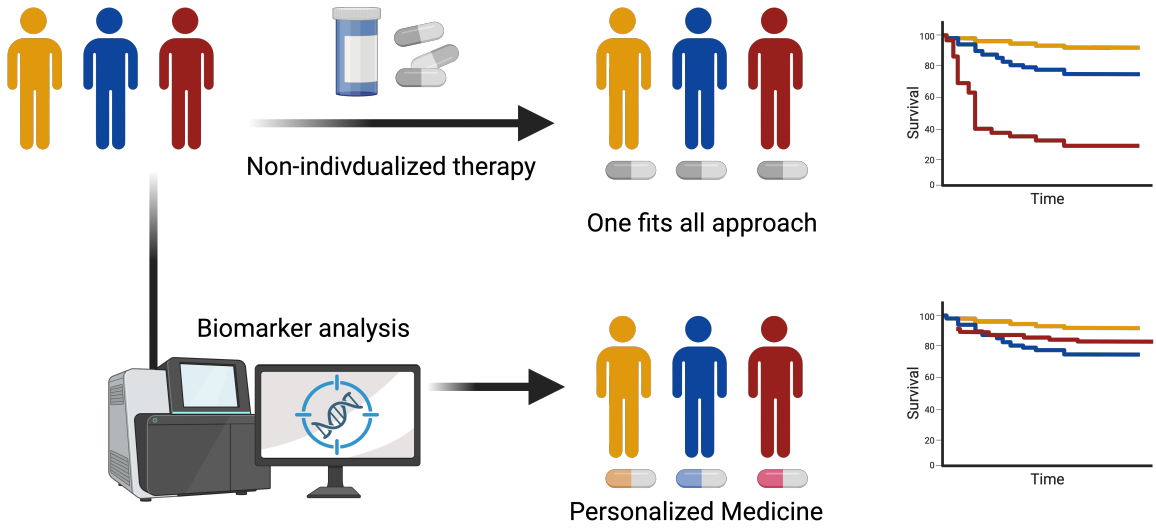

Figure 7. Towards clinically applicable biomarker panels in oncology. Schematic overview of current strategies aimed at identifying robust and practical biomarker panels to guide therapeutic decision-making in routine clinical practice. Created with BioRender.com.

- A **multi-omics analysis** integrates multiple molecular layers from the same individual, offering a comprehensive view of biological systems and the mechanisms underlying disease and drug resistance.
- A **longitudinal prospective cohort** is a study design in which a group of individuals is followed over time from a defined starting point, allowing the collection of sequential data to monitor disease progression, treatment response, and temporal changes in biological markers.

However, the analysis of such complex datasets relies heavily on advanced computational tools, including machine learning algorithms powered by Artificial Intelligence, which enable the identification of intricate biomarker signatures. Recent studies have shown that Machine Learning and integrative multi-omics approaches significantly improve the precision of treatment response prediction, paving the way for personalized medicine [69-72].

1.5. The Role of Artificial Intelligence

Artificial intelligence (AI) is emerging as a transformative tool across multiple scientific domains, including oncology, where it offers promising advances in cancer detection, diagnosis and treatment planning. Initially conceptualized in the mid-20th century—through Alan

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Turing's pioneering insights and the formal definition proposed by John McCarthy—AI has evolved from simple rule-based systems to complex, adaptive algorithms capable of recognizing patterns and learning from large datasets to solve specific tasks with increasing accuracy.

The scientific relevance of this technological shift was formally recognized in 2024, when the Nobel Prizes in Physics and Chemistry were awarded for foundational contributions to the field of AI. John J. Hopfield and Geoffrey Hinton received the Nobel Prize in Physics for their seminal work in Artificial Neural Networks and Machine Learning. In parallel, David Baker, Demis Hassabis, and John Jumper were honored in Chemistry for advancing protein structure prediction and rational design through AI-driven methodologies. These distinctions underscore the profound and cross-disciplinary impact of AI in data-driven analysis, including its growing integration in cancer research and healthcare.

In the field of oncology, AI is being progressively embedded within high-performance computing frameworks to enhance clinical decision-making processes. Its applications are broad, encompassing early cancer detection, tumor subtype classification, biomarker identification, the prediction of therapeutic responses and the discovery of novel therapeutic targets [73, 74]. Central to this technological evolution are Machine Learning and Deep Learning—two subfields of AI that enable autonomous model refinement and facilitate the analysis of high-dimensional, multi-modal datasets [75].

Machine Learning (ML) is a subfield of AI that enables systems to learn autonomously from data without explicit programming. ML algorithms identify patterns within datasets and use them to make predictions when exposed to new, unseen data. Common types of ML algorithms vary in complexity and interpretability; each suited to specific data structures and prediction tasks.

- Generalized Linear Models (GLM) extend linear regression to accommodate different types of outcome variables using link functions [76].
- Lasso regression is a regularized linear model that introduces an L1 penalty (a constraint that promotes sparsity in the parameter vector by driving some coefficients to zero) to perform variable selection and shrinkage, improving model interpretability and reducing overfitting [77].
- Support Vector Machines (SVM) are powerful classifiers that construct optimal hyperplanes to separate data points in high-dimensional spaces, particularly effective in cases of non-linear separability when combined with kernel functions [78]. These functions map the original input vectors into a higher-dimensional feature space.
- Random Forest (RF), an ensemble learning method based on decision trees, aggregate predictions from multiple trees to improve accuracy and robustness while reducing variance [79].

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- Extreme Gradient Boosting (XGBoost) constructs an ensemble of decision trees in a sequential manner, with each subsequent tree trained to minimize the residual errors of the aggregate model generated up to that point [80].

Deep Learning (DL) is a specialized branch of ML that employs artificial neural networks with multiple layers to model complex and non-linear relationships in data. These networks consist of interconnected computational nodes arranged in input, hidden, and output layers. DL models are particularly suited for unstructured or high-dimensional data and are central to many state-of-the-art AI applications such as image recognition, speech processing, and specific biomedical data analysis. Unlike traditional ML, DL models extract relevant features automatically, minimizing the need for manual feature engineering. Common types of neural networks used in DL include:

- Feedforward Neural Networks (FFNNs): The simplest form, where data flows in one direction from input to output [81].
- Recurrent Neural Networks (RNNs): Designed for sequential data, retaining memory of prior inputs to inform current predictions [82].
- Long Short-Term Memory networks (LSTMs): A type of RNN that incorporates mechanisms to preserve information across longer sequences, meaning they can remember dependencies that occur many time steps apart [83].
- Convolutional Neural Networks (CNNs): Optimized for spatial data, such as images, by applying convolutional and pooling layers to extract features [**ecungradient-based1998**].
- Generative Adversarial Networks (GANs): Comprise two neural networks—a generator and a discriminator—that compete to improve the quality and realism of generated data [84].

Furthermore, depending on the type of task to be addressed using the aforementioned AI algorithms, three main categories can be distinguished: supervised learning, unsupervised learning, and reinforcement learning.

Supervised learning relies on a labeled training dataset, where both input features (x) and corresponding target values (y) are provided. The model learns to map inputs to outputs by minimizing the error between predicted and true values. Once trained, the model can predict outputs for new, unseen data. Depending on the nature of the output variable, supervised learning problems are typically divided into:

- Regression algorithms, where the target variable is continuous.
- Classification algorithms, where the goal is to assign categorical labels.

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The model parameters are calibrated during training using known input–output pairs to optimize prediction performance (hyperparameter tuning), which requires rigorous evaluation of the model's ability to generalize. To achieve this, cross-validation is employed: the training dataset is repeatedly partitioned into complementary subsets, with one subset used for validation and the remaining for model fitting. Finally, an independent test set is used to provide an unbiased evaluation of the model's predictive performance on unseen data.

Unsupervised learning leverages unlabeled data to uncover hidden structures or patterns based solely on input features (x). Typical tasks include:

- Clustering, where similar data points are grouped together based on shared characteristics.
- Dimensionality reduction, which projects data from a high-dimensional space to a lower-dimensional representation to facilitate analysis or visualization.

These methods are particularly useful for exploratory data analysis and pattern discovery in complex datasets.

In **reinforcement learning**, the algorithm learns by interacting with an environment. The system is provided with a set of states, actions, and a reward function. Through trial and error, the model learns to take actions that maximize cumulative rewards over time. Unlike supervised learning, no explicit input–output pairs are given. Instead, the agent refines its behavior based on feedback from previous actions.

A hybrid approach, known as **semi-supervised learning**, involves partially labeled datasets, allowing the model to learn structure while guided by known outcomes.

Despite the significant advances in the application of AI to biomedical and clinical research, interpretability remains one of its most pressing challenges [85]. In the context of ML, while certain models—such as decision trees or GLMs—offer a high degree of transparency, more complex approaches like ensemble methods (e.g., Random Forests) introduce a level of opacity that increases with model complexity. This issue is further exacerbated in DL, where artificial neural networks function as true "black boxes." Their multi-layered and highly nonlinear architectures make it difficult to trace the internal representation of data and the logic underlying predictions. This lack of interpretability can undermine user trust, interfere with regulatory approval, and limit clinical integration—particularly in oncology, where understanding the rationale behind predictive outputs is essential for patient safety and informed decision-making [86].

Moreover, interpretability is not the only aspect in which they differ; these methods also vary substantially in their architectural design, data requirements, and computational complexity. Each method exhibits distinct advantages and limitations, and their suitability depends on the nature of the dataset, the complexity of the features, the volume of available data,

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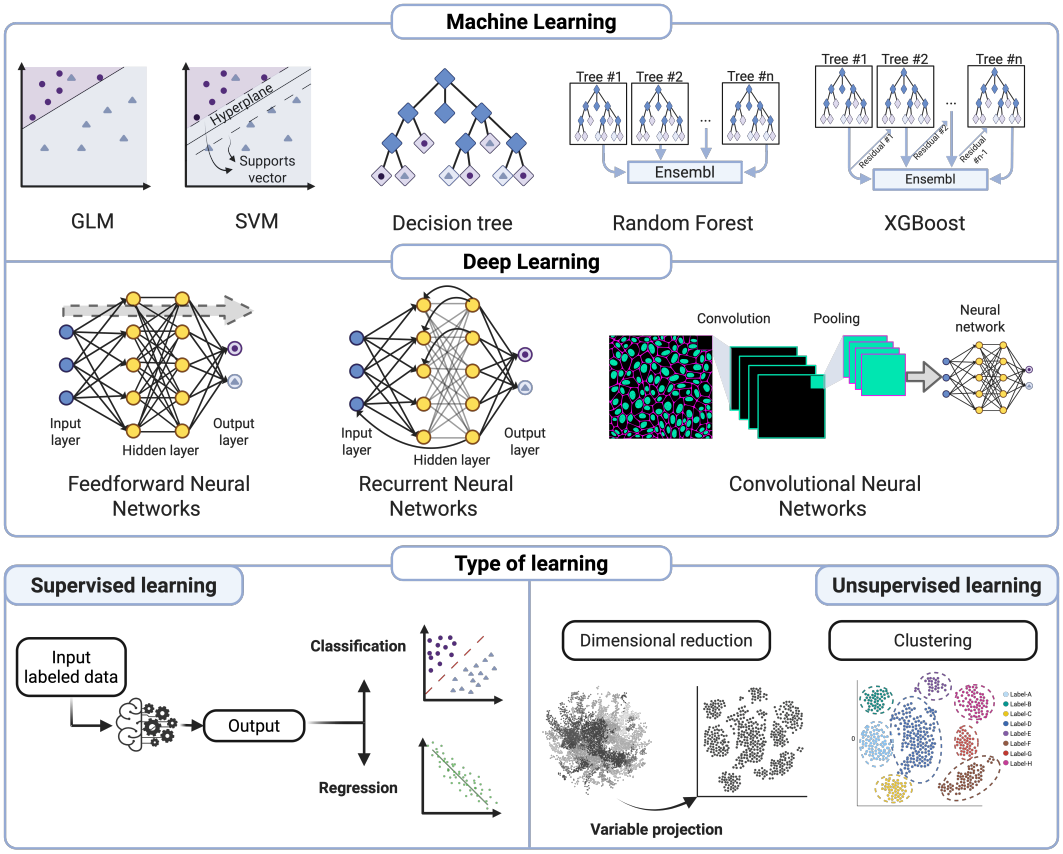

Figure 8. Schematic overview of Machine Learning (ML) and Deep Learning (DL), along with learning strategies. This figure presents representative machine learning algorithms—including generalized linear models (GLM), support vector machines (SVM), decision trees, Random Forest (RF), and extreme gradient boosting (XGBoost)—as well as deep learning architectures such as feedforward neural networks (FNN), recurrent neural networks (RNN), and convolutional neural networks (CNN). Both supervised and unsupervised learning strategies are illustrated, covering tasks such as classification, regression, dimensionality reduction, and clustering. Created with BioRender.com.

