TESE DE DOUTORAMENTO

DECIPHERING THE IMPORTANCE OF BIOMARKERS IN COLORECTAL AND UROTHELIAL CANCERS IN THE ERA OF PRECISION ONCOLOGY

Juan Ruiz Bañobre

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CONFLICT OF INTEREST

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ETHICAL ASPECTS

This study was approved by the Institutional Review Boards of all participating institutions and all experiments were conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All alive patients provided written informed consent before enrollment. Informed consent was waived for dead patients before study initiation.

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Tipo de estudo: Outros
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Versión: Versión 1 26/06/2019 y HIP/CI de la misma fecha
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Impossible is nothing

– Adidas, 2004

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RESUMO

¿Como un desastre ocorrido durante a Segunda Guerra Mundial provocou un avance no tratamento do cancro? Un derramo accidental de mostaza nitroxenada sobre as tropas dun barco bombardeado no porto de Bari (Italia) durante a Segunda Guerra Mundial foi determinante na historia da terapia contra o cancro. A observación de que tanto a medula ósea como os ganglios linfáticos destes homes expostos ao gas mostaza presentaban unha marcada aplasia, fomentou un interese crecente por examinar os potenciais efectos terapéuticos destes produtos químicos sobre os linfomas. Tras confirmar a capacidade da mostaza nitroxenada para inducir remisións tumorais, o seu uso como tratamento do linfoma estendeuse rapidamente. Ademais, a identificación de análogos do ácido fólico como resultado da investigación nutricional levada a cabo antes e durante a Segunda Guerra Mundial posibilitou o descubrimento do metotrexato como unha nova opción terapéutica para nenos con leucemia. Curiosamente, o metotrexato foi o primeiro medicamento capaz de curar un tumor sólido non hematolóxico, o coriocarcinoma de placenta. Ata ese momento, a cirurxía e a radioterapia dominaban o campo da terapia contra o cancro, logrando unha taxa de curación despois de tratamentos locais cada vez máis radicais cunha meseta ao redor do 33%, o que fixo pensar na existencia de micrometástases. Neste contexto nace o concepto de quimioterapia adxuvante, que en combinación coa cirurxía e/ou radioterapia podería abordar a presenza das devanditas micrometástases. O cancro de mama foi o primeiro tipo de tumor no que se investigou a terapia adxuvante. O éxito dos dous primeiros ensaios clínicos realizados provocaron un rebulir de estudos de adxuvancia en cancro de mama e outros tipos de tumores, incluído o cancro colorrectal. O uso de terapia adxuvante contribuíu a un descenso significativo da mortalidade por cancro, especialmente importante para os tumores de mama e colon.

Non obstante, houbo outro feito determinante para cambiar o panorama do desenvolvemento de medicamentos, a chegada da bioloxía molecular. Como consecuencia dunha mellor comprensión das alteracións moleculares nas células cancerosas, as probas aleatorias de fármacos substituíronse gradualmente por unha selección máis racional de fármacos dirixidos contra dianas moleculares específicas. A era da quimioterapia daba paso á era da terapia dirixida. Retrospectivamente, na historia da oncoloxía hai varios precedentes que se poden considerar os primeiros exemplos de terapia dirixida. Este é o caso da terapia hormonal para o cancro de próstata ou incluso máis recentemente, a síntese de 5-fluorouracilo, un análogo do uracilo que exerce os seus efectos anticancerosos a través da inhibición da timidilato sintasa e a interrupción da síntese do ARN. Máis tarde, o descubrimento de novos oncoxenes, xenes supresores de tumores e vías de sinalización esenciais para a bioloxía do cancro levou á identificación de novas dianas farmacolóxicas que actualmente centran o foco do desenvolvemento de medicamentos antineoplásicos. Todos estes logros foron posibles debido aos extraordinarios avances tecnolóxicos e computacionais na secuenciación de ácidos nucleicos que, xunto coa bioloxía experimental, facilitaron unha comprensión máis detallada e completa da bioloxía molecular dos tumores.

O concepto de terapia dirixida, tamén coñecida como terapia dirixida molecularmente, abarca diversas estratexias terapéuticas onde as drogas están deseñadas para atacar as células tumorais interferindo con moléculas concretas fundamentais para a supervivencia celular ou, máis recentemente, para promover o ataque do sistema inmunitario contra o cancro. A terapia dirixida inclúe principalmente anticorpos monoclonais, inhibidores da tirosina quinasa, inhibidores mTOR, inhibidores do proteasoma, terapias hormonais e, máis recentemente, conxugados anticorpo-medicamento e inhibidores da proteína KRAS. Todos estes avances na comprensión da bioloxía molecular e o desenvolvemento de medicamentos levaron a unha nova era onde o obxectivo principal é loitar contra as células cancerosas con máis precisión, dun xeito máis personalizado e potencialmente con menos efectos secundarios. Esta é a era da oncoloxía de precisión.

A medicina de precisión defínese como un enfoque sanitario co obxectivo primordial de identificar que intervencións son susceptibles de ser máis beneficiosas para os pacientes en función das características do individuo e da súa enfermidade. O termo oncoloxía de precisión normalmente refírese ao uso de terapias que se espera confiran beneficio a un subconxunto de pacientes cuxo cancro presenta características moleculares ou celulares específicas (máis frecuentemente cambios xenómicos e patróns de expresión de xenes ou proteínas). Non obstante, este concepto tamén inclúe o uso de marcadores de prognose, preditores de toxicidade e calquera parámetro, como factores ambientais e de estilo de vida, que permita adaptar o tratamento en cada caso. En resumo, a oncoloxía de precisión é un enfoque para o coidado do paciente baseado na idea de que a enfermidade dunha persoa non é necesariamente igual que a de outra persoa que aparentemente ten a mesma patoloxía. Tendo en conta que a definición de oncoloxía de precisión está intimamente ligada ao concepto de biomarcador, compre definir este termo. Un biomarcador defínese como unha característica que se mide como indicador de procesos biolóxicos normais, procesos patolóxicos ou respostas biolóxicas a unha exposición ou intervención, incluídos os tratamentos antineoplásicos. Os biomarcadores poden abranguer características moleculares, histolóxicas, radiográficas ou fisiolóxicas.

Por outra banda, para determinar se un biomarcador debe ser empregado nun proceso de toma de decisións, hai tres características importantes a ter en conta con antelación. A primeira delas é a validez analítica, a cal se define como a capacidade do biomarcador para medir con precisión e fiabilidade a variable de interese no laboratorio clínico e en exemplares representativos da poboación en cuestión. A validez analítica inclúe sensibilidade analítica, especificidade analítica, fiabilidade e solidez do ensaio. Todos estes elementos de validez analítica son elementos que se integran tamén na avaliación da súa validez clínica. A validez clínica defínese como a capacidade do biomarcador para predicir con precisión e confianza o trastorno ou fenotipo de interese definido clinicamente. A validez clínica inclúe a sensibilidade e especificidade clínica e os valores preditivos das probas positivas e negativas que teñen en conta a prevalencia do trastorno. En resumo, a validez clínica implica que o rendemento dun biomarcador é aceptable para o propósito proposto e identifica distintos subconxuntos dunha poboación, cada un deles con diferenzas significativas respecto ó outro. A última característica a ter en conta é a utilidade clínica do biomarcador, a cal se define coma o balance de beneficios e prexuízos asociados ó seu uso na práctica, incluíndo a mellora dos resultados clínicos medibles e a utilidade e valor engadido na xestión clínica e na toma de decisións en comparación co non uso do mesmo. Aínda que é improbable que exista utilidade clínica se o biomarcador non ten validez clínica, a validez clínica non implica utilidade clínica. Ademais, a utilidade clínica inclúe a efectividade (utilidade

no escenario clínico real) e o beneficio neto. Frecuentemente, tamén implica a avaliación da eficacia (evidencia da utilidade en contextos controlados como un ensaio clínico). Con isto ponse de manifesto que unha definición clara do escenario clínico é de gran importancia, xa que as características de rendemento dunha proba determinada poden variar dependendo do uso previsto da mesma.

Dentro dos diferentes tipos de biomarcadores descritos, esta tese céntrase no estudo daqueles con valor preditivo e prognóstico. Un biomarcador preditivo é aquel que se usa para identificar individuos máis propensos a experimentar un efecto favorable ou desfavorable debido á exposición a un axente. Os biomarcadores preditivos en oncoloxía úsanse habitualmente para identificar aqueles pacientes que son máis propensos a presentar (ou non) melloras na calidade de vida, aumento na súa supervivencia, ou nalgúns casos toxicidades ante a exposición a un determinado tratamento. Por outra banda, un biomarcador prognóstico é aquel que se usa para identificar a probabilidade de que aconteza un determinado suceso clínico; no caso da oncoloxía os sucesos de maior interese adoitan ser a recorrencia ou progresión da enfermidade e a morte. Ademais, nalgúns casos, os biomarcadores poden ser á vez prognósticos e preditivos. Os biomarcadores preditivos non se distinguen xeralmente daqueles que presentan únicamente utilidade prognóstica cando só se estudan pacientes que recibiron unha determinada terapia. Para determinar que un biomarcador é preditivo, compre realizar unha comparación entre dúas cohortes que inclúan pacientes con e sen o biomarcador, unha delas tratada co fármaco de interese e a outra cunha terapia de control, idealmente no contexto de ensaios clínicos aleatorizados.

A oncoloxía de precisión é un campo en rápida evolución en moitos aspectos diferentes. Tendo isto en conta e considerando o papel central dos biomarcadores na oncoloxía de precisión, esta tese ofrece varios aspectos importantes relativos aos biomarcadores preditivos e de prognose en dous escenarios clínicos importantes. En primeiro lugar, presenta unha revisión sistemática no eido dos biomarcadores preditivos para varios tratamentos na área do cancro colorrectal metastásico, de maneira que resume os fitos máis relevantes acadados; analiza e discute aspectos metodolóxicos, tendencias actuais e direccións futuras. En segundo lugar, debido á falta de biomarcadores no contexto do cancro colorrectal mucinoso, esta tese aborda o papel dos microRNAs desde un punto de vista biolóxico e prognóstico neste subtipo específico de cancro colorrectal. Por último, esta tese presenta un estudo retrospectivo multicéntrico que ademais de investigar a seguridade e eficacia da monoterapia con anticorpos anti-PD-1 e anti-PD-L1, explora se algún factor basal pretratamento inflúe nos resultados acadados nun contexto de práctica clínica diaria con este tipo de inmunoterapia en pacientes con carcinoma urotelial localmente avanzado irresecable ou metastásico.

Centrándonos no resultado do primeiro dos estudos recollidos nesta tese, unha revisión sistemática dos biomarcadores preditivos de beneficio clínico en cancro colorrectal metastásico, compre sinalar que a día de hoxe, a pesar do enorme esforzo adicado á identificación de biomarcadores preditivos para varios tratamentos empregados en pacientes con cancro colorrectal metastásico, ata o momento só tres destes marcadores foron introducidos na práctica clínica para o seu uso cotiá.

O primeiro dos biomarcadores, as mutacións no xen *RAS*, presentes en aproximadamente un 55% dos pacientes con cancro colorrectal metastásico, serve como biomarcador preditivo negativo e correlaciónase coa falta de eficacia cos tratamentos con anticorpos anti-EGFR. O rol das mutacións no xen *RAS* como marcador preditivo negativo foi inicialmente descuberto no contexto

