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Aitor
Rodríguez Casanova

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EPIGENETIC AND GENETIC PROFILING OF CELL-FREE DNA FOR PRECISION ONCOLOGY IN COLORECTAL AND BREAST CANCER

Author

Aitor Rodríguez Casanova

Supervisors: Rafael López López
Ángel Díaz Lagares

Tutor: Rafael López López

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SANTIAGO DE COMPOSTELA

CONFLICT OF INTEREST

I, Aitor Rodríguez Casanova, author of this thesis, declare that I am the author of one patent developed in the context of the present thesis: In vitro method for the diagnosis or prognosis of colorectal cancer or a pre-cancerous stage thereof; EP No. 19 382 290.5

I, Aitor Rodríguez Casanova, author of this thesis, have no other conflicts of interest to declare.

DECLARATION OF THE AUTHOR

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“La ciencia no tiene patria, porque el conocimiento pertenece a la humanidad y es la antorcha que ilumina el mundo.”

Louis Pasteur

"La vida no es fácil para ninguno de nosotros. Pero… ¡qué importa! Hay que perseverar y, sobre todo, tener confianza en uno mismo."

Marie Curie

A mi madre.

A mi padre.

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ABBREVIATIONS AND ACRONYMS

ABBREVIATIONS AND ACRONYMS

#	
5-FU	5-fluorouracil
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

A	
AA	Advanced adenomas
ACMG	American College of Medical Genetics and Genomics
ACP	Advanced colorectal polyp
ACVR2A	Activin Receptor Type-2A
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALK	Anaplastic lymphoma kinase
AMP	Association for Molecular Pathology
AN	Advanced neoplasia
APC	Adenomatous Polyposis Coli
APAF1	Apoptotic protease-activating factor 1
APCDD1	APC down-regulated 1
ASCL2	Achaete-Scute Family BHLH Transcription Factor 2
AUC	Area under the ROC curve
AXIN2	Axin 2
AZA	5-aza-2′-deoxycytidine

B	
BC	Breast cancer
BCAT1	Branched Chain Amino Acid Transaminase 1
BEAMing	Beads, Emulsions, Amplification and Magnetics
BMI	Body Mass Index
BOLL	Boule Homolog, RNA Binding Protein
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRCA1	BRCA1 DNA repair associated

BRCA2	BRCA2 DNA repair associated

C

C9orf50	Chromosome 9 Open Reading Frame 50
CCND2	Cyclin D2
CCS	Colon cancer subtype
CDK	Cyclin-dependent kinase
CEA	Carcinoembryonic Antigen
cfDNA	Cell-free DNA
CGIs	CpG islands
CH_3	Methyl group
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CLIP4	CAP-Gly Domain Containing Linker Protein Member 4
CMS	Consensus molecular subtypes
CNV	Copy number variant
CpG	Cytosine-phosphate-guanine dinucleotide
CRC	Colorectal cancer
CRCSC	CRC Subtyping Consortium
CTC	Circulating Tumor Cell

D

DAPK	Death associated protein kinase 1
DCC	DCC Netrin 1 Receptor
ddPCR	Digital droplet PCR
DKK1	Dickkopf WNT Signaling Pathway Inhibitor 1
DKK3	Dickkopf WNT Signaling Pathway Inhibitor 3
DMCpGs	Differentially methylated CpGs
dMMR	Deficient MMR
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dsDNA	double-stranded DNA

E

ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition

ER	Estrogen receptor
ESR1	Estrogen Receptor 1
ETV6	ETS Variant Transcription Factor 6
EYA4	EYA Transcriptional Coactivator and Phosphatase 4

F

FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration
FFPE	Formalin-Fixed Paraffin-Embedded
FGFR	Fibroblast Growth Factor Receptor
FIT	Fecal immunochemical test
FOLFIRI	5-Fluorouracil + irinotecan
FOLFOX	5-Fluorouracil + oxaliplatin
FOXA1	Forkhead Box A1

G

GATA3	GATA Binding Protein 3
GATA4	GATA Binding Protein 4
GATA5	GATA Binding Protein 5
gDNA	Genomic DNA
gFOBT	Guaiac fecal occult blood testing
GO	Gene ontology
GREM1	Gremlin 1, DAN Family BMP Antagonist
GRIA4	Glutamate Ionotropic Receptor AMPA Type Subunit 4
GSTP1	Glutathione S-Transferase Pi 1

H

HATs	Histone acetyltransferases
HDMs	Histone demethylases
HER2	Erb-b2 Receptor Tyrosine Kinase 2
HIC1	HIC ZBTB Transcriptional Repressor 1
HIF	Hypoxia-Inducible Factor
HLTF	Helicase Like Transcription Factor
HMTs	Histone methyltransferases
HNPCC	Hereditary nonpolyposis colorectal cancer
HPP1	Hyperpigmentation, Progressive, 1
HR	Hormone receptor

I

IKZF1	IKAROS Family Zinc Finger 1
ITGA4	Integrin Subunit Alpha 4
ITIH5	Inter-Alpha-Trypsin Inhibitor Heavy Chain 5

K

KCNQ5	Potassium Voltage-Gated Channel Q Member 5
KRAS	KRAS Proto-Oncogene, GTPase

L

LBBC	Luminal B Breast Cancer
LOD	Limit of Detection
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
LINC00473	Long Intergenic Non-Protein Coding RNA 473
LINC10606	Long Intergenic Non-Protein Coding RNA 10606
lncRNAs	Long non-coding RNAs

M

MAP3K14-AS1	MAP3K14 Antisense RNA 1
MAPK	Mitogen-Activated Protein Kinase 1
mBC	Metastatic BC
mCRC	Metastatic CRC
MGMT	O-6-Methylguanine-DNA Methyltransferase
MLH1	MutL Homolog 1
MLH3	MutL Homolog 3
MMPs	Matrix metalloproteinases
MMR	DNA mismatch repair
MRI	Magnetic Resonance Imaging
MSC	Musculin
MSH2	MutS Homolog 2
MSH3	MutS Homolog 3
MSH6	MutS Homolog 6
MSI	Microsatellite instability
MYB	MYB Proto-Oncogene, Transcription Factor
MYC	MYC Proto-Oncogene, BHLH Transcription Factor

N

ncRNA	Non-coding RNA
NDR	Nucleosome-depleted regions
ng	Nanogram
NGS	Next-generation sequencing
nM	Nanomolar
NOTCH	Notch Receptor 1
NPV	Negative predictive value
NPY	Neuropeptide Y
NRAS	NRAS Proto-Oncogene, GTPase
NTHL1	Nth Like DNA Glycosylase 1

O

OS	Overall survival

P

PARP1	Poly(ADP-ribose) polymerase-1
PCR	Polymerase chain reaction
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PITX2	Paired Like Homeodomain 2
PMN	Premetastatic niche
PMS2	PMS1 Homolog 2, Mismatch Repair System Component
PPV	Positive predictive value
PR	Progesterone receptor
PTEN	Phosphatase And Tensin Homolog
PTMs	Post-translational modifications
P4H	Prolyl-4-hydroxylases

Q

qMSP	Quantitative methylation-specific PCR
qPCR	Quantitative polymerase chain reaction

R

RAF	Rapidly accelerated fibrosarcoma
RARB	Retinoic Acid Receptor Beta
RARB2	Retinoic Acid Receptor Beta
RAS	Rat sarcoma virus
RASSF1A	Ras Association Domain Family Member 1
RFS	Relapse-free survival
RNA	Ribonucleic acid
RNF43	Ring Finger Protein 43
ROC	Receiver operating characteristic
ROS1	ROS Proto-Oncogene 1, Receptor Tyrosine Kinase
RPS20	Ribosomal Protein S20
RT	Room temperature

S

SAM	S-adenosyl-L-methionine
SDC2	Syndecan 2
SEOM	Spanish Society of Medical Oncology
SEPT9	Septin 9
SFRP2	Secreted Frizzled Related Protein 2
SMAD4	SMAD Family Member 4
SNAIL	Zinc finger protein SNAI1
sncRNAs	Small non-coding RNAs
SNPs	Single-nucleotide polymorphisms
SNV	Single nucleotide variant
SOC	Standard-of-care
SPAG6	Sperm Associated Antigen 6

T

TBCD	Tubulin Folding Cofactor D
TCGA	The Cancer Genome Atlas
TET	Ten-eleven translocation
TGFBR2	Transforming Growth Factor Beta Receptor 2
TNBC	Triple-negative breast cancer
TNM	Tumor, Node, Metastasis system
TP53	Tumor Protein P53
TSG	Tumor suppressor gene

TSS	Transcription start site
TST170	TruSight Tumor 170
TWIST	Twist-related protein 1

U

UICC	Union for International Cancer Control
UTR	Untranslated region

V

VAF	Variant allele frequency
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

W

WBCs	White blood cells
WES	Whole exome sequencing
WGA	Whole genome amplification
WGBS	Whole genome bisulfite sequencing
WGS	Whole genome sequencing
WIF1	WNT Inhibitory Factor 1
WNT1	Wnt Family Member 1
WT	Wild type

Z

ZEB	Zinc finger E-box-binding homeobox
ZNF750	Zinc Finger Protein 750

RESUMO *IN EXTENSO*

RESUMO *IN EXTENSO*

O cancro é un conxunto de enfermidades caracterizadas por aberracións xenéticas e epixenéticas que alteran as funcións celulares normais. A pesar dos avances no manexo do cancro, a incidencia global desta enfermidade alcanzou case 20 millóns de casos en 2020, destacando a necesidade de descubrir novos biomarcadores para o diagnóstico precoz, seguimento e selección do tratamento. O cancro de mama (CM) e o cancro colorrectal (CCR) están entre os cancros máis diagnosticados en todo o mundo, sendo o CM o máis prevalente. As proxeccións para 2023 da Sociedade Española de Oncoloxía Médica (SEOM) indican que o CCR e o CM serán os tipos máis comúns de tumores en España.

O CCR é a segunda causa principal de morte relacionada co cancro a nivel mundial. As mutacións xenéticas en vías clave de sinalización, como a de PI3K/Akt e Wnt, desempeñan roles fundamentais na carcinoxénese e a progresión. Ademais, o CM sitúase como a principal causa de morte relacionada con cancro entre as mulleres. A etioloxía complexa do CM implica factores xenéticos, hormonais e do estilo de vida. Estes tumores requiren dun enfoque multidisciplinario que abarque a prevención, o diagnóstico e a intervención temperá e o desenvolvemento continuo de novos tratamentos.

O cancro pode ser causado pola herdanza de variantes patoxénicas na liña xerminal, pero a maioría dos casos son provocados por factores non hereditarios como a aparición de certas variantes somáticas que poden activar oncoxenes, alterando as vías reguladoras e propiciando o desenvolvemento de tumores. As variantes xenéticas inclúen variantes dun só nucleótido (SNV), variantes do número de copias (CNV), fusións xénicas e inserción-delecións (indels). As SNVs, dentro das rexións codificantes de xenes, como *KRAS* ou *PIK3CA*, están vinculadas ao desenvolvemento do

cancro. Ademais, os indels con cambio de marco de lectura tamén contribúen á aparición de cancro (por exemplo, indels de *TGFBR2* e *ACVR2A* en CCR; e *BRCA2* e *GATA3* en CM). Por outra banda, as CNVs alteran a dose xenética, o que pode afectar ao cancro, como sucede coas amplificacións de *MYC* e *HER2*. Aínda que as fusións xénicas son menos comúns, están relacionadas con tumores agresivos. Os avances recentes en tecnoloxías de alto rendemento, como a secuenciación de próxima xeración (NGS), melloraron a nosa capacidade para comprender e abordar a complexidade do cancro.

A epixenética explora cambios herdables na actividade dos xenes que non involucran alteracións na secuencia de ADN. Estas modificacións reversibles desempeñan un papel clave na regulación da expresión xénica durante distintos procesos biolóxicos, incluída a diferenciación celular. Factores do estilo de vida como a dieta, o estrés e a exposición ambiental, inflúen nos cambios epixenéticos. Os mecanismos epixenéticos inclúen a metilación do ADN, as modificacións de histonas, o posicionamento dos nucleosomas e os ARN non codificantes. A metilación do ADN prodúcese mediante a adición de grupos metilo aos CpGs, o que pode silenciar a expresión xénica. Este proceso reversible ocorre en rexións xenómicas específicas, incluídas as illas CpG e as rexións "*shore*". As modificacións de histonas e o posicionamento dos nucleosomas influen na estrutura da cromatina e a accesibilidade xenética, alterando a expresión xénica. Ademais, as moléculas de ARN non codificantes, como miARNs e lncARNs, regulan a expresión xénica post-transcricionalmente. Patróns aberrantes de metilación do ADN e outras modificacións epixenómicas están implicados na carcinoxénese.

Os cambios na metilación do ADN, especialmente a hipometilación xeneralizada e a hipermetilación *de novo* nas illas CpG, asócianse coa inestabilidade xenómica e a inactivación de xenes supresores de tumores,

respectivamente. No caso do CCR, describiuse a hipermetilación de xenes como *APC*, a familia RASSF, *SEPT9* e *PTEN*. No CM, *BRCA1/2* e algúns xenes de receptores hormonais tamén se ven afectados pola metilación anormal do ADN, influíndo no desenvolvemento da enfermidade e a resposta terapéutica. No cancro, a interacción entre eventos xenéticos e epixenéticos é crucial para o inicio e a progresión da enfermidade. Comprender o complexo panorama xenético e epixenético do cancro proporciona información sobre os mecanismos da enfermidade e permite o descubrimento de novos biomarcadores.

A biopsia líquida xurdiu como unha técnica innovadora para o análise molecular de tumores, ofrecendo unha alternativa non invasiva ás biopsias de tecido tradicionais. Aínda que as biopsias de tecido son a proba de referencia, teñen limitacións, como a invasividad e os desafíos asociados coa heteroxeneidade tumoral e as metástases. A biopsia líquida, que utiliza principalmente sangue, permite o análise de compoñentes derivados do tumor, como as células tumorais circulantes (CTCs), o ADN libre circulante (cfDNA) e as vesículas extracelulares (EVs).

Un dos principais compoñentes da biopsia líquida é o cfDNA, que se orixina nas células a través de varios mecanismos, incluíndo a apoptose e a necrose. O cfDNA ten unha vida media curta, o que facilita o seu uso para estudar a progresión da enfermidade en tempo real. É importante destacar que a biopsia líquida permite o análise de alteracións xenéticas e epixenéticas, incluíndo variantes xénicas e cambios na metilación do cfDNA. A fase preanalítica, que implica unha coidadosa recollida da sangue e illamento do cfDNA, é crucial para manter a calidade da mostra. Utilízanse varias metodoloxías para detectar estas alteracións, como a PCR cuantitativa, a PCR dixital e a NGS.

A pesar da baixa concentración de moléculas de cfDNA presentes nas mostras de sangue, varias probas, incluíndo EpiProColon e Cologuard para o CCR, e o ensaio Therascreen *PIK3CA* RGQ PCR para o CM, foron aprobados pola FDA. A biopsia líquida está a contribuír á oncoloxía de precisión e representa unha ferramenta prometedora para a detección temperá do cancro e a monitorización da enfermidade. Nos últimos anos, a investigación sobre cfDNA permitiu o descubrimento de biomarcadores non invasivos para o diagnóstico, o prognostico, a monitorización da enfermidade e a selección de terapia en varios tipos de tumores.

No caso do CCR, as variantes "*hotspot*" en xenes como *KRAS*, *BRAF* e *PIK3CA* demostraron utilidade clínica. Para o prognostico, os niveis de cfDNA correlacionan cunha peor supervivencia libre de recaída (RFS) e supervivencia global (OS), mentres que as variantes de *KRAS* e *BRAF* relaciónanse cun mal prognostico. Ademais, a detección de variantes de *KRAS* axuda a determinar a resistencia á terapia anti-EGFR. De maneira semellante, no CM, o análise de cfDNA para detectar variantes en algúns xenes, como *HER2*, *ESR1* e *PIK3CA*, proporciona información prognostica valiosa e axuda a avaliar a resposta ao tratamento. Ademais, o análise das concentracións de cfDNA describiuse como un preditor fiable da OS e da supervivencia libre de progresión (PFS).

Os biomarcadores epixenéticos, especificamente os patróns de metilación en cfDNA, tamén ofrecen información sobre a detección temperá, o prognostico e a selección terapéutica para o CCR e o CM. No CCR, a metilación de *SEPT9* mostrou utilidade diagnóstica, e marcadores adicionais como *SDC2*, *RARB* e *RASSF1A,* contribúen á avaliación do prognostico e á monitorización da resposta ao tratamento. Para o CM, por exemplo, *RASSF1A*, *FOXA1*, *DKK3* e *BRCA1*, mostran cambios de metilación relacionados coa detección, o prognostico e a resposta terapéutica.

O descubrimento de biomarcadores en cfDNA mediante biopsia líquida abre un campo moi prometedor para avanzar na oncoloxía de precisión. Neste contexto, o obxectivo principal desta tese doutoral foi realizar o perfil xenético e epixenético de cfDNA en pacientes con CCR e CM utilizando enfoques a nivel xenómico para descubrir e avaliar biomarcadores non invasivos con utilidade clínica para a oncoloxía de precisión.

Para acadar este propósito, no primeiro capítulo desta tese, avaliamos a capacidade do panel de NGS TruSight Tumor 170 (TST170) para detectar variantes somáticas en cfDNA de pacientes con CCR. Os paneis de NGS demostraron ser útiles para a análise molecular de pacientes con cancro, permitindo a detección de variantes con implicacións clínicas e o descubrimento de novas dianas terapéuticas. O TST170 abrangue a rexión codificante de 170 xenes relacionados co cancro e permite a detección de SNVs, CNVs e Indels. Este panel está deseñado para utilizarse en mostras de tecido tumoral e non se usara previamente en mostras de cfDNA. Por iso, decidimos avaliar o rendemento do TST170 en cfDNA, abrindo a posibilidade de contar cunha nova ferramenta non invasiva para o estudo de variantes xenéticas na investigación do cancro ou nun entorno clínico. Utilizamos mostras de plasma de pacientes con CCR metastásico (mCCR) con variantes coñecidas no xene *KRAS*, tanto en tecido (analizado mediante pirosecuenciación) como en plasma (analizado mediante BEAMing).

O análise de cfDNA mediante TST170 mostrou a presencia de SNVs e indels en todos os pacientes analizados. Para avaliar a patoxenicidade e implicación clínica das variantes detectadas, utilizamos a plataforma VarSome Clinical que clasifica as variantes somáticas en catro categorías segundo o seu impacto clínico: nivel I, variantes cunha forte significancia clínica; nivel II, variantes con potencial significancia clínica; nivel III, variantes con significancia clínica descoñecida; e nivel IV, variantes benignas ou probablemente benignas. Utilizando este sistema, identificamos variantes de

nivel I no xene *KRAS* no 79% dos pacientes analizados e en *PIK3CA* no 26%. Ademais, detectáronse variantes de nivel II noutros xenes relevantes como *APC*.

Centrándonos nas mutacións detectadas en *KRAS*, un xene de gran importancia no CCR xa que determina o uso do tratamento anti-EGFR, observamos unha alta concordancia entre os datos obtidos en cfDNA por TST170 cos datos obtidos por pirosecuenciación en tecido (77%) como en cfDNA mediante BEAMing (94%). Ademais, para avaliar a sensibilidade do panel, utilizamos os datos de BEAMing para o xene NRAS. Así, o TST170 demostrou unha sensibilidade do 100% para o análise de cfDNA ao detectar o estado nativo (WT) deste xene en todos os pacientes analizados.

Dado que en algúns casos o análise do cfDNA utilizando TST170 mostrou discrepancias respecto ao cfDNA analizado por BEAMing ou o estado dos tecidos tumorais, volvemos a analizar as mostras dispoñibles de cfDNA utilizando PCR dixital (ddPCR). Esta técnica confirmou que as discrepancias estaban relacionadas coa baixa frecuencia alélica da variante (VAF), por debaixo do límite de detección que obtivemos na nosa cohorte para detectar variantes en *KRAS* mediante o TST170. Observamos que a causa desta baixa VAF podería estar directamente relacionada coa ausencia de metástases hepáticas e a resección previa do tumor primario.

En resumo, os resultados obtidos neste primeiro capítulo demostran a viabilidade de utilizar o panel TST170 para un análise xenético integral en cfDNA, sendo capaz de detectar variantes con importancia clínica, apoiando a aplicación deste panel en contornas clínicas e de investigación do cancro. A pesar de algunhas limitacións observadas, os achados xerais avalan o uso do análise dirixido de cfDNA mediante o panel TST170 como unha ferramenta prometedora e fácil de implementar para a detección non invasiva de variantes xenéticas en pacientes con mCCR. Este ensaio podería ser útil para

avanzar na oncoloxía de precisión e mellorar o resultado dos pacientes con CCR.

Ademais das alteracións xenéticas, as modificacións epixenéticas tamén son de gran importancia e utilidade no CCR. Por iso, no segundo capítulo desta tese doutoral propuxémonos identificar novos biomarcadores de metilación en cfDNA para o manexo do CCR. No capítulo II.A, exploramos a utilidade clínica da metilación do promotor do lncRNA *LINC00473* como biomarcador non invasivo para detectar o CCR e lesións precancerosas. Dado que a maior parte das mortes polo CCR se deben ás metástases, existe unha necesidade clínica de detectar esta enfermidade o antes posible, incluso no seu estado precanceroso. Aínda que existen técnicas de detección, como a proba inmunoquímica fecal (FIT) ou a colonoscopia, ambas teñen certas limitacións. Polo tanto, necesítanse con urxencia novas probas non invasivas para a detección temperá do CCR.

Os niveis de metilación de *LINC00473* analizáronse en liñas celulares de CCR e en oito cohortes clínicas independentes de pacientes, incluíndo catro cohortes de mostras de tecido colorrectal e catro cohortes de mostras de plasma. Primeiro, confirmouse a hipermetilación do promotor de *LINC00473* na liña celular HCT-116 mediante secuenciación xenómica por bisulfito. Ademais, para confirmar o efecto da hipermetilación, analizamos os niveis de expresión de *LINC00473* mediante RT-PCR, mostrando a diminución deste lncRNA nas células HCT-116 en comparación coa mucosa normal de colon non metilada.

Utilizando datos do *The Cancer Genome Atlas* (TCGA), verificamos que os niveis de metilación nos tumores colorrectais primarios (en todas as etapas da enfermidade, incluído o estadio I) eran maiores que no tecido normal. Ademais, esta hipermetilación produciu unha diminución significativa dos niveis de expresión de *LINC00473* nos tecidos tumorais. A análise da

curva ROC mostrou unha precisión moi alta para a detección do CCR en todos os estadios do tumor, cunha alta sensibilidade e especificidade. Estes resultados foron confirmados nunha segunda cohorte de tumores colorrectais primarios (estadios I a IV) e controis non tumorais pareados mediante pirosecuenciación.