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and the specific goals of the analysis. See Table 2 for a comparative overview of algorithm characteristics.

Table 2. Advantages and disadvantages of Machine Learning (ML) and Deep Learning (DL). This table summarizes the main strengths and limitations of ML and DL approaches.

	Machine Learning (ML)	**Deep Learning (DL)**
Advantages:	• Requires relatively smaller datasets to achieve acceptable performance. • Generally more interpretable and explainable, which is crucial in clinical settings. • Faster training times and lower computational cost, often executable on standard Central Processing Units (CPUs). • Includes well-established algorithms with proven reliability in structured data applications.	• Capable of automatically learning hierarchical feature representations, reducing the need for manual feature selection. • Highly effective in modeling non-linear, high-dimensional relationships. • Particularly suitable for large-scale, unstructured datasets (e.g., medical imaging, genomics, natural language). • State-of-the-art performance in many prediction and classification tasks.
Disadvantages:	• Performance can plateau with increasing data complexity or volume. • Often requires manual feature engineering and domain expertise for optimal results. • Less effective with unstructured data such as images, audio, or free text.	• Requires large amounts of labeled data for training to avoid overfitting. • Computationally intensive; often demands Graphics Processing Unit (GPU) acceleration and substantial memory resources. • Functions as a "black box,"making interpretation and explainability challenging. • Longer training times and higher implementation complexity.

Therefore, model selection should not be based on arbitrary or default choices, but rather on a systematic evaluation tailored to the research context. This includes benchmarking multiple algorithms through appropriate validation strategies—such as cross-validation—while balancing predictive performance with interpretability. This is particularly relevant in clinical and biological applications.

A representative example of AI applied to immunotherapy is the study by Charoentong et al. (2017) [69], which presented a large-scale computational framework to characterize

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tumor–immune interactions across 20 solid tumor types. Using a Random Forest classifier, the authors developed the Immunophenoscore (IPS), a ML-based scoring system designed to quantify tumor immunogenicity. The IPS integrates a wide array of immune-relevant parameters, including the expression of immune checkpoints, MHC molecules, effector cell markers, and immunosuppressive components. Although the IPS demonstrated potential in predicting response to ICIs, its clinical utility remains limited by the need of available validation datasets, and further refinement is needed to tailor its performance to specific tumor types.

In a complementary approach, Boehm et al. (2019) [70] introduced ForestMHC, a ML framework, aimed at improving the prediction of peptide presentation by MHC class I molecules. Accurate modeling of MHC-I ligand presentation is critical for the identification of immunogenic neoantigens and the design of personalized cancer vaccines. By training on the largest known set of mass spectrometry-derived MHC-I peptide data, ForestMHC outperformed existing prediction tools and provided novel insights into the influence of gene expression on antigen presentation.

A distinct application of DL in immunotherapy was proposed by Reiman et al. (2019) [71], who developed NEXT (Neural-based models for integrating gene EXpression and visual Texture features), a multimodal framework that combines transcriptomic and histopathological image data to model the tumor-immune microenvironment. Through integration of RNA-seq profiles and digitized pathology slides, the model enabled improved prediction of immune infiltration, with enhanced performance in identifying B cells and $CD8^+$ T cells. These results highlight the potential of DL architectures to capture both spatial and molecular information, supporting more accurate immunological profiling for clinical decision-making.

Despite the extensive efforts dedicated to identifying reliable biomarkers of response to ICIs in melanoma and NSCLC, no robust and externally validated predictive signature has yet emerged. A major limitation across many studies lies in the lack of consistency regarding how therapeutic response is defined, leading to variability in outcome interpretation and limited clinical applicability. To address these challenges, we have designed a study that applies standardized and biologically relevant criteria for response assessment, based on well-defined metrics of primary resistance as outlined in the recent consensus guidelines by the Society for Immunotherapy of Cancer (SITC) [87]. In addition, we aim to holistically explore approaches that have not yet been applied in this context, specifically unsupervised methods for identifying molecular subtypes associated with response to ICIs. Our approaches aim to improve the reproducibility and clinical relevance of biomarker discovery in immunotherapy, ultimately advancing the implementation of precision oncology.

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1.6. Our Clinical and Translational Protocol: A Multi-Omics Approach

This doctoral thesis is embedded within the IMMUNOMARK research project, a prospective clinical study designed to investigate predictive and prognosis biomarkers to ICIs therapies in metastatic cancer. The study comprised two patient cohorts: one with metastatic melanoma and the other with advanced NSCLC, resulting in a total of 180 patients treated with ICIs, either as monotherapy or in combination, and followed longitudinally (yielding a total of 348 samples).

In the melanoma cohort (n=89), treatment strategies involved anti-PD1 (nivolumab) mainly as monotherapy, with some use in combination strategies involving other investigational agents. In the NSCLC cohort (n=91), patients received anti-PD1 (nivolumab) or anti-PD-L1 (atezolizumab) either as monotherapy or in combination with chemotherapy or other investigational agents.

Across both cohorts, liquid biopsy samples were collected at predefined timepoints: prior to treatment initiation (T1), following the second treatment cycle (T2; approximately 2–3 weeks), and at six (T3) and twelve months (T4) post-initiation. When feasible, additional samples were obtained at the time of radiological or clinical progression. Clinical and imaging evaluations were performed at three, six, and twelve months to monitor therapeutic response. In parallel, a subset of patients contributed to a retrospective collection of solid tumor biopsies preserved in formalin-fixed paraffin-embedded (FFPE) blocks, allowing for complementary tissue-based molecular analyses.

The IMMUNOMARK protocol was initially funded by the European Commission through the Horizon 2020 program in 2018 (Grant No. 799818, H2020-MSCA-IF-2017) and subsequently supported by competitive programs from the Carlos III Health Institute, including Health Strategic and Technology Transfer Projects. Ethical approval was granted by the Provincial Research Ethics Committee of Málaga (Spain), under the title “Omics integration for precision cancer immunotherapy” (Approval No. 26/10/2017). All study procedures conformed to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines.

Eligibility criteria; patients were enrolled if they met the following inclusion criteria:

- NSCLC cohort: Histological confirmation of stage IIIB–IV NSCLC, age ≥18 years, adequate renal, hepatic, cardiac, and bone marrow function. Exclusion criteria included contraindications to ICIs or chemotherapy, uncontrolled infections, refusal to provide informed consent, coexisting neoplastic, and prior oncologic therapy of any kind.

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- Melanoma cohort: Histological diagnosis of metastatic melanoma, age ≥18 years, and adequate organ function based on standard laboratory values. Exclusion criteria were similar to those in the NSCLC group.

All clinical data were prospectively collected and extracted from complete electronic medical records, with no loss to follow-up reported.

In the collected samples, molecular analyses are performed on solid tumor biopsies when available, and through liquid biopsy components including PBMCs, cfDNA, and EVs. These molecular markers are systematically correlated with distinct clinical outcomes, including extreme response phenotypes (defined as sustained responders at 12 months and early progressors at 3 months), primary resistance according to SITC criteria [87] (non-resistant patients showed RECIST v1.1 response or stable disease ≥6 months; resistant patients progressed within 6 months after at least two ICI cycles), and overall patient survival. In a subset of patients, matched multi-omic analyses across these molecular layers are conducted within the same individual and longitudinally, enabling integrative profiling to better understand the mechanisms underlying response and resistance to immunotherapy. The design protocol is summarised in Figure 9.

1.7. Hypothesis and Objectives of the Thesis

Hypothesis: The central hypothesis of this doctoral thesis is that ML and multi-omics approaches provide a comprehensive and robust framework for the identification of predictive biomarkers in cancer immunotherapy. By integrating genomic, transcriptomic, and epigenetic information across both solid and liquid biopsies, it is possible to capture the biological complexity of tumor–immune interactions and accurately predict the response to ICIs.

General aim: To explore and validate multi-omic biomarkers associated with response and resistance to immunotherapy in metastatic melanoma and advanced NSCLC patients, through integrative bioinformatics and AI methodologies applied to clinically annotated solid and liquid biopsy samples.

Specific objectives: This thesis is structured as a compendium of four scientific articles, each aligned with a specific research objective derived from the central hypothesis:

- Objective 1: To identify transcriptomic signatures associated with extreme phenotypes of response to ICIs in metastatic melanoma, using differential expression and pathway-level analysis in solid tumor biopsies.
- Objective 2:
    1. To develop a predictive model of primary resistance to ICIs in metastatic cutaneous melanoma based on pre-treatment tumor tissue transcriptomic signatures.

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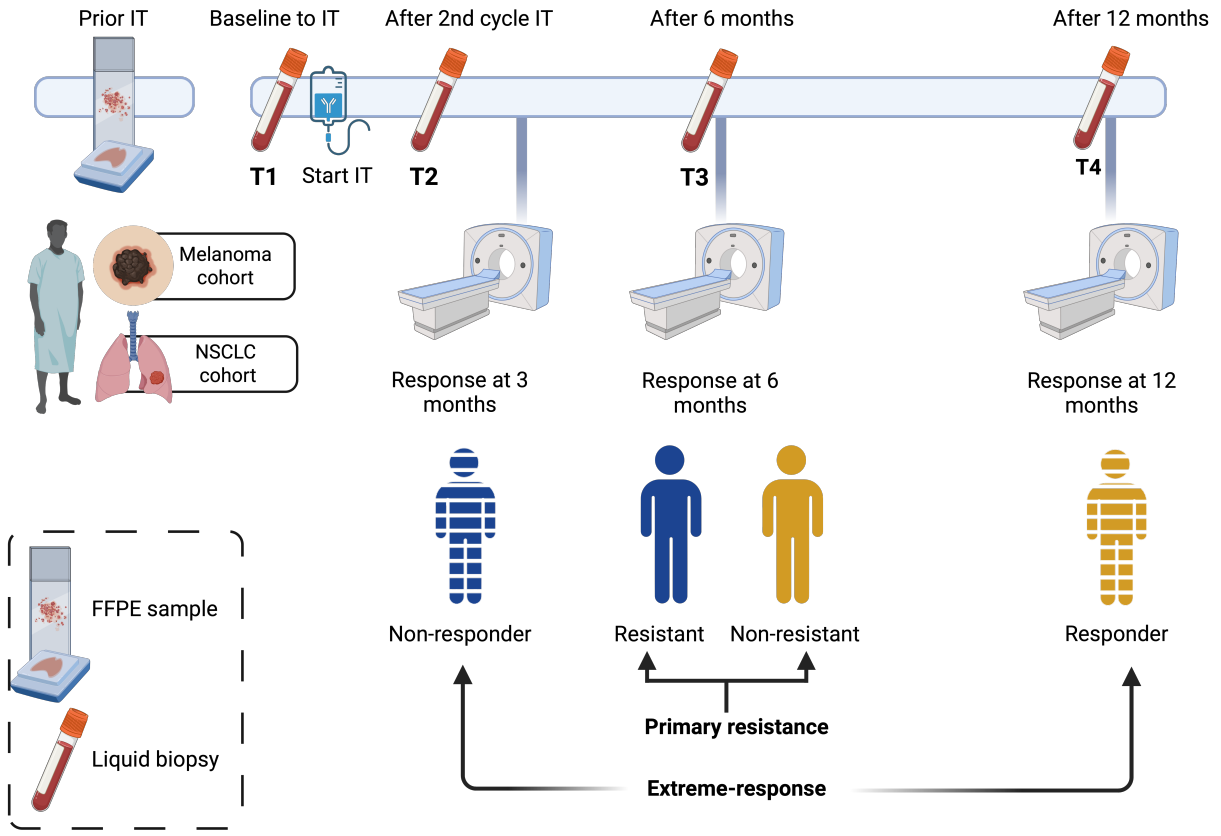

Figure 9. Overview of patient cohort design, sampling timeline, and response classification. This figure illustrates the experimental design, including the collection of solid tumor samples prior to immunotherapy (IT) and longitudinal peripheral blood sampling. Blood samples were obtained at four time points: baseline (T1), after the second cycle of IT (T2), at six months (T3), and at twelve months (T4). Patients were clinically monitored over time and stratified based on treatment outcomes into the following response categories: extreme-response (responders; disease control ≥12 months and non-responders; progression by month 3) and primary resistance (SITC criteria, 6 months of evaluation). Created with BioRender.com.