de estudos retrospectivos, sendo posteriormente validado retrospectivamente tamén nos ensaios clínicos pivotais dos fármacos cetuximab e panitumumab.
O segundo biomarcador é a presenza de inestabilidade de microsatélites no tumor, a cal se considera un factor preditivo positivo para un tipo de inmunoterapia baseada no bloqueo da molécula PD-1, os anticorpos anti-PD-1. Nos meses de maio e xullo do ano 2017, a Administración de Medicamentos e Alimentos dos Estados Unidos aprobou dúas terapias anti-PD-1, pembrolizumab e nivolumab respectivamente, para o tratamento de pacientes con cancro colorrectal metastásico con inestabilidade de microsatélites cuxa enfermidade progresara despois do tratamento cunha fluoropirimidina, oxaliplatino e irinotecán. Case un ano despois, en xullo do ano 2018, a mesma administración aprobou un réxime de tratamento baseado na combinación de nivolumab e ipilimumab, o cal supuxo unha terceira opción de tratamento inmunoterápico para pacientes con cancro colorrectal metastásico con inestabilidade de microsatélites, aproximadamente un 5% de tódolos pacientes con cancro colorrectal metastásico. Aínda que os pacientes con cancro colorrectal metastásico con inestabilidade de microsatélites teñen unha peor prognose, pénsase que os beneficios clínicos que obteñen da terapia anti-PD-1 é debido á presenza no tumor dunha elevada infiltración de linfocitos atraídos pola existencia de neoantíxenos asociados a mutacións. Seguindo a evolución lóxica no desenvolvemento de fármacos antineoplásicos, en maio do ano 2020 a Administración de Medicamentos e Alimentos dos Estados Unidos aprobou pembrolizumab como terapia de primeira liña para pacientes con cancro colorrectal metastásico con inestabilidade de microsatélites. Esta aprobación foi posible grazas aos resultados do estudo KEYNOTE-177 (NCT02563002), un ensaio clínico aleatorizado multicéntrico, internacional, aberto, con control activo que comparou a terapia de primeira liña con pembrolizumab fronte á quimioterapia en 307 pacientes con cancro colorrectal metastásico con inestabilidade de microsatélites. Este estudo demostrou unha mellora estatisticamente significativa na supervivencia libre de progresión da enfermidade, cunha mediana de 16.5 meses para o brazo de tratamento con pembrolizumab fronte a 8.2 meses para o brazo comparador con quimioterapia estándar. O impacto definitivo en supervivencia global deste novo tratamento e aínda descoñecido, sendo necesario un período de seguimento maior dos pacientes incluídos no ensaio clínico para acadar un resultado concluínte neste aspecto.
Por último, o terceiro biomarcador é a mutación *BRAF V600E*, considerado como un biomarcador preditivo positivo para os réximes de tratamento baseados no inhibidor da proteína con actividade tirosina quinasa BRAF. Este terceiro e último biomarcador da conta da velocidade á que se producen os avances no eido da oncoloxía de precisión, pois aínda que se presentan datos preliminares da súa utilidade na sección de material suplementario do primeiro estudo desta tese, a súa utilidade non foi plenamente establecida ata despois da finalización do mesmo. As mutacións no xen *BRAF* prodúcense nun 10–15% de tódolos cancros colorrectais e aproximadamente nun 7% dos casos de cancro colorrectal metastásico. Aínda que a maioría das mutacións no xen *BRAF* responsables da activación constitutiva da tirosina quinasa da proteína BRAF e da sinalización sostida da vía MAPK prodúcense no codón 600 (principalmente a mutación *V600E*), hai en torno a un 2% de casos de cancro colorectal metastásico que presentan mutacións atípicas no xen BRAF fóra deste codón, normalmente no 594. Sorprendentemente, aínda que a monoterapia con inhibidores BRAF demostrou ser eficaz no tratamento do melanoma *BRAF* mutado, esta foi ineficaz no cancro colorrectal metastásico coa mutación *BRAF V600E*. Os resultados de estudos preclínicos e ensaios clínicos en fase inicial demostraron que o bloqueo dual das proteínas BRAF e EGFR era factible e seguro e podía mellorar a eficacia terapéutica dos

inhibidores BRAF. Ademais, estudos preclínicos suxeriron que a inhibición combinada das proteínas con actividade tirosina quinasa BRAF e MEK era máis efectiva que o bloqueo dual de BRAF e EGFR. Esta estratexia comprobouse en posteriores ensaios clínicos de fase 1 e fase 2 que combinaron inhibidores BRAF con anticorpos monoclonais anti-EGFR e inhibidores da proteína MEK. Os resultados destes ensaios clínicos levaron á aprobación de encorafenib, un inhibidor da tirosina quinasa BRAF, usado en combinación con cetuximab para o tratamento de pacientes adultos con cancro colorrectal metastásico coa mutación *BRAF V600E*, en abril do ano 2020 por parte da Administración de Medicamentos e Alimentos dos Estados Unidos. A eficacia desta combinación de fármacos foi avaliada no estudo BEACON CRC, un ensaio clínico multicéntrico, aleatorizado, con control activo, de fase 3 (NCT02928224). Neste estudo, a combinación de encorafenib e cetuximab demostrou un beneficio estatisticamente significativo en termos de supervivencia global e supervivencia libre de progresión en comparación co brazo control con quimioterapia e cetuximab en pacientes con cancro colorrectal metastásico coa mutación *BRAF V600E* que progresaran a un ou dous réximes de tratamento previos. Este ensaio tamén avaliou a eficacia da tripla terapia con encorafenib, binimetinib (un inhibidor da proteína MEK) e cetuximab nun segundo brazo experimental, pero aínda que este réxime demostrou melloras en supervivencia global e supervivencia libre de progresión en comparación co brazo control, foi máis tóxico que o bloqueo dual das proteínas BRAF e EGFR. Outro inhibidor da tirosina quinasa BRAF, o vemurafenib, a pesar de amosar unha actividade clínica máis modesta neste tipo tumoral, foi recentemente incluído nas guías de práctica clínica da *National Comprehensive Cancer Network* como opción de tratamento para pacientes con cancro colorrectal metastásico coa mutación *BRAF V600E* cando se usa en combinación con irinotecan e cetuximab.

Entre os resultados do segundo dos estudos desta tese, centrado en cancro colorrectal, compre destacar a identificación dun panel de microARNs que se atopan diferencialmente expresados no tecido tumoral en función da presenza ou ausencia de mucina extracelular no tumor; a destacar, os niveis de expresión de miR-31 son maiores no cancro con diferenciación mucinosa, mentres que pola contra, miR-196-b, miR-592, miR-1247, miR-1269 e miR-552 presentan uns niveis de expresión máis baixos en aqueles tumores que carecen deste tipo de diferenciación. Por outra banda, un panel composto por estes 6 microARNs, ademais de ser útil para a diagnose de cancro colorrectal con diferenciación mucinosa, presenta unha destacada capacidade para predicir o risco de morte en pacientes con este tipo tumoral. Finalmente, a integración nun modelo matemático do panel de microARNs xunto coa estadiaxe TNM clásica, mellora a capacidade prognóstica de cada unha destas ferramentas por separado. Estes achados supoñen un avance significativo nun tipo tumoral no que o desenvolvemento de biomarcadores é, ata o día de hoxe, practicamente inexistente.

Por último, os resultados do terceiro estudo desta tese, ademais de confirmar a seguridade e eficacia da monoterapia con fármacos anti-PD-(L)1 nun escenario de práctica clínica diaria, sitúa a presenza de metástases peritoneais coma un factor independente de mala prognose, asociado a unha menor supervivencia global. Ademais, este estudo confirma a correlación entre o uso de inhibidores da bomba de protóns previo ao inicio da terapia con inmunoterapia, cunha menor efectividade do tratamento no contexto clínico avaliado. Para coñecer a natureza desta asociación, se é prognóstica e/ou preditiva, o impacto do uso de inhibidores da bomba de protóns deberá investigarse en cohortes procedentes de ensaios clínicos aleatorizados que permitan estudar o efecto do uso destes fármacos tanto en doentes tratados con inmunoterapia como con

outros axentes antineoplásicos. Finalmente, neste estudo establécese un modelo de avaliación de riscos fácil de usar no día a día da consulta médica composto por 5 factores clínicos e analíticos que permiten predicir a supervivencia global en pacientes con carcinoma urotelial metastásico tratados con anticorpos anti-PD-(L)1. De validarse noutros estudos, o modelo de avaliación de riscos proposto podería representar unha ferramenta útil non só para a práctica clínica diaria, senón tamén para a estratificación de pacientes en futuros ensaios clínicos con inmunoterapia.

Pouco a pouco, o manexo do cancro transita dun enfoque común no que todos os pacientes reciben un tratamento similar, independentemente das súas peculiaridades, a unha estratexia de medicina máis personalizada na que os pacientes se clasifican nun número crecente de subconxuntos en base ás características propias de cada individuo e ás alteracións moleculares específicas dos seus tumores. O propósito da oncoloxía de precisión é, mediante o uso de tratamentos molecularmente dirixidos, obter maiores beneficios terapéuticos en termos de supervivencia global e calidade de vida. Con este concepto na mente, a investigación sobre biomarcadores avanza integrando diferentes tipos de datos "ómicos", clínicos, epidemiolóxicos, e establecendo novas estratexias entre as que se inclúen as técnicas de biopsia líquida, que permiten non solo captar a heteroxeneidade temporo-espacial intra e intertumoral, senón tamén a toma de decisións en tempo real. Esta aproximación na que moitas disciplinas da ciencia traballan xuntas por un fin común, xeran novos coñecementos, avances tecnolóxicos e estratexias clínicas; deixando entrever un futuro cheo de esperanza no eido da diagnose, tratamento e seguimento dos pacientes con cancro.

1. INTRODUCTION

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1. INTRODUCTION

1.1. HISTORY OF ONCOLOGY

How a World War II (WWII) disaster led to a cancer treatment breakthrough? An accidental spill of sulfur mustards on troops from a bombed ship in Bari Harbor (Italy) during WWII was determinant in the history of cancer therapy[1,2]. Both, bone marrow and lymph nodes of those men exposed to the mustard gas were markedly depleted. This observation fostered a growing interest to examine the potential therapeutic effects of these chemicals on lymphomas, and after confirming the capacity of nitrogen mustard for induced marked remissions, its use as lymphoma treatment spread rapidly[3]. Moreover, the identification of folic acid analogues as a result of nutritional research before and during WWII led to the discovery of methotrexate, a new therapeutic option for children with leukemia[4]. Interestingly, methotrexate was the first drug able to cure a solid non-hematological tumor, the choriocarcinoma of the placenta[5]. Until that moment, surgery and radiotherapy dominated the field of cancer therapy, achieving a cure rate after ever more radical local treatments with a plateau at about 33%, which was blamed on the presence of heretofore-unappreciated micrometastases[6,7]. In this context borns the concept of adjuvant chemotherapy, which in combination with surgery and/or radiotherapy could deal with the presence of micrometastases. Breast cancer was the first tumor type where adjuvant therapy was investigated. The successful results of the first two clinical trials conducted set off a cascade of adjuvant studies in breast cancer[8,9] and other tumor types, including colorectal cancer (CRC). The use of adjuvant therapy has contributed to a significant decline in cancer mortality, especially important for breast and colorectal cancers[7].

However, something else happened that contribute to change the landscape of drug development, the arrival of molecular biology. As a consequence of a better understanding of molecular aberrations in cancer cells, random drug testing was gradually replaced by screening against specific critical molecular targets. The chemotherapy era was transitioning to the age of targeted therapy[7]. In retrospect, in the history of oncology, there are various precedents that can be considered the very first examples of targeted therapy. This is the case of hormonal therapy for prostate cancer[10] or even more recently, the synthesis of 5-fluorouracil[11], an analogue of uracil which exerts its anticancer effects through the inhibition of thymidylate synthase and the disruption of RNA synthesis. Later, the discovery of new oncogenes, tumor suppressor genes, and signaling pathways essential for cancer biology led to the identification of new drug targets that are currently the focus of cancer drug development[12]. All these achievements were possible due to the extraordinary technological and computational advances in nucleic acid sequencing which together with the experimental biology, facilitated a more detailed and comprehensive understanding of molecular biology[13].

The concept of targeted therapy, also known as molecularly targeted therapy, encompass many different therapeutic strategies where drugs are designed to tackle tumor cells by interfering with specific molecules of cancer cells, or even more recently, to unleash the attack of the immune system against cancer. Targeted therapy includes mainly monoclonal antibodies, tyrosine kinase inhibitors, mTOR inhibitors, proteasome inhibitors, hormonal therapies, and more recently

antibody-drug conjugates and KRAS inhibitors[14–17]. All these advances in the understanding of molecular biology and drug development have led to a new era where the primary goal is to fight cancer cells with more precision, in a more personalized way, and potentially with fewer side effects. This is the precision oncology era.

1.2. PRECISION ONCOLOGY: BIOMARKERS AND ENDPOINTS

In the era of precision oncology, biomarkers have become increasingly important given their relevance in the decision to implement, or not, effective therapeutic strategies that may have substantial toxicities[18]. In this context, effective, and concise communication is essential for efficient translation of promising research discoveries into approved clinical realities. Unclear definitions and inconsistent use of key terms can hinder the evaluation and interpretation of scientific evidence and may pose significant hurdles to advance of medical product development[19]. Moreover, recognizing drug development as a time- and cost-consuming endeavor, any efficiency that can be realized during the development and regulatory processes will speed access of approved therapies and devices to patients[20]. The expected positive impact of biomarkers specifically on drug development is substantial, and coordinated efforts to identify biomarkers are a focus of intensive research and debate[19–21]. In this line, there have been several efforts to standardize the criteria for biomarker research[22–24]. In 2009, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Initiative proposed three semantics to determine if a genetic test should be used to manage care[25]: analytical validity, clinical validity, and clinical utility, which have been adopted by both the National Academy of Medicine (NAM)[26] and ASCO for deliberations specifically regarding biomarkers in oncology[26,27]. In 2015, the Food and Drug Administration (FDA)-National Institutes of Health Joint Leadership Council identified the harmonization of terms used in translational science and medical product development as a priority need, with a focus on terms related to study endpoints and biomarkers. Working together with the goals of improving communication, aligning expectations, and improving scientific understanding, the two agencies developed the Biomarkers, EndpointS, and other Tools (BEST) Resource[19]. The first phase of BEST comprises a glossary that clarifies important definitions and describes some of the hierarchical relationships, connections, and dependencies among the terms it contains[19].