Para probar se a metilación de *LINC00473* tamén ocorrería en etapas precanceríxenas, analizamos o estado de metilación do promotor de *LINC00473* mediante pirosecuenciación en tecido de pólipos colorrectais premalignos, CCR e mucosa colorrectal normal pareada. Así, confirmamos que a metilación de *LINC00473* era significativamente maior nos pólipos e o CCR que nos controles sanos, coa curva ROC mostrando unha gran capacidade para diferenciar controles de pólipos. Estes datos foron confirmados por pirosecuenciación nunha cohorte máis grande de pólipos colorrectais e mucosa normal.

Co obxectivo de lograr un biomarcador de diagnóstico non invasivo, avaliamos a detección da hipermetilación de *LINC00473* en mostras de cfDNA. Primeiro, analizamos a metilación do seu promotor en cfDNA de plasma dunha cohorte de controis sans autodeclarados e pacientes con CCR mediante qMSP. Ademais, tamén avaliamos o cfDNA de plasma dunha cohorte de controis san autodeclarados e pacientes con pólipos colorrectais avanzados (ACPs). En ambos os casos, a análise mostrou un nivel significativamente maior de metilación no cfDNA das mostras de CCR e ACPs que nos controis sans. Ademais, a análise da curva ROC mostrou unha alta precisión diagnóstica para a detección do CCR e ACPs en ambas as cohortes analizadas.

Para confirmar a viabilidade de utilizar a metilación do promotor de *LINC00473* para a detección temperá non invasiva do CCR, analizamos retrospectivamente unha cohorte independente de mostras de cfDNA de

plasma obtidas antes dunha colonoscopia programada, como parte do cribado estándar do CCR, ou antes da cirurxía de colon para tumores primarios. Neste caso, utilizamos ddPCR para o análise de metilación. Consistentes cos nosos resultados previos, os niveis de metilación de *LINC00473* foron significativamente maiores en ACPs e CCR que en controis sans confirmados. É importante destacar que a metilación do promotor de *LINC00473* mostrou unha alta precisión para a detección de ACPs e CCR, cunha alta sensibilidade, especificidade, valor preditivo positivo e valor preditivo negativo.

Unha vez confirmada a utilidade de analizar o estado de metilación do promotor de *LINC00473* para detectar CCR e lesións precancerosas, quixemos avaliar se podería ser útil para detectar cambios na enfermidade durante o seguimento da mesma. Para iso, analizamos a metilación mediante ddPCR en diferentes momentos clinicamente relevantes. Os niveis de metilación do cfDNA de plasma de *LINC00473* diminuíron coa terapia efectiva e aumentaron coa progresión da enfermidade. Especialmente, en algúns casos, a metilación de *LINC00473* precede á detección de CEA na presenza de CCR, converténdoo nun biomarcador potencial para detectar a presenza de CCR durante o seguimento de pacientes metastásicos.

En conclusión, a aparición temperá da hipermetilación de *LINC00473* na carcinoxénese colorrectal e a súa detección en cfDNA de plasma demostran a viabilidade de utilizar a metilación de *LINC00473* como biomarcador non invasivo para a detección temperá do CCR. Integrar este novo biomarcador epigenético no panorama da oncoloxía de precisión promete mellorar a precisión dos diagnósticos de CCR, proporcionando aos médicos unha valiosa ferramenta non invasiva para a detección temperá desta enfermidade.

Ademais, no Capítulo II.B, analizamos o metiloma do cfDNA en pacientes con mCCR en relación a súa resposta a FOLFOX. A pesar de ser un dos tratamentos de primeira liña máis utilizados, algúns pacientes non responden a esta terapia. Isto destaca a urxente necesidade clínica de identificar biomarcadores que poidan axudar na selección da terapia máis efectiva para cada paciente. O obxectivo deste estudo foi identificar novos biomarcadores de metilación non invasivos en cfDNA para prever a resposta dos pacientes con mCCR á terapia baseada en FOLFOX.

Para lograr isto, seleccionamos a 20 pacientes con mCCR antes de comezar o tratamento con FOLFOX. Seguindo os criterios RECIST 1.1, avaliamos a resposta á terapia aproximadamente aos 3 e 6 meses, clasificando os pacientes segundo a súa mellor resposta ao tratamento. Establecemos un grupo de 12 pacientes con enfermidade non progresiva (nPD: enfermidade estable ou resposta parcial) e outro grupo de 8 pacientes con enfermidade progresiva (PD). Posteriormente, analizamos o metiloma do cfDNA dos pacientes a niveis basais utilizando un enfoque xenómico baseado na tecnoloxía do array de metilación Infinium MethylationEPIC (EPIC *array*). Identificamos 1.174 CpG diferencialmente metiladas (DMCpGs) entre os grupos nPD e PD, distribuídas ao longo de todos os cromosomas. Destacar que ao seleccionar as CpGs máis diferentes, identificamos unha firma epixenética (epifirma) de 406 CpGs capaz de diferenciar claramente os pacientes nPD e os PD. Ao realizar un análise de ontoloxía xénica (GO) dos xenes relacionados con esta firma, observamos a súa implicación en vías moleculares cruciais no desenvolvemento do cancro e coa resposta á quimioterapia, como as vías de sinalización PI3K/AKT, Wnt e Cadherina.

En resumo, este estudo realizada no Capítulo II.B representa un avance significativo na busca de biomarcadores preditivos no contexto do tratamento do mCCR con terapia FOLFOX. A epifirma non invasiva identificada en cfDNA ten un gran potencial como biomarcador preditivo para

estratificar aos pacientes con mCCR segundo a súa resposta a FOLFOX. Este estudo subliña o potencial de utilizar os *arrays* EPIC en cfDNA para descubrir novos biomarcadores epixenéticos non invasivos para o manexo de pacientes con cancro. Ademais, os xenes e as vías que se atoparon epixeneticamente desreguladas, en asociación coa resposta á terapia, serven como posibles obxectivos para superar a resistencia ao tratamento con FOLFOX en mCCR.

Ao igual que no CCR, a desregulación dos mecanismos epixenéticos no CM presenta importantes implicacións para o desenvolvemento, a progresión e a resposta terapéutica do cancro. No terceiro capítulo, o noso obxectivo foi analizar o metiloma do cfDNA de pacientes con CM luminal B metastásico (CMLB) utilizando un enfoque a nivel xenómico baseado na tecnoloxía do EPIC *array* para descubrir posibles biomarcadores non invasivos. Para lograr isto, analizamos a 9 pacientes con CMLB metastásico e 5 controis sans. A metodoloxía do EPIC *array* adoita requirir unha gran cantidade de ADN, o cal é un desafío obter a partir de plasma. Como novidade no noso estudo, utilizamos pequenas cantidades de cfDNA de mostras individuais de plasma, as cales foron amplificadas a nivel xenómico despois da modificación con bisulfito.

Este enfoque permitiunos identificar 28.799 DMCpGs entre CMLB e os controis non tumorais. Dado que a hipermetilación nos promotores pode alterar a expresión xénica, enfocamos o noso estudo nesas rexións. Así, identificamos unha epifirma de 1.467 DMCpGs capaz de diferenciar claramente aos pacientes con CMLB dos controis non tumorais. A continuación, para obter información relacionada coas vías funcionais involucradas na epifirma identificada, realizamos un análise GO baseado na base de datos PANTHER. Este análise revelou que as diferenzas de metilación no cfDNA de pacientes con CMLB e controis non tumorais estaban principalmente asociadas con xenes relacionados coa vía de sinalización

Wnt. Entre as DMCpGs na epifirma, encontramos 2 CpGs localizadas no xene *WNT1* que estaban hipermetiladas no cfDNA de pacientes con CMLB. Para confirmar esta metilación aberrante, seleccionamos a CpG de *WNT1* máis diferencialmente metilada e analizamos o seu estado de metilación mediante ddPCR no cfDNA da nosa cohorte.

A metilación de *WNT1* foi significativamente maior no cfDNA de pacientes con CMLB que nos controis non tumorais, diferenciando aos pacientes dos controis cunha alta sensibilidade e especificidade. Ademais, para avaliar se esta metilación aberrante tamén estaba presente nos tecidos tumorais, analizamos a metilación de *WNT1* mediante ddPCR en mostras de tumores primarios e metastásicos da nosa cohorte. Este ensaio revelou que a hipermetilación de *WNT1* estaba presente non só no cfDNA, senón tamén nas mostras pareadas de tumores primarios e metastásicos dos pacientes con CMLB analizados.

A continuación, utilizando datos públicos do TCGA, confirmamos que a metilación de *WNT1* era significativamente maior en todos os estadios de CMLB en comparación con controis sans. Un análise da curva ROC mostrou que a metilación de *WNT1* diferenciaba os tumores primarios luminais B (estadios I-IV) dos controis non tumorais cunha alta precisión diagnóstica. Ademais, confirmamos que a hipermetilación do promotor de *WNT1* tiña un efecto inhibidor na expresión xénica neste subtipo de CM. En conclusión, este estudo revelou unha epifirma única vinculada ao CMLB. Ademais, identificamos a hipermetilación do promotor de *WNT1* en cfDNA como un prometedor biomarcador non invasivo para o CMLB. Estes achados respaldan tamén a aplicación da tecnoloxía EPIC *array* para descubrir novos biomarcadores epixenéticos non invasivos no CM.

En resumo, esta tese destaca o estudo de biomarcadores xenéticos e epixenéticos mediante biopsia líquida para avanzar na oncoloxía de

precisión. A avaliación do panel TST170 en cfDNA mellora as opcións para o análise non invasivo de variantes xenéticas no CCR metastásico. A posibilidade de detectar variantes somáticas clinicamente relevantes con TST170, incluíndo *KRAS* e *PIK3CA*, apoia a utilidade deste panel para o análise xenético de cfDNA para personalizar as estratexias terapéuticas. A avaliación de mecanismos epixenéticos, como a metilación do ADN, demostrou a súa utilidade como biomarcadores para o diagnóstico, prognostico e selección da terapia máis axeitada, proporcionando novas perspectivas para a oncoloxía de precisión. Neste traballo, o análise da metilación de *LINC00473* abriu un camiño prometedor para a detección temperá do CCR e as súas lesións precancerosas. A precisión diagnóstica da metilación de *LINC00473* como biomarcador epixenético en biopsia líquida subliña o potencial deste tipo de enfoques non invasivos na detección do cancro. Ademais, nesta tese identificamos unha epifirma capaz de prever a resposta a FOLFOX, o que representa un prometedor biomarcador preditivo non invasivo para mellorar a selección de pacientes con mCCR tratados con esta terapia. Por outra banda, o perfilado do metiloma en cfDNA de CMLB metastásico revelou unha firma epixenética distintiva, coa hipermetilación do promotor de *WNT1* emerxendo como un posible biomarcador desta enfermidade. En conxunto, estes achados contribúen a mellorar o manexo non invasivo de pacientes con CCR e CM, avaliando o uso do análise xenético e epixenético na biopsia líquida para avanzar cara á oncoloxía de precisión.

RESUMEN *IN EXTENSO*

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El cáncer es un conjunto de enfermedades caracterizadas por aberraciones genéticas y epigenéticas que alteran las funciones celulares normales. A pesar de los avances en el manejo del cáncer, la incidencia global de esta enfermedad alcanzó casi 20 millones de casos en 2020, resaltando la necesidad de descubrir nuevos biomarcadores para el diagnóstico temprano, seguimiento y selección del tratamiento. El cáncer de mama (CM) y el cáncer colorrectal (CCR) se encuentran entre los cánceres más diagnosticados en todo el mundo, siendo el CM el más prevalente. Las proyecciones para 2023 de la Sociedad Española de Oncología Médica (SEOM) indican que el CCR y el CM serán los tipos más comunes de tumores en España.

El CCR es la segunda causa principal de muerte relacionada con el cáncer a nivel mundial. Las mutaciones genéticas en vías clave de señalización, como la de PI3K/Akt y Wnt, desempeñan roles fundamentales en la carcinogénesis y la progresión. Además, CM se sitúa como la principal causa de muerte relacionada con cáncer entre las mujeres. La etiología compleja del CM involucra factores genéticos, hormonales y del estilo de vida. Estos tumores requieren un enfoque multidisciplinario que abarque la prevención, el diagnóstico y la intervención temprana y el desarrollo continuo de nuevos tratamientos.

El cáncer puede ser causado por la herencia de variantes patogénicas en la línea germinal, pero la mayoría de los casos son provocados por factores no hereditarios como la aparición de ciertas variantes somáticas que pueden activar oncogenes, alterando las vías regulatorias y propiciando el desarrollo de tumores. Las variantes genéticas incluyen variantes de un solo nucleótido (SNV), variantes del número de

copias (CNV), fusiones génicas e inserciones-deleciones (indels). Las SNVs, dentro de las regiones codificantes de genes, como *KRAS* o *PIK3CA*, están vinculadas al desarrollo del cáncer. Además, los indels con cambio de marco de lectura también contribuyen a la aparición de cáncer (por ejemplo, indels de *TGFBR2* y *ACVR2A* en CCR; y *BRCA2* y *GATA3* en CM). Por otro lado, las CNVs alteran la dosis génica, lo que puede afectar al cáncer, como sucede con las amplificaciones de *MYC* y *HER2*. Aunque las fusiones génicas son menos comunes, están relacionadas con tumores agresivos. Los avances recientes en tecnologías de alto rendimiento, como la secuenciación de próxima generación (NGS), han mejorado nuestra capacidad para comprender y abordar la complejidad del cáncer.

La epigenética explora cambios heredables en la actividad de los genes que no involucran alteraciones en la secuencia de ADN. Estas modificaciones reversibles desempeñan un papel clave en la regulación de la expresión génica durante distintos procesos biológicos, incluida la diferenciación celular. Factores del estilo de vida como, la dieta, el estrés y la exposición ambiental, influyen en los cambios epigenéticos. Los mecanismos epigenéticos incluyen la metilación del ADN, las modificaciones de histonas, el posicionamiento de los nucleosomas y los ARN no codificantes. La metilación del ADN se produce mediante la adición de grupos metilo a los CpG, lo que puede silenciar la expresión génica. Este proceso reversible ocurre en regiones genómicas específicas, incluidas las islas CpG y las regiones "*shore*". Las modificaciones de histonas y el posicionamiento de los nucleosomas influyen en la estructura de la cromatina y la accesibilidad génica, alterando la expresión génica. Además, las moléculas de ARN no codificantes, como miARNs y lncARNs, regulan la expresión génica post-transcripcionalmente. Patrones aberrantes de metilación del ADN y otras modificaciones epigenómicas están implicados en la carcinogénesis.

Los cambios en la metilación del ADN, especialmente la hipometilación generalizada y la hipermetilación *de novo* en las islas CpG, se asocian con la inestabilidad genómica y la inactivación de genes supresores de tumores, respectivamente. En el caso del CCR, se ha descrito la hipermetilación de genes como *APC*, la familia RASSF, *SEPT9* y *PTEN*. En el CM, *BRCA1/2* y algunos genes de receptores hormonales también se ven afectados por la metilación anormal del ADN, influyendo en el desarrollo de la enfermedad y la respuesta terapéutica. En el cáncer, la interacción entre eventos genéticos y epigenéticos es crucial para el inicio y la progresión de la enfermedad. Comprender el complejo panorama genético y epigenético del cáncer proporciona información sobre los mecanismos de la enfermedad y permite el descubrimiento de nuevos biomarcadores.

La biopsia líquida ha surgido como una técnica innovadora para el análisis molecular de tumores, ofreciendo una alternativa no invasiva a las biopsias de tejido tradicionales. Aunque las biopsias de tejido son la prueba de referencia, tienen limitaciones, como la invasividad y los desafíos asociados con la heterogeneidad tumoral y las metástasis. La biopsia líquida, que utiliza principalmente sangre, permite el análisis de componentes derivados del tumor, como las células tumorales circulantes (CTCs), el ADN libre circulante (cfDNA) y las vesículas extracelulares (EVs).

Uno de los principales componentes de la biopsia líquida es el cfDNA, que se origina en las células a través de varios mecanismos, incluyendo la apoptosis y la necrosis. El cfDNA tiene una vida media corta, lo que facilita su uso para estudiar la progresión de la enfermedad en tiempo real. Es importante destacar que la biopsia líquida permite el análisis de alteraciones genéticas y epigenéticas, incluyendo variantes génicas y cambios en la metilación del cfDNA. La fase preanalítica, que implica una cuidadosa recolección de la sangre y aislamiento del cfDNA, es crucial para mantener

la calidad de la muestra. Se utilizan varias metodologías para detectar estas alteraciones, como la PCR cuantitativa, la PCR digital y la NGS.

A pesar de la baja concentración de moléculas de cfDNA presentes en las muestras de sangre, varias pruebas, incluyendo EpiProColon y Cologuard para el CCR, y el ensayo Therascreen *PIK3CA* RGQ PCR para el CM, han sido aprobados por la FDA. La biopsia líquida está contribuyendo a la oncología de precisión y representa una herramienta prometedora para la detección temprana del cáncer y la monitorización de la enfermedad. En los últimos años, la investigación sobre cfDNA ha permitido el descubrimiento de biomarcadores no invasivos para el diagnóstico, el pronóstico, la monitorización de la enfermedad y la selección de terapia en varios tipos de tumores.

En el caso del CCR, las variantes "*hotspot*" en genes como *KRAS*, *BRAF* y *PIK3CA*, han demostrado utilidad clínica. Para el pronóstico, los niveles de cfDNA se correlacionan con una peor supervivencia libre de recaída (RFS) y supervivencia general (OS), mientras que las variantes de *KRAS* y *BRAF* se relacionan con un mal pronóstico. Además, la detección de variantes de *KRAS* ayuda a determinar la resistencia a la terapia anti-EGFR. De manera similar, en el CM, el análisis de cfDNA para detectar variantes en algunos genes, como *HER2*, *ESR1* y *PIK3CA*, proporciona información pronóstica valiosa y ayuda a evaluar la respuesta al tratamiento. Además, el análisis cuantitativo de las concentraciones de cfDNA se ha descrito como un predictor fiable de la OS y la supervivencia libre de progresión (PFS).

Los biomarcadores epigenéticos, específicamente los patrones de metilación en cfDNA, también ofrecen información sobre la detección temprana, el pronóstico y la selección terapéutica para el CCR y el CM. En el CCR, la metilación de *SEPT9* ha mostrado utilidad diagnóstica, y marcadores adicionales, como *SDC2*, *RARB* y *RASSF1A,* contribuyen a la evaluación del

pronóstico y la monitorización de la respuesta al tratamiento. Para el CM, genes como *RASSF1A*, *FOXA1*, *DKK3* y *BRCA1*, exhiben cambios de metilación relacionados con la detección, el pronóstico y la respuesta terapéutica.

El descubrimiento de biomarcadores en cfDNA mediante biopsia líquida abre un campo muy prometedor para avanzar en la oncología de precisión. En este contexto, el objetivo principal de esta tesis doctoral fue realizar el perfil genético y epigenético de cfDNA en pacientes con CCR y CM utilizando enfoques a nivel genómico para descubrir y evaluar biomarcadores no invasivos con utilidad clínica para la oncología de precisión.

Para lograr este propósito, en el primer capítulo de esta tesis, evaluamos la capacidad del panel de NGS TruSight Tumor 170 (TST170) para detectar variantes somáticas en cfDNA de pacientes CCR. Los paneles de NGS han demostrado ser útiles para el análisis molecular de pacientes con cáncer, permitiendo la detección de variantes con implicaciones clínicas y el descubrimiento de nuevas dianas terapéuticas. El TST170 abarca la región codificante de 170 genes relacionados con el cáncer y permite la detección de SNVs, CNVs e indels. Este panel está diseñado para utilizarse en muestras de tejido tumoral y no se había utilizado previamente en muestras de cfDNA. Por lo tanto, decidimos evaluar el rendimiento de TST170 en cfDNA, abriendo la posibilidad de contar con una nueva herramienta no invasiva para el estudio de variantes génicas en la investigación del cáncer o en un entorno clínico. Utilizamos muestras de plasma de pacientes con CCR metastásico (mCCR) con variantes conocidas en el gen *KRAS*, tanto en tejido (analizado mediante pirosecuenciación) como en plasma (analizado mediante BEAMing).

El análisis de cfDNA mediante TST170 mostró la presencia de SNVs e indels en todos los pacientes analizados. Para evaluar la patogenicidad e

implicación clínica de las variantes detectadas, utilizamos la plataforma VarSome Clinical que clasifica las variantes somáticas en cuatro categorías según su impacto clínico: nivel I, variantes con una fuerte significancia clínica; nivel II, variantes con potencial significancia clínica; nivel III, variantes con significancia clínica desconocida; y nivel IV, variantes benignas o probablemente benignas. Utilizando este sistema, identificamos variantes de nivel I en el gen *KRAS* en el 79% de los pacientes analizados, y en *PIK3CA* en el 26%. Además, se detectaron variantes de nivel II en otros genes relevantes como *APC*.

Centrándonos en las mutaciones detectadas en *KRAS*, un gen de gran importancia en el CCR ya que determina el uso del tratamiento anti-EGFR, observamos una alta concordancia entre los datos obtenidos en cfDNA por TST170 con aquellos obtenidos por pirosecuenciación en tejido (77%) como en cfDNA mediante BEAMing, (94%). Además, para evaluar la sensibilidad del panel, utilizamos los datos de BEAMing para el gen NRAS. Así, el TST170 demostró una sensibilidad del 100% para el análisis de cfDNA al detectar el estado nativo (WT) de este gen en todos los pacientes analizados.

Dado que en algunos casos el análisis de cfDNA utilizando TST170 mostró discrepancias con respecto al cfDNA analizado por BEAMing o el estado de los tejidos tumorales, volvimos a analizar las muestras disponibles de cfDNA utilizando PCR digital (ddPCR). Esta técnica confirmó que las discrepancias estaban relacionadas con la baja frecuencia alélica de la variante (VAF), por debajo del límite de detección que obtuvimos en nuestra cohorte para detectar variantes en *KRAS* mediante TST170. Observamos que la causa de esta baja VAF podría estar directamente relacionada con la ausencia de metástasis hepáticas y la resección previa del tumor primario.

En resumen, los resultados obtenidos en este primer capítulo demuestran la viabilidad de utilizar el panel TST170 para un análisis genético integral en cfDNA, siendo capaz de detectar variantes con importancia clínica. Esto respalda la aplicación de este panel en entornos clínicos y de investigación del cáncer. A pesar de algunas limitaciones observadas, los hallazgos generales avalan el uso del análisis dirigido de cfDNA mediante el panel TST170 como una herramienta prometedora y fácil de implementar para la detección no invasiva de variantes genéticas en pacientes con mCCR. Este ensayo podría ser útil para avanzar en la oncología de precisión y mejorar el resultado de los pacientes con CCR.