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2. To correlate circulating immune cell composition data—derived from both bulk deconvolution and single-cell transcriptomics—with liquid biopsy profiles and clinical outcomes.

- Objective 3: To evaluate cfDNA in liquid biopsy as non-invasive predictors of immunotherapy response in advanced NSCLC.
- Objective 4: To integrate multiple omic layers from liquid biopsy (e.g., cfDNA and EV-derived miRNAs) into a unified analytical framework, with the aim of uncovering distinct molecular subtypes with hidden mechanisms of resistance to ICIs in advanced NSCLC.

The thesis integrates clinical, molecular, and computational approaches to explore tumor–immune interactions across both solid and liquid biopsy samples, with a translational perspective. Together, these four objectives address complementary aspects of biomarker discovery and validation, converging on a data-driven strategy to support precision oncology and guide clinical decision-making in cancer immunotherapy.

1.8. Structure and Scientific Contribution of the Thesis

This doctoral thesis is presented as a compendium of four original research articles, all of which are thematically aligned with the central objective. The four manuscripts included in this compendium are as follows:

- **Paper #1: Onieva JL**, Xiao Q, Berciano-Guerrero MÁ, et al. High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade. Int J Mol Sci. 2022;23(16):9124. doi:10.3390/ijms23169124
- **Paper #2: Onieva JL**, Pérez-Ruiz E, Vilkki V, et al. Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma. bioRxiv. 2025:670062. doi:10.1101/2025.08.13.670062
- **Paper #3:** Oliver J, **Onieva JL (equally contributed)**, Garrido-Barros M, et al. Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade. Cancers (Basel). 2023;15(13):3357. doi:10.3390/cancers15133357
- **Paper #4: Onieva JL**, Pérez-Ruiz E, Figueroa-Ortiz L, et al. Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC. medRxiv. 2025:25333961. doi:10.1101/2025.08.19.2533-3961

Following the regulations of the Málaga University Doctoral School, this thesis includes:

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i. a comprehensive introduction outlining the scientific context, the rationale for the study, and a review of the current state of the field;

ii. a global synthesis of the main findings derived from the collective work;

iii. a discussion and the final conclusions of the thesis; and

iv. the full texts of the published articles that constitute the core of this research project.

This structure ensures that the thesis not only presents novel and original scientific contributions but also reflects a coherent and integrated line of research, offering a significant advance in the field of precision oncology and immunotherapy biomarker discovery.

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Chapter 2: Summary of the Results

This section presents a comprehensive synthesis of the principal findings obtained from the original research articles comprising this doctoral thesis.

This dissertation consists of four interconnected studies designed to address the objectives outlined in the introduction. The first two studies focus on metastatic melanoma, while the latter two investigate advanced NSCLC. Across these works, various bioinformatic approaches were employed depending on the clinical endpoint under investigation—including extreme response, primary resistance, and overall survival. A central feature of this thesis is the integration of multi-modal data types across studies. Tissue-based transcriptomic analyses are employed in the first article; the second combines tissue and liquid biopsies to deepen immunogenomic insight; and the third and fourth rely exclusively on circulating biomarkers derived from blood.

To guide the reader through this multi-layered investigation, a summary figure illustrates the relationships among cohorts, methodologies, and omics layers, providing a visual bridge that connects the individual contributions of each paper into a cohesive analytical framework (Figure 10).

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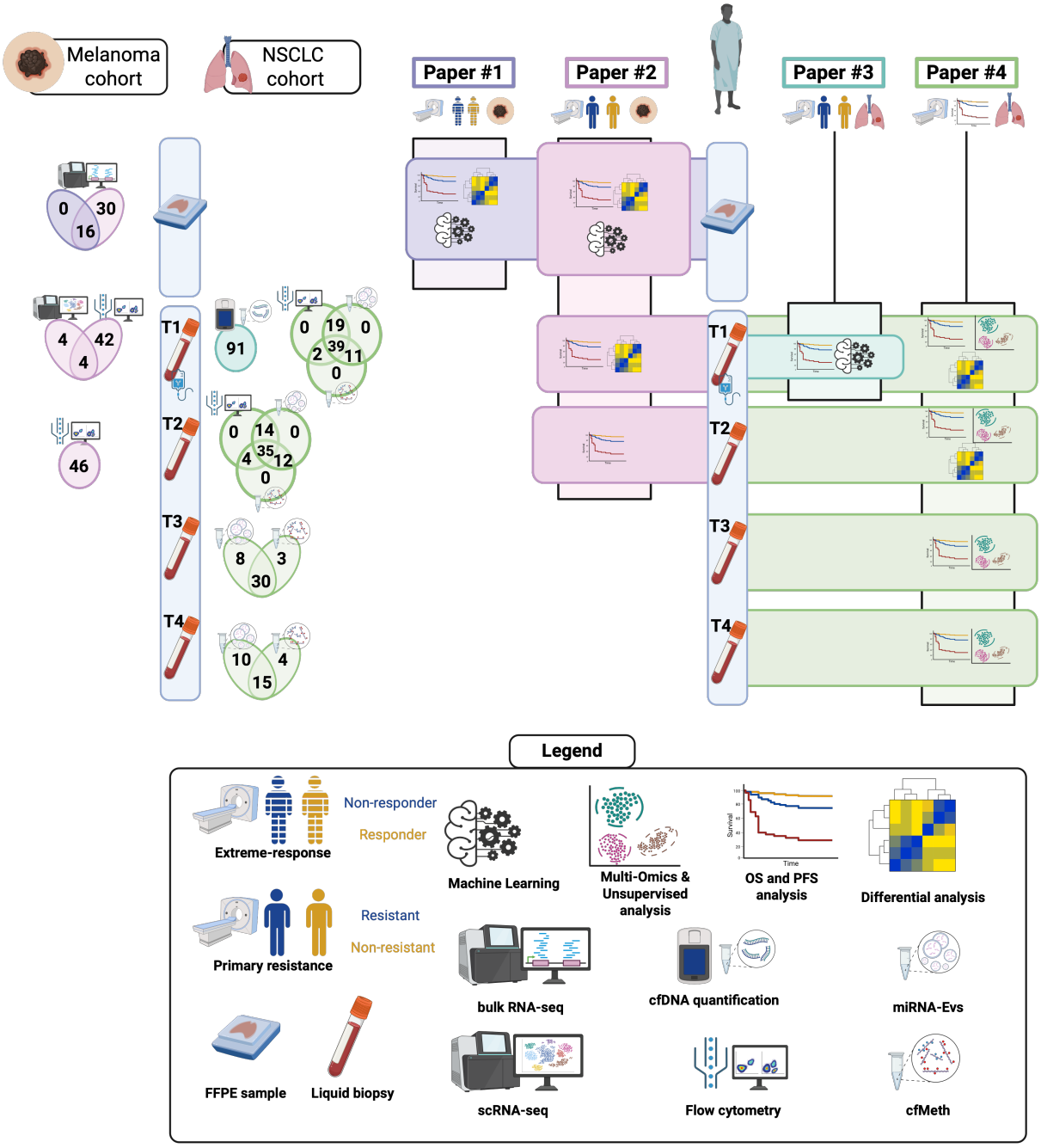

Figure 10. Integrated overview of cohorts, methodologies, and multi-omics layers across the four papers. The figure provides a schematic summary of the results of the doctoral thesis. It maps the relationships among patient cohorts, analytical methodologies, and the diverse omics data layers employed throughout the four interconnected studies. The Venn diagrams illustrate the number of samples shared across the different omics experiments. Colors correspond to each individual paper, while accompanying symbols indicate the specific experimental approaches performed in each case. Created with BioRender.com.

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Paper #1

In the first study, a transcriptomic analysis of solid tumors was conducted to identify predictive biomarkers of response to ICIs with nivolumab (anti-PD-1 therapy) in metastatic melanoma, with a specific focus on cutaneous melanoma. The cohort included 21 melanoma patients, of whom 16 had cutaneous subtype. Clinical response was classified based on extreme outcomes: patients experiencing disease progression within 3 months were defined as non-responders, whereas those achieving durable clinical benefit for at least 12 months were categorized as responders.

Through bulk RNA-sequencing, a gene expression signature comprising 140 differentially expressed genes was identified between responders and non-responders in the metastatic cutaneous melanoma cohort. This signature was predominantly enriched in genes associated with the B-cell lineage, including multiple immunoglobulin-related transcripts and canonical surface markers such as CD19 and CD22. Functional enrichment analysis using Canonical Pathways highlighted biological processes related to B-cell activation, immunoglobulin production, and complement activation pathways. Among the 140 differentially expressed genes, 55 were significantly associated with PFS, and 59 with OS, underscoring the prognostic potential of this transcriptional profile, in addition to their predictive extreme-response power. Technical validation of selected genes by quantitative real-time PCR (qRT-PCR) confirmed the overexpression of *TNFRSF11B*, *IGHA1*, and *IGLV6-57* in responders, and these results showed a strong concordance with RNA-seq data (Pearson's r ranging from 0.80 to 1, p-value <0.001).

To estimate the abundance of immune cell populations within the TME, two computational deconvolution methods—MCP-counter and CIBERSORTx—were applied. Both analyses revealed a significantly higher infiltration of B cells in responders, with a specific enrichment of Plasma cells. Complementary analyses of publicly available melanoma tissue single-cell RNA-seq data confirmed the presence of a "Naïve B cells IGK-high" subpopulation that was enriched in responding patients. This finding was further validated at the protein level by multiplex immunofluorescence, which demonstrated an increased density of $CD19^+/CD20^+$ B cells and $CD138^+$ Plasma cells in tumor samples from responders.

Notable, clonotype analysis of immune receptor repertoires revealed that responders exhibited a significantly greater number and diversity of B-cell receptor (BCR) clonotypes, with immunoglobulin kappa (IGK) being the most frequently expanded chain among them. In contrast, no significant differences were observed in TCR diversity between extreme response groups. Moreover, TMB inferred from RNA-seq did not correlate with clinical response in this cohort.

Finally, a ML classification model was constructed using a Random Forest algorithm based on a subset of 15 genes selected from the 140 DE genes. The model achieved high predictive performance in the training cohort (Area Under the Receiver Operating Characteristic

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curve, AUC-ROC=0.98), and maintained robust generalizability when tested across eight independent external melanoma cohorts treated with ICIs, yielding an AUC $\geq$ 0.8 in three of them. For benchmarking, the same three external cohorts were evaluated using TIDE (Tumor Immune Dysfunction and Exclusion), a publicly available computational framework for predicting ICI response in melanoma, which produced notably lower AUC values of 0.62, 0.45, and 0.23, respectively. These results demonstrates the superior predictive accuracy of our gene-based Random Forest model compared to existing approaches.

Overall, this study identifies a transcriptional signature enriched in B-cell-associated genes, along with the infiltration of intratumoral Plasma cells exhibiting high expression levels of *IGKC*, as key biomarkers associated with clinical response to anti-PD-1 immunotherapy in metastatic cutaneous melanoma. These findings suggest that B-cell-driven immune mechanisms play a critical role in modulating the efficacy of ICIs. By highlighting the predictive value of immune components beyond T lymphocytes, this work contributes to a more comprehensive understanding of the tumor-immune interaction and provides a foundation for the development of improved biomarker strategies to guide patient selection and therapeutic decision-making in melanoma immunotherapy. In the subsequent study, this cohort will be expanded to analyze primary resistance, as well as to incorporate liquid biopsy data such as scRNA-seq and flow cytometry.