1.2.1. PRECISION ONCOLOGY GLOSSARY

To better understand the concept behind precision medicine in oncology, besides to set an understandable definition of the term itself, it is clear the necessity of describing concepts and terminologies specifically related to this area that are often poorly defined. For this purpose, this introduction presents some of the most important definitions collected in the European Society for Medical Oncology (ESMO) Precision Medicine and BEST glossaries[19,28].

1.2.1.1. Precision Medicine

Precision Medicine is defined as a healthcare approach with the primary aim of identifying which interventions are likely to be of most benefit to which patients based upon the features of the individual and their disease. In cancer, the term usually refers to the use of therapeutics that are expected to confer benefit to a subset of patients whose cancer displays specific molecular or cellular features (most commonly genomic changes and gene or protein expression patterns). Nevertheless, the term also includes the use of prognostic markers,

predictors of toxicities and any parameter such as environmental and lifestyle factors that leads to treatment tailoring. Characterization approaches in the future are expected to encompass a wider range of technologies such as functional imaging or global phosphoprotein analyses[28]. In short, it is an approach to patient care that is based on the idea that one person´s disease is not necessarily exactly the same in someone else who seemingly has the same disease.

1.2.1.2. Biomarker

A biomarker is defined as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions. Biomarkers may include molecular, histologic, radiographic, or physiologic characteristics[19]. A biomarker is not a measure of how an individual feels, functions, or survives. This succinct but comprehensive description intended to correctly identify the biomarker, its biologic plausibility, and its measurement method[19]. While not exhaustive, these key concepts included in the biomarker description bring important details to evaluate information from multiple sources and set a homogeneous framework:

Biomarker Identity. The name of the biomarker includes the specific analyte, anatomic feature, or physiological characteristic that is measured. If applicable, the unique identifier for the biomarker and the commonly used acronym are useful information to ensure that two or more resources are referring to the same analyte. The specific source for the biomarker (for example plasma, serum, urine, tumor tissue, or computed tomography scan, among others) provides important context and determines not only measurement reference ranges but also the biomarker type itself[19].

Biologic Plausibility. A brief summary of the biological, physiological, or pathological pathway for the association of the biomarker with the disease or condition of interest provides a contextual linkage between a biomarker and its intended use. In addition, this information helps to delineate how multiple biomarkers may interplay as part of a common use[19].

Measurement Method. The measurement method that will be used to quantify the biomarker and the units of quantification is critical information when comparing information from independent platforms. This information is helpful throughout biomarker development, including early discovery. Sufficient detail should be included to facilitate the interpretation of the results across multiple resources[19].

On the other hand, in accordance with the EGAPP Initiative to determine if a test should be used to the decision-making process, there are three important biomarker features to consider in advance:

Analytical validity

Analytical validity of a biomarker test is defined by EGAPP as its ability to accurately and reliably measure the variable of interest in the clinical laboratory, and in specimens representative of the population of interest[25,29]. Analytic validity includes analytic sensitivity, analytic specificity, reliability, and assay robustness[29]. All these elements of analytic validity are themselves integral elements in the assessment of clinical validity[29,30]. Many evidence-based processes assume that evaluating clinical validity will address any analytic problems, and do not formally consider analytic validity[31]. As technologies are rapidly evolving, and validation data are limited in some circumstances, it is important to consider that review of analytic validity can to determine whether clinical validity can be improved by addressing test performance in new

scenarios[25].

Clinical validity

Clinical validity of a biomarker test is defined by EGAPP as its ability to accurately and reliably predict the clinically defined disorder or phenotype of interest. Clinical validity includes clinical sensitivity and specificity, and predictive values of positive and negative tests that take into account the disorder prevalence[25]. In short, the term clinical validity implies that the performance of a biomarker is acceptable for its intended purpose, and identifies different portions of one population, each of which has significant differences from the other[18,19].

Clinical utility

Clinical utility defines the balance of benefits and harms associated with the use of the biomarker test in practice, including improvement in measureable clinical outcomes, and usefulness and added value in clinical management and decision-making compared with not using the biomarker[25]. Whereas it is unlikely that clinical utility would exist if the biomarker does not have clinical validity, clinical validity does not imply clinical utility[18]. Clinical utility includes effectiveness (utility in real clinical scenario), and the net benefit. Frequently, it also involves assessment of efficacy (evidence of utility in controlled settings like a clinical trial). A clear definition of the clinical scenario is of major importance, as the performance characteristics of a given test may vary depending on the intended use of the test.

1.2.1.2.1. Types of Biomarkers

Although BEST glossary establishes several biomarker categories (susceptibility/risk biomarker, diagnostic biomarker, prognostic biomarker, predictive biomarker, monitoring biomarker, pharmacodynamic/response biomarker, safety biomarker)[19], for the purpose of this thesis only predictive and prognostic biomarkers will be discussed in detail.

Predictive Biomarker

A predictive biomarker is that one used to identify individuals who are more likely than similar individuals without the biomarker to experience a favorable or unfavorable effect from exposure to a medical product or an environmental agent[19]. The effect could be a symptomatic benefit, improved survival, or a toxicity or adverse event[19].

A common example of use of a predictive biomarker in medical product development is predictive enrichment of the study population for a randomized controlled clinical trial of an investigational therapy, in which the biomarker is used either to select patients for participation or to stratify patients into biomarker positive and biomarker negative groups, with the primary endpoint being the effect in the biomarker positive group. If the biomarker is in fact predictive of a favorable outcome, then the effect of the investigational therapy compared to a control therapy (or placebo) will be greater (or present at all) in patients with the biomarker or some level of the biomarker. The notion of a predictive biomarker applies to a wide variety of interventions, including drugs, biologics, medical devices or procedures, and behavioral or dietary modifications for treatment or prevention of diseases or conditions[19].

The utility of predictive biomarkers is not limited to a clinical trial setting, as these biomarkers can also assist in informing patient care decisions, such as determining who might benefit from a particular treatment or selecting among multiple interventions. In the latter

situation, evidence that a biomarker predicts the comparative effectiveness of an intervention should be accompanied by specification of the alternative interventions involved in the comparison[19].

Predictive biomarkers for effects of interventions may be characteristics of the individual's biological constitution (host characteristics) or features of the disease process or other medical condition. Biomarkers representing host characteristics are present irrespective of the individual's disease or medical condition status, such as germline DNA, human leukocyte antigen (HLA) type or dihydropyrimidine dehydrogenase phenotype, renal or hepatic function, or metabolic characteristics. Predictive biomarkers for drugs are often chosen initially based on the mechanism of action of the drug and understanding of pathophysiology, but they could also be identified empirically based on previous studies. Understanding the impact on outcome of both host and disease or condition characteristics is important for efficient development and optimal application of interventions[19].

Establishing that a biomarker is predictive for an intervention's effect generally requires a comparison of the intervention to a control treatment in individuals with and without the biomarker, usually in randomized trials. Although studying only biomarker positive patients would establish effectiveness of a particular intervention it does not specifically demonstrate the predictive role of the biomarker. It is therefore generally appropriate to stratify patients in the randomized trial by presence or absence of the biomarker (if dichotomous). Randomization to treatment and control groups is usually important because demonstrating that individuals who are positive for a biomarker and receive an investigational therapy experience a better outcome than those who receive the same therapy but are negative for the biomarker does not establish that the biomarker is predictive. Differences in outcome associated with the biomarker could be due to prognostic abilities of the biomarker and may be present irrespective of the therapy received. The greater differences between treatment and control in the biomarker positive compared to biomarker negative groups are what establish the biomarker as predictive[19].

Studies designed to evaluate a predictive biomarker should usually include patients with a range of biomarker values (or positive and negative for binary biomarkers). Sometimes there is sufficient prior evidence to strongly suggest that an investigational therapy will not be effective (or could even be harmful) in a certain subgroup of individuals defined by a biomarker; these circumstances may require excluding patients who are negative for the biomarker from trials of the investigational therapy. When a biomarker identifies a subgroup of patients who will benefit most from an investigational therapy, enrichment of a trial with individuals from that subgroup will provide increased statistical power for detection of the (larger) effect of that therapy; use of such an enrichment strategy will also affect the intended population to receive the therapy after its regulatory approval[19].

Prognostic Biomarker

A prognostic biomarker is that one used to identify an increased (or decreased) likelihood of a future clinical event in an identified population[19]. Prognostic biomarkers are measured at a defined baseline, which may include a background treatment. Many familiar examples of prognostic biomarkers occur in clinical contexts where an individual is diagnosed with a disease or condition and there is interest in assessing the likelihood of a future clinical event. Examples of future events include death, disease progression, disease recurrence, or development of a new medical condition. In oncology, biomarkers such as tumor size, number of lymph nodes positive

for tumor cells, and presence of metastasis have traditionally been used to indicate prognosis. Increasingly, molecular indicators or signatures measured on tumors are being used in lieu of, or in addition to, these clinicopathologic characteristics. The prognostic biomarker's association with outcome is present without reference to different interventions. However, the presence or strength of a prognostic association may vary depending on the specific clinical setting and particular endpoint of interest, so it is important that prognostic biomarkers be described in the proper context[19].

Prognostic biomarkers are often used as eligibility criteria in clinical trials to identify patients who are more likely to have clinical events or disease progression. Thus, they are widely used as enrichment factors in drug development. Many clinical trials of medical interventions have as their endpoint either an event rate or time-to-event. The statistical power for a time-to-event endpoint to assess treatment effect in a controlled clinical trial is driven by the planned effect size and the planned number of events. Enrichment with patients who have a higher likelihood of experiencing an event will therefore increase statistical power. In a treatment setting, prognostic biomarkers can contribute to decisions about whether or how aggressively to intervene with the treatment[19].

Prognostic versus Predictive Biomarker Complexity

A variety of factors influence a patient clinical outcome, including intrinsic characteristics of the patient, disease, or medical condition, and the effects of any treatments that the patient receives.

Prognostic biomarkers and predictive biomarkers cannot generally be distinguished when only patients who have received a particular therapy are studied. Some biomarkers are both prognostic and predictive. Prognostic biomarkers are often identified from observational data and are regularly used to identify patients more likely to have a particular outcome[19].

To identify a predictive biomarker, as it was detailed in the previous specific section, there generally should be a comparison of a treatment to a control in patients with and without the biomarker. However, there are circumstances in which preclinical and early clinical data provide such compelling evidence that a new treatment will not work in patients without the biomarker that definitive clinical trials are performed only in populations enriched for the putative predictive biomarker[19]. Moreover, this prognostic-predictive complexity is also partly driven by the search for more effective therapies for patients who have a poor prognosis with standard treatments[32]. In this manner, genetic alterations classically associated with a poor prognosis in some cancer types, are now targets of some of the most promising targeted therapies and consequently, predictive biomarkers in their respective scenarios.

1.2.1.3. Endpoints in Precision Oncology

To fully understand the nature of precision oncology in general, and biomarkers in particular, it seems essential to properly set the concept of endpoint and related terminology.

1.2.1.3.1. Types of Endpoints

Endpoint

An endpoint is a precisely defined variable intended to reflect an outcome of interest that is statistically analyzed to address a particular research question. A precise definition of an endpoint typically specifies the type of assessments made, the timing of those assessments, the

assessment tools used, and possibly other details, as applicable, such as how multiple assessments within an individual are to be combined[19].

Surrogate Endpoint

A surrogate endpoint is that one used in clinical trials as a substitute for a direct measure of how a patient feels, functions, or survives. A surrogate endpoint does not measure the clinical benefit of primary interest in and of itself, but rather is expected to predict that clinical benefit or harm based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence[19].

From a regulatory standpoint, surrogate endpoints and potential surrogate endpoints can be characterized by the level of clinical validation: validated surrogate endpoint, reasonably likely surrogate endpoint, candidate surrogate endpoint[19].

1.2.1.3.2. Principal Endpoints in Oncology

In the next paragraphs, the more relevant endpoints used in oncology are described based on the information provided by the US FDA guideline *Clinical trial endpoints for the approval of cancer drugs and biologics: guidance for industry*[33].

Overall Survival

Overall survival (OS) is defined as the time from randomization until death from any cause and is measured in the intent-to-treat population. Survival is considered the most reliable cancer endpoint, and when studies can be conducted to adequately assess survival, it is usually the preferred endpoint. This endpoint is precise and easy to measure without bias, documented by the date of death. Survival improvement should be analyzed as a risk-benefit analysis to assess clinical benefit. OS should be evaluated in randomized controlled studies. Data derived from externally controlled trials are seldom reliable for time-to-event endpoints, including OS. Apparent differences in outcome between external controls and current treatment groups can arise from differences other than drug treatment, including patient selection, improved imaging techniques, or improved supportive care. Randomized studies minimize the effect of these known and unknown differences by providing a direct outcome comparison. Demonstration of a statistically significant improvement in OS can be considered to be clinically significant if the toxicity profile is acceptable and has often supported new drug approval. Difficulties in performing and analyzing survival studies include long follow-up periods in large trials and subsequent cancer therapy potentially confounding survival analysis[33].