Además de las alteraciones genéticas, las modificaciones epigenéticas también son de gran importancia y utilidad en el CCR. Por lo tanto, en el segundo capítulo de esta tesis doctoral nos propusimos identificar nuevos biomarcadores de metilación en cfDNA para el manejo del CCR. En el capítulo II.A, exploramos la utilidad clínica de la metilación del promotor del lncARN *LINC00473* como biomarcador no invasivo para detectar el CCR y lesiones precancerosas. Dado que la mayoría de las muertes por CCR se deben a las metástasis, existe una necesidad clínica de detectar esta enfermedad lo antes posible, incluso en su estado precanceroso. Aunque existen técnicas de detección, como la prueba inmunoquímica fecal (FIT) o la colonoscopia, ambas tienen algunas limitaciones. Por lo tanto, se necesitan con urgencia nuevas pruebas no invasivas para la detección temprana del CCR.

Los niveles de metilación de *LINC00473* se analizaron en líneas celulares de CCR y en ocho cohortes clínicas independientes de pacientes, incluyendo cuatro cohortes de muestras de tejido colorrectal y cuatro cohortes de muestras de plasma. Primero, se confirmó la hipermetilación del promotor de *LINC00473* en la línea celular HCT-116 mediante secuenciación genómica por bisulfito. Además, para confirmar el efecto de la hipermetilación,

analizamos los niveles de expresión de *LINC00473* mediante RT-PCR, mostrando la disminución de este lncRNA en las células HCT-116 en comparación con la mucosa normal de colon no metilada.

Utilizando datos de *The Cancer Genome Atlas* (TCGA), verificamos que los niveles de metilación en los tumores colorrectales primarios (en todas las etapas de la enfermedad, incluida es estadio I) eran mayores que en el tejido normal. Además, esta hipermetilación produjo una disminución significativa de los niveles de expresión de *LINC00473* en los tejidos tumorales. El análisis de la curva ROC mostró una precisión muy alta para la detección del CCR en todas las etapas del tumor, con una alta sensibilidad y especificidad. Estos resultados fueron confirmados en una segunda cohorte de tumores colorrectales primarios (estadios I a IV) y controles no tumorales pareados mediante pirosecuenciación.

Para probar si la metilación de *LINC00473* también ocurría en etapas precancerosas, analizamos el estado de metilación del promotor de *LINC00473* mediante pirosecuenciación en tejido de pólipos colorrectales premalignos, CCR y mucosa colorrectal normal pareada. Así, confirmamos que la metilación de *LINC00473* era significativamente mayor en los pólipos y el CCR que en los controles sanos, con la curva ROC mostrando una gran capacidad para diferenciar controles de pólipos. Estos datos fueron confirmados por pirosecuenciación en una cohorte más grande de pólipos colorrectales y mucosa normal.

Con el objetivo de lograr un biomarcador de diagnóstico no invasivo, evaluamos la detección de la hipermetilación de *LINC00473* en muestras de cfDNA. Primero, analizamos la metilación de su promotor en cfDNA de plasma de una cohorte de controles sanos autodeclarados y pacientes con CCR mediante qMSP. Además, también evaluamos el cfDNA de plasma de una cohorte de controles sanos autodeclarados y pacientes con pólipos

colorrectales avanzados (ACPs). En ambos casos, el análisis mostró un nivel significativamente mayor de metilación en el cfDNA de las muestras de CCR y ACPs que en los controles sanos. Además, el análisis de la curva ROC mostró una alta precisión diagnóstica para la detección de CCR y ACPs en ambas cohortes analizadas.

Para confirmar la viabilidad de utilizar la metilación del promotor de *LINC00473* para la detección temprana no invasiva de CCR, analizamos retrospectivamente una cohorte independiente de muestras de cfDNA de plasma obtenidas antes de una colonoscopia programada, como parte del cribado estándar de CCR, o antes de la cirugía de colon para tumores primarios. En este caso, utilizamos ddPCR para el análisis de metilación. Consistentes con nuestros resultados previos, los niveles de metilación de *LINC00473* fueron significativamente mayores en ACPs y CCR que en controles sanos confirmados. Es importante destacar que la metilación del promotor de *LINC00473* mostró una alta precisión para la detección de ACPs y CCR, con una alta sensibilidad, especificidad, valor predictivo positivo y valor predictivo negativo.

Una vez confirmada la utilidad de analizar el estado de metilación del promotor de *LINC00473* para detectar CCR y lesiones precancerosas, quisimos evaluar si podría ser útil para detectar cambios en la enfermedad durante el seguimiento de la misma. Para ello, analizamos la metilación mediante ddPCR en diferentes momentos clínicamente relevantes. Los niveles de metilación del cfDNA de plasma de *LINC00473* disminuyeron con la terapia efectiva y aumentaron con la progresión de la enfermedad. Especialmente, en algunos casos, la metilación de *LINC00473* precede a la detección de CEA en la presencia de CCR, convirtiéndolo en un biomarcador potencial para detectar la presencia de CCR durante el seguimiento de pacientes metastásicos.

En conclusión, la aparición temprana de la hipermetilación de *LINC00473* en la carcinogénesis colorrectal y su detección en cfDNA de plasma demuestran la viabilidad de utilizar la metilación de *LINC00473* como biomarcador no invasivo para la detección temprana de CCR. Integrar este nuevo biomarcador epigenético en el panorama de la oncología de precisión promete mejorar la precisión de los diagnósticos de CCR, brindando a los médicos una valiosa herramienta no invasiva para la detección temprana de esta enfermedad.

Además, en el Capítulo II.B, analizamos el metiloma de cfDNA en pacientes con mCCR en relación con su respuesta a FOLFOX. A pesar de ser uno de los tratamientos de primera línea más comúnmente utilizados, algunos pacientes no responden a esta terapia. Esto destaca la urgente necesidad clínica de identificar biomarcadores que puedan ayudar en la selección de la terapia más efectiva para cada paciente. El objetivo de este estudio fue identificar nuevos biomarcadores de metilación no invasivos en cfDNA para predecir la respuesta de los pacientes con mCCR a la terapia basada en FOLFOX.

Para lograr esto, seleccionamos a 20 pacientes con mCCR antes de comenzar el tratamiento con FOLFOX. Siguiendo los criterios RECIST 1.1, evaluamos la respuesta a la terapia aproximadamente a los 3 y 6 meses, clasificando a los pacientes según su mejor respuesta al tratamiento. Establecimos un grupo de 12 pacientes con enfermedad no progresiva (nPD: enfermedad estable o respuesta parcial) y otro grupo de 8 pacientes con enfermedad progresiva (PD). Posteriormente, analizamos el metiloma del cfDNA de los pacientes a nivel basal utilizando un enfoque genómico basado en la tecnología del array de metilación Infinium MethylationEPIC (EPIC *array*). Identificamos 1.174 CpG diferencialmente metiladas (DMCpGs) entre los grupos nPD y PD, distribuidos a lo largo de todos los cromosomas. Destacar que al seleccionar las CpGs más diferentes, identificamos una firma

epigenética (epifirma) de 406 CpGs capaz de diferenciar claramente a nivel basal entre los pacientes nPD y los PD. Al realizar un análisis de ontología génica (GO) de los genes relacionados con esta firma, observamos su implicación en vías moleculares cruciales en el desarrollo del cáncer y con otras involucradas en la respuesta a la quimioterapia, como las vías de señalización PI3K/AKT, Wnt y Cadherina.

En resumen, el estudio llevado a cabo en el Capítulo II.B representa un avance significativo en la búsqueda de biomarcadores predictivos en el contexto del tratamiento del mCCR con la terapia FOLFOX. La epifirma no invasiva identificada en cfDNA tiene un gran potencial como biomarcador predictivo para estratificar a los pacientes con mCCR según su respuesta a FOLFOX. Este estudio subraya el potencial de utilizar los *arrays* EPIC en cfDNA para descubrir nuevos biomarcadores epigenéticos no invasivos para el manejo de pacientes con cáncer. Además, los genes y las vías que se encontraron epigenéticamente desregulados en asociación con la respuesta a la terapia sirven como posibles objetivos para superar la resistencia al tratamiento con FOLFOX en mCCR.

Al igual que en el CCR, la desregulación de los mecanismos epigenéticos en el CM presenta importantes implicaciones para el desarrollo, la progresión y la respuesta terapéutica del cáncer. En el tercer capítulo, nuestro objetivo fue analizar el metiloma del cfDNA de pacientes con CM luminal B metastásico (CMLB) utilizando un enfoque a nivel genómico basado en la tecnología del EPIC *array* para descubrir posibles biomarcadores no invasivos. Para lograr esto, analizamos a 9 pacientes con CMLB metastásico y 5 controles sanos. La metodología del EPIC *array* suele requerir una gran cantidad de ADN, lo cual es un desafío obtenerlo a partir de plasma. Como novedad en nuestro estudio, utilizamos pequeñas cantidades de cfDNA de muestras individuales de plasma, las cuales fueron amplificadas a nivel genómico después de la modificación con bisulfito.

Siguiendo este enfoque, identificamos 28.799 DMCpGs entre CMLB y los controles no tumorales. Dado que la hipermetilación en los promotores puede alterar la expresión génica, enfocamos nuestro estudio en esas regiones. Así, identificamos una epifirma de 1.467 DMCpGs capaz de diferenciar claramente a los pacientes con CMLB de los controles no tumorales. A continuación, para obtener información relacionada con las vías funcionales involucradas en la epifirma identificada, realizamos un análisis GO basado en la base de datos PANTHER. Este análisis reveló que las diferencias de metilación en el cfDNA de pacientes con CMLB y controles no tumorales estaban principalmente asociadas con genes relacionados con la vía de señalización Wnt. Entre las DMCpGs en la epifirma, encontramos 2 CpGs ubicadas en el gen *WNT1* que estaban hipermetiladas en el cfDNA de pacientes con CMLB. Para confirmar esta metilación aberrante, seleccionamos la CpG de *WNT1* más diferencialmente metilada y analizamos su estado de metilación mediante ddPCR en el cfDNA de nuestra cohorte.

La metilación de *WNT1* fue significativamente mayor en el cfDNA de pacientes con CMLB que en los controles no tumorales, diferenciando a los pacientes de los controles con una alta sensibilidad y especificidad. Además, para evaluar si esta metilación aberrante también estaba presente en los tejidos tumorales, analizamos la metilación de *WNT1* mediante ddPCR en muestras de tumores primarios y metastásicos de nuestra cohorte. Este ensayo reveló que la hipermetilación de *WNT1* estaba presente no solo en el cfDNA, sino también en las muestras pareadas de tumores primarios y metastásicos de los pacientes con CMLB analizados.

A continuación, utilizando datos públicos de TCGA, confirmamos que la metilación de *WNT1* era significativamente mayor en todos los estadios de CMLB en comparación con controles sanos. Un análisis de la curva ROC mostró que la metilación de *WNT1* diferenciaba los tumores primarios luminales B (estadios I-IV) de los controles no tumorales con una alta

precisión diagnóstica. Además, confirmamos que la hipermetilación del promotor de *WNT1* tenía un efecto inhibidor en la expresión génica en este subtipo de CM.

En conclusión, este estudio reveló una epifirma única vinculada al CMLB. Además, identificamos la hipermetilación del promotor de *WNT1* en cfDNA como un prometedor biomarcador no invasivo para el CMLB. Estos hallazgos respaldan también la aplicación de la tecnología EPIC *array* para descubrir nuevos biomarcadores epigenéticos no invasivos en el CM.

En resumen, esta tesis destaca el estudio de biomarcadores genéticos y epigenéticos mediante biopsia líquida para avanzar en la oncología de precisión. La evaluación del panel TST170 en cfDNA mejora las opciones para el análisis no invasivo de variantes genéticas en el CCR metastásico. La posibilidad de detectar variantes somáticas clínicamente relevantes con TST170, incluyendo *KRAS* y *PIK3CA*, apoya la utilidad de este panel para el análisis genético de cfDNA para personalizar las estrategias terapéuticas. La evaluación de mecanismos epigenéticos, como la metilación del ADN, han demostrado su utilidad como biomarcadores para el diagnóstico, pronóstico y selección de la terapia más adecuada, proporcionando nuevas perspectivas para la oncología de precisión. En este trabajo, el análisis de la metilación de *LINC00473* ha abierto un camino prometedor para la detección temprana del CCR y sus lesiones precancerosas. La precisión diagnóstica de la metilación de *LINC00473* como biomarcador epigenético en biopsia líquida subraya el potencial de este tipo de enfoques no invasivos para la detección del cáncer. Además, en esta tesis identificamos una epifirma capaz de predecir la respuesta a FOLFOX, lo que representa un prometedor biomarcador predictivo no invasivo para mejorar la selección de pacientes con mCCR tratados con esta terapia. Por otra parte, el perfilado del metiloma en cfDNA de CMLB metastásico reveló una firma epigenética distintiva, con la hipermetilación del promotor de *WNT1*

emergiendo como un posible biomarcador de esta enfermedad. En conjunto, estos hallazgos contribuyen a mejorar el manejo no invasivo de pacientes con CCR y CM, respaldando el uso del análisis genético y epigenético en la biopsia líquida para avanzar hacia la oncología de precisión.

SUMMARY *IN EXTENSO*

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Cancer is a group of diseases characterized by genetic and epigenetic aberrations that disrupt normal cellular functions. Despite advances in cancer management, global cancer incidence reached nearly 20 million cases in 2020, highlighting the need to discover new biomarkers for early diagnosis, follow-up, and treatment selection. Breast cancer (BC) and colorectal cancer (CRC) are among the most diagnosed cancers worldwide, with BC being the most prevalent. Projections for 2023 by the Spanish Society of Medical Oncology (SEOM) indicate CRC and BC as the most common types of tumors in Spain.

CRC is the second leading cause of cancer-related death globally. Genetic mutations in key pathways, such as PI3K/Akt and Wnt signaling, play pivotal roles in carcinogenesis and disease progression. Besides, BC stands as the leading cause of cancer-related deaths among women. The complex etiology of BC involves genetic, hormonal and lifestyle factors. These tumors require a multidisciplinary approach encompassing prevention, early diagnosis and intervention and the continual development of new treatments.

Cancer can be produced by the inheritance of pathogenic germline variants, but the majority of cases are produced by non-hereditary factors, like the appearance of certain somatic variants that may activate oncogenes, disrupting regulatory pathways and leading to tumor development. Gene variants include single nucleotide variants (SNVs), copy number variants (CNVs), gene fusions and insertion-deletions (indels). SNVs, within coding regions of genes, including *KRAS* or *PIK3CA*, are linked to cancer development. In addition, frameshift indels also contribute to cancer formation (e.g., *TGFBR2* and *ACVR2A* indels in CRC; and *BRCA2* and *GATA3* indels in BC). On the other hand, CNVs disrupt gene dosage, which may impact

cancer, as it happens with *MYC* and *HER2* amplifications. Although gene fusions are less common, they are related with aggressive tumors. Recent advances in high-throughput technologies, such as next-generation sequencing (NGS), have improved our ability to understand and address the complexity of cancer.

Epigenetics explores heritable changes in the activity of genes that do not involve alterations in DNA sequences. These reversible modifications play a vital role in regulating gene expression during biological processes, including cell differentiation. Lifestyle factors like diet, stress and environmental exposure, influence epigenetic changes. The epigenetic machinery involves DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs. DNA methylation is produced by adding methyl groups to CpGs, which can silence the gene expression. This reversible process occurs in specific genomic regions, including CpG islands and shore regions. Histone modifications and nucleosome positioning influence chromatin structure and gene accessibility, altering gene expression. Moreover, non-coding RNAs, such as miRNAs and lncRNAs, regulate gene expression post-transcriptionally. Aberrant patterns of DNA methylation and other epigenomic modifications are implicated in carcinogenesis.

Changes in DNA methylation, particularly widespread hypomethylation and *de novo* hypermethylation at CpG islands, are associated with genomic instability and inactivation of tumor suppressor genes, respectively. In CRC, the hypermethylation of genes, including *APC*, RASSF family, *SEPT9* and *PTEN* has been described. In BC, *BRCA1/2* and some hormone receptor genes are also affected by aberrant DNA methylation, influencing the development of disease and therapeutic responses. In cancer, the interaction between genetic and epigenetic events is crucial for the initiation and progression of the disease. Understanding the intricate genetic

and epigenetic landscape of cancer provides insights into disease mechanisms and enables the discovery of new biomarkers.

Liquid biopsy has emerged as an innovative technique for the molecular analysis of tumors, offering a non-invasive alternative to traditional tissue biopsies. Although tissue biopsies are the gold standard, they have limitations, such as invasiveness, and challenges associated with tumor heterogeneity and metastases. Liquid biopsy, which primarily uses blood, allows the analysis of tumor-derived components, namely circulating tumor cells (CTCs), cell-free DNA (cfDNA) and extracellular vesicles (EVs).

One of the main components of liquid biopsy is cfDNA, which originates from cells through various mechanisms, including apoptosis and necrosis. CfDNA has a short half-life, making it easy to use to study disease progression in real time. Importantly, liquid biopsy allows the analysis of genetic and epigenetic alterations, namely gene variants and methylation changes, in cfDNA. The preanalytical phase, which involves careful blood collection and cfDNA isolation, is crucial to maintain sample quality. Various methodologies are used to detect these alterations, including quantitative PCR, digital droplet PCR and NGS.

Despite the low concentration of cfDNA molecules present in blood samples, several tests, including EpiProColon and Cologuard for CRC, and the Therascreen *PIK3CA* RGQ PCR assay for BC, have been approved by the FDA. Liquid biopsy is contributing to precision oncology and represents a promising tool for early cancer detection and monitoring of the disease. In recent years, research on cfDNA has led to the discovery of non-invasive biomarkers for cancer diagnosis, prognosis, disease monitoring and therapeutic selection in several tumor types.

In CRC, hotspot variants in genes, such as *KRAS*, *BRAF* and *PIK3CA,* have shown clinical utility. For prognosis, cfDNA levels correlate with

worse relapse-free survival (RFS) and overall survival (OS), while *KRAS* and *BRAF* variants are related with poor prognosis. In addition, the detection of *KRAS* variants helps determine resistance to anti-EGFR therapy. Similarly, in BC, the analysis of cfDNA to detect variants in some genes, including *HER2*, *ESR1*, and *PIK3CA*, provides valuable prognostic information and helps evaluate response to treatment. Also, quantitative analysis of cfDNA concentrations has been described as a reliable predictor of OS and progression-free survival (PFS).

Epigenetic biomarkers, specifically methylation patterns in cfDNA, also offer insights into early detection, prognosis, and therapeutic selection for CRC and BC. In CRC, *SEPT9* methylation has shown diagnostic utility, and additional markers like *SDC2*, *RARB*, and *RASSF1A* contribute to the assessment of prognosis and monitoring response to treatment. For BC, genes such as *RASSF1A*, *FOXA1*, *DKK3* and *BRCA1* exhibit methylation changes related to detection, prognosis and therapeutic response.

The discovery of biomarkers in cfDNA using liquid biopsy opens a very promising field to advance in precision oncology. In this context, the main objective of this doctoral thesis was to perform the genetic and epigenetic profiling of cfDNA in patients with colorectal and breast cancer using genome-wide approaches to discover and evaluate non-invasive biomarkers with clinical utility for precision oncology.

To achieve this purpose, in the first chapter of this thesis, we evaluated the ability of the TruSight Tumor 170 NGS panel (TST170) to detect somatic variants in cfDNA of CRC patients. NGS panels have proven to be useful for the molecular analysis in cancer patients, allowing the detection of variants with clinical implications and the discovery of new therapeutic targets. The TST170 covers the coding region of 170 cancer-related genes and allows the detection of SNVs, CNVs and indels. This is a panel designed to be used

on tumor tissue samples that had not been previously used in cfDNA samples. Therefore, we decided to evaluate the performance of TST170 in cfDNA, opening the possibility of having a new non-invasive tool for the study of gene variants in cancer research or in a clinical setting. Thus, we used plasma samples from patients with metastatic CRC (mCRC) with known variants in the *KRAS* gene, both in tissue (analyzed by pyrosequencing) and plasma (analyzed by BEAMing) samples.

The analysis of cfDNA with TST170 showed the presence of SNVs and indels in all patients analyzed. To assess the pathogenicity and clinical implication of the detected variants, we used the VarSome Clinical platform that classifies somatic variants into four categories based on their clinical impact: tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants with unknown clinical significance; and tier IV, benign or likely benign variants. Using this system, we identified Tier I variants in the *KRAS* gene in 79% of the patients analyzed, and in *PIK3CA* in 26%. In addition, Tier II variants were detected in other relevant genes such as *APC*.

Focusing on the mutations detected in *KRAS*, a gene of great importance in CRC, since it determines the use of anti-EGFR treatment, we observed a high concordance between the data obtained in cfDNA by TST170 with those obtained in tissue by pyroseqeuncing (77%) and in cfDNA using BEAMing (94%). Additionally, to evaluate the sensitivity of the panel, we used cfDNA data obtained for the NRAS gene by BEAMing. Thus, TST170 demonstrated 100% sensitivity for cfDNA analysis by detecting wild type (WT) status for this gene in all patients tested.

Since in some cases the analysis of cfDNA using TST170 showed discrepancy with respect to the cfDNA analyzed by BEAMing or the status of tumor tissues, we reanalyzed the available cfDNA samples using droplet

digital PCR (ddPCR). This technique confirmed that the discrepancies were related to the low variant allele frequency (VAF), below the limit of detection obtained in our cohort to detect *KRAS* variants by TST170. We observed that the cause of this low VAF could be directly related to the absence of liver metastasis and the previous resection of the primary tumor.

In summary, the results obtained in this first chapter demonstrate the feasibility of using the TST170 panel for comprehensive genetic analysis in cfDNA, being able to detect variants with clinical importance, supporting the application of this panel in clinical settings and in cancer research. Despite some observed limitations, the overall findings support the use of targeted NGS analysis of cfDNA with the TST170 panel as a promising and easy to implement tool for the non-invasive detection of genetic variants in patients with mCRC. This assay could be useful to advance in precision oncology and improve the outcome of CRC patients.