Paper #2

In this second study, an integrative transcriptomic and immunophenotypic analysis was conducted with the aim of identifying predictive biomarkers of primary resistance to ICIs in patients with metastatic cutaneous melanoma. The study utilized RNA sequencing data from pre-treatment FFPE tumor samples obtained from a discovery cohort of 46 patients. Through differential gene expression analysis, a robust transcriptional signature composed of 82 genes was identified, enabling the stratification of patients into resistant and non-resistant groups. These genes were functionally enriched in immune-related pathways, particularly those involved in intercellular communication between innate and adaptive immunity, and processes linked to B-cell activation.

To evaluate its predictive potential, a ML model was trained on this gene expression signature and subsequently validated in an independent external metastatic cutaneous melanoma cohort of 54 patients. The model achieved an AUC-ROC of 0.814, demonstrating solid discriminatory power and potential clinical utility for identifying patients likely to exhibit primary resistance to ICIs prior to treatment initiation.

To deepen the immunological context of the transcriptomic findings, PBMCs from a subset of patients (n = 8) were analyzed using scRNA-seq. This high-resolution technique enabled the characterization of 12 distinct circulating immune cell populations. Subsequently, these single-cell transcriptomic data was used to estimate the relative abundance of these immune

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cell populations within the tumor tissue from the FFPE RNA-seq cohort of 46 patients. This was achieved by deconvoluting bulk RNA-seq profiles using reference signatures derived from such scRNA-seq using CIBERSORTx. The inferred cell proportions were compared between patients with and without primary resistance. Among these, four immune subsets—Plasma cells, Pre-B cells, Naive $CD4^+$ T cells, and Memory $CD4^+$ T cells—showed a significant correlation with primary resistance, exhibiting higher levels in non-resistant patients. Based on the abundance of these subpopulations, an immune-based prognostic scoring signature (ImmuneSignature) was modeled, capable of stratifying patients also in terms of long-term survival. A high ImmuneSignature was associated with improved prognosis, whereas a low ImmuneSignature correlated with poorer clinical outcomes. The prognostic relevance of this signature was independently validated in a large external cohort of 268 metastatic cutaneous melanoma patients, confirming its robustness potential. Notably, the signature was also mirrored in liquid biopsy using flow cytometry data from pre- and post-treatment (T1 and T2) samples (n = 46 in each timepoint). However, the selection of antibodies did not allow for phenotyping the four populations, limiting this blood-based ImmuneSignature surrogate to only two populations ($CD4^+$ T cells—$CD4^+$— and Plasma cells—$CD20^-$, $CD27^+$, $CD138^+$). Particularly, no significant associations were observed in T1 samples, whereas T2 samples, collected after administration of the second cycle doses, yielded significant results in the survival analysis. This concordance highlights the feasibility of applying the ImmuneSignature through clinically accessible methodologies, thereby enhancing its generalizability and supporting its potential utility as a biomarker of long-term survival in the context of ICIs.

The convergence of transcriptomic profiling and single-cell resolution data highlights the value of integrating tumor-intrinsic features with systemic immune surveillance metrics. The identification of B-cell-related elements, such as Plasma and Pre-B cells, in combination with distinct $CD4^+$ T-cell subtypes, represents a conceptual expansion of current biomarker paradigms. These findings provide novel mechanistic insights into the drivers of primary resistance. Collectively, this integrative framework lays the groundwork for more precise patient stratification and opens new avenues for targeted interventions aimed at mitigating resistance to ICIs in melanoma.

Findings from this study highlighted the potential of liquid biopsy as a prognostic tool, prompting a shift in focus toward its clinical utility. Accordingly, the last two studies were designed to further investigate the prognostic value of liquid biopsy-derived biomarkers in the context of ICI therapy in advanced NSCLC.

Paper #3

The third study explored the prognostic relevance of baseline total cfDNA levels, measured using a simple, non-invasive fluorometric assay, in patients with advanced NSCLC undergoing treatment with ICIs, either as monotherapy or in combination with chemotherapy.

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The analysis was performed across two independent cohorts to ensure reproducibility and validation. The discovery cohort comprised 61 patients, while the validation cohort included an additional 31 patients. Both cohorts included patients with advanced NSCLC who had blood samples collected before the initiation of ICIs.

In the discovery cohort, a predefined threshold of ≥ 0.55 ng/µL for cfDNA concentration effectively stratified patients by survival outcomes. Individuals with high baseline cfDNA levels experienced a significantly shorter median OS of 3.8 months, compared to 17.5 months in patients with lower cfDNA concentrations (Log-rank test, p-value $= 3.3 \times 10^{-4}$). These findings were independently corroborated in the validation cohort, where high cfDNA levels were also associated with inferior prognosis (median OS: 5.9 months vs. 24.3 months; Log-rank test, p-value $= 0.038$) when applying the same threshold.

Importantly, multivariate Cox regression analysis, including cfDNA concentration, age at treatment, ECOG, LDH and PD-L1 expression, confirmed that cfDNA levels served as an independent factor of survival. Model performance was evaluated using Uno's time-dependent area under the curve (Uno's AUC), a metric specifically designed for censored survival data. The cfDNA-based model alone achieved a summary Uno's AUC of 0.71, clearly outperforming the clinical model based solely on conventional variables (Uno's AUC = 0.55). Moreover, the integration of cfDNA levels with age, ECOG, LDH, and PD-L1 expression further improved predictive performance, resulting in a combined model with a summary Uno's AUC of 0.74. Notably, this multivariable model, based on variables collected prior the initiation of ICI therapy, showed time-dependent improvements in prognostic accuracy, reaching peak Uno's AUCs of 0.94 at month 2 and 0.83 at month 14, demonstrating its robust performance in both early and later stages of follow-up.

This study provides strong evidence supporting the use of baseline total cfDNA as a non-invasive, easily implementable, and cost-effective prognostic biomarker in the context of ICI therapy. By offering a blood-based surrogate for the systemic disease activity, cfDNA quantification has the potential to enhance patient stratification, improve clinical decision-making, and ultimately contribute to personalized treatment strategies readily applicable in advanced NSCLC. However, to achieve a more holistic molecular characterization, it is necessary to incorporate omics-level data, as addressed in the final study.

Paper #4

The last study presents a novel approach for molecular subtype identification in advanced NSCLC by integrating cfDNA methylation and extracellular vesicle-associated microRNA (EV-miRNA) profiles derived from liquid biopsies. These molecular layers were selected based on their regulatory functions and their potential utility as non-invasive biomarkers traceable in peripheral blood. As observed in the previous studies, not all patients progress at the same rate or follow uniform clinical trajectories after ICI treatment. To better capture this

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variability and broaden our analytical perspective, we analyzed OS and PFS as continuous and integrative endpoints in a prospective cohort of 79 advanced NSCLC patients. An unsupervised approach was applied to identify patient subgroups with distinct survival profiles. To further explore the temporal dynamics of treatment response, plasma samples were collected longitudinally at four clinically relevant time points: pre-treatment (T1), after the second cycle (T2), at six months (T3), and at one year (T4).

The multi-omics data generated from cfDNA methylation and EV-miRNAs were integrated using Multi-Omics Factor Analysis (MOFA2), yielding latent molecular subtypes termed MOFA-Derived Clusters (MDCs), defined independently at both T1 (MDC-T1) and T2 (MDC-T2). MOFA2 integration revealed distinct contribution patterns between the two omic layers across models MDC-T1 and MDC-T2. In T1, the total variance explained was comparable (miRNA: 23 %; cfDNA methylation: 25 %). Latent factor analysis showed that Factors 1 (F1) and 3 (F3) reflected input from both layers, while Factor 2 (F2) was predominantly influenced by miRNAs. In contrast, T2 showed a greater contribution from cfDNA methylation (50 %) compared to EV-miRNAs (25 %). In this model, some factors were clearly dominated by one modality (e.g., EV-miRNAs in F2, cfDNA methylation in F3), while others displayed more balanced input. These patterns underscore the complementary and subtype-specific roles of miRNA and cfDNA methylation in defining the molecular architecture.

The resulting MDCs captured distinct molecular phenotypes that were reflective of divergent survival trajectories and biological process: three clusters in T1 (MDC1-T1, MDC2-T1, MDC3-T1) and three clusters in T2 (MDC1-T2, MDC2-T2, MDC3-T2). At T1, the three clusters exhibited progressively divergent outcomes: MDC1-T1 was associated with the poorest prognosis (median PFS: 4.54 months; median OS: 7.64 months), followed by MDC2-T1 with intermediate survival (median PFS: 12.23 months; median OS: 19.71 months), and MDC3-T1 showing the most favorable profile (median PFS: 28.89 months; median OS: 40.79 months). In contrast, the T2-derived model delineated two major prognostic groups: MDC1-T2 and MDC2-T2 clustered within a low-survival category (median PFS: 4.66 and 7.71 months; median OS: 7.78 and 9.9 months), while MDC3-T2 was characterized by significantly improved survival (median PFS: 28.89; median OS: 40.79). The projection of these molecular clusters onto subsequent time points (T3 and T4) revealed that early molecular features retained prognostic relevance over time, underscoring the longitudinal stability and predictive potential of the early signatures.

To characterize the biological drivers of each cluster, differential expression analysis of EV-derived miRNAs and differential methylation regions analysis of cfDNA methylation data were performed independently. The top-ranked features from each omics layer were examined for their functional relevance, revealing the enrichment of hallmark pathways such as angiogenesis, cytoskeletal remodeling, and immune signaling. Furthermore, each MDC also exhibited distinct immunological profiles when evaluated in relation to circulating immune cell populations, identified via flow cytometry. In the T1 model, MDC3-T1 exhibited a

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distinct immune composition characterized by reduced frequencies of $CD4^+$ T cells and regulatory T cells, along with a marked enrichment of cytotoxic $CD8^+$ T cells. This immune profile—dominated by effector cytotoxicity and reduced immunosuppression—may underlie the favorable clinical outcomes observed in this group. In the T2 model, $CD4^+$ T cell frequencies were again significantly different across subtypes, with MDC3-T2 showing higher levels compared to MDC1-T2. Additionally, exhausted $CD8^+PD1^+$ T cells displayed a subtype-dependent distribution, being most abundant in MDC1-T2, intermediate in MDC3-T2, and lowest in MDC2-T2. In contrast, $CD3^+$PD-$L1^+$ T cells exhibited an inverse pattern, peaking in MDC2-T2 and declining in MDC1-T2.

To assess the generalizability of the identified MDCs, we developed predictive models capable of accurately assigning new samples to their corresponding subtypes. However, due to the lack of external datasets with matched multi-omic profiles, each molecular layer—cfDNA methylation and EV-miRNAs—was validated independently using two external NSCLC cohorts: EV-miRNA dataset (n=54) and cfDNA methylation dataset (n=57). For this purpose, separate ML-based classifiers were constructed for each time point and omic layer, using the subtype-specific features identified in our cohort. This stepwise validation approach enabled evaluation of the reproducibility and predictive performance of each molecular signature across independent patient populations. Nonetheless, we acknowledged a key limitation: the absence of multi-omic external datasets prevented validation of an integrated model, thereby precluding assessment of the full predictive potential of the combined molecular profile. Notably, the MDC-T1 model showed stratification of PFS exclusively in the cfDNA methylation validation cohort. In contrast, the MDC-T2 model demonstrated robust and consistent validation across both cfDNA methylation and EV-miRNA layers, effectively stratifying patients by PFS. These findings reinforce the clinical relevance and robustness of the mainly in T2 subtypes, underscoring their potential utility as early on-treatment biomarkers—captured as early as 2 to 3 weeks following ICI initiation (post-second cycle).

In summary, this work demonstrates the feasibility and translational relevance of multi-omic liquid biopsy–based framework capable of revealing clinically actionable molecular subtypes in advanced NSCLC patients undergoing ICI therapy. By integrating dynamic and minimally invasive biomarker data, the study advances a precision oncology model that enables early and accurate patient stratification. Moreover, this approach facilitates a more holistic and systematic understanding of the treatment impact, made possible by the synergistic combination of liquid biopsy and multi-omic analysis. The MDC signatures represent a step forward in the realization of personalized immuno-oncology, offering a powerful tool for predicting therapeutic outcomes and guiding clinical decision-making.