Disease-Free and Event-Free Survivals

Disease-free survival (DFS) is defined as the time from randomization until disease recurrence or death from any cause. The most frequent use of this endpoint is in the adjuvant setting after definitive surgery or radiotherapy. DFS also can be an important endpoint when a large percentage of patients achieve complete responses (CRs) with chemotherapy. Although OS is a conventional endpoint for most adjuvant settings, DFS can be an important endpoint in situations where survival may be prolonged, making an OS endpoint impractical. An endpoint that is similar to DFS but is differentiated from it in that randomization takes place before definitive surgery or radiotherapy in the adjuvant setting is event-free survival (EFS). EFS is defined as time from randomization to any of the following events: progression of disease that precludes surgery, local or distant recurrence, or death due to any cause. Treatment effect

measured by DFS or EFS can be a surrogate endpoint to support accelerated approval, a surrogate endpoint to support traditional approval, or it can represent direct clinical benefit based on the specific disease, context of use, magnitude of the effect, the disease setting, available therapy, and the risk-benefit relationship. Important considerations in evaluating DFS or EFS as a potential endpoint include the estimated size of the treatment effect and proven benefits of standard therapies. Moreover, the schedule for follow-up assessments and visits should be carefully delineate. Unscheduled assessments can occur for many reasons and differences between study arms in the frequency, timing, or reason for unscheduled assessments can introduce bias. Bias can be minimized by blinding patients and investigators to the treatment assignments, as appropriate. Application of the definition of DFS or EFS in a study can be complicated, particularly when deaths are noted without prior tumor progression documentation. These events can be scored either as disease recurrences or as censored events. Although all methods for statistical analysis of deaths have some limitations, considering deaths from all causes as recurrences can minimize bias. DFS or EFS can be overestimated using this definition, especially in patients who die after a long period without observation. Bias can be introduced if the frequency of long-term follow-up visits is dissimilar between the study arms or if dropouts are not random because of toxicity. Some analyses count cancer-related deaths as DFS or EFS events and censor non-cancer deaths. This method can introduce bias in the attribution of the cause of death. Furthermore, any method that censors observations on patients, whether at death or at the last visit, assumes that the patients with censored observations have the same risk of recurrence as patients with non-censored observations who have not yet experienced the event[33].

Objective Response Rate

Objective response rate (ORR) is defined as the proportion of patients with tumor size reduction of a predefined amount and for a minimum time period. Response duration usually is measured from the time of initial response until documented tumor progression. Generally, the FDA has defined ORR as the sum of partial responses plus CRs. When defined in this manner, ORR is a direct measure of a drug antitumor activity, which can be evaluated in a single-arm study. Stable disease should not be a component of ORR. Stable disease can reflect the natural history of disease, whereas tumor reduction is a direct therapeutic effect. Also, stable disease can be more accurately assessed by time to progression (TTP) or progression-free survival (PFS) analysis. If available, standardized criteria should be used to ascertain response. A variety of response criteria have been considered appropriate, being the most widely used revised Response Evaluation Criteria In Solid Tumors (RECIST) guideline (version 1.1)[34]. The response criteria should be predefined in the protocol before the start of the study. The significance of ORR is assessed by its magnitude and duration, and the percentage of CRs. Treatment effect measured by ORR can be a surrogate endpoint to support accelerated approval, a surrogate endpoint to support traditional approval, or it can represent direct clinical benefit based on the specific disease, context of use, magnitude of the effect, the number of CRs, the durability of response, the disease setting, the location of the tumors, available therapy, and the risk-benefit relationship[33].

Complete Response

CR is defined as no detectable evidence of tumor. CR is generally measured through imaging studies or through histopathologic assessment. Treatment effect measured by CR can be a surrogate endpoint to support accelerated approval, a surrogate endpoint to support traditional

approval, or it can represent direct clinical benefit based on the specific disease, context of use, magnitude of the effect, effect duration, disease setting, location of disease, available therapy, and the risk-benefit relationship[33].

Time to Progression and Progression-Free Survivals

TTP and PFS have served as primary endpoints for drug approval. TTP is defined as the time from randomization until objective tumor progression; TTP does not include deaths. PFS is defined as the time from randomization until objective tumor progression or death, whichever occurs first. The precise definition of tumor progression is important and should be carefully detailed. Compared with TTP, PFS is the preferred regulatory endpoint. PFS includes deaths and thus can be a better correlate to OS. In TTP analysis, death events are censored, either at the time of death or at an earlier visit representing informative censoring (nonrandom pattern of loss from the study). PFS assumes that death events are randomly related to tumor progression. PFS can reflect tumor growth and be assessed before the determination of a survival benefit. Importantly, its determination is not confounded by subsequent therapy. For a given sample size, the magnitude of effect on PFS can be larger than the effect on OS. Data are usually insufficient to allow a robust evaluation of the correlation between effects on OS and PFS. Cancer trials are often small, and proven survival benefits of existing drugs are generally modest. Treatment effect measured by PFS can be a surrogate endpoint to support accelerated approval, a surrogate endpoint to support traditional approval, or it can represent direct clinical benefit based on the specific disease, context of use, magnitude of the effect, the disease setting, location of metastatic sites, available therapy, the risk-benefit relationship, and the clinical consequences of delaying or preventing progression in key disease sites such as the brain or spine, or delaying administration of more toxic therapies. It is important to carefully define tumor progression criteria in the protocol. Although there are no standard regulatory criteria for defining progression, RECIST criteria is currently the most frequently used[33].

Time to Treatment Failure

Time to treatment failure (TTF) is defined as a composite endpoint measuring time from randomization to discontinuation of treatment for any reason, including disease progression, treatment toxicity, and death. TTF is generally not recommended as a regulatory endpoint for new molecular-targeted therapy approval[33].

Specific Symptom Endpoints

Symptom improvement is a direct measure of clinical benefit rather than a surrogate endpoint. A decrease in the severity of cancer symptoms has been used to support traditional approval of anti-cancer agents where anti-tumor activity has also been demonstrated. The use of a symptom palliation endpoint requires that the population be symptomatic at baseline, which can be problematic in many cancer trials where patients can often be asymptomatic at baseline. This endpoint can also be subject to open label response bias, the magnitude of which is not well described[33].

Time to progression of cancer symptoms is a direct measure of clinical benefit rather than a potential surrogate endpoint. Because few cancer trials are blinded, symptom assessments can also be subject to response bias. A delay between tumor progression and the onset of cancer symptoms can occur. Often alternative treatments are initiated before achieving the symptom

endpoint, confounding this analysis. In addition, tumor symptoms can be difficult to differentiate from drug toxicity[33].

Blood or Bodily Fluid-Based Biomarkers

Generally, biomarkers assayed from blood or body fluids have not served as primary endpoints for cancer drug approval, although the FDA has accepted blood-based markers as elements of a composite endpoint for solid tumors. The occurrence of certain clinical events such as a significant decrease in performance status or bowel obstruction, in conjunction with marked increases in CA-125 was considered progression in ovarian cancer patients. In addition, blood-based biomarkers can be useful in identifying prognostic factors and in selection of patients and stratification factors to be considered in study designs[33].

Emerging Endpoints

In addition to the endpoints already discussed in this section, the US FDA recognizes that advances in science are facilitating development of novel oncology products, which may also result in the identification of additional endpoints that may be used to support their approval. One example is metastasis-free survival, which has been used as a clinical endpoint for traditional approval for a therapy in non-metastatic castration-resistant prostate cancer[33].

2. HYPOTHESES AND OBJECTIVES

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2. HYPOTHESES AND OBJECTIVES

Although precision medicine in oncology is rapidly evolving in many different aspects, there are still several areas that deserve specific attention. Following, the hypotheses and objectives behind the works developed along the next sections of this thesis are described.

Section **4.1. COLORECTAL CANCER** presents a systematic and comprehensive review on the state of affairs for predictive biomarkers in metastatic CRC (mCRC), as well as a retrospective study which offers new insights into the potential clinical significance of microRNAs (miRNAs) in mucinous carcinoma (MC), a less well-investigated CRC subtype which represents 10–15% of all CRC diagnoses[35,36].

A. Predictive biomarkers in metastatic colorectal cancer

Although the backbone of treatment in patients with mCRC has historically been chemotherapy, over the last few decades targeted molecular therapies against EGFR and angiogenic factors have been introduced into daily clinical practice. Furthermore, new treatment options such as anti-programmed cell death 1 (PD-1) antibodies have recently been added to the mCRC armamentarium. Historically, and in parallel with drug development, multiple research efforts have been undertaken to discover and implement molecular biomarkers to guide therapeutic strategies. This becomes even more important in today's clinical scenario in which multiple therapeutic options are available, and therefore treatment selection aims not only to improve patient survival, but also to spare patients from unnecessary toxicity and reduce the economic burden of expensive treatments.

Hypothesis

A systematic review of published studies will offer not only an updated perspective of the biomarker landscape in the mCRC setting from a molecular and clinical viewpoint, but also some insights regarding needs to face in the near future.

Objectives

To summarize the most relevant milestones achieved in the field of biomarkers for approved therapies in patients with mCRC.

To describe, analyze and quantify the most important clinical and methodological aspects behind the biomarker development for approved therapies in patients with mCRC.

B. Clinical significance of a microRNA signature for the identification and predicting prognosis in colorectal cancers with mucinous differentiation

Accumulating evidence suggests that mucinous carcinomas (MCs) represent a distinct entity, particularly in the context of CRC and present a unique clinical challenge – both from diagnostic and prognostic risk-stratification purposes. However, the currently used definition to categorize a CRC as mucinous is arbitrary and results in a considerable diagnostic inter-observer

variability[37–40]. In addition, the prognostic significance of mucinous CRC subtype is not clear[39,40]. On the other hand, although different studies have indicated that the expression of various mucin-associated genes is regulated by specific miRNAs[41], no systematic studies have thus far interrogated miRNA expression profiles in CRCs with a mucinous subtype. While quite a few studies have investigated the role of miRNAs in non-mucinous CRC, the biological and prognostic significance of miRNAs in mucinous CRCs remains unclear.

Hypothesis

MiRNAs are useful biomarkers to improve the biological understanding of MCs and to risk stratification of patients with this tumor subtype.

Objectives

To examine the specific miRNA expression profiles of MC specimens and identify a panel of miRNA biomarkers differentially expressed between MC and non-MC tumors.

To evaluate and confirm the clinical importance of these miRNA biomarkers in various independent patient cohorts.

To examine the association between the expression levels of a panel of the selected miRNAs and OS in mucinous CRCs.

To construct an integrative clinical and molecular model for prognostication in mucinous CRC patients.

Section **4.2. UROTHELIAL CANCER** presents a multicenter retrospective study involving previously treated or untreated metastatic urothelial carcinoma (mUC) patients under anti-PD-1 or anti-programmed death ligand 1 (PD-L1) [PD-(L)1] therapy in in the context of routine clinical practice and clinical trials between June 2016 and February 2020 from 7 Galician medical centers.

Hypothesis

Monotherapy with anti-PD-(L)1 antibodies is a safety and effective treatment option in daily clinical-practice for mUC patients, and its efficacy is influenced by many baseline clinical and analytical factors.

Objectives

To confirm safety and efficacy of anti-PD-(L)1 monotherapy in daily clinical-practice.

To identify pretreatment factors influencing anti-PD-(L)1 monotherapy outcomes.

To develop and internally validate an independent prognostic model for OS.

3. MATERIAL AND METHODS

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3. MATERIAL AND METHODS

Material and methods are described in detail in the articles presented in the next sections.

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4. RESULTS

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4. RESULTS

4.1. COLORECTAL CANCER

A. Predictive Biomarkers in Metastatic Colorectal Cancer

Article 001 *- Title: Predictive Biomarkers in Metastatic Colorectal Cancer: A Systematic Review.*

Article 002 *- Title: DNA Mismatch Repair Deficiency and Immune Checkpoint Inhibitors in Gastrointestinal Cancers.*

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Article 001 *- Title: Predictive Biomarkers in Metastatic Colorectal Cancer: A Systematic Review.*

Authors: ***Juan Ruiz-Bañobre****, Raju Kandimalla, Ajay Goel.*

Specific contribution of the PhD candidate to the article: Conception and design of the study, analysis and interpretation of data, drafting of the manuscript and revision after peer-review.

Journal: JCO Precision Oncology.
ISSN: 2473-4284 (online).
Publisher: Wolters Kluwer Health.
Indexed in Web of Science – JCR 2019 impact factor: not available.
Indexed in Scopus – SJR 2019 impact factor: 2.59 – Q1, Cancer Research.

https://doi.org/10.1200/PO.18.00260

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Article 002 *- Title: DNA Mismatch Repair Deficiency and Immune Checkpoint Inhibitors in Gastrointestinal Cancers.*

Authors: ***Juan Ruiz-Bañobre****, Ajay Goel.*

Specific contribution of the PhD candidate to the article: Conception and design of the study, analysis and interpretation of data, drafting of the manuscript and revision after peer-review.