In addition to genetic alterations, epigenetic modifications are also of great importance and usefulness in CRC. Therefore, in the second chapter of this doctoral thesis we wanted to identify novel cfDNA methylation biomarkers for the management of CRC. Thus, in the chapter II.A we explored the clinical utility of the promoter methylation of the lncRNA *LINC00473* as a non-invasive biomarker to detect CRC and precancerous lesions. Because most deaths from CRC are due to metastasis, it is a clinical need to detect this disease as soon as possible, even in its precancerous state. There are screening techniques to detect CRC such as the fecal immunochemical test (FIT) or colonoscopy, but they have some limitations. Therefore, novel non-invasive tests for early detection of CRC are urgently needed.

Methylation levels of *LINC00473* were analyzed in CRC cell lines and in eight independent clinical cohorts of patients, including four cohorts of colorectal tissue samples and four cohorts of plasma samples. First, the

hypermethylation of the *LINC00473* promoter was confirmed in the CRC cell line HCT-116 by bisulfite genomic sequencing. Furthermore, to confirm the effect of hypermethylation, we analyzed the expression levels of *LINC00473* by RT-PCR, showing the downregulation of this lncRNA in HCT-116 cells with respect to normal unmethylated colon mucosa.

Using data from The Cancer Genome Atlas (TCGA), we verified that methylation levels in primary colorectal tumors (in all the stages of the disease, including stage I) were higher than in normal tissue. Furthermore, this hypermethylation produced a significant downregulation of *LINC00473* expression levels in tumor tissues. ROC curve analysis showed a very high accuracy for the CRC detection across all tumor stages, with high sensitivity and specificity. These results were confirmed in a second cohort of primary colorectal tumors (stages I to IV) and matched non-tumor controls by bisulfite pyrosequencing.

To test whether *LINC00473* methylation also occurred in precancerous stages, we analyzed the methylation status of the *LINC00473* promoter by bisulfite pyrosequencing in tissues from premalignant colorectal polyps, CRC and matched normal colorectal mucosa. Thus, we confirmed that *LINC00473* methylation was significantly higher in polyps and CRC than in healthy controls, with the ROC curve showing a high capacity to differentiate controls from polyps. These data were confirmed by pyrosequencing in a larger cohort of colorectal polyps and normal mucosa.

With the aim of achieving a non-invasive diagnostic biomarker, we evaluated the detection of *LINC00473* hypermethylation in cfDNA samples. First, we analyzed its promoter methylation in plasma cfDNA from a cohort of self-reported healthy controls and CRC patients using qMSP. Furthermore, we also evaluated the plasma cfDNA of a cohort of self-declared healthy controls and patients with advanced colorectal polyps (ACPs). In both cases,

the analysis showed a significantly higher level of methylation in the cfDNA of CRC and ACP samples than in the healthy controls. Furthermore, ROC curve analysis showed high diagnostic accuracy for the detection of CRC and ACPs in both cohorts analyzed.

To confirm the feasibility of using *LINC00473* promoter methylation for the non-invasive early detection of CRC, we retrospectively analyzed an independent cohort of plasma cfDNA samples obtained either prior to a scheduled colonoscopy, as part of standard CRC screening, or prior to colonic surgery for primary tumors. In this case, we used ddPCR for methylation analysis. Consistent with our previous results, *LINC00473* methylation levels were significantly higher in ACP and CRC than in confirmed healthy controls. Of note, *LINC00473* promoter methylation showed high accuracy for the detection of ACP and CRC, with high sensitivity, specificity, positive predictive value, and negative predictive value.

Once the usefulness of measuring the methylation status of the *LINC00473* promoter to detect CRC and precancerous lesions was confirmed, we wanted to evaluate whether it could be useful to detect changes in the disease during the follow-up of patients. Then, we analyzed methylation by ddPCR at different clinically relevant time points. Plasma cfDNA methylation levels of *LINC00473* decreased with effective therapy and increased with disease progression. Notably, in some cases, *LINC00473* methylation precedes CEA in detecting the presence of CRC, making it a potential biomarker for detecting the presence of CRC during the follow-up of metastatic patients.

Altogether, the early appearance of *LINC00473* hypermethylation in colorectal carcinogenesis and its detection in plasma cfDNA demonstrates the feasibility of using *LINC00473* methylation as a non-invasive biomarker for the early detection of CRC. Integrating this novel epigenetic biomarker into the

precision oncology landscape holds promise for improving the accuracy of CRC diagnoses, providing clinicians with a valuable non-invasive tool for the early detection of this disease.

Besides, in Chapter II.B, we focused on analyzing the cfDNA methylome of mCRC patients in relation to FOLFOX response. Despite being one of the most commonly used first-line treatments, some patients do not respond to this therapy. This highlights the urgent clinical need to identify biomarkers that can assist in selecting the most effective therapy for each patient. The objective of this study was to identify novel non-invasive methylation biomarkers in cfDNA to predict the response of mCRC patients to FOLFOX-based therapy.

To achieve this, we selected 20 mCRC patients before starting treatment with FOLFOX. Following RECIST 1.1 criteria, therapy response was evaluated at approximately 3 and 6 months, classifying patients based on their best response to treatment. We established a group of 12 patients with non-progressive disease (nPD: stable disease or partial response) and another group of 8 patients with progressive disease (PD). Subsequently, we analyzed the cfDNA methylome of the patients at baseline using a genome-wide approach based on Infinium MethylationEPIC array (EPIC array) technology. We identified 1,174 differentially methylated CpGs (DMCpGs) between the nPD and PD groups, distributed across all chromosomes. Notably, by selecting the most DMCpGs, we identified an epigenetic signature (episignature) of 406 CpGs able to clearly differentiate at baseline between patients with nPD and PD. Performing a gene ontology (GO) analysis of the genes involved in this signature, we observed their association with crucial molecular pathways in cancer development and in chemotherapy response, such as PI3K/AKT, Wnt and Cadherin signaling.

In summary, the study conducted in Chapter II.B represents a significant advance in the search for predictive biomarkers in the context of mCRC treatment with FOLFOX therapy. The identified non-invasive cfDNA episignature holds great promise as a predictive biomarker for stratifying mCRC patients based on their response to FOLFOX. This study further underscores the potential of using EPIC arrays in cfDNA to discover new non-invasive epigenetic biomarkers for managing cancer patients. Moreover, the genes and pathways found to be epigenetically deregulated in association with therapy response serve as potential targets for overcoming resistance to FOLFOX treatment in mCRC.

As in CRC, deregulation of epigenetic mechanisms in BC presents important implications for cancer development, progression and therapeutic response. In the third chapter, we aimed to profile the cfDNA methylome of metastatic luminal B breast cancer (LBBC) patients using a genome-wide approach based on EPIC array technology to discover potential non-invasive biomarkers. To achieve this, we analyzed 9 patients with metastatic LBBC and 5 healthy controls. EPIC array methodology usually requires a high amount of DNA, which is challenging to obtain from plasma. As a novelty in our study, we used small amounts of cfDNA from individual plasma samples, which were genome-wide amplified after bisulfite modification.

Following this approach, we identified 28,799 DMCpGs between LBBC and non-tumor controls. Since hypermethylation in the promoters can alter gene expression, we focused our study on these regions. Thus, we identified an episignature of 1,467 DMCpGs able to clearly differentiate LBBC patients from non-tumor controls. Next, to obtain information related to the functional pathways involved in the identified episignature, we performed a GO enrichment analysis based on the PANTHER database. This analysis revealed that methylation differences in the cfDNA of LBBC patients and non-tumor controls were mainly associated with genes related to the Wnt signaling

pathway. Among the DMCpGs in the episignature, we found 2 CpGs located in the *WNT1* gene that were hypermethylated in the cfDNA of LBBC patients. To confirm this aberrant methylation, we selected the most DMCpGs of *WNT1* and analyzed its methylation status by ddPCR in the cfDNA of our cohort.

The methylation of *WNT1* was significantly higher in cfDNA of LBBC patients than in non-tumor controls, differentiating LBBC from controls with high sensitivity and specificity. Additionally, to evaluate if this aberrant methylation was also present in tumor tissues, we analyzed the methylation of *WNT1* by ddPCR in samples of primary and metastatic tumors from our cohort. This assay revealed that *WNT1* hypermethylation was present not only in cfDNA but also in the paired primary and metastatic tumor samples of the LBBC patients analyzed.

Next, using public data from TCGA, we confirmed that the methylation of *WNT1* was significantly higher in all stages of LBBC patients compared to healthy controls. A ROC curve analysis showed that *WNT1* methylation differentiated luminal B primary tumors (stages I-IV) from non-tumor controls with high diagnostic accuracy. Furthermore, we confirmed that the hypermethylation of the *WNT1* promoter had a significantly downregulatory effect on gene expression in this BC subtype. In conclusion, this exploratory study revealed a unique episignature linked to LBBC. Additionally, we identified the hypermethylation of the *WNT1* promoter in cfDNA as a promising non-invasive biomarker for LBBC. These findings also support the application of EPIC array technology for discovering novel non-invasive epigenetic biomarkers in BC.

In summary, this thesis highlights the study of genetic and epigenetic biomarkers by liquid biopsy to advance in precision oncology. The evaluation of the TST170 panel in cfDNA enhances the options for the non-invasive analysis of genetic variants in mCRC. The possibility of detecting clinically

relevant somatic variants with TST170, including *KRAS* and *PIK3CA*, supports the utility of this panel for the genetic profiling of cfDNA to personalize treatment strategies. The evaluation of epigenetic mechanisms, such as DNA methylation, have shown utility as biomarkers for diagnosis, prognosis and selection of the most appropriate therapy, providing new insights for precision oncology. In this work, the analysis of *LINC00473* methylation, has opened a promising avenue for the early detection of CRC and its precancerous lesions. The robust diagnostic accuracy of *LINC00473* methylation as an epigenetic biomarker in liquid biopsy underscores the potential of this type of non-invasive approaches for cancer detection. In addition, in this thesis we identified an episignature able to predict the response to FOLFOX, which represents a promising non-invasive predictive biomarker to improve the selection of mCRC patients treated with this therapy. Moreover, the profiling of the cfDNA methylome in metastatic LBBC revealed a distinctive epigenetic signature, with *WNT1* promoter hypermethylation emerging as a potential biomarker of this disease. Altogether, these findings contribute to improve the non-invasive management of colorectal and breast cancer patients, supporting the use of genetic and epigenetic analysis in liquid biopsy to advance towards precision oncology.

INTRODUCTION

INTRODUCTION

1. Cancer

Cancer is a complex group of diseases characterized by different genetic and epigenetic alterations that lead to the disruption of genes [1]. In 2000, Hanahan and Weinberg introduced the hallmarks of cancer, describing the capabilities acquired by cells during tumor development [2]. These hallmarks encompass sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. In 2011, they added to these hallmarks the ability to avoid immune destruction, as well as to deregulate cellular energetics. They also described the genome instability and generation of mutations, and the tumor-promoting inflammation as important features of this disease [3]. In addition, other relevant new hallmarks of cancer have been described in 2022, like the non-mutational epigenetic reprogramming [4].

The understanding of cancer genomics and epigenomics has undergone exponential growth in recent years, thanks to large-scale studies such as The Cancer Genome Atlas (TCGA) launched in 2005. TCGA applied high-throughput technologies to characterize multiple types of tumors based on microarrays and next-generation sequencing (NGS) methods, including RNA-seq, DNA-seq, and DNA methylation arrays [5]. Furthermore, the ability to analyze the molecular profile at the single-cell level has facilitated the acquisition of deeper insights into tumor heterogeneity and clonal evolution [6]. By using this type of techniques, numerous studies have shown the involvement of genetic and epigenetic factors in tumor initiation, progression, and treatment response [7,8].

Despite these advances, it was estimated that in 2020 there would be almost 20 million new cases of cancer in the world, and close to 10 million deaths. This situation underscores cancer as one of the leading causes of death worldwide [9]. In addition, it is expected that in 2040 the number of new cases will reach 20.4 million, which highlights the clinical need to continue looking for new diagnostic methods and biomarkers, as well as potential therapeutic targets [10].

Among the most frequently diagnosed cancers worldwide, breast cancer (BC) ranks first as the most diagnosed cancer, with colorectal cancer (CRC) holding the third position of cases [9]. According to the Spanish Society of Medical Oncology (SEOM) report, in Spain, the most frequently diagnosed tumors in 2023 are expected to be CRC (15.30%) and BC (12.53%) [11].

In recent years, the discovery and utilization of cancer biomarkers has revolutionized the field of oncology, playing a crucial role in improving our understanding of cancer and developing personalized treatment strategies. The clinical applications of these biomarkers are extensive, including risk assessment, screening and early detection, accurate diagnosis, patient prognosis, prediction of response to therapy, cancer surveillance and monitoring of therapy response. The ultimate goal of these biomarkers is to achieve precision oncology to optimize prevention, screening and treatment strategies, contributing to benefit the outcome of cancer patients [12].

1.1 Colorectal cancer

1.1.1 Epidemiology

In addition to being the third most diagnosed cancer (10% of all tumors), CRC is the second leading cause of cancer-related deaths worldwide (9.4%), accounting for about 1.9 million new cases and almost 950,000 deaths

in 2020 [9] (**Figure 1**). When examining the data by gender, CRC stands as the third most diagnosed type of cancer among men (10.6%), while in women it is the second (9.4%), surpassed only by BC (24.5%). In terms of mortality, CRC ranks as the third leading cause of cancer-related death in both men (9.3%) and women (9.5%) [13].

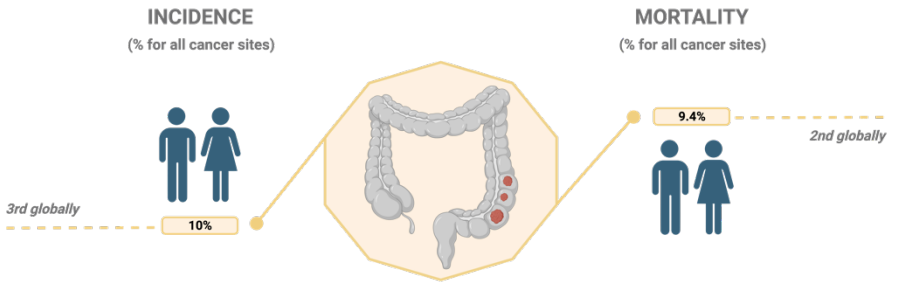

Figure 1. **Incidence and mortality of colorectal cancer in 2020.** CRC is the third most diagnosed cancer globally and the second in terms of the number of cancer-related deaths.

The incidence of CRC exhibits a strong correlation with the level of human development. More than two-thirds of all cases and approximately 60% of all deaths occur in countries with a high or very high human development index [14]. However, the rates of both CRC incidence and mortality continue to experience significant growth in numerous low- and middle-income countries. By 2030, the global burden of CRC is expected to increase by 60%, resulting in over 2.2 million new cases and 1.1 million annual deaths, directly linked to economic development [15].

Between 2018 and 2023, the mortality rate in Europe due to CRC exhibited a decline of 5.5% in men and 8.7% in women [16]. Conversely, in recent years, CRC incidence increased by 4% [17], with this rise being more prominent among young adults [18]. The falls in CRC mortality are

predominantly attributed to advances in early diagnosis, screening, treatment, and overall disease management [19].

The risk of developing CRC is associated with a variety of factors. Some of these factors are modifiable, including diet, physical activity, and lifestyle habits, such as smocking and alcohol consumption [20]. These modifiable factors could potentially contribute to as much as 47% of all CRC cases [21]. Other factors are non-modifiable, namely age, personal history of adenomatous polyps and inflammatory bowel disease, as well as family history [22,23].

1.1.2 Development

The development of CRC involves a complex series of changes in genes, tissues, and morphological structures that accumulate over time [24]. The mutations observed in CRC affect various pathways that control cancer progression, including PI3K/Akt, Wnt, TP53, and MAPK pathways. Other genes frequently altered in the development of CRC are *KRAS*, *SMAD4*, *BRAF*, and *PTEN* [24,25].

Among all the CRC cases, approximately 5 to 10% are attributed to hereditary syndromes [26]. The most common of these syndromes are the familial adenomatous polyposis (FAP), caused by mutations in the tumor suppressor gene *APC*; and the hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome, which is caused by germline mutations in DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) [27], and also by aberrant hypermethylation [28,29]. Additionally, mutations in genes like *NTHL1*, *MSH3*, *MLH3*, *GREM1*, *RNF43* and *RPS20* contribute to an increased predisposition to polyposis and CRC [30].

CRC can arise from one or a combination of three different mechanisms: microsatellite instability (MSI), CpG island methylator

phenotype (CIMP), and chromosomal instability (CIN), accounting for 15%, 17%, and 85% of cases, respectively [31]. The CIN pathway is characterized by imbalances in the number of chromosomes, leading to aneuploidy tumors and loss of heterozygosity. Mechanisms underlying CIN include alterations in chromosome segregation, telomere dysfunction and DNA damage response [31,32]. Moreover, the MSI pathway results from a hypermutable phenotype due to loss of DNA repair mechanisms, leading to the accumulation of mutations in microsatellite regions [33,34]. Besides, epigenetic deregulation responsible for the CIMP, is another common feature in CRC. The main characteristic of CIMP tumors is the hypermethylation of gene promoters, leading to genetic silencing and loss of expression [35,36].

In most cases of CRC (70%–90%), the conventional adenoma-carcinoma model is followed. This involves the transformation of an abnormal crypt into a benign adenomatous polyp, which eventually becomes sporadic CRC over approximately 10–15 years [37]. These phenotypic transitions are associated with the mutational signature "*APC-KRAS-TP53*" and are mainly influenced by the CIN pathway. Initial mutations affect the *APC* gene, impacting chromosome distribution during cell division. Subsequent *KRAS* oncogene mutations have downstream effects on cell growth, differentiation, motility, and survival. TP53 gene mutations can result in its loss of function, impacting a wide range of cellular functions that contribute to cancer development [38].

Alternatively, around 10% of CRCs follow a serrated neoplasia pathway associated with the CIMP phenotype. This pathway often starts with *BRAF* gene mutations, leading to altered growth signals and suppressed apoptosis [39]. Common epigenetic changes seen in this type of CRC involve abnormal methylation of gene promoter regions, which inhibits gene transcription. This affects many genes, including those controlling growth-promotion and tumor suppressor genes [38].

MSI can occur in both adenomatous and serrated polyps. It is linked to inherited mutations in DNA mismatch repair genes as well as sporadic mutations caused by abnormal methylation of *MLH1* promoter regions [33,40]. MSI leads to uneven replication of repetitive DNA sequences in noncoding regions (microsatellites), increasing susceptibility to additional genetic mutations [41].

1.1.3 Classification

The classification of CRC is complex, encompassing factors such as origin, tumor location and histology.

Regarding the primary tumor location, CRC can be located in four major anatomic sites: the right or ascending colon, the middle or transverse colon, the left or descending colon and the sigmoid colon [42]. The location has already been described as a prognostic factor in metastatic colorectal cancer (mCRC).In this sense, colon tumors in the rightl region have a worse survival prognosis [43], exhibit high levels of genome-wide promoter hypermethylation (CIMP), MSI due to deficient DNA mismatch repair mechanisms (dMMR) [33], and frequent *BRAF* mutations. On the other hand, left-sided colorectal tumors show better prognosis and are more likely to have CIN [44,45].

Related with the histology of the tumor, more than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa. Other rare types of colorectal carcinomas include neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas [46]. These histopathological criteria have modest prognostic value [47].

Molecular pathway classification distinguishes CRC into the three groups previously described: CIN, MSI, and CIMP [31]. Additionally, there are

CRC subtypes according to the cell of origin (stem-like, inflammatory, transit-amplifying, goblet-like, enterocytes) [48] and gene expression patterns (Colon Cancer Subtype 1, 2, 3 - CCS1, CCS2, CCS3) [49], providing additional insights into the heterogeneity and molecular landscape of CRC.

Despite numerous efforts to categorize CRC over time, its inherent heterogeneity presents challenges for creating clinically relevant classifications. The CRC Subtyping Consortium (CRCSC) has recently introduced four consensus molecular subtypes (CMS): CMS1 or MSI Immune subtype (14%), CMS2 or Canonical subtype (37%), CMS3 or Metabolic subtype (13%), and CMS4 or Mesenchymal subtype (23%). Some samples display mixed characteristics, indicating an intermediate phenotype or intra-tumoral heterogeneity [50].

1.1.4 Screening

Despite advances in screening techniques, like colonoscopies for individuals at risk of developing CRC, along with non-invasive tests, such as the fecal immunochemical test (FIT) and guaiac fecal occult blood testing (gFOBT), detecting CRC remains a challenge [51,52]. The difficulties for early detection of this tumor favors a high mortality, since a cure is not possible for most individuals with mCRC [53]. Although colonoscopies are considered a gold standard for early CRC detection and allow the removal of precancerous polyps, they may be rejected by the patient due to their invasiveness, contributing to the persistent challenge of late-stage diagnoses [54].

Non-invasive tests, including FIT and gFOBT, designed to detect blood in the stool, provide alternative and non-invasive screening approaches [52]. FIT is generally preferred over gFOBT as it tends to be more effective in detecting lower levels of blood, thereby enhancing its ability to identify patients in the early stages of the disease [51]. Additionally, false positives can occur

due to various factors, such as hemorrhoids, certain medications, or dietary choices, potentially causing psychological stress and anxiety in patients concerning the possibility of having cancer [55,56].

However, even with these screening options, approximately 60-70% of CRC cases diagnosed in symptomatic patients are still identified at advanced stages [57]. This late-stage diagnosis results in a 5-year relative survival rate below 20% [58], underscoring the clinical need for more sensitive, specific and non-invasive screening tools. New blood-based biomarkers could improve early detection and ultimately enhance the prognosis for CRC patients [59,60].

1.1.5. Treatment and resistance

Patients with localized CRC are mainly treated with surgery, which allows excellent oncological outcome [61]. In certain cases, particularly rectal cancer, neoadjuvant therapy (including radiotherapy and/or chemotherapy) is also used to reduce tumor mass, block tumor invasion and improve the rate of tumor resection [62]. Additionally, adjuvant chemotherapy can be used to high-risk patients or those with locally advanced disease, as studies indicate a lower recurrence rate and better survival [62].

In the case of mCRC, its management depends on factors such as tumor and disease characteristics, patient-related considerations such as co-morbidities and expectations, and treatment preferences [63]. Current treatment options for mCRC encompass: i) cytotoxic chemotherapy (like 5-fluorouracil (5-FU), capecitabine, irinotecan, oxaliplatin, and trifluridine-tipiracil); ii) targeted therapies (including anti-EGFR agents like cetuximab and panitumumab, and anti-VEGF/VEGFR agents like bevacizumab); and also, iii) immunotherapy based on anti-PD1 antibodies (pembrolizumab and nivolumab) [64].

The first-line treatment of mCRC is usually based on 5-FU, either as monotherapy or in combination with systemic treatments, specifically FOLFOX (5-FU and oxaliplatin) and FOLFIRI (5-FU and irinotecan) regimens [64]. These chemotherapy agents work to inhibit DNA synthesis and repair, and they can be synergistically employed with targeted therapies to enhance the treatment's effectiveness.