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Chapter 3: General Dicussion

The development of ICIs has established a new paradigm in the treatment of advanced malignancies such as melanoma and NSCLC. These advancements, alongside the impact of NGS and the evolving framework integrating modalities such as liquid biopsy and AI, provide an ideal landscape for the objectives addressed within this thesis: to identify and characterize multi-omic biomarkers associated with response and resistance to immunotherapy in melanoma and NSCLC patients through the application of integrative bioinformatics and ML methodologies.

The four studies presented in this thesis are structured to systematically address key challenges in the discovery of immunotherapy biomarkers. The first two studies (Papers #1 and #2) focused on a cohort of metastatic melanoma patients, for whom both solid and liquid biopsy samples were available. This section aims to address challenges such as the development of robust ML-based predictive models validated in external cohorts, as well as the standardization of sampling time points for assessing treatment response and resistance [88]. The subsequent studies, corresponding to Papers #3 and #4, were centered on advanced NSCLC and used only liquid biopsy samples. Therefore, in this phase we gained deep insight in the potential of utilizing blood-derived biomarkers along with the exploitation on our multi-omics longitudinal analysis pipeline.

Given the lack of consensus on resistance definition when this thesis project commenced [89], the first study focused on the discovery of biomarkers in metastatic melanoma patients treated with ICIs, utilizing an extreme-phenotype approach. Patients exhibiting early progression (within 3 months) were compared with those achieving sustained clinical benefit (for at least 12 months). This strategy enabled a comprehensive transcriptomic profiling of the baseline TME, revealing molecular signatures associated with extreme outcomes. The second study was conducted in accordance with the consensus established by the SITC on the definition of primary resistance [87]. In this analysis, the solid tissue metastatic cutaneous melanoma cohort was expanded to enhance analytical robustness. Complementing this, single-cell analysis of PBMCs of a subset of the cohort enabled a refined characterization of circulating immune cells, which was leveraged to deconvolute the bulk transcriptomic immune signatures observed in the TME. This integrative approach facilitated the molecular

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alignment of a single omics layer across spatially distinct biological compartments. The development of an externally validated predictive model, together with a scoring system based on four relevant immune cell populations identified in tumor tissue and subsequently validated in external cohorts and liquid biopsy samples, underscores the scientific and translational relevance of the findings.

In the third and fourth study, the limited availability of tumor tissue in patients with advanced NSCLC led us to rely in the use of liquid biopsy as the primary source for analysis. Firstly, we assessed the prognostic utility of cfDNA through a simple quantitative approach, without incorporating molecular profiling. Remarkably, this analysis identified a subset of patients with significantly improved OS and PFS. These results motivated the fourth study, wherein we applied an unsupervised algorithm to stratify patients based on survival outcomes. A multi-omics approach was implemented, incorporating EVs-miRNA and cfDNA methylation profiles, integrated using multi-omics factor analyses, followed by data-driven clustering to uncover latent molecular subtypes. Additionally, both baseline and post-treatment at second cycle (to capture systemic drug-related dynamics in an early stage, 2 or 3 weeks after ICI initiation) liquid biopsies were analyzed in parallel. This strategy enabled the identification of distinct molecular subtypes with differential survival trajectories, highlighting the potential of liquid biopsy-based omics to inform clinical decision-making in NSCLC.

Throughout the development of this doctoral thesis, we have emphasized the critical role of the immune system in delineating the mechanisms underlying responses to ICIs, highlighting its primary contribution to predictive and prognostic modeling systems. The findings obtained in the melanoma cohort (Papers #1 and #2), centered on tumor tissue, underscore the previously reported importance of B cells within the TME, consistent with observations described in studies conducted by Helmink et al. and Cabrita et al. [90, 91]. Of particular interest is the specific association between Plasma cell and B cell infiltration and favorable responses to ICIs, which emerged consistently across both of our first two analyses.

B cells and Plasma cells contribute to antitumor immunity through a variety of mechanisms. Plasma cells produce tumor antigen-specific antibodies that mediate antibody-dependent cellular cytotoxicity and enhance phagocytosis of tumor cells [92]. B cells facilitate antigen presentation to both $CD4^+$ and $CD8^+$ T cells and secrete pro-inflammatory cytokines such as IFN-γ and IL-12, which support cytotoxic responses [93]. These mechanisms collectively act to potentiate immune activation, particularly when inhibitory pathways, such as those mediated by PD-(L)1, are blocked [90, 91, 94]. Furthermore, although our data only suggested the presence of tertiary lymphoid structures (TLSs), previous studies have demonstrated a strong association between TLSs enriched with B cells and Plasma cells and improved clinical outcomes in both melanoma and NSCLC patients treated with anti-PD1 therapies [95, 96].

Our second study extended these observations by identifying two additional immune cell populations, together with B cells and Plasma cells: Naive $CD4^+$ T cells and Memory $CD4^+$

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T cells. At cellular level, specific abundance distributions of these four immune populations in tumor tissue, referred to as ImmuneSignature, were associated with longer OS. In addition to the previously discussed contribution of B cells and Plasma cells, the two newly identified subpopulations emerged as key players in ICIs response, with functions related to effective antitumor immunity [97], as well as in activation-induced cell death and differentiation into effector memory phenotypes [98]. Notably, in alignment with our ImmuneSignature, previous studies have shown that high B cell density within TLSs is linked to increased $CD4^+$ T cell clonality [99]. Moreover, at the gene level, a coordinated activity among some of these immune cell types became apparent, where we observed a significant overexpression of the gene *CXCL13* in primary non-resistant patients. *CXCL13* is central to orchestrating immune responses mediated by B and T cells, notably by recruiting follicular helper $CD4^+$ T cells and B cells to the TME, thereby promoting inflammation, the accumulation of other immune populations, and the formation of TLS [100].

These insights extend beyond tumor tissue to peripheral blood, where surrogate markers for immune activity have been identified through flow cytometry. Despite the lack of finely tuned flow cytometry surface markers for all subsets in our second study, we were able to validate the prognostic value of this cell-based signature using liquid biopsy samples in a partial implementation of the tissue-based one (Pre-B cells, Plasma Cells, Naive $CD4^+$ T cells and Memory $CD4^+$ T cells): $CD4^+$ T cell ($CD4^+$) and Plasma B cell ($CD20^-$, $CD27^+$, $CD138^+$). Taken together, our data and the supporting literature highlight the multifaceted and clinically significant role of predominantly B cell lineages, as well as $CD4^+$ T cells, in shaping the tumor immune microenvironment and modulating the efficacy of ICIs.

Additionally, both studies were specifically designed to develop binary classification predictive models: extreme response in the first study and primary resistance in the second. Given the high dimensionality of the data relative to the sample size used for training, a challenge commonly referred to as the "curse of dimensionality" [101], algorithms such as logistic regression with Lasso regularization and Random Forest were explored to reduce the number of variables included in the models. Also, cross-validation was applied in each model to optimize hyperparameters and minimize overfitting. Finally, to ensure unbiased predictive performance and avoid data leakage (it occurs when information from the validation set is unintentionally incorporated into the training process, leading to biased model performance), the models were validated on independent external cohorts that were not involved in any stage of model development.

To date, numerous studies have sought to develop predictive models for ICI response in melanoma [102-104]. However, the heterogeneity in response criteria employed across these studies has hindered direct comparison and benchmarking of their predictive performance. Furthermore, the majority have lacked robust external validation cohorts, relying instead on internal training metrics [102] or external validation limited to relatively small test sets

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[103]. Additional challenges have arisen from the use of heterogeneous patient cohorts, encompassing diverse melanoma subtypes, which may confound the generalizability of predictive signatures [104]. In contrast, our first study was rigorously benchmarked against established algorithms such as TIDE [105], demonstrating superior predictive accuracy. In our second study, we leveraged a patient classification framework based on the consensus guidelines from the SITC and homogenous subtype cohort (metastatic cutaneous melanoma), resulting in robust predictive metrics and a classification system with enhanced reproducibility. This approach not only improves the reliability of response prediction but also facilitates clinical translation by aligning model outputs with standardized immunotherapy response criteria.

In contrast to the first two studies, the analysis of the advanced NSCLC cohort focused primarily on evaluating the prognostic potential of liquid biopsy markers in patients treated with ICIs. Consequently, the development of predictive models was not a primary focus at this stage, while exploring the potential of less invasive biomarkers and the integration of multiple omics layers played a more prominent role.

Firstly, rather than focusing solely on the extensively studied ctDNA [106], we broadened our scope to include the entire pool of cfDNA. In cancer patients, cfDNA comprises DNA fragments originating not only from tumor cells but also from the TME and immune cells involved in the antitumor response, released through mechanisms such as necrosis, secretion, apoptosis, and NETosis [107]. In our third study, we found that high baseline concentrations of cfDNA identify patients with advanced NSCLC who are unlikely to benefit from ICIs, potentially reflecting complex biological processes such as increased tumor metabolism and aggressiveness [108]. Despite its prognostic value, a deeper understanding of the molecular mechanisms underlying differential responses to ICI therapy requires multi-omic approaches. This need provided the rationale for our fourth study.

The integrative multi-omics approach of the last study, including DNA methylation and miRNA analyses, enabled the identification and characterization of distinct molecular subtypes, providing an alternative framework to define response-associated profiles in this context. In contrast to the transcriptomic approach used in the first studies, we shift our focus to an earlier window of molecular layers, those involving miRNAs and DNA methylation, which play key roles in regulatory mechanisms [109]. Prior to this analysis, no studies have reported such a significant survival stratification in NSCLC using this multi-layered perspective coupled with unsupervised factor analysis. In the context of multi-omics, previous approaches have primarily focused on bulk tumor tissue to classify responders versus non-responders, providing valuable but limited insights into the systemic molecular mechanisms underlying ICI response [110, 111]. Furthermore, while cfDNA methylation and EV-derived miRNAs have each been independently studied as prognostic and predictive biomarkers in ICI-treated NSCLC, their combined integration into a unified liquid biopsy–based multi-omic framework had not been explored preceding our work [112-115]. Therefore, we focused on the integration of two biologically interrelated [109] liquid biopsy–derived omics layers: cfDNA methylation

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and EV-miRNAs. cfDNA methylation provides a window into epigenetic remodeling processes and reveals key features of the tumor immune landscape, offering insights into immune activation, suppression, and tumor-intrinsic signaling [116]. In parallel, EV-derived miRNAs reflect active cellular communication within the TME and have been shown to modulate immune responses [117].

The differential contribution of miRNA and cfDNA methylation data across latent factors highlights the non-redundant and complementary nature of these circulating biomarkers. In T1, the balanced variance explained by both omic layers suggests a synergistic contribution to the overall molecular signal, whereas in T2, the dominant role of cfDNA methylation indicates that epigenetic reprogramming may capture more robust features associated with specific biological states or disease subtypes. This dynamic redistribution of variance and factor composition between models suggests that the relative informational value of each omic layer may evolve across disease stages or subtypes. The identification of MDCs at both baseline and early on-treatment timepoints offers valuable prognostic insights while also reflecting dynamic biological processes influenced by ICI therapy. Moreover, in our analysis, we observed findings consistent with existing literature, including the presence of $PD1^+$ $CD8^+$ T cells, which has been associated with improved immunotherapy responses [118], as well as an increase in $CD4^+$ T cell populations within subtypes characterized by better survival outcomes [50]. Interestingly, the circulating $CD4^+$ T cells were consistently observed across both studies, in Paper #2, focused on metastatic melanoma, and Paper #4, conducted in advanced NSCLC. In both contexts, $CD4^+$ T cells were identified as key components, firstly as an element of the ImmuneSignature, and secondly it was consistently associated with the MDC subtype which exhibited more favorable prognostic features. These convergent findings suggest a potentially shared immune mechanism across tumor types. Further refined analyses will be necessary to determine whether other immune cell populations highlighted in each study are also conserved across malignancies treated with ICIs. Such insights could reinforce the role of $CD4^+$ T cells—and potentially other subsets—as cross-tumor biomarkers and contribute to a deeper understanding of immune-mediated mechanisms underlying response to ICI therapy.