Journal: Gastroenterology.
ISSN: 0016-5085.
Publisher: Elsevier.
Indexed in Web of Science – JCR 2019 impact factor: 17.373 – D1, Gastroenterology and Hepatology.
Indexed in Scopus – SJR 2019 impact factor: 6.85 – Q1, Gastroenterology.

https://doi.org/10.1053/j.gastro.2018.11.071

This article complements *article 001*, specifically in the section about immune checkpoint inhibitors development in mCRC, predictive biomarkers, and mechanisms of resistance.

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B. Clinical significance of a microRNA signature for the identification and predicting prognosis in colorectal cancers with mucinous differentiation

Article 003 *- Title: Clinical significance of a microRNA signature for the identification and predicting prognosis in colorectal cancers with mucinous differentiation.*

Authors: ***Juan Ruiz-Bañobre****, Roshni Roy, Miren Alustiza Fernández, Óscar Murcia, Rodrigo Jover, Miguel Pera, Francesc Balaguer, Rafael López-López, Ajay Goel.*

Specific contribution of the PhD candidate to the article: Conception and design of the study, analysis and interpretation of data, drafting of the manuscript and revision after peer-review.

Journal: Carcinogenesis.
ISSN: 0143-3334 (online) / 0143-3334 (print).
Publisher: Oxford University Press.
Indexed in Web of Science – JCR 2019 impact factor: 4.603 – Q2, Oncology.
Indexed in Scopus – SJR 2019 impact factor: 1.66 – Q1, Medicine.

https://doi.org/10.1093/carcin/bgaa097

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4.2. UROTHELIAL CARCINOMA

Article 004 *- Title: Rethinking Prognostic Factors In Locally Advanced Or Metastatic Urothelial Carcinoma In The Immune Checkpoint Blockade Era: A Multicenter Retrospective Study.*

Authors: ***Juan Ruiz-Bañobre***[#]*, Aurea Molina-Díaz, Ovidio Fernández-Calvo, Natalia Fernández-Núñez, Ana Medina-Colmenero, Lucía Santomé, Martín Lázaro-Quintela, María Mateos-González, Noelia García Cid, Rafael López-López; Sergio Vázquez, Urbano Anido-Herranz.*

[#]*Corresponding author.*

Journal: ESMO Open.
ISSN: 2059-7029 (online).
Publisher: Elsevier.
Indexed in Web of Science – JCR 2019 impact factor: 5.329 – Q1, Oncology.
Indexed in Scopus – SJR 2019 impact factor: 2.02 – Q1, Cancer Research.

Specific contribution of the PhD candidate to the article: Conception and design of the study, analysis and interpretation of data, drafting of the manuscript and revision after peer-review.

https://doi.org/10.1016/j.esmoop.2021.100090

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4.3. OTHER STUDIES CONDUCTED DURING THE DOCTORAL THESIS PERIOD

In addition to the specific results derived from the main research projects, the following research studies were also conducted during the doctoral thesis period, framed within the fields of medical oncology, biomarkers, epigenetics, nano-oncology, and immunotherapy. Following, these contributions are presented in chronological order:

1. Book Chapter: ***Ruiz-Bañobre J***, Goel A. 2021. Genomic and epigenomic biomarkers in colorectal cancer: From diagnosis to therapy. Advances in Cancer Research. Academic Press, Elsevier. ISBN 0065-230. https://doi.org/10.1016/bs.acr.2021.02.008

2. Original Research Article: Arias Ron D, Areses Manrique MC, Mosquera Martínez J, García González J, Afonso Afonso FJ, Lázaro Quintela M, Fernández Núñez N, Azpitarte Raposeiras C, Amenedo Gancedo M, Santomé Couto L, García Campelo MR, Muñoz Iglesias J, ***Ruiz-Bañobre J***, et al. Efficacy and safety of Nivolumab in older patients with pretreated lung cancer: A subgroup analysis of the Galician lung cancer group. *J Geriatr Oncol*. 2020. doi:https://doi.org/10.1016/j.jgo.2020.11.010

3. Original Research Article: García-González J, ***Ruiz-Bañobre J***, Afonso-Afonso FJ, et al. PD-(L)1 Inhibitors in Combination with Chemotherapy as First-Line Treatment for Non-Small-Cell Lung Cancer: A Pairwise Meta-Analysis. *J Clin Med*. 2020;9(7). doi:10.3390/jcm9072093

4. Poster presentation: Pérez Martelo M, Fernández Álvarez J, Abdulkader Nallib I, Brozos Vázquez E, Vázquez Rivera F, Vidal Ínsua Y, Candamio Folgar S, López López R, ***Ruiz-Bañobre J***. P-89 Validation of the prognostic significance of the dNLR (2.2) in a population-based cohort of metastatic colorectal cancer patients treated with oxaliplatin-based first-line therapy. *Ann Oncol*. 2020;31:S118-S119. doi:10.1016/j.annonc.2020.04.171

5. Original Research Article: ***Ruiz-Bañobre J***, Areses-Manrique MC, Mosquera-Martínez J, et al. Evaluation of the lung immune prognostic index in advanced non-small cell lung cancer patients under nivolumab monotherapy. *Transl Lung Cancer Res Vol 8, No 6 (December 2019) Transl Lung Cancer Res*. 2019

6. Original Research Article: Reimondez-Troitiño S, González-Aramundiz JV, ***Ruiz-Bañobre J***, et al. Versatile protamine nanocapsules to restore miR-145 levels and interfere tumor growth in colorectal cancer cells. *Eur J Pharm Biopharm*. 2019;142:449-459. doi:https://doi.org/10.1016/j.ejpb.2019.07.016

7. Book Chapter: Vázquez-Ríos AJ, Alonso-Nocelo M, López-Bouzo B, ***Ruiz-Bañobre J***, de la Fuente-Freire M. 2018. Chapter 8: Nanotheranostics and Their Potential in the Management of Metastatic Cancer Handbook of Nanomaterials for Cancer Theranostics. Elsevier. ISBN 9780128133392

8. Book Chapter: López-López R, ***Ruiz-Bañobre J***, Muinelo-Romay L. 2018. Capítulo 3: Biopsia tisular versus biopsia líquida 50 Preguntas Clave en Oncología de Precisión. Permanyer. ISBN 9788417221416

9. Original Research Article: Vidal J, Muinelo L, Dalmases A, Jones F, Edelstein D, Iglesias M, Orrillo M, Abalo A, Rodríguez C, Brozos E, Vidal Y, Candamio S, Vázquez F, ***Ruiz-Bañobre J***, et al. Plasma ctDNA RAS mutation analysis for the diagnosis and treatment monitoring of metastatic colorectal cancer patients. *Ann Oncol*. 2017;28(6):1325-1332

10. Letter to the Editor: ***Ruiz-Bañobre J***[#], Garcia-Gonzalez J. Anti-PD-1/PD-L1-induced psoriasis from an oncological perspective. *J Eur Acad Dermatol Venereol*. 2017;31(9):e407-e408. doi:10.1111/jdv.14217

11. Case Report: ***Ruiz-Bañobre J***[#], Abdulkader I, Anido U, Leon L, Lopez-Lopez R, Garcia-Gonzalez J. Development of de novo psoriasis during nivolumab therapy for metastatic renal cell carcinoma: immunohistochemical analyses and clinical outcome. *APMIS*. 2017;125(3):259-263. doi:10.1111/apm.12658

12. Original Research Article: ***Ruiz-Bañobre J***[#], Pérez-Pampín E, García-González J, et al. Development of psoriatic arthritis during nivolumab therapy for metastatic non-small cell lung cancer, clinical outcome analysis and review of the literature. *Lung Cancer*. 2017;0(0). doi:10.1016/j.lungcan.2017.04.007

13. Letter to the Editor: ***Ruiz-Bañobre J***[#], Anido U, García-González J. Re: Francesco Piva, Matteo Santoni, Marina Scarpelli, et al's Letter to the Editor re: Daniel M. Geynisman. Anti-programmed Cell Death Protein 1 (PD-1) Antibody Nivolumab Leads to a Dramatic and Rapid Response in Papillary Renal Cell Carcinoma with Sarcom. *Eur Urol*. July 2016. doi:10.1016/j.eururo.2016.06.038 13

14. Case Report: ***Ruiz-Bañobre J***[#], Anido U, Abdulkader I, Antunez-Lopez J, Lopez-Lopez R, Garcia-Gonzalez J. Long-term Response to Nivolumab and Acute Renal Failure in a Patient with Metastatic Papillary Renal Cell Carcinoma and a PD-L1 Tumor Expression Increased with Sunitinib Therapy: A Case Report. *Front Oncol*. 2016;6:250. doi:10.3389/fonc.2016.00250

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5. GENERAL DISCUSSION

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5. GENERAL DISCUSSION

Precision oncology is a rapidly evolving field in many different aspects. Taking this in mind and considering the central role of biomarkers in precision oncology, this thesis affords various important aspects regarding predictive and prognostic biomarkers in two important clinical scenarios. First, this thesis presents a systematic and comprehensive review on the field of mCRC that summarizes the most relevant milestones achieved in the field of predictive biomarkers to various treatments; analyzes and discusses methodological aspects, current trends, and future directions in this exciting area. Second, because of the lack of biomarkers in the context of MC, this thesis affords the role of miRNAs from a biological and prognostic viewpoint in this specific CRC subtype. Lastly, this thesis presents a multicenter retrospective study in mUC patients under anti-PD-(L)1 monotherapy which investigates the safety and efficacy of anti-PD-(L)1 antibodies, and explores pretreatment factors influencing therapeutic outcomes in mUC in daily clinical practice, a context where prognostic biomarkers for risk stratification are an unmet medical need.

5.1. Predictive biomarkers in metastatic colorectal cancer

Despite the tremendous body of effort devoted for the identification of predictive biomarkers for various treatments used in patients with mCRC, thus far only three of such markers have translated into routine clinical practice. The first one, the mutations in the *RAS* gene, serves as a negative predictive biomarker that is present in ~55% of mCRC patients[42] and correlates with the lack of efficacy to anti-EGFR antibody treatments. The identification of *RAS* mutations as a negative predictive marker, which was initially based on retrospective studies, was subsequently retrospectively validated in cetuximab and panitumumab pivotal clinical trials. The second marker is the tumor MSI status, which has emerged as a predictive marker for anti-PD-1 drugs. In May and July of 2017, the US FDA approved the anti-PD-1 therapies pembrolizumab and nivolumab for the treatment of patients with MSI-H mCRC for whom the disease has progressed after treatment with fluoropyrimidine, oxaliplatin, and irinotecan. Almost a year later, in July 2018, a nivolumab plus ipilimumab combination regimen was approved, which opened three novel treatment options for patients with MSI-H or dMMR mCRC (patients with MSI-H or dMMR mCRC represent approximately 5% of all patients with mCRC)[43]. Although patients with MSI-positive mCRC have worse prognosis, it is thought that they derive clinical benefit from anti-PD-1 therapy because of a large proportion of lymphocytic infiltration and the presence of mutation-associated neoantigens[44–48]. This exciting discovery has led to universal MSI testing for the management of patients with mCRC.

Not surprisingly, in May 2020 the US FDA approved pembrolizumab as first-line therapy for patients with MSI-H/dMMR mCRC. This approval was based on the results of the KEYNOTE-177 study (NCT02563002), a multicenter, international, open-label, active-controlled, randomized trial that compared first-line therapy with pembrolizumab vs. chemotherapy in 307 patients with MSI-H/dMMR mCRC. This study demonstrated a statistically significant improvement in PFS, with a median PFS of 16.5 months vs. 8.2 months for pembrolizumab compared to chemotherapy standard-of-care. Longer-term analysis is needed to

assess the effect on OS. Moreover, in June 2020 the US FDA granted accelerated approval to pembrolizumab for the treatment of patients with any unresectable or metastatic solid tumor with high mutational burden (as determined by the FDA-approved test, the FoundationOne CDx assay) whose cancer has progressed after previous treatment and has no satisfactory alternative treatment options [49]. Several clinical trials evaluating the combination of anti-PD-1 therapy with chemotherapy are ongoing for previously untreated MSI-H/dMMR mCRC patients with the goal of improving on results from previous studies and further extending survival of these patients. Meanwhile, other different immunotherapeutic approaches are being evaluated for treatment of microsatellite stable (MSS) CRC, which is less responsive to immune checkpoint inhibition than MSI-H mCRC. Although all of these results represent substantial therapeutic advances in the treatment of mCRC, they also emphasize the growing need for more precise predictive biomarkers to support more rational development of immunotherapies. A more comprehensive understanding of the intersection between genomics, epigenomics, and immunology in mCRC seems essential for meeting this need for new strategies.