Related with target therapies, CRC involves molecular targets including the epidermal growth factor receptor (EGFR), which is often overexpressed in most CRC cases, as well as the vascular endothelial growth factor (VEGF) and its corresponding receptor (VEGFR). Cetuximab and panitumumab block the binding of EGF to EGFR, thereby inhibiting the activation of the Ras/Raf/MAPK pathway [53,65], a critical pathway for cell proliferation, migration, angiogenesis, and resistance to apoptosis. These treatments are recommended for wild type *KRAS* and *NRAS* tumors, as mutations in these genes and also the *BRAF* V600E mutation, can lead to the constitutive activation of this pathway. However, approximately 40% of mCRC cases harbor these mutations and therefore do not benefit from these treatments [64,66].

Tumor cells and their associated stroma produce VEGF and other proangiogenic factors, which are then released into circulation. These factors bind to VEGFR, subsequently triggering the development of new blood vessels that facilitate ongoing tumor growth [67]. Therapies like bevacizumab work to prevent the ligand-receptor interaction, preventing the activation of signaling pathways responsible for stimulating angiogenesis [64].

Despite an initial response to these treatments, a significant number of patients eventually develop resistance and experience disease recurrence. Immunotherapy has emerged as a novel strategy in CRC treatment, offering the advantage of reduced adverse effects and improving quality of life [68].

The U.S. Food and Drug Administration (FDA) approved pembrolizumab and nivolumab to be employed in the treatment of mCRC patients who present dMMR and high levels of microsatellite instability (MSI-H). While approximately 15% of all CRC cases demonstrate dMMR-MSI-H characteristics, this proportion reduces to only 5% in stage IV cases. Nonetheless, these stage IV cases exhibit a strong positive response to immune checkpoint blockade [69,70].

1.2 Breast cancer

1.2.1 Epidemiology

Breast cancer has recently surpassed lung cancer to become the most frequently diagnosed form of cancer, with an estimated 2.3 million new cases reported annually (11.7% of total cases). It ranks as the fifth leading cause of death, responsible for nearly 700,000 deaths (6.9%) [9] (**Figure 2**). Focusing specifically on women, BC holds the distinction of being the most commonly diagnosed cancer (24.5%) and the leading cause of cancer-related death (15.5%) [13], being the metastatic disease the responsible for the majority of deaths in these patients. Incidences of BC tend to be more prevalent in high-income countries compared to low-income ones [71]. Disparities in education, economic status, environmental conditions, dietary habits, lifestyle factors, and cultural practices contribute to variations in BC rates worldwide. Globalization and expanding economies could potentially exacerbate the incidence of BC. Projections indicate that by 2040, the burden of BC could increase to over 3 million new cases and 1 million deaths annually [72].

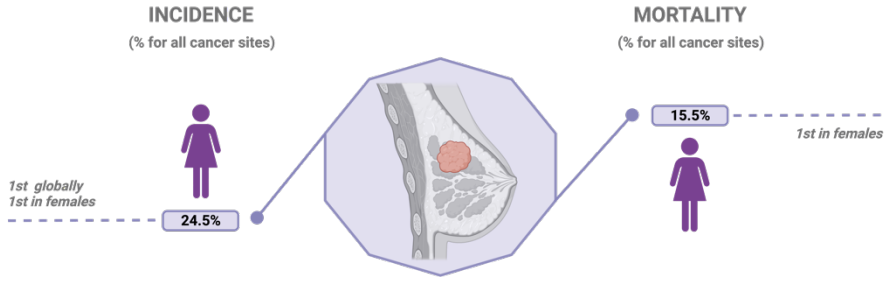

Figure 2. **Incidence and mortality of breast cancer in 2020.** BC is the most diagnosed cancer in women and globally, and it is the leading cause of cancer-related deaths in women.

In Europe, the cumulative risk of a BC diagnosis before the age of 75 stands at 8%, while the risk of death from BC before the same age is 1.6%. Despite the rising incidence, modest reductions in BC mortality are projected (-4.63% less between 2018 and 2023) [16], and the 5-year relative survival rate is around 80% [73]. This may be due to effective diagnosis, better treatment, and disease management, as well as high participation in organized mammography screening programs. BC is estimated to be the most prevalent cancer diagnosed across all age groups in Europe (35% of cases in women up to 44 years, 34% for those aged 45–64, and 23% among women aged 65 years or older). It also ranks as the primary cause of cancer-related mortality in each of these age categories (25%, 19%, and 15%, respectively) [17].

1.2.2. Development

BC arises from genetic changes in tumor suppressor and oncogenic genes that drive breast epithelial cells toward a malignant phenotype [71]. The development of BC involves dynamic changes in the mammary gland, influenced by genetic and hormonal factors [74]. These genetic alterations

and hormonal influences contribute to the initiation and progression of BC, leading to the complex and multifaceted nature of the disease [74,75].

There are several factors, some of them potentially modifiable [76,77], that are associated to the development of this disease, including environmental, reproductive (notably, early menarche escalates risk; conversely, early first childbirth and increased full-term births mitigate it), and lifestyle factors (for instance, positive correlation with BC exists for increased body mass index (BMI).

Inheritance plays a relatively minor role; autosomal dominant mutations in *BRCA1* and *BRCA2* genes, constituting about 5% to 10% of all BC diagnoses [77]. These genes function in DNA double-strand break repair via homologous recombination. A deletion mutation in either corresponds astonishingly to a 10-fold increase in BC risk. Approximately 2% of BC patients carry a *BRCA1* mutation. Such cases are often triple negative and are diagnosed at a younger age than sporadic BC. A 60% of women with a *BRCA1* mutation will develop BC by age 70. On the other hand, *BRCA2*-associated tumors tend to be of higher grade, with approximately 55% of carriers developing BC by age 70 [76]. Mutations in other genes, such as *TP53* and *PTEN*, also elevate the risk of BC [71].

1.2.3. Classification

BC is a complex disease with different types, each having unique characteristics. BC subtypes are classified based on the immunohistochemical expression of hormone receptors: estrogen receptor positive (ER+), progesterone receptor positive (PR+), human epidermal growth factor receptor 2 positive (HER2+), and triple-negative breast cancer (TNBC), which lacks the expression of any of these receptors [78]. Concurrently, classical clinicopathological variables including the proliferation

index (Ki67), tumor size, histological grade, and nodal involvement have been used for patient prognosis and management. The proliferative activity measured by Ki67 mirrors the cancer's aggressiveness, therapeutic responsiveness, and time to recurrence. Elevated Ki67 expression correlates with decreased survival rates [79].

Of fundamental diagnostic importance is the estrogen receptor (ER), prevalent in approximately 70-75% of invasive breast carcinomas. The progesterone receptor (PR), noted in over 50% of ER+ cases, is also of significance. Augmented PR expression associates positively with overall survival (OS), time to recurrence, time of treatment efficacy or progression. Conversely, reduced PR levels generally associate a more aggressive disease course, poor recurrence, and unfavorable prognosis [80].

Approximately 15-25% of BC cases involve HER2 overexpression, and its status is important for the choice of treatment. HER2 overexpression materializes as an early event during breast carcinogenesis. HER2 amplification leads to disproportionate activation of proto-oncogenic signaling pathways, resulting in uncontrolled cell proliferation [81,82].

Based on their gene expression patterns, BC can be classified into the following molecular subtypes: Luminal, HER2-positive, and TNBC [83] (**Table 1**). Luminal BC, which makes up approximately two-thirds of all cases, includes the subtypes Luminal A and Luminal B. Luminal A is identified by the presence of hormonal receptors and the absence of HER2, along with a low proliferation index [ER+ | PR+ | HER2- | Ki67-]. Clinically, these cases are low-grade, slow-growing, and have a favorable prognosis, with a lower incidence of relapse and higher survival rates [76,81]. On the other hand, Luminal B exhibits a high proliferation index, hormonal receptor expression, and the potential presence of HER2 [ER+ | PR+/- | HER2+/- | Ki67+]. These cases are of higher grade and have a less favorable prognosis [76,81]. The HER2

overexpression subtype constitutes 10–15% of BC and is characterized by a lack of hormonal receptors and an overexpression of HER2 [ER- | PR- | HER2+]. These cases have a worse prognosis compared to Luminal tumors and require targeted treatments aimed at the HER2/neu protein [81,84]. Lastly, TNBC does not express any of the aforementioned markers. It accounts for about 15-20% of all BC and is characterized by its aggressiveness, early relapse, high proliferation rate, DNA repair gene alterations, and increased genomic instability [81,85]. In recent years, the integration of new molecular information has enabled the identification and characterization of new subtypes of TNBC, including basal-like and normal-like [86,87].

Table 1. Breast cancer subtypes according to their expression patterns.

Subtype	Frequency (%)	ER	PR	HER2	Ki67	Prognosis
Luminal A	50	+	+	-	-	Good
Luminal B	20	+	+/-	+/-	+	Middle
HER2+	15	-	-	+	+	Middle/Bad
TNBC	15	-	-	-	+/-	Bad

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; Ki67, marker of proliferation Ki-67.

1.2.4. Screening

Early detection of BC through screening is crucial for improving the chances of a cure, as a smaller tumor size is a predictor of lower disease stage and better outcomes [88,89]. Current medical efforts are focused on reducing BC mortality through prevention, increased awareness (including self-breast examination), and early detection with screening methods [89].

Randomized controlled trials and observational studies support the effectiveness of mammography as a screening technique, reporting reductions in BC mortality ranging from 25% to 40% [89,90]. In the European

Union, it is recommended to undergo mammography screening for women aged 50 to 69, as this age group has shown the highest reduction in mortality, with a more limited impact among women aged 40 to 49 [88]. For women with a family history of BC or those at high risk, annual screening with Magnetic Resonance Imaging (MRI) in combination with mammography is recommended [88,90]. In addition to mammography and MRI, clinical trials are evaluating the use of ultrasound as a supplementary screening method [88,89].

1.2.5 Treatment and resistance

The primary aims of treating nonmetastatic BC include eliminating tumors from the breast and nearby lymph nodes while preventing metastatic recurrence. Local treatment involves surgical removal of tumors, axillary lymph node sampling, and potentially postoperative radiation [91]. Systemic therapy can be given before (neoadjuvant) or after (adjuvant) surgery, dependent on the BC subtype. Hormone receptor-positive (HR+) tumors typically receive endocrine therapy, sometimes combined with chemotherapy. HER2+ tumors receive trastuzumab (anti-HER2) immunotherapy along with chemotherapy, and if HR+ as well, endocrine therapy [92]. Current treatment options for triple-negative BC are limited to surgery and adjuvant chemotherapy, but targeted therapies and immunotherapy could play a key role in the future [93].

Metastatic breast cancer (mBC) treatment aims to extend life and alleviate symptoms, but cure remains rare. Similar systemic therapy categories as in nonmetastatic cases are employed, with surgery and radiation mainly used for symptom relief [91]. In metastatic HR+/HER2− BC, early treatment should be endocrine therapy based, typically with incorporation of a cyclin-dependent kinase (CDK) 4/6 inhibitor [94,95].

Newer targeted therapies for the treatment of mBC such as poly(ADP-ribose) polymerase-1 (*PARP1*) inhibitors for patients with *BRCA1* or *BRCA2* mutations [96], phosphatidylinositol 3-kinase (*PI3K*) inhibitors for HR+/HER2− and *PIK3CA*-mutated BC [97] and anti-PD-L1 and anti-PD-1 antibodies especially for TNBC patients [98,99], are being evaluated.

Although therapies for breast cancer have significantly decreased the risk of disease recurrence and mortality, the existence of both *de novo* and acquired resistance mechanisms limits the effectiveness of treatments, presenting a substantial challenge for patient management [100-102].

1.3 Staging

Numerous cancer-staging systems are used worldwide, but the TNM system stands out as the most clinically useful staging system. It was developed by the American Joint Committee on Cancer (AJCC) in partnership with the Union for International Cancer Control (UICC).

The Eighth Edition of the AJCC Cancer Staging Manual, published in October 2016, is a comprehensive compilation of current information on the staging of adult cancers at various anatomic sites [103]. While based on the extent of anatomic disease, the Eighth Edition also takes a more modern personalized approach. This shift involves the integration of biological and molecular markers to guide treatment decisions at both the population and individual levels [104].

This system categorizes cancers based on the size and how far the main tumor has grown into adjacent tissues (T), whether regional lymph nodes are affected (N), and the presence or absence of distant metastases (M) [105]. The primary tumor's categories go from T0 to T4, depending on whether the tumor exists, its size, and how far it has grown. For regional lymph nodes, the categories range from N0 to N3, which depends on whether regional nodes

are invaded and how extensive the invasion is. Finally, the categories for distant metastases are either M1 or M0, indicating whether there are distant metastases present or not. Additionally, factors like the grade of cancer cell differentiation and evidence-based factors that predict prognosis and outcomes can also help determine the stage of cancer [103,105].

Importantly, AJCC 8th edition emphasis on personalized approaches for diagnosing and treating CRC. It delves into molecular markers, including mutations like mismatch repair deficiency, microsatellite instability, and RAS pathway mutations (*KRAS*, *BRAF*, *NRAS*) [106]. The update also stipulates determining the status of ER and PR receptors, along with HER2 expression, for all breast invasive carcinomas whenever possible [107]. This enhances the understanding of the individual characteristics of cancer.

2. The metastatic process

Metastasis refers to the spread of cancer cells from the primary tumor to distant sites in the body. This process is of great importance in the development of cancer, profoundly influencing both the prognostic prospects and the therapeutic pathways available to people affected by this disease. Unfortunately, nowadays, the metastatic disease still remains incurable and contributes to more than 90% of cancer-related deaths [108].

Metastasis involves a sequence of steps that are closely linked. It begins with cancer cells emerging from the primary tumor and infiltrating adjacent stromal tissue (**local invasion**). These invading cells then penetrate the microvasculature of the blood and lymphatic systems (**intravasation**). Subsequently, the must survive and travel largely through the bloodstream to microvessels in distant tissues and leaves the bloodstream (**extravasation**). Subsequent phases involve acclimation of metastatic cells to the new microenvironment to improve their viability and proliferative capacity, and

finally the establishment of a secondary macroscopic tumor (**colonization**) [109,110].

Local invasion is the first step in the metastatic cascade. The main mechanism that allows this step is the epithelial-mesenchymal transition (EMT), a genetic reprogramming process that mediates the conversion of cells from epithelial to mesenchymal phenotype and gives cells a marked increase in motility, invasiveness, and the ability to degrade components of the extracellular matrix (ECM) [108]. All this together gives the cells an invasive phenotype. EMT is actually a group of cell-biological programs that are coordinated by a number of EMT-inducing transcription factors (EMT-TFs), notably SNAIL, TWIST, and ZEB [108]. Mechanistically, these EMT-TFs are regulated at the transcriptional level by DNA methylation, histone modifications, and RNA-mediated epigenetic regulation [111].

Hypoxic signaling mediated by hypoxia-inducible transcription factors (HIFs) induces migratory and invasive tumor cell behaviors via multiple mechanisms, including the activation of the same transcription factors, promoting EMT and epithelial plasticity [112]. Furthermore, alterations in the cytoskeletal structure within cancer cells, coupled with the influence of adhesive interactions, the secretion of extracellular matrix metalloproteinases (MMPs) [113], and the involvement of cathepsins [114], collectively propel the process of cancer cell invasion and migration through the stroma. HIF signaling also promotes invasion by upregulating proteolytic enzymes like MMPs, cathepsins, lysyl oxidases, and prolyl-4-hydroxylases (P4H) [112]. Cancer cells exhibit various migration patterns, including solitary migration, wherein individual cells carve a route through the extracellular matrix, locomotion along collagen fibers, and coordinated migration as clusters that forge ahead from tumor invasion front [114].

Cancer cells that successfully infiltrate the surrounding stromal environments of primary tumors can **intravasate** either into blood or lymphatic vessels. Once these tumor cells are within the lumina of these vessels, they are known as circulating tumor cells (CTCs) [110]. While a portion of CTCs travel alone, others travel together in clusters. Within CTC clusters, elements of the original microenvironment, such as stromal cells and immune components, may contribute to the heterogeneity of the pool and enhance its survival. These clusters present a greater potential for metastasis compared to solitary cells. [113]. Moreover, the clustering of CTCs triggers specific modifications in DNA methylation, promoting characteristics associated with stemness and metastasis formation [115].

Upon entering circulation, these tumor cells confront an array of stress factors. These encompass loss of attachment to their substrate, exposure to hydrodynamic flow, and shear stress [114]. Additionally, carcinoma cells circulating in the bloodstream become susceptible to immune attacks, particularly from NK cells which target them for elimination [114]. Interestingly, interactions between circulating carcinoma cells and other cell types within the bloodstream can paradoxically facilitate their survival and eventual **extravasation** at distant sites [108]. These interactions notably involve platelets, neutrophils, macrophages, and endothelial cells.

Arresting in the capillaries, CTCs are faced with two pathways: they can extravasate the endothelium in a process of transendothelial migration or grow within the vessel limits before finally colonize a premetastatic niche (PMN) [108,113].

During the final phase of the metastatic cascade known as **colonization**, cells that extravasate at the target site must undergo adjustments to accommodate an environment that diverges from their original location in terms of ECM composition [116], stromal cells, soluble factors,

vascularization, and metabolic as well as nutritional elements. However, it is known that the target site's environment undergoes modifications before the arrival of tumor cells, preparing distant sites to be more suitable for their establishment. Various secreted tumor-derived factors along with bone marrow-derived cells collaborate to trigger the development of the PMN [113], where tumor cells then proliferate. These factors encompass signals that promote cell division and evasion of growth suppressors, protection against apoptosis and immune response, induce angiogenesis, and reconfigure energy metabolism [113].

In addition to tumor-derived factors, exosomes play a pivotal role in metastasis. They influence the pre-metastatic microenvironment by enhancing the expression of pro-inflammatory genes and releasing immunosuppressive cytokines [117]. Additionally, exosomes stimulate phenotype-specific differentiation and attract distinct types of stromal cells, further facilitating the process [118].

Although a substantial number of tumor cells enter the bloodstream, the metastatic process remains highly inefficient [114]. Tumor cells need the capability to withstand the harsh conditions of the bloodstream, successfully extravasate, and then adapt to their new tissue and microenvironment. Furthermore, the mere survival of tumor cells in their new environment does not guarantee their subsequent proliferation to generate metastases [114].

3. Gene variants

The human genome consists of 6,200 Mbp of DNA in a diploid genome organized into 23 pairs of chromosomes. DNA strands are constructed from nucleotides, which are units containing one of these four nucleobases: cytosine (C), guanine (G), adenine (A), and thymine (T). Although the sequence of the human genome has been almost completely

determined by DNA sequencing, its full understanding remains incomplete [119]. The culmination of the Human Genome Project in 2004 marked a new phase in human genetics, ushering in an era of exploring the entirety of the genome [120]. Various global initiatives, including the HapMap Project and the 1000 Genomes Project, were subsequently launched to collect data about extensive collections of reference human genomes [121]. With respect to the reference sequence of DNA, genome of individuals and populations may undergo variations leading to the presence of gene variants. According to the frequency in the population, a gene variant can be classified as single-nucleotide polymorphisms (SNPs), with a frequency > 1%, or as mutation, when the frequency is < 1% [122]. Rare genetic variations generally exert more pronounced impacts on diseases when compared to common genetic variations [119,123]. Recent advancements in technology, such as NGS techniques, and the elucidation of diverse forms of individual genetic diversity [124], have opened doors to highly detailed analyses of the human genome's involvement in various diseases, including cancer [125,126].

It is important to distinguish between two types of gene variants: germline and somatic. Germline DNA is the genetic material present in every cell of an individual and is inherited from both parents [127]. Germline variants can be linked to an increased risk of cancer and follow a hereditary pattern [128]. However, it is crucial to note that only a small percentage of cancer cases is associated with these inherited genetic changes [129].

On the other hand, somatic variants exist exclusively within cancer cells [130]. These types of variants are responsible for the majority of cancers [131]. Somatic variants can activate oncogenes (genes promoting cell growth), inactivate tumor suppressor genes (genes preventing cell overgrowth), disrupt molecular pathways regulating cell growth, and allow tumor cells to escape from immune system control, leading to cancer development [132,133]. Somatic mutations arise before and during tumor

development, leading to the evolution of different clones of tumor cells [134]. This tumor heterogeneity makes treatment more challenging, as it implies that some clones may be more invasive or exhibit resistance to certain therapies [132,134].

3.1 Types of gene variants

The main types of gene variants include single nucleotide variants (**SNVs**), insertion-deletions (**indels**), gene **fusions** and copy number variants (**CNVs**) (**Figure 3**).

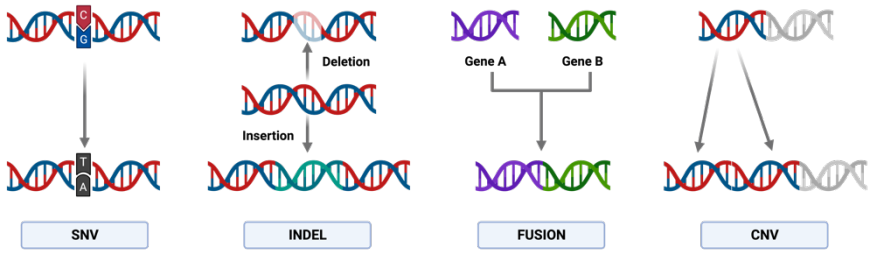

Figure 3. Main types of gene variants. Schematic representation of single nucleotide variants (SNVs), insertion-deletions (indels), gene fusions and copy number variants (CNVs).

A **SNV** is a type of genetic variant involving a single nucleotide change in DNA, often referred to as a point mutation. Detecting a single nucleotide mismatch within a given nucleic acid sequence can present significant technical challenges [135]. SNVs arise at a rate of approximately 1 per every 100–300 bases within the human genome, a phenomenon that could hold notable clinical and biological implications [136]. This nucleotide change can lead to a missense mutation, which results in coding for a different amino acid in the protein sequence, potentially affecting its function. On the other hand,

nonsense mutations introduce a premature stop codon, leading to the production of a truncated and typically non-functional protein [137].

Indels encompasses two types of genetic variants: insertions and deletions. Insertions involve the addition of one or more nucleotides to a genetic sequence, while deletions imply their removal [138]. These gene variants can occur anywhere in the genome and can have various effects on gene function and the resulting protein [138,139]. Depending on the location and size of the indel, they can cause frameshifts. These frameshift variants can have very severe effects on amino acid sequence, because a single shift impacts all triplets following downstream [140], which can lead to the production of an abnormal protein or the complete absence of a functional protein [139,141].