This doctoral thesis demonstrates a significant advancement in the predictive and prognostic performance of biomarkers for ICI response in metastatic melanoma and advanced NSCLC through the use of ML and multi-omics approaches. Relative to established clinical biomarkers and previously reported predictive models, our framework achieved superior accuracy and robustness in patient stratification, outperforming benchmarks such as TIDE and establishing an ImmuneSignature validated in a large independent cohort. The incorporation of longitudinal data obtained via non-invasive liquid biopsy enabled the detection of dynamic molecular changes throughout treatment, improving model sensitivity and facilitating the identification of immunotherapy-associated molecular subtypes in NSCLC. Furthermore, the integration of clinical variables and comprehensive patient follow-up across all analyses

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highlights the advantage of a holistic modeling strategy. From a methodological perspective, this study demonstrates the feasibility and added value of applying supervised ML algorithms for ICI response prediction and biomarker discovery, as well as leveraging unsupervised multi-omics integration approaches on plasma-derived data. Collectively, these results validate our hypothesis that multi-layered molecular profiling, combined with AI, can elucidate biomarkers to predict therapeutic response and support the development of personalized immunotherapy strategies.

This work presents several limitations that should be acknowledged. In the first melanoma study, the cohort size was relatively limited. Although we attempted to mitigate this constraint by validating the results in several external cohorts, the small sample size remains an important consideration when interpreting the findings and drawing conclusions. This limitation motivated the design of a second study with an expanded cohort; nevertheless, it also presented challenges, most notably the inability to further increase the single-cell RNA-seq cohort derived from PBMCs, which limited our capacity to capture the full heterogeneity of circulating immune populations. Additionally, the flow cytometry panels used were not specifically designed to capture the complete immune subsets identified through deconvolution analysis, thereby restricting validation at the protein level. Mechanistic validation through functional assays or spatial transcriptomics would have further strengthened our conclusions but was not feasible within the scope of this thesis. In the NSCLC analyses, the lack of available matched tumor tissue limited our ability to correlate blood-based findings with tissue-derived immune profiles. This reflects a broader challenge in advanced-stage NSCLC, where obtaining sufficient tumor material for research is often impractical. Furthermore, although we adopted a multi-omic approach using cfDNA methylation and EV-miRNAs, the absence of external validation cohorts with matched multi-layered molecular data restricted validation to individual omics layers separately. This represents a constraint in assessing the generalizability of the integrative model across independent datasets. Finally, further integration of additional omics layers, such as genomics or transcriptomics, could enhance our understanding of the underlying mechanisms driving each defined subtype, but this remains to be explored.

In addition to addressing some of the limitations described, future work will focus on conducting more extensive analyses using longitudinal data to capture dynamic changes over time. From a computational perspective, further optimization and simplification of ML models will be essential to derive minimal biomarker panels with strong predictive power and clinical interpretability. Incorporating explainable AI approaches could improve clinical adoption. Translationally, efforts should focus on developing cost-effective, standardized assays suitable for routine clinical use. Ultimately, prospective trials will be needed to demonstrate clinical utility and cost-effectiveness compared to existing standards.

Regarding the translational relevance of the four studies, it is important to consider their potential integration into clinical practice. For Papers #1 and #2, the clinical applicability

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lies in the use of tumor biopsy-derived markers. However, this approach depends on solid tumor biopsies, which are inherently invasive and may not always be feasible. Consequently, additional research is required to determine which type of solid biopsy provides the most informative readout and to clarify how the timing of tissue acquisition may influence the reliability of biopsy-derived markers for immunotherapy stratification. In contrast, the second component of Paper #2, in which we examined the monitoring of immune dynamics through liquid biopsy collected immediately before and during treatment, represents a more feasible and clinically scalable approach. This strategy relies on flow cytometry–based methodologies already widely implemented in routine clinical practice, thereby facilitating its potential adoption into real-world workflows. In Paper #3, cfDNA quantification emerges as a technically simple, rapid, and cost-effective method with clear translational potential. Notably, several commercially available platforms already offer robust fluorometric assays for cfDNA measurement, enabling standardized and reproducible quantification across laboratories. Conversely, the findings from Paper #4 require additional refinement before reaching clinical applicability. In particular, it will be necessary to define a reduced and clinically actionable panel of markers capable of reliably identifying the MOFA2-derived subtypes observed, thus enabling the development of targeted assays suitable for routine clinical implementation.

Taken together, the findings presented in this thesis highlight the value of integrative multi-omics and immune profiling approaches in uncovering clinically relevant biomarkers of response and resistance to ICIs across distinct tumor types. From the in-depth characterization of solid tumor biopsies in metastatic melanoma to the application of minimally invasive liquid biopsy in advanced NSCLC, the studies demonstrate the potential of combining transcriptomic, epigenetic, and cellular features to improve patient stratification. While each analysis has its own limitations, the consistent convergence of molecular signals across platforms, cohorts, and biological compartments reinforces the robustness of the insights gained. These results pave the way for future research aimed at refining personalized immunotherapy strategies, and underscore the critical role of multi-dimensional data integration in advancing precision medicine.

Research Impact

This doctoral thesis addresses a highly relevant topic in the field of cancer immunotherapy by integrating clinical and multi-omic molecular data through a multidisciplinary approach. The primary aim was to identify predictive and prognostic biomarkers that could improve patient selection and therapeutic outcomes in metastatic melanoma and advanced NSCLC treated with ICIs. Across the four studies included in this thesis, diverse methodologies, from transcriptomic profiling of solid tumor tissue to integrative multi-omics analyses of liquid

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biopsies, were employed to uncover immune-related mechanisms of response and resistance. The main contributions arising from this research can be summarized as follows:

1. **Identification of immune signatures in ICI response:** This thesis demonstrates that integrative transcriptomic and multi-omic analyses of solid and liquid biopsies reveal distinct immune signatures, particularly B cell and $CD4^+$ T cell–related profiles, associated with immunotherapy outcomes in metastatic melanoma and advanced NSCLC. These signatures enhance the understanding of ICI response mechanisms and provide a foundation for biomarker development.
2. **Advancement of predictive modeling:** ML models developed from bulk RNA-seq and multi-omic data accurately predict primary resistance to ICIs in metastatic melanoma and identify molecular subtypes with differential survival in advanced NSCLC. These models underscore the power of data-driven approaches to improve clinical decision-making and personalize immunotherapy strategies.
3. **Biomarker detection in liquid biopsy can be used for non-invasive monitoring:** The research establishes the prognostic potential of liquid biopsy approaches, including cfDNA and EV-miRNAs, in advanced NSCLC. These non-invasive methods enable patient stratification and monitoring, offering a practical alternative when solid tissue is unavailable, thus advancing precision oncology.
4. **Multi-omic data integration improves the prediction of clinical outcomes:** This thesis underscores the transformative potential of combining transcriptomic and epigenomic from circulating biomarker data, demonstrating its capacity to reveal actionable molecular insights. This integrative approach not only enhances the precision of immunotherapy but also sets a precedent for developing standardized, multi-omic diagnostic platforms in clinical oncology.

By bridging multi-omic technologies, AI, and clinical applicability, this thesis contributes to the evolving paradigm of precision immunotherapy and paves the way for more personalized and effective cancer treatment strategies.

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Chapter 5: Conclusions

This doctoral thesis has explored the application of bioinformatic analyses, machine learning techniques, and multi-omics data integration to identify predictive and prognostic biomarkers of cancer immunotherapy response. The main conclusions derived from the research presented are summarized below:

1. Two gene signatures—a 15-gene panel predicting extreme responders and an 82-gene panel predicting primary resistance to ICIs in metastatic cutaneous melanoma—were developed and validated with supervised machine learning models, achieving AUC >0.8 in external validation cohorts.

2. B cell subpopulations with high *IGKC* expression and clonal IGK BCR expansion were significantly enriched in the TME in extreme responders.

3. An ImmuneSignature score based on four immune cell populations (Plasma cells, Pre-B cells, Naive $CD4^+$ T cells, and Memory $CD4^+$ T) was established and validated in metastatic cutaneous melanoma tissue, identifying a subgroup with significantly improved survival following ICI therapy.

4. A liquid biopsy surrogate of the ImmuneSignature ($CD4^+$ T cells and Plasma Cells—$CD20^-$, $CD27^+$, $CD138^+$) measured in blood after the second immunotherapy cycle demonstrated potential as a minimally invasive biomarker for treatment monitoring.

5. cfDNA quantification was validated as a non-invasive prognostic biomarker in advanced NSCLC, with a threshold of <0.55 ng/µL effectively stratifying patients with better progression-free and overall survival, surpassing traditional clinical markers.

6. Multi-omics integration of extracellular vesicle-derived microRNAs and cfDNA methylation identified three molecular subtypes in advanced NSCLC with distinct survival outcomes, where high-survival subtypes showed enrichment of circulating PD-1^+ $CD8^+$ and increased $CD4^+$ T cells.

7. High expression of microRNAs *hsa-miR-451a* and *hsa-miR-4732-5p*, along with hypermethylation in specific regions of the genes *BLK* and *ADARB2*, was consistently

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observed in molecular subtypes associated with improved overall survival prior to immunotherapy initiation and after the second treatment cycle (weeks 2–3), suggesting their potential as early and persistent prognostic biomarkers.

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Research Articles

The following section includes the four research articles that constitute this doctoral thesis. Each article is presented in its original published or accepted version, with formatting adjustments made to ensure consistency with the overall style and structure of this dissertation. No modifications have been made to the scientific content of the manuscripts.

- **Paper #1: Onieva JL**, Xiao Q, Berciano-Guerrero MÁ, et al. High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade. Int J Mol Sci. 2022;23(16):9124. doi:10.3390/ijms23169124
- **Paper #2: Onieva JL**, Pérez-Ruiz E, Vilkki V, et al. Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma. bioRxiv. 2025:670062. doi:10.1101/2025.08.13.670062
- **Paper #3:** Oliver J, **Onieva JL (equally contributed)**, Garrido-Barros M, et al. Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade. Cancers (Basel). 2023;15(13):3357. doi:10.3390/cancers15133357
- **Paper #4: Onieva JL**, Pérez-Ruiz E, Figueroa-Ortiz L, et al. Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC. medRxiv. 2025:25333961. doi:10.1101/2025.08.19.25333961

For each article, a concise overview is presented, encompassing the list of authors, publication details, journal metrics, and the specific author contributions of the work. The four publications are subsequently included.

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Paper #1: High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade.

Authors: Juan Luis Onieva, Qingyang Xiao, Miguel-Ángel Berciano-Guerrero, Aurora Laborda-Illanes, Carlos de Andrea, Patricia Chaves, Pilar Piñeiro, Alicia Garrido-Aranda, Elena Gallego, Belén Sojo, Laura Gálvez, Rosario Chica-Parrado, Daniel Prieto, Elisabeth Pérez-Ruiz, Angela Farngren, María José Lozano, Martina Álvarez, Pedro Jiménez, Alfonso Sánchez-Muñoz, Javier Oliver, Manuel Cobo, Emilio Alba, Isabel Barragán.

Citation:

- Published in *International Journal of Molecular Sciences*, Vol. 23, No. 16, article number 9124.
- Published August 15, 2022.
- DOI: https://doi.org/10.3390/ijms23169124

Journal metrics:

- Impact Factor (JCR, 2022): 5.6
- Quartile: Q1 (Area: BIOCHEMISTRY & MOLECULAR BIOLOGY — 66/285)
- Indexed in: Google Scholar, Crossref, BibCnrs, CABI Digital Library, CAPlus / SciFinder, CNKI, CNPIEC, Dimensions, EBSCO, Elsevier Databases (Scopus, Embase, Reaxys), FSTA, Gale, J-Gate, National Library of Medicine (PubMed, MEDLINE, PMC), OpenAIRE, OSTI (U.S. Department of Energy), PATENTSCOPE, ProQuest, Web of Science (SCIE, Current Contents - Physical, Chemical & Earth Sciences).

PhD candidate's contribution: J.L.O. contributed substantially as first author by designing and implementing the bioinformatic analysis workflow, performing the data analysis, and collaborating in the preparation of the manuscript draft. Their role ensured the reproducibility and rigor of the computational approach, as well as the integration of the analytical results into the scientific narrative.

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Paper #2: Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma.

Authors: Juan Luis Onieva, Elisabeth Pérez-Ruiz, Ville Vilkki, Miguel Berciano-Guerrero, Laura Figueroa-Ortiz, Manuel Zalabardo, Beatriz Martínez-Gálvez, Isabel Barragán, Antonio Rueda-Domínguez.