Recently, Grasso et al.[50] reported the results of a large-scale genomic analysis (TCGA, Nurses' Health Study, and Health Professionals Follow-up Study cohorts) involving 1211 primary CRC tumor specimens. Mutations in genes involved in immune modulatory pathways, as well as in the neoantigen-presentation machinery (mainly *B2M* and *HLA*), significantly correlated with MSI-H. Along with *JAK1/2* and *IFN-gamma receptor 1* mutations, similar alterations have been observed in melanoma, non-small cell lung cancer, and CRC and deemed to be genetic drivers of primary or acquired resistance to immune checkpoint blockade, reflecting their role as a mechanism of adaptive resistance against T-cell tumor infiltration[45,50–54]. The interaction between somatic alterations and the immune system is complex, as indicated by a recent study in which 11 out of 13 *B2M*-mutant CRC patients achieved mCRC control with anti-PD-1 or anti-PD-L1 agents, despite the presence of a mutation that, theoretically, conferred primary resistance to ICI[55]. On the other hand, for both MSS and MSI-H tumors, active WNT/β-catenin signaling was inversely associated with tumor T-cell infiltration, providing evidence of the existence of an anti-immune response mechanism beyond the MSI profile[50].

Lastly, the third marker is the *BRAF V600E* mutation as a predictive biomarker for BRAF inhibitor (BRAFi)-based regimens. *BRAF* mutations occur in 10–15% of all CRCs and in ~7% of all mCRC[56,57]. Although most *BRAF* mutations occur in codon 600 (mainly *BRAF V600E*), which leads to constitutive BRAF kinase activity and sustained MAPK pathway signaling, 2% of mCRCs have atypical *BRAF* mutations that are outside of codon 600, usually in codon 594[58]. Surprisingly, although monotherapy with BRAFi has proven effective in the treatment of *BRAF*-mutant melanoma, it was ineffective in *BRAF V600E*-mutant CRCs. Preclinical evidence demonstrated that despite transient inhibition of pERK by BRAFi such as vemurafenib, rapid ERK reactivation occurs through EGFR-mediated activation of RAS and CRAF[59]. Furthermore, the fact that *BRAF V600E*-mutant CRCs express higher levels of pEGFR than do *BRAF*-mutant melanomas, positions them for EGFR-mediated resistance [59]. Collectively, these findings provided rationale to test dual BRAF and EGFR blockade. Results from preclinical studies and early phase clinical trials, have demonstrated this strategy is feasible and safe, and can potentially improve therapeutic efficacy of BRAFi. Moreover, preclinical studies have suggested that combined inhibition of BRAF and MEK was more effective than dual BRAF and EGFR blockade. This strategy was tested in subsequent phase 1 and phase 2 clinical trials that combined BRAF inhibitors with both anti-EGFR monoclonal antibodies and MEK inhibitors[59–61]. Results of

these trials led to US FDA approval (in April 2020) of encorafenib, a BRAF tyrosine kinase inhibitor, used in combination with cetuximab for the treatment of adult patients with *BRAF V600E*-mutated mCRC. The efficacy of this combination of drugs was evaluated in the BEACON CRC study[62], a phase 3 randomized, active-controlled, open-label, multicenter trial (NCT02928224). In this trial, encorafenib plus cetuximab demonstrated a clinical and statistically significant OS and PFS benefit compared to the control arm of either irinotecan or FOLFIRI plus cetuximab in patients with *BRAF V600E*-mutated mCRC who had progressed on one or two prior regimens. This trial also evaluated the efficacy of triple-therapy with encorafenib, binimetinib (a MEK inhibitor [MEKi]), and cetuximab in a second experimental arm, but although this regimen showed an improved OS and PFS compared to the control arm, it was more toxic than the dual BRAF and EGFR blockade and had similar efficacy. Another BRAFi, vemurafenib, which has more modest clinical activity, was recently included in the NCCN guidelines as a treatment option for patients with *BRAF V600E*-mutated mCRC when used in combination with cetuximab/panitumumab plus irinotecan[63,64]. Inclusion in the guidelines was based on results of the randomized phase 2 Southwest Oncology Group (SWOG) 1406 trial, in which the triple-therapy (vemurafenib, cetuximab, and irinotecan) demonstrated improved PFS and ORR as compared with cetuximab plus irinotecan[64]. In addition to the previously described regimens, based on the results of a phase 1 study[61], the NCCN Panel has recommended the combination of dabrafenib (BRAFi) plus trametinib (MEKi) plus either cetuximab or panitumumab as another treatment option beyond the first line setting for *BRAF V600E*-mutated mCRC[63].

Moreover, other well-described predictive biomarkers used in the management of several tumor types, have shown promising utility in selecting mCRC patients for various targeted therapy-based regimens:

1) HER-2 Blockade – Regarding the role of HER-2 amplification/overexpression as a predictive biomarker, a large body of evidence, accrued primarily from breast and gastric cancer patients, supports the role of *HER-2* amplification or overexpression as a predictive biomarker for anti-HER-2-based therapies. Therefore, there is renewed interest in evaluating HER-2 as a clinically actionable target in mCRC. Although initial mCRC clinical trials interrogating the anti-HER-2 monoclonal antibody trastuzumab in combination with other chemotherapeutic agents (either FOLFOX or irinotecan) closed early due to lack of patient accrual, mechanistic insights gained from preclinical analyses of *HER-2*-amplified mCRC patient-derived xenografts have led to improved design of new clinical trials[65–67]. Three phase 2 clinical trials evaluated dual HER-2 blockade in a biomarker-selected subset of heavily pretreated mCRC patients. Study treatment included trastuzumab plus lapatinib (HERACLES trial, NCT03225937), pertuzumab and trastuzumab (MyPathway trial, NCT02091141), or the antibody-drug conjugate trastuzumab deruxtecan (DESTINY-CRC01, NCT03384940). Results of these studies demonstrated an impressive ORR of ~30–45%[68–70]. These data have paved the way for development of ongoing phase 2 clinical trials evaluating the efficacy of new anti-HER-2 agents, such as S1613 (NCT03365882), trastuzumab-emtansine (NCT03418558), or tucatinib (NCT03043313) in this clinical scenario comprising ~5% of RAS wild-type mCRC patients[71]. Furthermore, determining the utility of ctDNA analyses in monitoring therapeutic efficacy and in identifying mechanisms of resistance to dual HER-2 blockade is also an attractive area of study[72].

2) Tyrosine Kinase Inhibitors – New drugs that target tyrosine kinase (TK) fusions in genes such as *NRTK1/2/3*, *RET*, *ALK*, and *ROS1* are showing promising preliminary results in phase 1 and 2 clinical trials that include patients with CRC. One agent, LOXO 101 (larotrectinib),

is a selective tropomyosin receptor kinase (TRK) inhibitor that demonstrated tumor-agnostic efficacy in patients with *NTRK* fusion-positive malignancies (including four patients with CRC who achieved a partial response)[73]. A second agent, entrectinib, an ALK, ROS1, TRKA, TRKB, and TRKC selective inhibitor, demonstrated clinical activity in patients who had fusions in the previously described TK genes[74]. Patients who responded to entrectinib included two patients whose mCRC harbored *CAD-ALK* or *LMNA-NTRK1* gene fusions[75,76]. Anticipating potential resistance mechanisms to larotrectinib based on evidence from other pan-TK inhibitors, Drilon et al.[77] developed LOXO-195 (selitrectinib), a potent and selective TRK kinase inhibitor designed to have a molecular structure that would overcome typical TRK resistance mutations. LOXO-195 was initially evaluated in a mCRC patient whose cancer had an *LMNA-NTRK1* rearrangement with a G595R larotrectinib-resistance mutation. This patient successfully achieved a durable partial response[77]. Although the prevalence of rearrangements in TK genes in mCRC patients may be as low as 1.5%, the accelerated development of TK inhibitors offers new hope for some heavily pretreated mCRC patients who have no other therapeutic options[78]. Given these promising results, the US FDA granted accelerated approval to larotrectinib (November 2018) and entrectinib (August 2019) for patients with *NTRK* gene fusion-positive solid tumors without a known acquired resistance mutation. The Committee for Medicinal Products for Human Use of the European Medicines Agency has also recommended the granting of a conditional marketing authorization for larotrectinib (July 2019) and entrectinib (May 2020) for the same indication.

3) KRAS Inhibitors – *KRAS* is one of the most commonly altered oncogenes in human cancers, and was long considered an undruggable target because of the small size of abnormal KRAS proteins, the presence of few binding sites, and the rapid, tight binding of active KRAS to GTP. However, recent data have suggested that KRAS may be targetable. For example, preliminary data on the activity of AMG510 (sotorasib), a small covalent inhibitor, have shown that it rapidly and irreversibly occupies *KRAS G12C* and extinguishes its activity through a unique interaction with the P2 pocket[79]. The *KRAS G12C* mutation occurs in ~4% of CRC[80]. In a recent phase 1 trial, sotorasib showed encouraging anti-tumor activity in heavily pretreated patients who had advanced, *KRAS G12C*-mutated solid tumors[17]. A total of 129 patients were included in this study, 42 of whom had CRC. Within CRC patients, sotorasib treatment yielded an ORR and disease control rate (DCR) of 7.1% and 73.8%, respectively. The median duration of stable disease was 5.4 months and the median PFS was 4.0 months. Although sotorasib showed promising anticancer activity in patients with heavily pre-treated solid tumors bearing the *KRAS G12C* mutation, inconsistency was seen in tumor response between patients with non-small cell lung cancer and those with CRC, which the authors suggested indicated either that *KRAS G12C* is not the dominant oncogenic driver for CRC or that other pathways, such as the WNT or EGFR pathways, mediate oncogenic signaling beyond *KRAS*. These hypotheses are supported by solid preclinical evidence[81–83], and therefore, clinical trials that combine sotorasib with other agents that block additional pathways have already been initiated (i.e., NCT04185883 and NCT04303780). Although many *KRAS* G12C inhibitors in addition to sotorasib are under development, to date only adagrasib, an irreversible covalent inhibitor, has shown promising antitumor activity in *KRAS G12C*-mutated CRC. Furthermore, inhibitors for mutations other than *KRAS G12C* are being developed. For example, initial preclinical data for MRTX1133, a new, first-in-class *KRAS G12D* inhibitor, have demonstrated significant tumor regression in preclinical animal models[84]. Thus, through development of a range of inhibitors, effective means of targeting KRAS are emerging.

Nonetheless, the discovery and validation of novel predictive biomarkers that can assist in decision-making has been a challenging endeavor, resulting in a long list of failed predictive markers. As highlighted by the results of our study, this task seems particularly even more daunting in terms of conventional chemotherapy and antiangiogenic drugs. In CRC, since the use of single-agent chemotherapeutic regimens have shown limited efficacy, and the majority of current treatment options include various combinations of drugs, biomarker discovery for a specific drugs is not surprisingly more complicated due to the interactions between different cytotoxic agents[63]. Similar concerns remain for developing predictive biomarkers for therapeutic response to bevacizumab, since: a) it is also not used as a single agent in the clinic[63], b) the poor understanding of its mechanism(s) of action[85], and c) the very reason that angiogenesis is an intriguingly adaptive process which involves numerous factors[86]. Presumably, the inherent complexity of angiogenesis has been a significant hurdle in the attempts to develop response predictive biomarkers for other multi-targeted antiangiogenic drugs such as aflibercept or regorafenib. Additional insights into the tumor microenvironment, including the role of tumor-associated stromal cells, could possibly shed light on this tortuous process in the future. On the other hand, and based on the results of our study, the gap between the discovery phase and subsequent biomarker development steps looks evident, highlighting the necessity of the implementation of a robust worldwide platform to move forward predictive biomarker validation. These facts, together with the lack of effort to undertake external validation of initial findings, block the advancement of the majority of the presumed predictive biomarkers in the clinic.