Gene fusions, also known as chromosomal rearrangements or gene rearrangements, occur when two previously separated genes become linked together, creating a hybrid gene [142]. This fusion can result from structural alterations in the DNA, such as chromosomal translocations, inversions, or other rearrangements [143,144]. Gene fusions can lead to the formation of abnormal proteins or regulatory elements [145,146].

CNVs refer to a type of genetic variant in which the number of copies of a particular DNA segment varies among individuals or compared to a reference genome [147]. CNVs can involve deletions, duplications, or rearrangements of relatively large sections of DNA, ranging from a few hundred to thousands of base pairs [148,149].

3.2 Gene variants in cancer: CRC and BC

SNVs occurring within coding regions of genes are closely linked to the origins of various somatic disorders like cancer [150,151]. Some SNVs related to the development of CRC include the gene variants of *KRAS*, such

as G12V and G12D, which are associated with therapy resistance and poor survival [152-154]. In addition, the presence of the gene variant *BRAF* V600E in CRC is associated with a more aggressive tumor phenotype [155-157]. In the case of BC, *PIK3CA* H1047R has been identified in various BC subtypes and is linked to cellular proliferation and survival mechanisms [158-160]. Similarly, *HER2* V777L is associated with more aggressive forms of BC and drug resistance [158,161].

Frameshift indels in the *TGFBR2* and *ACVR2A* genes can disrupt TGF-beta signaling pathways, contributing to the development and progression of CRC. These indels have been detected in the majority of MSI+ CRC [162,163]. Although not many indels have been described in BC, some of them have been identified as pathogenic in *BRCA2* [164], and indels in the *GATA3* gene are associated with increased tumor growth [165].

Gene fusions are uncommon in CRC, however, a small subset of patients exhibit gene fusions involving *ROS1* and *ALK* genes [166-168], which could be associated with tumor growth and aggressiveness. Regarding BC, the most frequent fusions involve genes, such as *ESR1*, *ETV6*, *MYB*, *NOTCH*, and the FGFR family, among others [169,170], correlating with more aggressive subtypes of this disease.

CNVs can disrupt normal gene dosage [149], leading to altered gene expression and potentially contributing to cancer. In CRC, certain CNVs are frequent, like amplifications in the *MYC* genes [171,172], which constitute a common mechanism of chemotherapy resistance; and amplifications in the *HER2* gene [173-175], which is associated with a worse prognosis and resistance to anti-EGFR therapy. Similarly, in BC, CNV-type variants also occur. For instance, *HER2* gene may have CNVs , which are associated with high invasiveness, a high risk of recurrence, rapid progression, and poor outcomes [176-178]. The presence of CNVs in other genes, such as

BRCA1/*BRCA2,* are linked to a higher likelihood of tumor development [179,180], and in the case of *FGFR1*, they are associated with a poorer response to endocrine therapy [181,182].

4. Epigenetics

The term “epigenetics” refers to the study of changes in the activity and expression of genes that are hereditary but do not involve alterations to the DNA sequence [183]. This mechanism plays a crucial role in regulating gene expression for numerous biological processes, and importantly, it is a reversible process [183,184].

Most of these heritable changes manifest during cell differentiation. These changes are also mitotically stable, meaning they are maintained through multiple rounds of cell division [185]. This allows cells to develop distinct identities while containing the same genetic content. These mechanisms hold a pivotal role in regulating gene expression in many biological processes, including tissue differentiation, embryonic development, imprinting, X-chromosome inactivation, and the silencing of repetitive elements [184,186].

Epigenetic modifications are closely intertwined with factors such as age and lifestyle. Lifestyle components like diet, physical exercise, stress, and consumption of toxic substances have a profound impact on these modifications [187-190]. Additionally, environmental factors like air pollution and chemical exposure also contribute to epigenetic changes [191,192].

4.1 The epigenetic machinery

The epigenetic machinery (**Figure 4**) exhibits several levels of regulation and encompasses multiple mechanisms, including DNA methylation and hydroxymethylation, post-translational modifications of

histones, nucleosome positioning, and non-coding RNAs (ncRNAs) [193-195]. Specifically, DNA methylation stands out as one of the extensively studied epigenetic factors, showing significant implications in the development and progression of cancer [196].

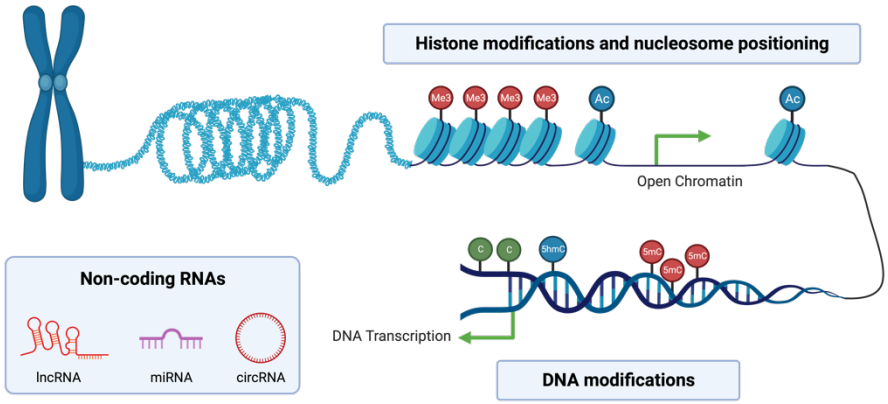

Figure 4. The epigenetic machinery. Representation of the fundamental epigenetic mechanisms: I) DNA modifications (methylation and hydroxymethylation), II) post-translational modifications of histones and nucleosome positioning, and III) non-coding RNAs. Me3, histone trimethylation; Ac, histone acetylation; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; C, cytosine; lncRNA, long non-coding RNA; miRNA, microRNA; circRNA, circular RNA.

4.1.1 DNA methylation

DNA methylation is a process involving the covalent modification of DNA through the addition of a methyl group (CH_3) to the 5´ carbon of cytosines within cytosine-phosphate-guanine (CpG) dinucleotides, resulting in the formation of 5-methylcytosine (5mC) [185,187]. This process is under the enzymatic control of DNA methyltransferase (DNMT) enzymes, namely DNMT1, DNMT3A, and DNMT3B (**Figure 5**). These enzymes catalyze the

transfer of methyl groups from S-adenosyl-L-methionine (SAM) to cytosines [197,198].

The formation of the DNA methylation profile entails a *de novo* methylation process, regulated by the enzymes DNMT3A and DNMT3B. Conversely, DNMT1 plays a crucial role for maintaining methylation patterns during cell division, ensuring the fidelity of inheritance [184,197]. While DNA methylation predominantly takes place in specific regions of the genome, such as gene promoters, characterized by a high density of CpG dinucleotides known as CpG islands (CGIs) [184,199], it also manifests in various other genomic areas. This broader methylation distribution serves to maintain chromosome structure and integrity, while also preventing potential damage from mobile genetic elements [187,198,200].

In relation to CGIs, regions adjacent to them are also susceptible to methylation. Regions located up to 2 kb upstream or downstream from CGIs are referred to as CGI shores, which exhibit lower CpG density [201,202]. The methylation of these CGI shores is closely linked to transcriptional inactivation. Interestingly, most of the tissue-specific DNA methylation appears to take place not at CGIs, but rather at CGI shores [187,201]. Moreover, regions known as CGI shelves, are situated between 2 and 4 kb from CGIs, while the open sea regions are located at a distance exceeding 4 kb from CGIs [202,203].

DNA methylation can be enhanced (hypermethylation) or repressed (hypomethylation) in different regions of the genome, playing an important role in regulating gene expression [196,204,205]. Typically, the methylation occurring at CGIs in a gene promoter is inversely correlated with its transcriptional activity. This links methylation to the suppression of gene expression, whereas the absence of methylation is associated to active gene expression [197,199]. DNA methylation has the capacity to inhibit gene

expression through the recruitment of methyl-CpG-binding domain proteins or can also directly inhibit the recruitment of DNA-binding proteins to their target sites [199,206]. Conversely, the absence of methylation creates a chromatin structure that promotes the gene expression [199].

DNA methylation is a reversible epigenetic mechanism that can be reversed by TET (ten-eleven translocations) enzymes (**Figure 5**), which facilitate the transformation of 5mC to 5-hydroxymethylcytosine (5hmC), regulating gene expression patterns through DNA hydroxymethylation [207,208]. Furthermore, the dynamic interplay of DNA methylation with hydroxymethylation, modulated by TET enzymes, emerges as a pivotal regulator, allowing precise fine-tuning of gene expression in response to diverse internal and external signals, thus underscoring its central role in cellular homeostasis and disease [207].

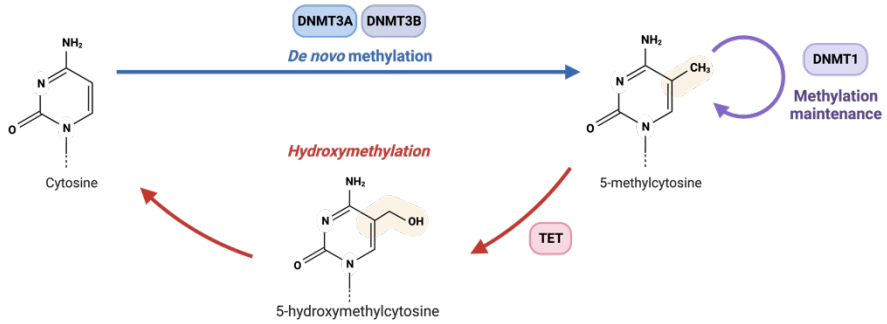

Figure 5. DNA methylation/demethylation process. Cytosines in DNA can be methylated *de novo* by the enzymes DNMT3A and DNMT3B. Additionally, the enzyme DNMT1 is responsible for maintaining methylation during cell division. In contrast, the TET enzyme can reverse this process through hydroxymethylation.

It is also important to note that epigenetic-based drugs (epidrugs), including DNA methyltransferase inhibitors (DNMTi) like 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine), holds therapeutic potential by reversing aberrant gene methylation [208,209].

4.1.2 Nucleosome positioning and histone modifications

Nucleosomes, the fundamental functional units of chromatin, are composed of 147 DNA base pairs wrapped around an octamer containing two copies each of four core histone proteins (H2A, H2B, H3, and H4) [210]. Histones, with a primary structure of a globular domain and an N-terminal tail extending from the nucleosome, facilitate DNA binding, protein interactions, and the covalent attachment of molecules [211]. Post-translational modifications (PTMs) namely acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation occur mainly in the histone tail within nucleosomes and are subject to enzymatic regulation [211,212]. These play a crucial role in normal development and are associated to processes such as transcriptional regulation, DNA repair, replication, alternative splicing, and chromatin condensation [211].

Histone PTMs, including processes as acetylation and methylation, are implicated in either activating or silencing genes [213]. Enzymes like histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) regulate the balance of these PTMs [195]. Nucleosome positioning has a significant impact on chromatin accessibility, influencing the binding of RNA polymerase and transcription factors to regulatory elements, particularly around transcription start sites (TSSs) [187,214]. The addition or removal of a nucleosome before the TSS is associated with gene activation or repression, respectively [215]. Chromatin-remodeling proteins play a crucial role in controlling nucleosome positioning, thereby influencing gene expression. Active genes often show

nucleosome-depleted regions (NDR) around their TSS, enhancing the accessibility of transcription regulatory proteins [216].

In the context of cancer, disruptions commonly occur in epigenetic mechanisms related to both nucleosome positioning and PTMs [213,217]. Mutations affecting histone-modifying enzymes can play a role in the development of cancer by disturbing the balance of these PTMs [213]. Nevertheless, specific FDA-approved epidrugs have demonstrated the ability to reverse histone modifications in certain tumor types, presenting a promising avenue for therapeutic intervention [218,219].

4.1.3 Non-coding RNAs

Non-coding transcripts, comprising around 98% of the transcriptome, play a crucial role in gene regulation [220]. These transcripts are broadly categorized based on their nucleotide length into: (i) small ncRNAs (sncRNAs), which are shorter than 200 nt and include microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs); and (ii) long ncRNAs (lncRNAs), which are longer than 200 nt and encompass long intergenic ncRNAs (lincRNAs) and long intronic ncRNAs (intronic lncRNAs) [195,221]. Some ncRNAs, such as enhancer RNAs (eRNAs) and circular RNAs (circRNAs), exhibit variable lengths, and may belong to both categories [220].

Among sncRNAs, miRNAs, single-stranded molecules ranging from 18 to 25 nucleotides, stand out as the most widely studied [220]. They regulate gene expression post-transcriptionally by either blocking transcription or triggering mRNA degradation [222]. Consequently, a single miRNA has the capacity to regulate the expression of numerous genes [223].

Recent findings highlight that lncRNAs constitute the majority of non-coding transcripts [224]. Despite lacking the ability to code proteins, lncRNAs

share certain mRNA-like characteristics, including multiexonic gene structures, polyadenylation, the presence of 5' caps, and transcription by RNA polymerase II [225]. They play a crucial role in gene expression, particularly in splicing and transcriptional regulation by forming complexes with transcription regulatory proteins [225,226]. Furthermore, lncRNAs may act as sponges (sponge effect), effectively capturing and sequestering miRNAs. This interaction influences the capacity of miRNAs to inhibit gene expression [226].

4.2 Interplay between epigenetic mechanisms

Epigenetic modifications form a complex regulatory network through dynamic interactions among various components. The dynamic and adaptable nature of chromatin structure is essential for its timely and spatial regulation [227]. The organization of DNA into chromatin is highly influenced by the interplay of epigenetic mechanisms, including DNA methylation, histone modifications, and ncRNAs [227,228].

For example, misregulation in DNA methylation can occur if DNMTs are not targeted correctly, with histone modifications playing a crucial role in guiding this process [229]. Recent research highlights the contribution of sncRNAs to alter the DNMTs activities and impact DNA methylation patterns [230]. On the other hand, methylation can also affect non-coding genes, thus regulating the expression of ncRNAs [231].

Emerging evidences have indicated the regulatory crosstalk between lncRNAs and DNA methylation [232]. The transcription of lncRNAs is regulated by promoter methylation, similar to protein-coding genes [231,233]. Additionally, lncRNAs can interact with enzymes involved in DNA methylation, influencing the methylation pattern of downstream genes and thereby regulating their expression [234]. This interplay underscores the multifaceted nature of epigenetic regulation.

4.3 DNA methylation and cancer

Cancer initiation and proliferation are the result of a complex interplay between epigenetic and genetic events, with increasing recognition of the important role attributed to epigenetic modifications in cancer research [235,236]. Particularly noteworthy is the frequent appearance of altered methylation patterns, closely related to the onset and progression of diseases [237]. Consequently, this phenomenon is being investigated as a promising biomarker for the early detection of malignancies. These methylation patterns exhibit tissue-specific characteristics, allowing DNA methylation to be used as a tool to classify different types of cancer [238].

Disruption of normal epigenetic processes is an established hallmark of the initiation and progression of tumorigenesis [235,237]. Two main categories of DNA methylation changes emerge in this context when tumor cells are compared with non-cancer cells [239] (**Figure 6**): i) Generalized genome-wide hypomethylation, which is associated with oncogene activation and genomic instability. This instability includes chromosome instability, and a higher prevalence of aneuploidies, both of which are recurring features in cancer genomes [236,238];ii) *De novo* hypermethylation in CGIs. In particular, hypermethylation of promoter CGIs is a frequent phenomenon that contributes to the inactivation of tumor suppressor genes (TSG), which control cell proliferation [236,239].

In addition to CGIs, recent studies indicate that CGI shores often undergo DNA hypermethylation in cancer. This phenomenon was initially identified in human colon cancer [240] and subsequently confirmed in other cancer types, including breast and lung tumor samples [241].

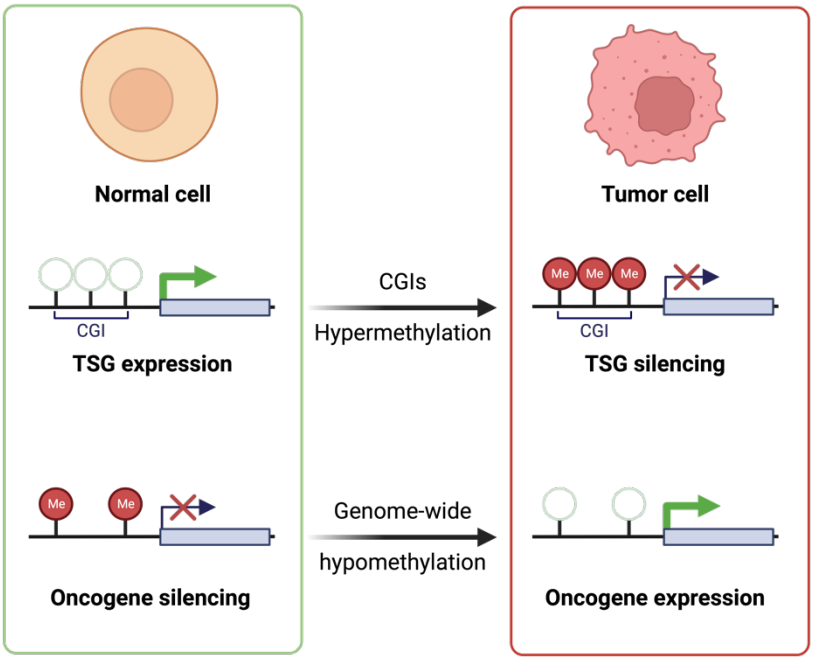

Figure 6. Role of DNA methylation in tumorigenesis. In normal cells, the promoter CGIs in TSGs are hypomethylated and can be expressed, while promoters of oncogenes are hypermethylated and not expressed. In cancer cells, TSGs are silenced by promoter hypermethylation of CGIs, while oncogenes are actively transcribed due to promoter hypomethylation. CGIs, CpG islands; Me, DNA methylation; TSG, Tumor suppressor gene.

4.3.1 DNA methylation in CRC

DNA methylation plays a significant role in the development and progression of CRC, presenting 10–40% lower levels of overall methylation compared to normal colon tissue [242]. This phenomenon mainly arises from the loss of methylation within repetitive elements like LINE-1, which is implicated in enhancing genomic instability and contributing to the initiation of CRC [243,244]. In addition to this global hypomethylation, CRC shows

hypermethylation in a specific subset of gene promoters [245]. It is worth noting the CIMP subgroup that presents a higher methylation rate in particular genes [246,247].

In CRC, promoter methylation affects critical components of signaling pathways, such as Wnt pathway. Within this context, the promoter of the tumor suppressor gene *APC* is hypermethylated in approximately 20% of CRCs [248,249]. Furthermore, methylation-associated downregulation is observed in Wnt target genes including receptor components (*LGR5*), negative regulators (*APCDD1*, *DKK1*, and *AXIN2*), and downstream effectors of the pathway (*ASCL2*), contributing to CRC and linking to poorer outcomes [250-252]. In addition, promoter methylation of *RASSF* gene family is very prevalent in CRC. These genes modulate the growth inhibitory and proapoptotic effects, thus their inactivation promotes RAS-driven tumorigenesis [253-255]. Similarly, hypermethylation of the promoter region of the *SEPT9* gene inhibits its expression, compromising its tumor suppressor functions. *SEPT9* expression decreases when colorectal adenoma progresses to CRC and exhibits significantly lower expression compared to normal epithelial cells [256,257]. Additionally, approximately 20% of MSI and 2% of MSS CRCs experience promoter methylation and loss of expression of *PTEN*, a key negative regulator of PI3K signaling [258,259]. Moreover, the transcription factors *GATA4* and *GATA5* undergo frequent promoter hypermethylation and transcriptional silencing in most CRCs. This epigenetic alteration inhibits TSG, contributing to tumor progression and suggesting their potential as non-invasive biomarkers for the detection of colorectal cancer [260-262].

4.3.2 DNA methylation in BC

DNA methylation also has relevant implications in the development and progression of BC. Similar to CRC, DNA methylation changes in BC can

lead to altered gene expression patterns that contribute to tumorigenesis. These methylation changes may be different among BC subtypes. For example, Luminal BC shows higher methylation frequency than TNBC [263], and ER+ tumors have higher overall DNA methylation than ER- tumors [264].

Regarding TSGs, *BRCA1*, which is associated with hereditary BC, can be affected by promoter hypermethylation, leading to decreased expression of the gene [265]. *BRCA1* is involved in DNA repair and maintenance of genomic stability. Methylation-driven inactivation of *BRCA1* can increase the risk of BC development [266,267] and is also associated with treatment response [268]. Methylation can also influence the expression of hormone receptor genes, such as estrogen receptor (ER) and progesterone receptor (PR) genes [269]. Hypermethylation of these promoter regions can lead to reduced expression of the receptors, with consequences for the effectiveness of hormonal therapies against BC cells [269,270]. There are other genes regulated by methylation in BC that are linked to disease progression, prognosis, tumor aggressiveness, and response to various therapies. These include *RASSF1A* [271], *GSTP1* [272], *HIC1* [273,274], *CCND2* [275], *DAPK*, and *RARβ* [276]. Furthermore, the loss of *PTEN* function due to promoter hypermethylation is observed in nearly 40% of cases of invasive BC. This can trigger Akt pathway activation, apoptosis inhibition, and increased cell survival [263,277].

5. Liquid biopsy

Analysis of the molecular profile of tumors has become a central aspect of patient care and treatment options. Until now, tumor tissue biopsies continue to be the gold standard for performing these molecular tests [278]. However, there are several limitations attached to this procedure. The collection of tissue samples is a highly invasive process, and it is not always

available. Additionally, depending on the tumor location, the ability to obtain tumor tissues can also present challenges. Importantly, the existence of variability within the tumor (tumor heterogeneity) [279], can lead to unreliable results in biomarker identification, particularly when only one sample is analyzed. Furthermore, the presence of multiple tumor sites adds complexity to the molecular characterization of the disease [280].

In the last decade, liquid biopsy has gained importance as a transformative approach to overcome these existing limitations in cancer diagnosis and follow-up [195,281]. This non-invasive technique makes it possible to analyze the molecular composition of tumor-derived components that are present in different body fluids (**Figure** 7), allowing the identification of multiple types of biomarkers for the diagnosis, prediction of the course of the disease, and even the evaluation of the effectiveness of therapeutic interventions [282,283].

Liquid biopsy has revolutionized the management of cancer patients, offering a more complete and real-time understanding of the disease compared to traditional methods based on tissue biopsies [280]. Due to its minimally invasive characteristics, liquid biopsy provides the possibility to obtain serial samples throughout the patient's follow-up. This enables the observation of disease progression, facilitates the identification of optimal treatments, and facilitates therapeutic adjustments in response to the appearance of new molecular alterations that confer resistance [284]. Besides, early cancer screening is considered another potential application [285]. Consequently, liquid biopsy contributes to achieving a more personalized and precision medicine [286].