Citation:

- Published in *bioRxiv*.
- Posted August 15, 2025.
- DOI: https://doi.org/10.1101/2025.08.13.670062
- Status: Preprint (not peer-reviewed).

Journal metrics:

- Indexed in: Google Scholar, Crossref, Semantic Scholar, Europe PubMed Central, and Preprint Citation Index (connected to the Web of Science).

PhD candidate's contribution: As the first author, J.L.O. led the conceptualization, investigation, formal bioinformatic analysis, and data curation efforts for this study. J.L.O. was primarily responsible for performing and validating the bioinformatic analyses, writing the original draft of the manuscript, coordinating the presentation of results, and ensuring the accuracy and coherence of data interpretation throughout the study.

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Paper #3: Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade.

Authors: Juan Luis Onieva, María Garrido-Barros, Manuel Cobo-Dols, Beatriz Martínez-Gálvez, Ana Isabel García-Pelícano, Jaime Dubbelman, José Carlos Benítez, Juan Zafra Martín, Alejandra Cantero, Elisabeth Pérez-Ruiz, Antonio Rueda-Domínguez, Isabel Barragán.

Citation:

- Published in *Cancers*, Vol. 15, No. 13, article number 3357.
- Published June 26, 2023.
- DOI: https://doi.org/10.3390/cancers15133357

Journal metrics:

- Impact Factor (JCR, 2023): 4.5
- Quartile: Q1 (Area: ONCOLOGY — 78/322))
- Indexed in: Google Scholar, Crossref, BibCnrs, CABI Digital Library, CAPlus / SciFinder, CNKI, CNPIEC, Dimensions, EBSCO, Elsevier Databases (Scopus, Embase), Gale, J-Gate, National Library of Medicine (PubMed, PMC), OpenAIRE, OSTI (U.S. Department of Energy), ProQuest, Web of Science (SCIE, Biological Abstracts, BIOSIS Previews).

PhD candidate's contribution: J.L.O., as an equally contributing first author, performed the statistical analyses and contributed significantly to data interpretation. Additionally, J.L.O. worked closely with senior authors in drafting and revising the manuscript, participating in the study design and ensuring a rigorous scientific approach throughout the publication.

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Paper #4: Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC.

Authors: Juan Luis Onieva, Elisabeth Pérez-Ruiz, Laura C Figueroa-Ortiz, José Miguel Jurado, Beatriz Martínez-Gálvez, José Carlos Benítez, Isabel Barragán, Antonio Rueda-Domínguez.

Citation:

- Published in *medRxiv*.
- Posted August 24, 2025.
- DOI: https://doi.org/10.1101/2025.08.19.25333961
- Status: Preprint (not peer-reviewed).

Journal metrics:

- Indexed in: Google Scholar, Crossref, Semantic Scholar, Europe PubMed Central, and Preprint Citation Index (connected to the Web of Science).

PhD candidate's contribution: As first author, J.L.O. contributed the initial conceptual framework and was responsible for designing the work, conducting investigations, performing formal data analysis, and curating the dataset. J.L.O. also led the preparation of the original manuscript draft, synthesizing complex data and coordinating with co-authors to produce a comprehensive and precise scientific article.

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International Journal of
Molecular Sciences

Article

High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade

Juan Luis Onieva[1,2,3,4], Qingyang Xiao[5,6], Miguel-Ángel Berciano-Guerrero[1,3], Aurora Laborda-Illanes[1,2,3,4], Carlos de Andrea[7,8], Patricia Chaves[1,2,3], Pilar Piñeiro[1,2,3], Alicia Garrido-Aranda[1,2,3], Elena Gallego[3,9], Belén Sojo[1,2,3], Laura Gálvez[1,3], Rosario Chica-Parrado[1,2,3], Daniel Prieto[3,9], Elisabeth Pérez-Ruiz[1,3], Angela Farngren[6], María José Lozano[3,10], Martina Álvarez[1,2,3], Pedro Jiménez[1,3], Alfonso Sánchez-Muñoz[1,3], Javier Oliver[1,2,3,*], Manuel Cobo[1,3,*], Emilio Alba[1,2,3] and Isabel Barragán[1,2,3,6,*]

[1] Medical Oncology Intercenter Unit, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina, Regional and Virgen de la Victoria University Hospitals, 29010 Malaga, Spain
[2] Cancer Molecular Biology Laboratory, Translational Research in Cancer Immunotherapy Group, Health and Medical Research Centre (CIMES), University of Malaga, Marques de Beccaria 3, 29010 Malaga, Spain
[3] Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010 Malaga, Spain
[4] Facultad de Medicina, Campus de Teatinos s/n, Universidad de Málaga, 29071 Malaga, Spain
[5] Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institutet, 17177 Stockholm, Sweden
[6] Group of Pharmacoepigenetics, Department of Physiology and Pharmacology, Karolinska Institutet, 17177 Stockholm, Sweden
[7] Department of Anatomy, Physiology and Pathology, University of Navarra, 31008 Pamplona, Spain
[8] Department of Anatomy and Pathology, University of Navarra, 31008 Pamplona, Spain
[9] Department of Pathology, Clinical University Hospital, Campus de Teatinos, 29010 Malaga, Spain
[10] Department of Pathology, University of Malaga (UMA), 29010 Malaga, Spain
[*] Correspondence: javier.oliver@ibima.eu (J.O.); manuelcobodols@yahoo.es (M.C.); isabel.barragan@ibima.eu (I.B.)

Citation: Onieva JL, Xiao Q, Berciano-Guerrero MÁ, et al. High IGKC-Expressing Intratumoral Plasma Cells Predict Response to Immune Checkpoint Blockade. Int J Mol Sci. 2022;23(16):9124. doi:10.3390/ijms23169124

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Abstract: Resistance to Immune Checkpoint Blockade (ICB) constitutes the current limiting factor for the optimal implementation of this novel therapy, which otherwise demonstrates durable responses with acceptable toxicity scores. This limitation is exacerbated by a lack of robust biomarkers. In this study, we have dissected the basal tumor microenvironment composition at the gene expression and cellular levels that predict response to Nivolumab and prognosis. BCR, TCR and HLA profiling were employed for further characterization of the molecular variables associated with response. The findings were validated using a single-cell RNA-seq data of metastatic melanoma patients treated with ICB, and by multiplex immunofluorescence. Finally, machine learning was employed to construct a prediction algorithm that was validated across eight metastatic melanoma cohorts treated with ICB. Using this strategy, we have unmasked a major role played by basal intratumoral Plasma cells expressing high levels of *IGKC* in efficacy. *IGKC*, differentially expressed in good responders, was also identified within the top response-related BCR clonotypes, together with IGK variants. These results were validated at gene, cellular and protein levels; $CD138^{+}$ Plasma-like and Plasma cells were more abundant in good responders and correlated with the same RNA-seq-defined fraction. Finally, we generated a 15-gene prediction model that outperformed the current reference score in eight ICB- treated metastatic melanoma cohorts. The evidenced major contribution of basal intratumoral *IGKC* and Plasma cells in good response and outcome in ICB in metastatic melanoma is a groundbreaking finding in the field beyond the role of T lymphocytes.

Keywords: Immunotherapy; Biomarkers; Melanoma

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Article

Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma

Juan Luis Onieva[1,2], Elisabeth Pérez-Ruiz[2,3,*], Ville Vilkki[1], Miguel Berciano-Guerrero[2,4], Laura Figueroa-Ortiz[1,2], Manuel Zalabardo[5], Beatriz Martínez-Gálvez[1], Isabel Barragán[1,6,*,§], Antonio Rueda-Domínguez[1,2,§],

[1] Group of Translational Research in Cancer Immunotherapy and Epigenetics (B-05), Medical Oncology Unit of Virgen de la Victoria Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[2] Facultad de Medicina, Campus de Teatinos s/n, Universidad de Málaga, 29071 Malaga, Spain.
[3] Group of Translational Research in Cancer Immunotherapy and Epigenetics (B-05), Medical Oncology Unit of Regional Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[4] Group New Horizons for Cancer Patients (B-23), Medical Oncology Unit of Regional Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[5] Group of Cancer Clinical and Translation Research (B-01), Medical Oncology Unit of Virgen de la Victoria Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, University of Malaga, 29010, Malaga, Spain.
[6] Group of Pharmacoepigenetics, Department of Physiology and Pharmacology, Karolinska Institutet, 171 65, Stockholm, Sweden.
[*] Corresponding authors. isabel.barragan@ki.se; isabel.barragan@ibima.eu; eliperu@gmail.com.
[§] Contributed equally.

Citation: Onieva JL, Pérez-Ruiz E, Vilkki V, et al. Integrating Tumor and Immune Cell Transcriptomics to Predict Immune Checkpoint Inhibitor Primary Resistance in Metastatic Melanoma. bioRxiv. 2025:670062. doi:10.1101/2025.08.13.670062

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Abstract

Background: The emergence of immune checkpoint inhibitors (ICIs) has transformed the treatment landscape of metastatic melanoma. However, despite its success, reliable biomarkers for predicting primary resistance are not available in clinical practice. This study seeks to identify predictors of primary resistance based on novel gene expression signatures using pre-treatment multidimensional profiling in melanoma patients.

Methods: The transcriptomic profile of the tumor microenvironment was analyzed using tissue samples from 46 metastatic cutaneous melanoma patients collected prior to the initiation of ICIs therapy. A primary resistance predictive model was trained with the Discovery FF-PE RNA-seq sub-cohort and validated using an independent external cohort of 54 samples. Additionally, liquid biopsy samples from peripheral blood mononuclear cells were analyzed in 8 patients using single-cell RNA sequencing (scRNA-seq) and in 46 patients using flow cytometry to characterize the distribution and abundance of the different immune cell populations.

Results: We identified an 82-gene transcriptomic signature composed of tumor- and immune-related genes that stratifies metastatic cutaneous melanoma patients based on primary resistance to ICIs, with key markers including *CXCL13, WDR63, MZB1, FDCSP, IGKC* and *GRIK3*. This signature was enriched for pathways related to B cell activation and immune cell communication and achieved an AUC of 0.814 in predictive modeling. Immune deconvolution guided by scRNA-seq revealed four immune cell subsets (Plasma cells, Pre-B cells, Memory $CD4^+$ T cells, and Naive $CD4^+$ T cells) as prognostic indicators of resistance. Some of these subpopulations were validated by flow cytometry before and after treatment.

Conclusions: We propose a transcriptomic biomarker signature that accurately predicts primary resistance to ICIs in metastatic cutaneous melanoma. Through the integration of immune deconvolution with circulating immune cell profiles, we derived an ImmuneSignature linked to patient survival. By combining these approaches, we provide a framework for enhancing the prediction of immunotherapy outcomes and offer a novel strategy for identifying therapeutic targets to overcome resistance. Our findings lead to more effective and personalized immunotherapy guidance.