Another important question worthy of discussion in any biomarker discovery effort is the origin of tumor tissue samples ─ whether primary tumor tissue or metastatic lesions. An interesting example of this important concept is the TS expression as a predictive biomarker to 5-FU based chemotherapy, since its efficacy has been discordant depending on the tumor tissue origin[87,88]. This concept is highly congruent with tumor heterogeneity, which is a possible source of discrepancy even when the molecular marker is analyzed in a different region of the same source[89]. Besides spatial heterogeneity, tumors are dynamic entities that continue to evolve over time, especially if they are under selective pressure[90]. For this reason, the time from sample acquisition to biomarker analysis is of significant clinical relevance ─ an issue that is often overlooked in most studies. Since only ~20% of CRC patients present with a metastatic disease at the time of diagnosis, it is often the practice or only option available to analyze archival tissues from the primary tumor to identify biomarkers ─ which is not always an optimal or preferred choice[91].Patient selection is gaining importance, which is evidenced by the recent initiative, the US National Cancer Institute's Exceptional Responder Program[92,93]. Consideration of extreme phenotypes such as long-term responders and extremely early progressors for biomarker discovery can facilitate successful identification of molecular alterations that better correlate with clinical phenotypes. For instance, in the majority of studies presented in this work, there was no consideration of PFS as a selection criterion, and many studies included patients with stable disease in the non-responders. In general, improved ORRs and longer PFS are superior indicators of the true efficacy of any drug intervention, while inclusion of gain in OS as a selection feature may inadvertently introduce bias. In addition, new biomarker-driven study designs such as basket or umbrella trials, which assign a treatment according to tumor molecular characteristics, not only are going to improve clinical drug development, but will also facilitate improved biomarker validation. While analysis of clinical specimens with robust follow-up data from retrospective series or randomized trials are of tremendous value, a well-designed biomarker discovery phase

followed by technical validation in subsequent prospective clinical cohorts using longitudinally collected specimens is much needed to establish clinically translatable predictive biomarkers. Additionally, although many surgical specimens are of suitable quality, needle biopsy-derived metastatic lesions often yield lower amount of DNA/RNA required for robust sequencing experiments[94,95]; hence having access to liquid biopsy-based predictive markers would be transformative in overcoming this limitation in mCRC patients. Furthermore, in addition to helping the clinicians in quicker and easier decision-making, liquid biopsy biomarkers will improve patient compliance and eliminate the concerns surrounding intra-tumor heterogeneity associated with tumor/biopsy specimens, and may also help in disease monitoring as well as predicting secondary resistance. The international community has to consolidate initiatives to improve biomarker development studies, and more importantly undertake conscious efforts to validate the results gathered from retrospective studies in prospective randomized multicenter cohorts. Such efforts will guarantee improved success and will decrease the economic burden by allowing precision treatment of cancer patients. Furthermore, a significant majority of patients will be spared from unnecessary toxicity and side effects of treatments that will not benefit them clinically. Lastly, the implementation of novel high-throughput molecular analytical techniques and the integration of multi-omic approaches together with clinical and epidemiological data using machine-learning algorithms will definitely hasten the biomarker development in the coming years[96].

In spite of a large body of attempts over the last decades, there remain only three well-established predictive biomarkers ─ mutations in the *RAS* gene, the MSI status, and the *BRAF V600E* mutation (after the completion of our systematic review, as we previously discussed, the clinical utility of *BRAF V600E* was confirmed in a phase 3 randomized clinical trial) ─ that currently guide treatment decisions in patients with mCRC (**Figure 1**). Although the past efforts in this context may not have been as rewarding, we currently are a frontier, where the future looks quite promising. The integration of high-throughput deep techniques, together with the advent of machine-learning algorithms and novel clinical trial designs will definitely revolutionize predictive biomarkers for response to cancer therapeutics, as we usher into the new era of precision oncology.

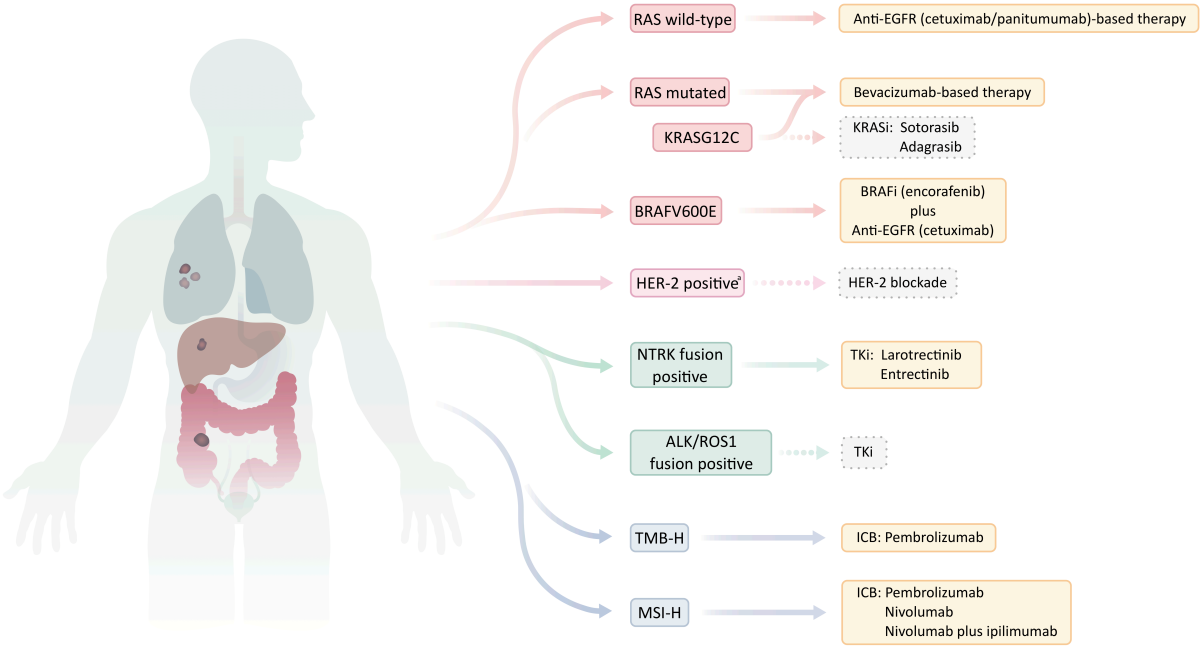

Figure 1. Predictive biomarkers for metastatic CRC treatment. Summary of currently known molecular tumor alterations that enable improved clinical decision-making regarding use of therapies that are tailored to the metastatic CRC (mCRC) patient. The therapies shown go beyond classical chemotherapeutic agents. Excluding *RAS* mutations for EGFR-targeted therapies, which are considered a negative predictive biomarker, the molecular alterations shown represent positive predictors of benefit with the indicated targeted therapies. Although bevacizumab is considered a useful therapeutic option in combination with chemotherapy in mCRC independent of any particular molecular alteration, currently there is no consistent predictive biomarker to guide bevacizumab use. Although many of these molecular alterations are applicable only to a minority of mCRC patients, collectively these low-prevalence actionable characteristics support a new-targeted therapeutic horizon for many patients. Color-coded boxes: yellow, US FDA-approved therapy; grey, not US FDA approved therapy.
[a]*HER2* amplification or overexpression.
Abbreviations: BRAFi, BRAF inhibitors; CRC, colorectal cancer; ICB, immune checkpoint blockade; KRASi, KRAS inhibitors; MSI-H, microsatellite instability high; TKi, tyrosine kinase inhibitors; TMB-H, tumor mutation burden high.

5.2. Clinical significance of a microRNA signature for the identification and predicting prognosis in colorectal cancers with mucinous differentiation

Accumulating evidence suggests that mucinous carcinoma represent a distinct entity, particularly in the context of CRC, and present a unique clinical challenge - both from a diagnostic and prognostic risk-stratification purposes. However, the current used definition to categorize a CRC as mucinous is arbitrary, and results in a considerable diagnostic inter-observer variability[37–40]. In addition, the prognostic significance of mucinous CRC subtype is not clear[39,40]. We addressed this important gap in knowledge based upon the growing recognition for the critical role of miRNAs and other non-coding RNAs in the regulation of various biological processes in carcinogenesis[97–99]. Apart from their significant role in the CRC pathogenesis, previous studies have also elegantly demonstrated the potential of miRNAs as biomarkers in multiple cancer types including CRC[25]. While quite a few studies have investigated the role of miRNAs in non-mucinous CRC, the biological and prognostic significance of miRNAs in mucinous CRCs

remains unclear. Therefore, in our study we attempted to address these key unresolved, important clinical questions. First, we used a systematic and comprehensive miRNA-seq-based analysis to examine the specific miRNA expression profiles of MC specimens from the TCGA dataset. This approach allowed us to identify a panel of 6 miRNA biomarkers that were differentially expressed between MC vs. non-MC cancers. Second, given the fact that several studies have suggested that the presence of mucinous differentiation rather than its proportion defines this tumor subtype better[40,100,101], we next evaluated and confirmed the clinical importance of these miRNA biomarkers in two independent patient cohorts. In line with our biomarker discovery phase findings, we successfully validated the clinical significance of these biomarkers in both clinical cohorts. In addition, consistent with our results, miRNA dysregulation in mucinous CRCs has been previously shown by others. For instance, miR-31[102], miR-10b[102], miR-205[103] and miR-373[103] have been shown to be upregulated, and miR-139-5p[102], miR-143[102] and miR-106[104] appear to be downregulated in tumor tissues of these cancers. Nevertheless, most of these previously published studies were limited due to the lack of a systematic and comprehensive discovery approach, as well as the absence of independent validation of these findings. Furthermore, our comprehensive discovery approach resulted in the identification of several previously unreported mucinous differentiation-associated miRNAs (miR-196-b, miR-592, miR-1247, miR-1269 and miR-552), which together with miR-31 emerged to be superior in the identification of mucinous CRCs with higher accuracy. Not surprisingly, when we investigated downstream gene targets of the miRNAs of our panel using a miRNA-mRNA regulatory network analysis, we identified several genes involved in major oncogenic pathways such as p53, PI3K/AKT and Wnt/β-catenin; highlighting the biological and functional significance of our miRNA biomarkers in terms of their role in CRC pathogenesis of neoplasms with mucinous differentiation.

In addition to their diagnostic potential, given the lack of studies assessing prognostic markers in CRC with mucinous differentiation, we examined the association between the expression levels of our six miRNAs and the overall survival in mucinous CRCs. Interestingly, we observed a correlation for high miRNA-based scores and a poor OS. To further appreciate the clinical significance of our biomarkers, we also evaluated its prognostic potential together with the TNM staging, which is one of the most well-established prognostic factors in cancer. Since AICc yielded smaller values for our miRNA-based scores in both cohorts, a slightly better model's performance of the miRNA-based score was confirmed. Next, building on these findings, we constructed an integrative clinical and molecular model for prognostication in CRC with mucinous differentiation patients. The model comprising the combination of the miRNA-based score and TNM stage, demonstrated the best fitting and its utility in prognostication in our cohorts, as evidenced by the significant degree of correlation for a high integrative clinical and molecular score with a worse prognosis. Our work, along with a recent study supporting the prognostic role of tumor-infiltrating lymphocyte in this specific tumor subtype[40], represent the first steps in the development of prognostic biomarkers in CRC with mucinous differentiation.

One of the potential limitations of our study is the use of retrospective cohorts with limited sample size and cancer-specific survival information. Therefore, large-scale, prospective studies will be required in future to definitively confirm the results of the present study. Nevertheless, we were able to validate our results in two independent clinical cohorts of CRC patients. Furthermore, while pathway analysis of downstream targets of these miRNAs showed some insights into potential biological relevance of the miRNAs that we have identified, further

mechanistic studies may help reveal the specific biological functions of these miRNAs in mucinous CRCs.

In summary, in this study we have performed a systematic and comprehensive analysis of the miRNA expression profiles in MC and non-MC, and have identified a panel of miRNAs that are differentially expressed. Subsequently, we interrogated their clinical significance and demonstrated their diagnostic and prognostic utility in CRC with mucinous differentiation. Finally, we established a clinical and molecular integrative model for determining survival outcomes in CRCs with mucinous differentiation combining the miRNA-based risk scores together with TNM staging. Collectively, our study suggests that a panel of miRNAs possess the clinical potential as biomarkers for the identification of CRC with mucinous differentiation, as well as for predicting prognosis in these patients.

5.3. Rethinking prognostic factors in locally advanced or metastatic urothelial carcinoma in the immune checkpoint blockade era: a multicenter retrospective study

The treatment landscape of mUC has changed dramatically since the US FDA approved atezolizumab in May 2016. This approval was based on the results of the multicenter single-arm phase 2 trial IMvigor210, where atezolizumab showed, in a cohort of 310 mUC patients with disease progression during or following platinum-based chemotherapy, similar ORR and longer duration of response (DoR) compared with historical chemotherapy controls. Three subsequent additional approvals of nivolumab, durvalumab, and avelumab were also based solely on the same surrogate endpoints in early-phase single-arm clinical trials. In May 2017, pembrolizumab was approved by the US FDA in the same setting of mUC, being to date the unique anti-PD-(L)1 drug approved based on the positive results of an open-label randomized phase 3 trial in the post-platinum context. Unfortunately, another randomized phase 3 trial, the IMvigor211, failed to demonstrate a statistically significant OS advantage of atezolizumab compared to chemotherapy, although the DoR and safety profile was favorable to this drug. Furthermore, the safety and efficacy of atezolizumab have been recently confirmed in the SAUL study, a single-arm multicenter open-label phase 3B trial conducted in a patient population more similar to the real-world setting. Apart from the SAUL trial, few studies have investigated the safety and efficacy of anti-PD-(L)1 antibodies in daily clinical practice[105–111]. Taking this into account, we conducted a multicenter retrospective study in a cohort of 119 mUC patients treated with different anti-PD-(L)1 drugs. In our study, the safety and efficacy were consistent with previously reported experiences, and in line with the SAUL trial, patients with either brain metastases or Eastern Cooperative Oncology Group Performance Status (ECOG-PS) 2 had also worse efficacy outcomes[107,110].