In addition, beyond the ease of sample collection, liquid biopsy has a major additional great advantage compared to tissue biopsy. It allows a

comprehensive assessment of the different cell subpopulations constituting the tumor, thus facilitating accurate tumor characterization [287].

The most used biological fluid in liquid biopsy is blood, but others, such as saliva, urine, ascitic fluid, pleural effusion and cerebrospinal fluid, can also be used [195,283]. These fluids receive tumor materials from both the primary tumor and the different metastatic sites. These materials encompass circulating tumor cells (CTCs) [183], circulating nucleic acids, including circulating tumor DNA (ctDNA) and circulating tumor RNA (mRNA and non-coding RNA) [288], as well as extracellular vesicles (EVs) that carry RNA, proteins, and DNA inside [289] (**Figure 7**).These circulating elements are an important source of promising non-invasive biomarkers for the study of cancer [195,290-292].

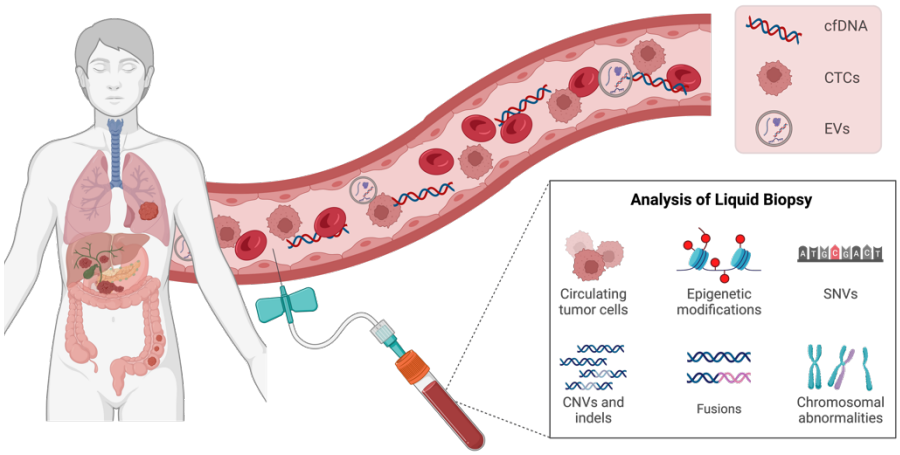

Figure 7. Components and applications of liquid biopsy. Liquid biopsy enables the analysis of tumor-derived material, such as cfDNA, CTCs and EVs from biological fluids, mainly blood.

5.1 Cell-free DNA

Cell-free DNA (cfDNA) refers to the fragmented DNA which is released from cells through different mechanisms and is present in biofluids (**Figure 8**). This phenomenon was initially observed by Mandel and Métais in 1948, who described the presence of DNA and RNA in the blood of patients and healthy individuals [293]. While cfDNA from healthy cells is usually present at modest levels in plasma, studies have indicated that the concentration of cfDNA can increase in response to conditions such as inflammation [294], surgical procedures, acute trauma [292], age and physical exercise [286,295]. In 1977, Leon et al. provided the first comprehensive characterization of cfDNA in the field of oncology. They noted elevated levels of cfDNA in cancer patients compared to healthy individuals, suggesting its potential utility as a non-invasive and dynamic diagnostic biomarker for identifying tumors and assessing their characteristics [296].

Most cfDNA fragments are around 180 bp in size, suggesting that cfDNA is associated with nucleosome, since the DNA that wraps around the histone octamers (147 bp) and the linker DNA (20 – 50 bp) represent the length of the cfDNA. This size suggests an apoptotic origin [297,298]. Likewise, larger fragments can be found in the bloodstream that may have their origin in necrosis processes [298,299]. It is also necessary to consider other possible sources of cfDNA that have been less studied so far, like pyroptosis, phagocytosis, mitotic catastrophe, and NETosis [300].

Other known mechanisms by which cells release nucleic acids into circulation is through active secretion. This is an intercellular communication process in which extracellular vesicles, such as exosomes, release the DNA they contain inside [289,301]. It has been described that a significant portion of cfDNA in the blood is associated with the membranes of exosomes, providing protection against nuclease-mediated degradation [302]. In

addition, some cells actively release DNA/RNA-lipoprotein complexes, called virtosomes [303], which do not have a membrane. These complexes can enter other cells causing immunological changes and transforming healthy cells into cancer cells [304].

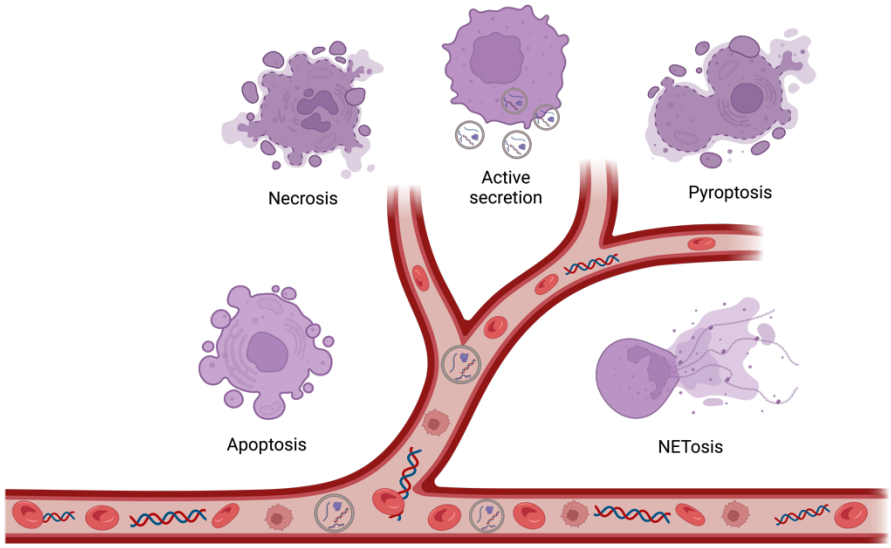

Figure 8. Mechanisms involved in the release of cfDNA. CfDNA can be released passively from cells (normal and tumor) as free fragments, or actively as part of extracellular vesicles and lipoprotein complexes.

In cancer patients, the highest levels of cfDNA in plasma result from the rapid division of tumor cells, resulting in substantial release of cfDNA due to cell necrosis or apoptosis [305,306]. The subset of cfDNA originating from tumor cells, known as circulating tumor DNA (ctDNA), typically represents a fraction ranging from 0.01% to 10% of the total cfDNA pool [307]. This percentage depends on factors such as tumor stage, vascularization, and metastases [308].

In addition, ctDNA preserves the genetic and epigenetic alterations present in the tumor, which allows its characterization [195]. Furthermore, cfDNA has a short half-life of between 16 minutes and a few hours, due to its rapid clearance through the liver and kidneys [309]. This allows ctDNA to be used to monitor disease status in real-time [290,310]. Advances in detecting and characterizing the ctDNA have facilitated the integration of this approach into clinical practice for the management of specific types of tumors, such as CRC [311,312] and BC [313,314]. Thus, the FDA has approved several liquid biopsy tests, including the methylation assays EpiProColon and Cologuard for CRC [315], and the Therascreen PIK3CA RGQ PCR assay for BC [313]. Recently, two NGS assays, namely FoundationOne Liquid CDx and Guardant360 CDx have also received FDA approval for clinical use to analyze gene variants of cfDNA, providing a valuable support for cancer management [316].

5.2 Analysis of epigenetic and genetic alterations in cfDNA

5.2.1 Pre-analytical considerations

The pre-analytical phase refers to all steps preceding the analytical process. Various pre-analytical variables, such as sample storage, the type of tube used for blood extraction, and cfDNA isolation methods, can influence the quality and quantity of cfDNA, affecting the performance of the molecular tests used for the analysis [317].

First, careful blood extraction is crucial to avoid hemolysis because cfDNA may be diluted by DNA released from nucleated blood cells [318]. Standard EDTA tubes are suitable for liquid biopsy analysis since EDTA anticoagulant prevent DNA degradation by chelating bivalent cations. However, processing the sample within a 4-hour timeframe is critical to prevent white blood cell lysis, reducing the quantity of non-tumor-derived DNA

[318,319]. Alternative tubes, such as Streck cfDNA BCT (Streck) or PAXgene Blood ccfDNA (Qiagen), incorporate preservative agents to reduce white blood cell lysis, allowing blood storage at room temperature for up to 7 days before centrifugation [318,319].

Plasma is the optimal choice for cfDNA isolation, as although serum may contain a higher DNA content, studies reveal that this is due to genomic DNA contamination derived from leukocyte lysis during the clotting process [318,320]. Plasma isolation involves a two-step centrifugation: an initial centrifugation not exceeding 1900 g for 10 minutes, followed by a subsequent centrifugation of the plasma at least at 6000 g to precipitate cellular debris. Resultant plasma samples can be stored at -80 °C until use [318,320].

Given the small quantity and highly fragmented nature of cfDNA in plasma, the use of commercial kits for extraction and purification is strongly recommended [318]. Finally, prior to subsequent analysis, it is imperative to quantify cfDNA using highly sensitive fluorometric methods [320].

5.2.2 Methodologies

The greatest challenges presented by the analyses of cfDNA are due to the short length of these molecules and their low concentration in plasma [321]. In addition, in the case of the study of cancer through cfDNA analysis, these challenges are compounded by the high sequence similarity between cancer-derived and healthy human DNA, the low representation of ctDNA in the cfDNA isolated from the sample and the large number of markers that must be simultaneously analyzed to achieve high clinical sensitivity [308,321]. Analysis of cfDNA allows detection of epigenetic (e.g., changes in DNA methylation) and genetic alterations (e.g., SNVs, CNVs and fusions, among others) [322,323].

Different techniques have been developed to study these alterations at both locus-specific and genome-wide levels [324] (**Table 2**). At the locus-specific scale, PCR-based techniques are the most commonly used, allowing for the detection of one or a few alterations with high sensitivity and specificity. Among these approaches, quantitative PCR (qPCR) is one of the most used techniques, but it has limited sensitivity (> 1%) for its application in liquid biopsy samples [325]. A variation of this technique, quantitative methylation-specific PCR (qMSP), allows analysis of methylation at the locus-specific level [183].

More sensitive techniques include digital droplet PCR (ddPCR), which achieves better sensitivities (0.01-1.0%) [322,326], and BEAMing (Beads, Emulsions, Amplification, and Magnetics), a PCR-based technique that combines PCR with flow cytometry and can detect alterations at levels as low as 0.01% [327]. In addition, methyl-BEAMing [328,329] and ddPCR [330], have also been used for methylation analysis in cfDNA.

For the genome-wide analysis, the techniques are usually based on NGS. Among these approaches, cfDNA can be analyzed using specific NGS panels that cover a high number of targeted genes [331,332] or a high number of CpG sites of multiple cancer-related genes [333]. Another option is analyzing the entire genome through whole genome sequencing (WGS) [334,335] or whole exome sequencing (WES) [334,336]. Among the most common NGS panels currently available for analyzing gene variants in cfDNA are the TruSight Oncology 500 ctDNA panel, which includes 523 cancer-associated genes (Illumina) [337], the AVENIO ctDNA panels (Roche) [338], as well as panels approved by the FDA such as FoundationOne Liquid CDx and Guardant360 CDx [316].

Methods based on WES and WGS enable the detection of all potential DNA aberrations [323]. Additionally, whole-genome bisulfite sequencing

(WGBS) enables the assessment of the complete methylome in liquid biopsies [339,340]. In addition to NGS methods, DNA methylation can also be examined at a genome-wide level in liquid biopsies using methylation microarrays [341,342].

Table 2. Methodologies for the genetic and epigenetic analysis of cfDNA.

Approach	Method	Feature	Sensitivity
Locus-specific	qPCR	Gene variants	High
	qMSP	Methylation	High
	ddPCR	Gene variants/Methylation	Very high
	BEAMing	Gene variants	Very high
	Methyl-BEAMing	Methylation	Very high
Genome-wide	NGS panels	Gene variants/Methylation	High
	WES	Gene variants	High
	WGS	Gene variants	High
	WGBS	Methylation	High

QPCR, quantitative PCR; qMSP, quantitative methylation-specific PCR; ddPCR, digital droplet PCR; BEAMing, Beads, Emulsions, Amplification, and Magnetics; NGS, Next-generation sequencing; WES, whole exome sequencing; WGS, whole genome sequencing; WGBS, whole genome bisulfite sequencing.

6. Genetic and Epigenetic biomarkers in cfDNA

Even though the use of tissue sample remains the current gold standard for biomarker analysis, the possibility of sampling bias is notable [343]. This arises from the challenges in acquiring an adequate amount of appropriately high-quality material for cancer genome profiling and from the sampling biases that emerge due to genetic heterogeneity [343]. Over the past few years, advances in cfDNA research have highlighted its potential in identifying biomarkers for precision oncology, including diagnosis, prognosis, disease monitoring, and therapy selection [344,345].

6.1 Genetic biomarkers

6.1.1 Genetic biomarkers in CRC

The analysis of genetic biomarkers in cfDNA represents a crucial tool for the diagnosis, prognosis and therapy selection of CRC patients [346].

Regarding diagnosis, cfDNA levels are significantly higher in CRC patients compared to healthy individuals, which could be useful for the detection of the disease [347,348]. Additionally, the detection of hotspot variants in genes of plasma, such as *KRAS*, *BRAF*, and *PIK3CA* has shown potential utility for non-invasive CRC diagnosis [349,350]. In addition, cfDNA levels have been reported to have strong prognostic value [351], correlating with worse relapse-free survival (RFS) and OS [352]. Moreover, the presence of *KRAS* and *BRAF* variants especially *BRAF* V600E, serves as robust biomarkers indicating a poor prognosis in CRC patients [353,354].

Analysis of cfDNA is also valuable for therapy selection. CRC patients should be tested for *KRAS* variants as they indicate primary resistance to anti-EGFR therapy [355]. Thus, cfDNA analysis proves to be a promising non-invasive surrogate for assessing primary resistance to anti-EGFR treatment [312]. The analysis of *KRAS* variants has demonstrated accuracy in several clinical scenarios involving mCRC patients, including the initial determination of RAS status, evaluation of treatment efficacy, and monitoring the emergence of RAS variants as mechanisms of resistance to anti-EGFR therapy [312,356]. In line with this, the OncoBEAM RAS CRC assay is the only CE-IVD certified test designed to identify RAS variants in cfDNA. This assay utilizes BEAMing technology to accurately detect up to 34 variants spanning exons 2, 3, and 4 of the *KRAS* and *NRAS* genes [356].

6.1.2 Genetic biomarkers in BC

In the context of BC, genetic biomarkers have been also extensively studied with the aim of improving early detection, assessment of prognosis and personalization of treatment. Some studies have demonstrated that the identification of variants in genes, such as *PIK3CA* and *TP53,* could be useful for early detection of BC [357,358].

Some genetic biomarkers in cfDNA of particular interest include the detection of gene variants in *HER2* [359,360], *ESR1* [361,362] and *TP53* [363,364],providing valuable information for prognosis and therapy response . Moreover, the *PIK3CA* oncogene, which is altered in approximately 40% of breast cancer patients [365], may be analyzed in cfDNA of plasma samples [363,366]. The presence of particular variants of this gene in cfDNA have been proposed as predictive biomarkers for the effectiveness of PI3K inhibitors [366], as well as a predictive biomarker of recurrence [367].

Furthermore, the quantitative analysis of cfDNA concentrations may be also useful to reflect the response to treatment [368], and as a reliable predictor of OS and progression-free survival (PFS) [369,370].

6.2 Epigenetic biomarkers

The profiling of methylation changes that occur both at the beginning of the tumorigenesis process, and throughout the evolution of the disease, allows the possibility to identify epigenetic biomarkers for early detection, prognosis and therapy selection.

6.2.1 Epigenetic biomarkers in CRC

The methylation of *SEPT9* is among the most widely studied epigenetic biomarkers in cfDNA for the early detection of CRC [371,372]. The EpiproColon® assay became the first FDA-approved test for *SEPT9*

methylation detection in plasma via real-time PCR [60,372]. Nonetheless, further studies revealed that the detection of SEPT9 methylation has a low accuracy for detecting both CRC (sensitivity of 48.2%) and advanced precancerous lesions (sensitivity of 11.2%) [373]. By using ColoDefense® to examine blood samples, the incorporation of *SDC2* methylation assessment along with *SEPT9*, enabled sensitive and specific detection of advanced adenomas (AA) and CRC [374]. Another diagnostic tool, SpecColon, combines SFRP2 and SDC2 methylation analysis, exhibiting heightened sensitivity and specificity in identifying both AA and CRC [375]. In addition, the TriMeth test (*C9orf50*, *KCNQ5* and *CLIP4*) has shown an average sensitivity of 85% in detecting CRC across all disease stages [376].

In the context of prognosis, hypermethylation of *HPP1* and *HLTF* indicates an unfavorable prognosis and high mortality rates [377,378], while methylation of *RARB* and *RASSF1A* has been linked to disease aggressiveness [379]. For CRC patients, methylation of cfDNA can also be useful to monitor tumor burden and assess therapy response. For instance, the analysis of the methylation status of the gene pair *BCAT1*/*IKZF1* in cfDNA of plasma exhibited superior sensitivity in detecting CRC recurrence compared to CEA levels [380,381]. Likewise, plasma methylation of *SEPT9*, *DCC*, *BOLL*, and *SFRP2* demonstrated stronger correlation with tumor burden than CEA and CA-19-9 [382]. The hypermethylation of *WIF1* and *NPY* identified 80% of mCRC cases and 45% of localized CRC cases, and dynamic changes in methylation during the disease facilitated the follow-up of tumor progression [383].

The analysis of methylation in liquid biopsy can also be useful for monitoring treatment response in patients with CRC. For instance, the methylation assessment of five genes (*EYA4*, *GRIA4*, *ITGA4*, *MAP3K14-AS1*, and *MSC*) in cfDNA by ddPCR enabled the monitoring of tumor burden and the detection of treatment resistance [384]. Additionally, *MGMT* methylation

in cfDNA has shown to be useful as a potential predictive biomarker for chemotherapy response in CRC [328].

6.2.2 Epigenetic biomarkers in BC

In the context of BC, methylation analysis of cfDNA is a promising approach for identifying highly sensitive and specific molecular biomarkers for screening and early detection. In line with this, *RASSF1A* exhibits higher methylation levels in cfDNA of BC patients compared to healthy controls [385]. This gene has also been studied in combination with other genes, such as *APC and FOXA1*, whose combination is able to increase the diagnostic accuracy of BC [386]. Moreover, methylation of tumor-specific genes like *ITIH5*, *DKK3* and *RASSF1A* could serve as valuable biomarkers for early BC detection [387]. Other study has focused on the cfDNA methylation of *SPAG6*, *LINC10606*, and *TBCD*/*ZNF750*, demonstrating their high capability to distinguish TNBC patients from controls [388].

In term of prognosis, hypermethylation of *RASSF1A*, *BRCA1* and *RARβ2* in cfDNA indicates a poor prognosis [389,390]. Furthermore, the simultaneous detection of methylation in *RASSF1A*, *RARβ2* and *GSTP1* is strongly associated with unfavorable outcomes [389]. *GSTP1* methylation in cfDNA correlates with an aggressive phenotype in ER-positive BC [272] and SOX17 methylation stands as another independent prognostic factor, associated with poorer survival rates [391]. Hypermethylation of *PITX2* in plasma cfDNA has also emerged as an indicator of unfavorable OS [392].

Regarding therapy response, detecting methylation of *RASSF1A* and *WIF-1* in cfDNA of locally advanced BC patients is highly predictive of neoadjuvant chemotherapy efficacy [393]. Similarly, hypermethylation of *PITX2* serves as a predictor for the response to anthracycline-based therapy [392]. In line with this, *ESR1* methylation status in cfDNA has shown utility as a biomarker of resistance to endocrine therapy [394].

HYPOTHESIS

HYPOTHESIS

Cancer continues to be one of the leading causes of death worldwide, despite the advances and developments in recent years in both diagnosis and treatment. Colorectal and breast cancer are two of the most frequent tumor types, which are usually diagnosed at advanced stages of the disease, causing a significant increase in mortality rates. Furthermore, the effectiveness of treatments is influenced by the molecular profile of cancer patients. These observations highlight the need to use novel biomarkers and approaches that improve the clinical management of CRC and BC, providing precision oncology for these patients. In this regard, genome-wide analyses of genetic and epigenetic alterations represent promising strategies for the discovery and evaluation of non-invasive biomarkers. Despite their potential, some of these methodologies have been relatively underexplored in the field of liquid biopsy. Although potential biomarkers have been identified in cfDNA from highly prevalent tumors, such as colorectal and breast cancer, many of them lack significant sensitivity and specificity to be used in the clinic. Therefore, there is still the unmet clinical need of having novel non-invasive biomarkers and tools that improve the management of these tumor types. In this context, we propose that the identification and evaluation of genetic/epigenetic biomarkers in cfDNA by using genome-wide approaches could be relevant to improve the clinical management of CRC and BC patients. The possibility of improving the non-invasive early detection, monitoring and therapy selection, will contribute to achieving a more personalized medicine for cancer patients.

OBJECTIVES

OBJECTIVES

The main objective of this doctoral thesis was to perform a genetic and epigenetic profiling of cfDNA in patients with colorectal and breast cancer using genome-wide approaches to discover and evaluate non-invasive biomarkers with clinical utility for precision oncology. To achieve this aim, the following specific objectives were pursued:

Objective 1. To evaluate the performance of the TruSight Tumor 170 (TST170) next-generation sequencing panel in cfDNA of mCRC patients. This study aimed to determine the utility of this panel for the non-invasive detection of somatic variants in patients with mCRC.

Objective 2. To identify novel methylation biomarkers in cfDNA for the management of CRC. In particular, we aimed i) to investigate the clinical utility of the promoter methylation of the lncRNA *LINC00473* as a non-invasive biomarker for the early detection of CRC and associated precancerous lesions; and ii) to discover novel methylation biomarkers of therapy response in CRC patients following a genome-wide cfDNA methylation approach.

Objective 3. To discover novel non-invasive epigenetic biomarkers associated to BC by means of the genome-wide methylation profiling of cfDNA.