Keywords: Melanoma; Predictive markers; Prognostic markers; Omics integration; Immune Checkpoint Inhibitors; Treatment response; Primary resistance

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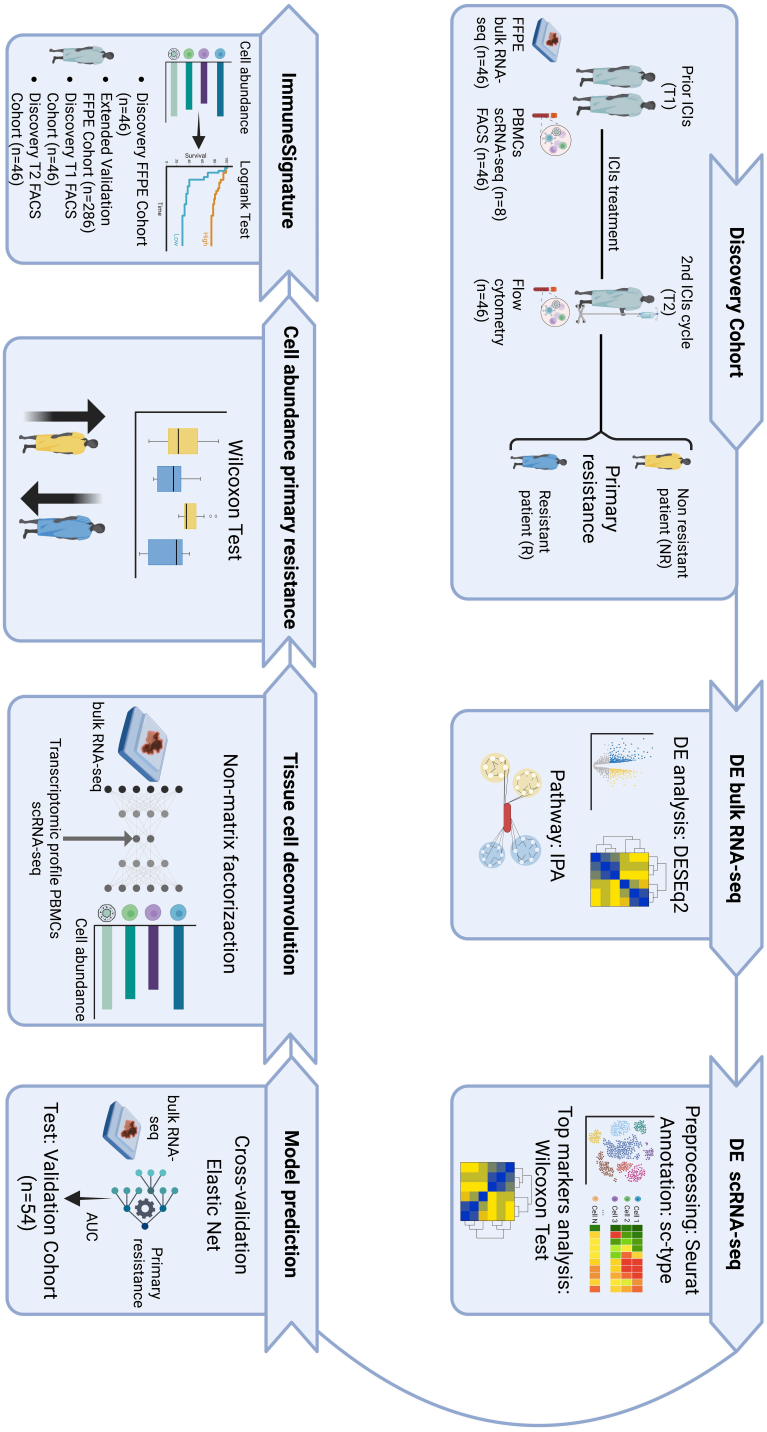

Graphical abstract

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cancers

Article

Fluorometric Quantification of Total Cell-Free DNA as a Prognostic Biomarker in Non-Small-Cell Lung Cancer Patients Treated with Immune Checkpoint Blockade

Javier Oliver[1,†], Juan Luis Onieva[1,2,†], María Garrido-Barros[1,2], Manuel Cobo-Dols[1], Beatriz Martínez-Gálvez[1], Ana Isabel García-Pelícano[1], Jaime Dubbelman[1], José Carlos Benítez[1], Juan Zafra Martín[1,2,3], Alejandra Cantero[1], Elisabeth Pérez-Ruiz[1,*], Antonio Rueda-Domínguez[1,‡] y Isabel Barragán[1,4,* ‡]

[1] Medical Oncology Service (Group of Translational Research in Cancer Immunotherapy), Regional and Virgen de la Victoria University Hospitals, Institute of Biomedical Research in Malaga and BIONAND Nanomedicine Platform (IBIMA BIONAND Platform), C/Marqués de Beccaría n○3, 29010 Málaga, Spain
[2] Faculty of Medicine, Campus de Teatinos s/n, Universidad de Málaga, 29071 Málaga, Spain
[3] Department of Radiation Oncology, Virgen de la Victoria University Hospital, 29010 Málaga, Spain
[4] Group of Pharmacoepigenetics, Department of Physiology and Pharmacology, Karolinska Institutet, 171 77 Stockholm, Sweden
[*] Correspondence: eliperu@gmail.com (E.P.-R.); isabel.barragan@ki.se (I.B.)
[†] These authors contributed equally to this work.
[‡] These authors contributed equally to this work.

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Simple Summary: The current study investigated the potential use of fluorometric cfDNA quantification as a prognostic biomarker in advanced non-small cell lung cancer (NSCLC) patients treated with an Immune Checkpoint Blockade (ICB). Results showed that a cfDNA cut-off of 0.55 ng/µL before the ICB determines the overall survival of patients with a log rank p-value of $3{,}3 \times 10^{-4}$. High cfDNA concentrations identify patients with advanced NSCLC who do not benefit from ICB. The determination of cfDNA is a simple test that could select a group of patients in whom new therapeutic strategies are needed.

Abstract: The present study aimed to investigate the potential of basal cell-free fluorometric DNA (cfDNA) quantification as a prognostic biomarker in advanced non-small cell lung cancer (NSCLC) patients treated with an Immune Checkpoint Blockade (ICB). A discovery and validation cohort of 61 and 31 advanced lung cancer patients treated with ICB were included in this study. Quantification of cfDNA concentration was performed before the start of the treatment and patients were followed up for a median of 34 (30–40) months. The prognostic predicted value of cfDNA was evaluated based on ROC, and Cox regression was conducted via univariate and multivariate analyses to estimate the hazard ratio. We observed that a cfDNA cut-off of 0.55 ng/µL before the ICB determines the overall survival of patients with a log rank p-value of $3{,}3 \times 10^{-4}$. That represents median survivals of 3.8 vs. 17.5 months. Similar results were obtained in the validation cohort being the log rank p-value $3{,}8 \times 10^{-2}$ with median survivals of 5.9 vs. 24.3. The univariate and multivariate analysis revealed that the cut-off of 0.55 ng/µL before ICB treatment was an independent predictive factor and was significantly associated with a better survival outcome. High cfDNA concentrations identify patients with advanced NSCLC who do not benefit from the ICB. The determination of cfDNA is a simple test that could select a group of patients in whom new therapeutic strategies are needed.

Keywords: cfDNA; Immunotherapy; NSCLC; Prognosis

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Article

Integrative Multi-Omic Profiling of cfDNA Methylation and EV-miRNAs Identifies Immunotherapy-Outcome Molecular Subtypes in NSCLC

Juan Luis Onieva[1,2], Elisabeth Pérez-Ruiz[2,3,*], Laura C Figueroa-Ortiz[1,2], José Miguel Jurado[4], Beatriz Martínez-Gálvez[1], Jose Carlos Benitez[1], Isabel Barragán[1,5,*,§], Antonio Rueda-Domínguez[1,2,§],

[1] Group of Translational Research in Cancer Immunotherapy and Epigenetics (B-05), Medical Oncology Unit of Virgen de la Victoria Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[2] Facultad de Medicina, Campus de Teatinos s/n, Universidad de Málaga, 29071 Malaga, Spain.
[3] Group of Translational Research in Cancer Immunotherapy and Epigenetics (B-05), Medical Oncology Unit of Regional Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[4] Medical Oncology Unit of Regional Hospital, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina–IBIMA Plataforma Bionand, 29010, Malaga, Spain.
[5] Group of Pharmacoepigenetics, Department of Physiology and Pharmacology, Karolinska Institutet, 171 65, Stockholm, Sweden.
[*] Corresponding authors. isabel.barragan@ki.se; isabel.barragan@ibima.eu; eliperu@gmail.com.
[§] Contributed equally.

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Abstract:

Background: Non-small cell lung cancer (NSCLC) patients exhibit heterogeneous responses to immunotherapy (IT) with high resistance rates, highlighting the need for precise biomarkers predictive of treatment outcomes.

Methods: In a prospective cohort study, we longitudinally assessed liquid biopsy samples from NSCLC patients undergoing IT at four distinct time points (T1 pre-treatment, T2 post-second cycle, T3 six months, and T4 one year). We profiled plasma-derived cell-free DNA methylation and extracellular vesicle-associated microRNAs from 79 metastatic NSCLC patients treated with immune checkpoint inhibitors (ICIs). High-dimensional omics data were integrated using Multi-Omics Factor Analysis (MOFA2) to uncover latent molecular subtypes, which we termed MOFA-Derived Clusters (MDCs), independently established at baseline (MDC-T1) and post-second cycle (MDC-T2). Differential expression and methylation analyses, pathway enrichment, and immune phenotyping via flow cytometry were used to characterize the molecular and immunological landscape of each MDC. External validation was performed using independent NSCLC cohorts for miRNAs (Genova et al., 2024, n=54) and methylation (SMC-Cohort, GSE119144, n=57).

Results: MDCs captured divergent survival outcomes and reflected biologically coherent processes including angiogenesis, cytoskeletal remodeling, and immune signaling. Projection of MDCs onto later time points (T3, T4) supported the temporal relevance of early molecular signatures. MDCs also displayed immunological correlates via circulating immune cell subsets. Importantly, MDC classifiers demonstrated consistent survival stratification in external cohorts, particularly MDC-T2.

Conclusions: This study defines a multi-omic, liquid biopsy–based framework for molecular subtyping in NSCLC to manage ICI treatment. Our MDC signatures reveal clinically meaningful, treatment-informative biology and offer a path toward minimally invasive patient stratification in immuno-oncology.

Keywords: Multi-omics; Immunotherapy; Prognostic/Predictive biomarkers; miRNAs; Methylation; Non-small cell lung cancer; Cell-free DNA

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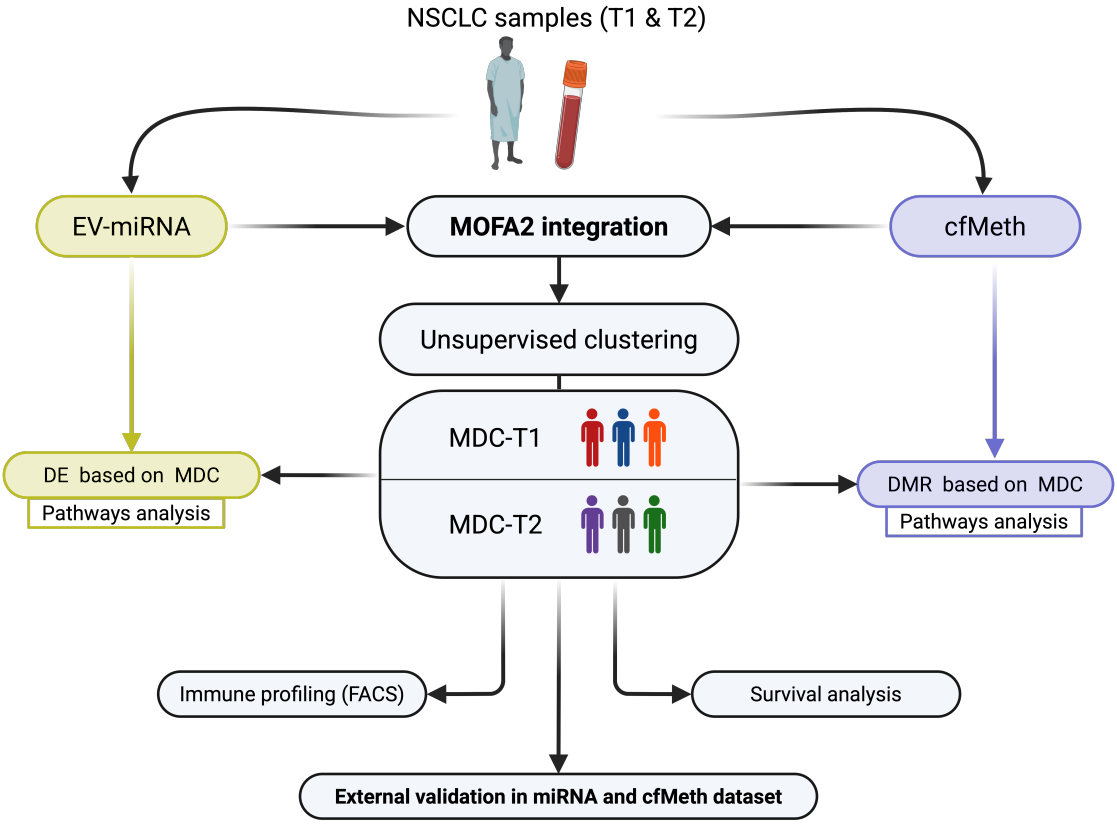

Graphical abstract

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