Identifying prognostic factors is of paramount importance as we move forward with the development of different immunotherapeutic agents. While several studies in mUC have evaluated prognostic factors in the platinum and post-platinum chemotherapy settings[112–118], not many studies have been conducted to evaluate specifically baseline pre-treatment factors influencing anti-PD-(L)1 therapy outcomes[109,119,120]. To address this gap in knowledge, we examined the influence of 29 pretreatment factors with perceived clinical importance on main ICI-efficacy endpoints. Among the studied baseline prognostic factors, three of them require special attention, the presence of peritoneal and liver metastases, and the use of proton-pump inhibitors (PPI).

Despite being associated with a poor prognosis in other tumor types such as gastric or colorectal cancers[121,122], the influence of the presence of peritoneal metastases has never been systematically evaluated in mUC. Herein, we described for the first time to the best of our knowledge the negative impact of peritoneal cancer spread on OS in a series of mUC patients treated with anti-PD-(L)1 antibodies. Although current evidence is scarce, one of the main biological aspects that potentially can explain the lack of efficacy of ICIs in this context is the extremely aberrant tumor vasculature observed in peritoneal carcinomatosis[123]. An abnormal structure and function of tumor vessels drives an immunosuppressive tumor microenvironment characterized by hypoxia, acidosis, and high interstitial pressure. This situation generates a physicochemical barrier that makes difficult the tumor infiltration by immune cells and the delivery of many different types of therapeutic molecules[124–126]. Interestingly, in our series the percentage of cases with more metastatic sites involved was higher among those patients with peritoneal metastases, which probably underlines a more aggressive disease. On the other hand, although many different single-institution studies have correlated the plasmacytoid urothelial carcinoma variant with higher rates of peritoneal involvement[127], in our series we did not find differences in the distribution of distinct histological subtypes based on the presence of peritoneal metastases.

Regarding the impact of liver metastases on systemic immunotherapy efficacy, recently Yu et al.[128] have reported a detrimental effect in preclinical mouse models and patients. The authors found that patients with liver metastases present a reduced number of peripheral T cells and tumoral T cell diversity and function, which means a limited benefit from immunotherapy independent of many other well-established predictive factors. Moreover, in preclinical models, activated CD8+ T cells underwent apoptosis following their interaction with FasL+CD11b+F4/80+ monocyte-derived macrophages presented in the liver[128]. Similarly, in our series the presence of liver metastases was independently associated with worse survival outcomes. Moreover, the percentage of patients with a dNLR ≥3 was higher among those with metastatic liver involvement. In accordance with the findings of Yu et al.[128], a higher dNLR could reflect a relative small number of peripheral lymphocytes in this subgroup of patients with liver metastases.

Although previously investigated in a small retrospective study by Mukherjee et al.[129], the first solid correlation regarding the negative impact of PPI use on ICIs efficacy was reported by Homicsko et al. in 2018[130]. The authors retrospectively analyzed 140 melanoma patients from the Checkmate 069 and found an independent significant detrimental effect of baseline PPI use on ipilimumab plus nivolumab efficacy, which was subsequently validated in an independent cohort of 68 advanced melanoma patients treated with anti-PD-1 monotherapy in the first-line setting. Recently, the same negative correlation has been described in a pooled post hoc analyses of the POPLAR and OAK studies, two randomized clinical trials which demonstrated the superior efficacy of atezolizumab over docetaxel in advanced NSCLC[131]. OS and PFS were significantly shorter for PPI users in the atezolizumab group, although tests for interaction between PPI use and treatment (atezolizumab *vs* docetaxel) were not statistically significant. Correlation with ORR and DCR was not evaluated. Similarly, in our study, the use of PPI was associated not only with worse OS and PFS but also with lower DCR and ORR. Moreover, this correlation was confirmed after adjusting for various confounding factors in multivariate Cox and logistic regression analyses respectively. Even lacking an external validation cohort, the clinical coherence and internal validation of these data reinforces the strength of our findings.

Furthermore, our study confirms the results previously reported by Morales-Barrera et al.[132], who described a trend toward better outcomes in non-PPI users in a cohort of 95 mUC patients treated with anti-PD-(L)1 drugs alone or in combination with an anti-CTLA-4 antibody.

During the past few years, the gut microbiome has emerged as an important mediator associated with responsiveness to ICI therapy[133]. Following the initial evidence in preclinical animal models for the key role in mediating anti-CTLA-4 and anti-D-L1 tumor responses, the importance of certain intestinal commensals has been subsequently substantiated in humans with different cancer types[134,135]. A high diversity of the gut microbiome and abundance of certain commensal bacteria of the intestinal microbiome such as *Faecalibacterium spp* and *Akkermansia muciniphila* have been associated with improved ICI-efficacy outcomes in various scenarios[136,137]. This positive effect seems to be mediated by a systemic and tumoral modulation of the immune system driven by a favorable gut microbiome. On the other hand, there is available evidence suggesting the role of PPI in altering the functionality of the immune system through gut microbiome modulation[138–140]. Together, these data may provide a rational explanation for the negative impact of PPI use on anti-PD-(L)1 efficacy. Considering the use of PPI a modifiable risk factor, these data should encourage physicians to carefully evaluate in advance the PPI use in mUC patients candidates to anti-PD-(L)1 monotherapy. The prognostic impact of other co-medications such as antibiotics or steroids was not confirmed in our study, despite seeing a trend toward higher risk of death and disease progression among patients commencing the use of these drugs before anti-PD-(L)1 initiation.

To understand the clinical influence of the different independent prognostic factors altogether, we developed a simple model to segregate patients into three categories based on risk of death: favorable, intermediate and poor prognostic groups. Among the factors traditionally included in the two best established prognostic models in mUC[112,113], only presence of liver metastases and ECOG-PS were retained in our model. Again, ECOG-PS appears as the most consistent prognostic factor in oncology, regardless of line and type of therapy[113]. The other 3 baseline prognostic factors retained in our model were the use of PPI, albumin level, and presence of peritoneal metastases. Interestingly, we confirmed the best performance of our model compared to the three-factor prognostic model proposed by Bellmunt et al.[113]. Our work, along with a recent study conducted by Sonpavde et al.[120], represent the first steps in the development of clinical prognostic models in mUC in the immune checkpoint blockade era.

Together with the aspects already discussed, one of the potential limitations of our study is the use of only one retrospective cohort with limited sample size. Although the effect size of the described significant correlations was rather big, and a substantial number of clinical and analytical important factors were considered in multivariate analyses, validation in other independent retrospective datasets and prospective cohorts from randomized clinical trials will help to confirm their prognostic significance and to clarify their specific predictive nature in the ICI scenario.

This study, besides confirming the safety and efficacy of anti-PD-(L)1 monotherapy in a daily clinical-practice scenario, positions the presence of peritoneal metastases as an independent prognostic factor for OS in mUC. Furthermore, this study confirms the correlation between the use of PPI before ICI therapy initiation with poor efficacy endpoints among these patients. Whether the association is prognostic and/or predictive should be investigated further in larger prospective cohorts from randomized clinical trials. Finally, we established an easy-to-use risk-assessment model composed of 5 readily available clinico-analytical factors which allow for

predicting OS in mUC patients treated with anti-PD-(L)1 antibodies. If validated in further studies, our risk-assessment model may represent a useful tool not only for daily clinical practice but also for patient stratification in future ICI-based clinical trials.

Gradually, cancer management is moving from a "one-size-fits-all" approach to a more personalized medicine strategy in which increasing numbers of subsets of patients can obtain long-term survival benefits targeting low prevalence driver molecular alterations. With this concept of precision oncology in mind, biomarker research is moving forward, bringing together many types of "omics" data and establishing novel liquid biopsy-based strategies to capture temporo-spatial tumor heterogeneity (**Figure 2**). The future of cancer diagnosis and treatment is promising as many scientific disciplines come together, generating new knowledge and strategies that will improve patient care and outcomes.

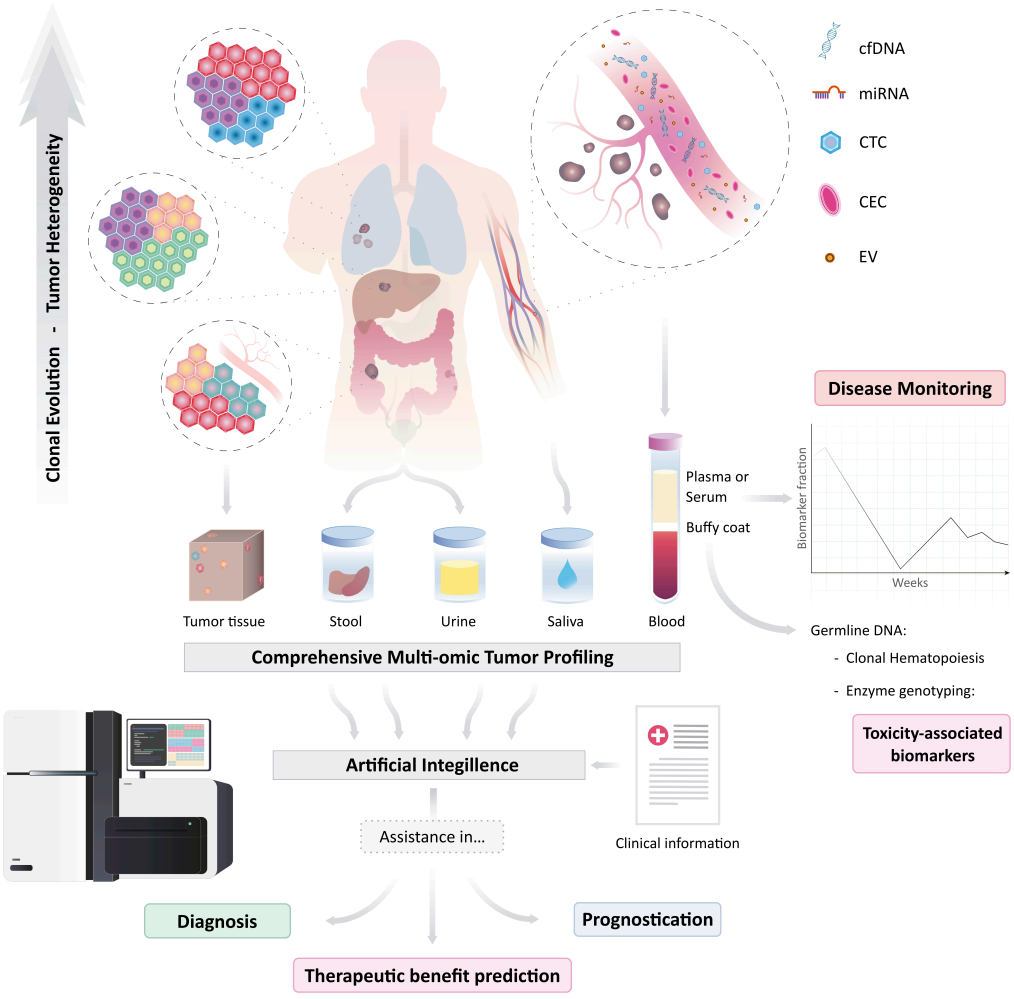

Figure 2. Schematic and general overview of the different specimen sources and biomarkers involved in precision oncology.
Abbreviations: CEC, circulating endothelial cells; cfDNA, cell-free DNA; CRC, colorectal cancer; CTC, circulating tumor cell; EV, extracellular vesicle; miRNA, microRNA.

6. GENERAL CONCLUSIONS

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6. GENERAL CONCLUSIONS

1. In spite of a large body of attempts over the last decades, there are only three well-established predictive biomarkers ─ mutations in the *RAS* gene, the MSI status, and the *BRAF V600E* mutation ─ that currently guide treatment decisions in patients with mCRC.
2. The integration of high-throughput deep techniques, together with the advent of machine-learning algorithms and novel clinical trial designs will revolutionize predictive biomarkers for response to cancer therapeutics in mCRC, as we usher into the new era of precision oncology.
3. There is a novel six-miRNA panel associated with mucinous differentiation in CRC patients; miR-31 is upregulated, and miR-196-b, miR-592, miR-1247, miR-1269, and miR-552 are downregulated in tumor tissue specimens of this CRC subtype.
4. The six-miRNA biomarker panel exhibits a robust diagnostic potential for the identification of CRC patients with mucinous differentiation.
5. The six-miRNA panel is an independent predictor for OS in CRC patients with mucinous differentiation.
6. In mucinous CRC, the integrative risk-assessment model comprising the combination of miRNA-based risk scores and TNM stage, improves the prognosis prediction in comparison to each component independently.
7. Monotherapy with anti-PD-(L)1 antibodies is a safe and effective treatment option in daily clinical-practice for mUC patients.
8. Peritoneal metastases represent an independent prognostic factor for OS in patients with mUC under anti-PD-(L)1 monotherapy.
9. The use of PPI correlates with poor therapeutic outcomes with anti-PD-(L)1 monotherapy in mUC patients.
10. The new three-risk category prognostic model, which includes ECOG-PS, PPI use, albumin level, presence of liver metastases, and presence of peritoneal metastases, enables OS prediction in mUC patients under anti-PD-(L)1 monotherapy.

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7. REFERENCES

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