CHAPTER I

Evaluation of a targeted next-generation sequencing panel for the non-invasive detection of variants in circulating DNA of colorectal cancer

Evaluation of a Targeted Next-Generation Sequencing Panel for the Non-Invasive Detection of Variants in Circulating DNA of Colorectal Cancer

This chapter has been adapted from: **Rodriguez-Casanova A**[1,2,3,†], Bao-Caamano A[1,3,†], Lago-Lestón RM[4], Brozos-Vázquez E[5], Costa-Fraga N[1,3], Ferreirós-Vidal I[4], Abdulkader I[6], Vidal-Insua Y[5], Rivera FV[5], Candamio Folgar S[5,7], López-López R[2,5,7], Muinelo-Romay L[4,7], Diaz-Lagares A[1,7]. **Evaluation of a Targeted Next-Generation Sequencing Panel for the Non-Invasive Detection of Variants in Circulating DNA of Colorectal Cancer.** J Clin Med. 2021 Sep 29;10(19):4487. DOI: 10.3390/jcm10194487.

[1]Epigenomics Unit, Cancer Epigenomics, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), Santiago de Compostela, Spain.

[2]Roche-Chus Joint Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), Santiago de Compostela, Spain.

[3]Universidade de Santiago de Compostela (USC), Santiago de Compostela, Spain.

[4]Translational Medical Oncology Group (Oncomet), Liquid Biopsy Analysis Unit, Health Research Institute of Santiago (IDIS), 15706 Santiago de Compostela, Spain.

[5]Translational Medical Oncology Group (Oncomet), Health Research Institute of Santiago (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), 15706 Santiago de Compostela, Spain.

[6]Department of Pathology, University Clinical Hospital of Santiago (CHUS/SERGAS), 15706 Santiago de Compostela, Spain.

[7]Centro de Investigación Biomédica en Red Cáncer (CIBERONC), 28029 Madrid, Spain.

[†]These two authors share first authorship.

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Abstract

Background: Molecular profiling of cfDNA has shown utility for the management of CRC. TruSight Tumor 170 (TST170) is a NGS panel that covers 170 cancer-related genes, including *KRAS*, which is a key driver gene in CRC.

Methods: We evaluated the capacity of TST170 to detect gene variants in cfDNA from a retrospective cohort of 20 metastatic CRC patients with known *KRAS* variants in tumor tissue and in cfDNA previously analyzed by pyrosequencing and BEAMing, respectively.

Results: The cfDNA of most of the patients (95%) was successfully sequenced. We frequently detected variants with clinical significance in *KRAS* (79%, 15/19) and *PIK3CA* (26%, 5/19) genes. Variants with potential clinical significance were also identified in another 27 cancer genes, such as *APC*. The type of *KRAS* variant detected in cfDNA by TST170 showed high concordance with those detected in tumor tissue (77%), and very high concordance with cfDNA analyzed by BEAMing (94%). The variant allele fractions for *KRAS* obtained in cfDNA by TST170 and BEAMing correlated strongly.

Conclusions: This proof-of-principle study indicates that targeted NGS analysis of cfDNA with TST170 could be useful for non-invasive detection of gene variants in metastatic CRC patients, providing an assay that could be easily implemented for detecting somatic alterations in the clinic.

1. Introduction

Colorectal cancer (CRC) is the third most frequent cancer worldwide and the second leading cause of cancer mortality [395]. To date, certain gene alterations have been identified in metastatic colorectal cancer (mCRC) as clinically useful biomarkers. Among these, variants in specific codons of the Kirsten RAS (*KRAS*) oncogene are of particular interest due to their ability to predict tumor response to anti-epidermal growth factor receptor (EGFR)-targeted therapies [396]. In addition, other genetic alterations in relevant genes, such as *NRAS*, *BRAF*, and *PIK3CA*, are associated with mCRC [154].

Liquid biopsy has emerged in recent years as a non-invasive method for analysis of the molecular landscape of solid tumors using different types of biological fluids, including blood [397-400]. One of the most common strategies is analysis of cfDNA present in blood to detect tumor-specific alterations in the fraction originating from tumor cells (called circulating tumor DNA (ctDNA)) [401]. Molecular profiling of cfDNA in liquid biopsies can be performed by several strategies, including digital PCR (dPCR) and next-generation sequencing (NGS). In this sense, BEAMing (Beads, Emulsions, Amplification and Magnetics) represents a highly sensitive dPCR method for identifying and quantifying hotspot variants in cancer-related genes of cfDNA, such as *KRAS* and *NRAS* [356,402]. This technology was used in the first assay clinically validated for determining the mutation status of *KRAS* in CRC (the OncoBEAM RAS CRC test, Sysmex Inostics) [327]. However, a limitation of dPCR is that it is unable to analyze a great number of genes in the same assay, which can be solved by NGS approaches [403,404].

Targeted NGS represents a reliable technology for characterizing tumors with the potential to screen for large cancer gene panels in both tissue and cfDNA samples. In this sense, some recent works have demonstrated the utility of using this type of approach to detect gene variants in CRC patients

[405-407]. The use of NGS for cfDNA analyses may facilitate the detection of driver genes and provide valuable information on tumor heterogeneity and clonal evolution. In addition, this approach may reveal novel therapeutic targets for the application of personalized therapy and represents a promising tool for the management of CRC patients [406].

The TruSight Tumor 170 (TST170, Illumina) is an enrichment-based targeted next-generation sequencing (NGS) panel that covers the coding regions of 170 cancer-related genes. DNA analysis with TST170 allows for the detection of somatic variants (SNVs, indels and CNVs). TST170 has been shown to be useful for molecular profiling of tumor tissues [408,409] and it covers a different set of cancer genes with respect to the other available targeted NGS assays previously used in CRC [406]. To our knowledge, TST170 has not been used before to address cfDNA characterization in cancer patients. The use of this assay in cfDNA would open the possibility of having a new non-invasive tool for the study of gene variants in cancer research or in a clinical setting. Therefore, the aim of this study was to evaluate the capacity of the TST170 panel to detect gene variants in cfDNA of mCRC patients. To achieve this aim, cfDNA of a retrospective cohort of mCRC patients with known *KRAS* variants in tumor tissue and cfDNA was analyzed by NGS with the TST170 panel. Collectively, the results obtained in this study indicate that cfDNA can be assayed by TST170 to identify cancer-associated gene variants in mCRC patients. This non-invasive approach could contribute to improving cancer research and precision oncology.

2. Materials and methods

2.1 Study participants

Patients were recruited for this retrospective study between September 2016 and January 2019 in the Medical Oncology Department at the University Clinical Hospital of Santiago de Compostela (CHUS), Spain, after signing the pertinent informed consent form approved by the Galician Ethical Committee (Ref. 2015/746). The cohort was composed of 20 patients diagnosed with mCRC and with known *KRAS* variants present in plasma cfDNA analyzed by BEAMing.

2.2 Blood and tissue samples

Blood samples were collected before therapeutic intervention in 10 mL Cell-Free DNA BCT tubes (Streck, La Vista, NE, USA), and processed within the next 3 days. Plasma was obtained after centrifugation of blood samples at 1600× g for 10 min followed by centrifugation at 6000× g for 10 min. Plasma was stored at −80 °C until use. All tumor tissues used for diagnoses were obtained according to standard-of-care (SOC) procedures.

2.3 Isolation of cfDNA from plasma

DNA extraction from 1–3 mL of plasma was performed using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cfDNA was eluted in 75 µL of kit-supplied elution buffer. Concentration was assessed using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) before sample storage at −20 °C.

2.4 Analysis of *KRAS* variants in tumor tissues

The profile of *KRAS* variants in tumor tissues was analyzed in FFPE samples. Quantitative detection of *KRAS* variants in codons 12, 13, and 61 in genomic DNA was analyzed with a Therascreen® KRAS Pyro® Kit by pyrosequencing in a PyroMark® Q24 system (Qiagen) according to the manufacturer's recommendations.

2.5 Detection of gene variants in cfDNA by digital PCR

KRAS/*NRAS* hotspot variants were analyzed in cfDNA by BEAMing using the OncoBEAM™ RAS CRC IVD assay (Sysmex Inostics, Hamburg, Germany) as previously described [410]. *KRAS* variants (p.G12D, p.G12S, p.G13D) were also analyzed by droplet digital PCR (ddPCR) following the manufacturer's recommendations in a QX200 System (Bio-Rad, Hercules, CA, USA) (**Supplementary Table 1**). Specific gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, IA, USA) were used as positive controls. The percentage of variant allele fractions (VAFs) was calculated as the fractional abundance of variant alleles with respect to wild type (WT) alleles.

2.6 Analysis of cfDNA by targeted NGS with TST170

The list of genes detected by TST170 is shown in **Supplementary Table 2**. cfDNA libraries for sequencing were prepared using a TST170 kit (Illumina, San Diego, CA, USA) following the manufacturer's reference guides. For library preparation, 35–100 ng of cfDNA was used (**Supplementary Table 3**). As a quality control, libraries were made using 40 and 100 ng of Structural Multiplex cfDNA Reference Standard (Horizon Discovery, Waterbeach, UK). The workflow recommended for TST170 was followed, omitting the shearing step. Libraries were sequenced in a NextSeq

500 (Illumina) and data analyzed on BaseSpace Sequence Hub (Illumina). Variant calling was performed using the TST170 v2.0 app, which is a Docker-based software package that analyzes sequencing reads from libraries prepared with the TST170 sequencing panel. For variant interpretation, the cloud-based interpretation and reporting platform Variant Interpreter v2.9 (Illumina) was used. FastQ files were aligned to the human reference genome (Human, UCSC hg19) with the TST170 app using the Burrows–Wheeler aligner (BWA). Variants were processed by BaseSpace Variant Interpreter; variant calling was performed using the variant caller Pisces v5.2, and variants were annotated using the BaseSpace Annotation Engine v3.1. The default software quality filter was used to exclude low-confidence variants. The clinical significance of variants according to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) was evaluated using the somatic option of VarSome Clinical 9.4 (Saphetor).

2.7 Statistical analysis

Quantitative variables are shown as means ± standard deviation (SD), and frequencies as percentages (%). Linear regression and Bland–Altman analysis were performed for comparison between methods. Differences between groups were assessed with Student's t-test according to the normality of the distribution. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). The sensitivity (S) and specificity (Sp) of TST170 to detect gene variants in cfDNA were determined according to the following calculations: $S\ (\%) = (TP/TP + FN) \times 100$ and $Sp\ (\%) = (TN/TN + FP) \times 100$, where TP, FN, TN, and FP represent true positives, false negatives, true negatives, and false positives, respectively.

3. Results

3.1 Patient characteristics

To evaluate the capacity of TST170 to detect gene variants in cfDNA, we selected a cohort of 20 mCRC patients with known *KRAS* variants, which is one of the genes included in the TST170 panel and previously analyzed in cfDNA by BEAMing. In particular, all the selected patients of this cohort had *KRAS* variants in cfDNA, and most of them also had *KRAS* variants in the corresponding paired tumor tissues. The clinical characteristics of the patients are displayed in **Table 3** and **Supplementary Table 4**. Six patients were female and 14 males. The average age was 65 ± 15 years. Most of the patients had tumors with adenocarcinoma histology and left colon or rectum localization and had not received prior systemic therapy before plasma collection.

Table 3. Clinical characteristics of mCRC patients included in the study.

Characteristics	Patients (N=19)
Age (years)	
<60	5 (26%)
60-69	5 (26%)
70-79	7 (37%)
>80	2 (11%)
Gender	
Female	6 (32%)
Male	13 (68%)
Histology	
Adenocarcinoma	18 (95%)
Mucinous adenocarcinoma	1 (5%)

Table 3. Continuation.

Characteristics	Patients (N=19)
Primary tumor location	
Right colon	8 (42%)
Left colon/rectum	11 (58%)
Metastatic sites	
1	9 (47%)
≥2	10 (53%)
Metastatic location	
Liver	10 (53%)
Lung	10 (53%)
Peritoneum	5 (26%)
Resected primary tumor	
Yes	10 (53%)
No	9 (47%)
Previous treatment[1]	
Yes	6 (32%)
No	13 (68%)
MSI status[2]	
Negative	16 (84%)
Unknown	3 (16%)
***KRAS* status in tissue**	
Wildtype	1 (5%)
Mutated	16 (84%)
Unknown	2 (11%)
***KRAS* status in cfDNA**	
Wildtype	0 (0%)
Mutated	19 (100%)
***NRAS* status in cfDNA**	
Wildtype	15 (79%)
Mutated	1 (5%)
Unknown	3 (16%)
Tissue biopsy location	
Primary tumor	14 (74%)
Metastasis	2 (10%)
Unknown	3 (16%)

[1]Surgery/systemic treatment before plasma collection. [2]MSI, microsatellite instability.

3.2 Analysis of variants in cfDNA of mCRC patients using the TST 170 targeted panel

To evaluate the feasibility of using TST170 to detect variants in cfDNA, a total of 20 mCRC patients were analyzed. As a control, we used a cfDNA reference standard, which contains validated variants, including SNVs, indels, and CNVs. Importantly, the TST170 panel was able to detect all variants expected in the control (**Supplementary Table 5**). Among the whole mCRC cohort, only one patient's sample yielded a mean coverage <500× with the TST170 assay. This patient's sample was not considered for further analysis. The cfDNA analysis of the other 19 patients by TST170 provided high-depth sequencing with a mean coverage of ~2500× (range: 620×–4595×).

TST170 detected SNVs and indels in the cfDNA of all patients analyzed (**Figure 9A**). Among the observed variants, we detected frameshift, inframe, missense, and stop gain variants (**Figure 9B**, **Supplementary Table 6**), but these were not evenly distributed among patients. Although all patients showed more than one of these types of variants, some patients (37%, 7/19) displayed two types of variants, some patients (26%, 5/19) displayed three types, and others (37%, 7/19) displayed four types. Missense variants were the most frequent, and the only type present in all patients. On the other hand, the analysis of cfDNA by TST170 was also able to identify CNVs in 1 out of the 19 patients analyzed (5%, 1/19) (**Supplementary Table 7**).

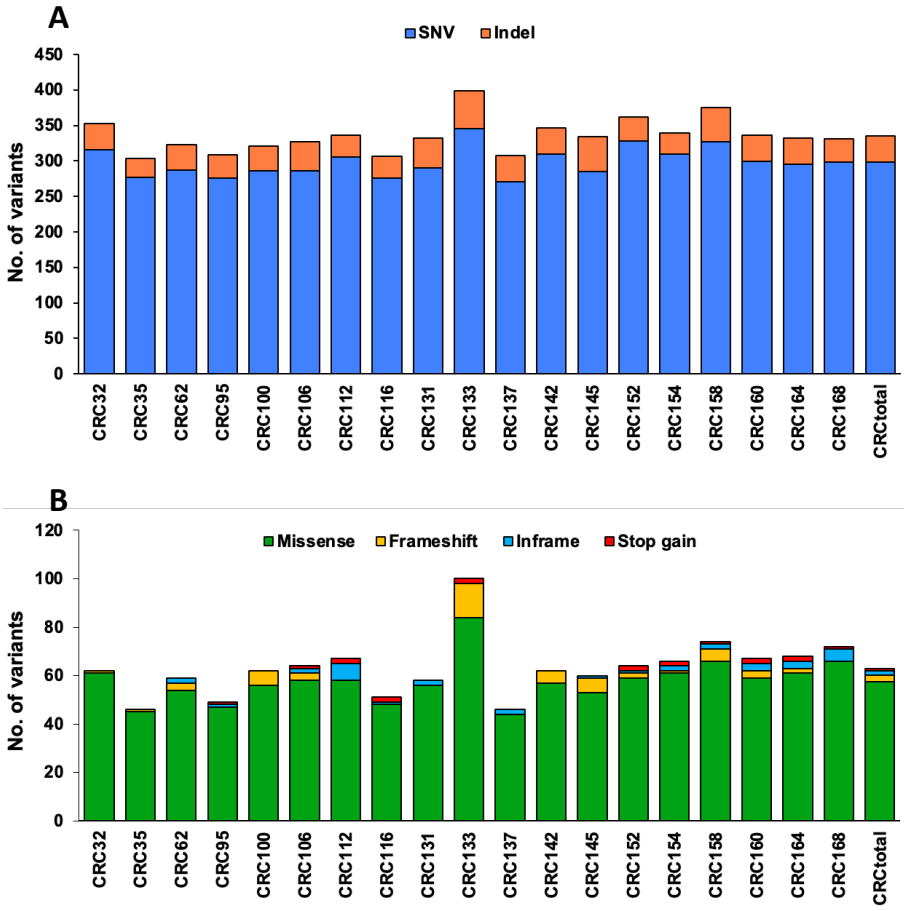


Figure 9. Overview of the variant distribution detected in cfDNA of CRC patients by TST170. A, Global distribution of SNVs and indels in mCRC patients. **B,** Distribution of SNVs and indels in mCRC patients according to variant type. The term "CRCtotal" represents the mean for all CRC patients analyzed of total variants detected per patient.

3.3 Identification of cancer-associated variants with clinical significance in cfDNA of mCRC patients

One of the key challenges in precision oncology is the identification and pathological interpretation of cancer-associated variants detected by sequencing [411]. Thus, to evaluate the pathogenicity of the variants detected in cfDNA by TST170, we used the somatic option of the *in silico* pipeline from VarSome Clinical, which follows the four-tier system recommended by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP). This system classifies somatic variants into four categories based on their clinical impact: tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants with unknown clinical significance; and tier IV, benign or likely benign variants [411]. Using this approach, we observed that 0.4% of all the variants detected in cfDNA of our cohort by TST170 were classified as tier I and 1.5% as tier II, while 3.8% were considered tier III and 94.3% were tier IV (**Supplementary Figure 1**). Importantly, we frequently detected tier I variants in *KRAS* (79%, 15/19) and *PIK3CA* (26%, 5/19) genes of our CRC cohort. In addition, tier II variants were detected in another 27 cancer-relevant genes, including *APC*, which was the gene with the most tier II variants identified (**Figure 10**).

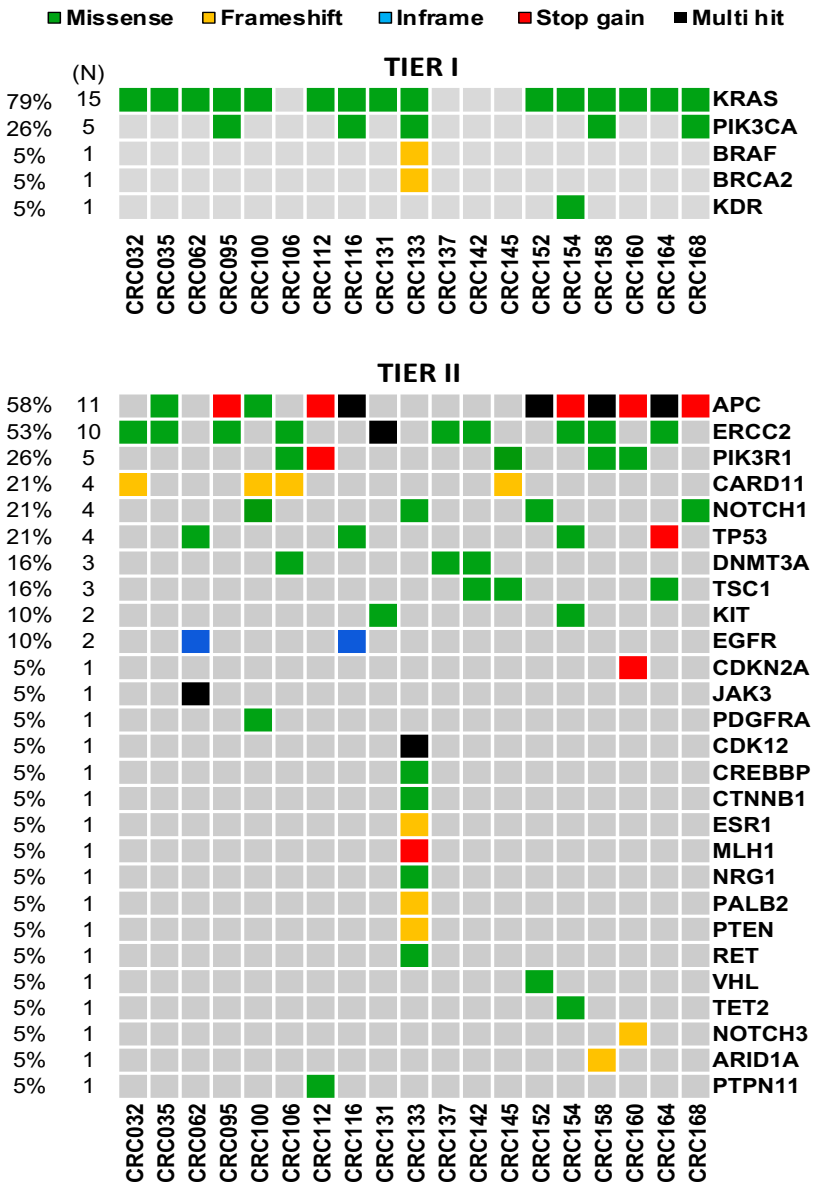
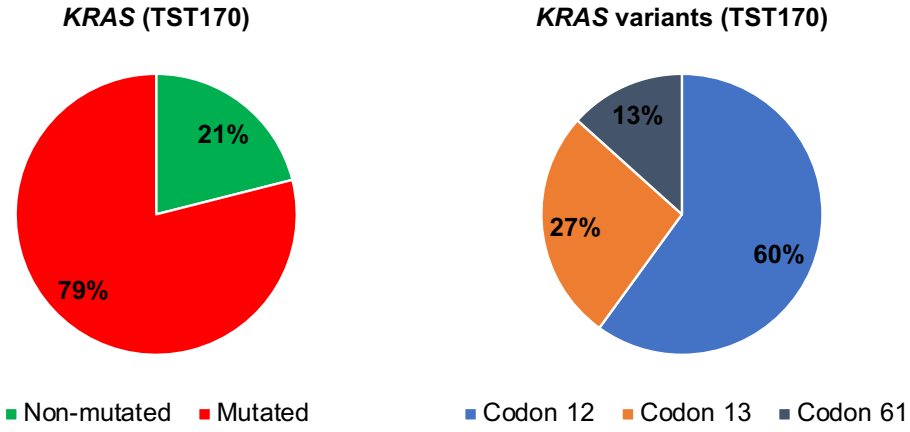


Figure 10. Spectrum of variants in cfDNA of CRC patients according to the cancer-associated clinical significance. The oncoplots show the frequency and distribution of the tier I and II variants detected in cfDNA of CRC patients. "Multi hit" indicates that more than one variant in a gene was found in the same patient. The number (N) and percentage of patients is shown on the left.

3.4 Analysis of *KRAS* variants in cfDNA of mCRC patients and concordance with tissue analysis

The genetic status of *KRAS* is clinically relevant in CRC patients [396]; therefore, to verify the ability of TST170 to detect gene variants in cfDNA, we focused this study on this gene. Among the *KRAS* variants identified in cfDNA of our cohort by TST1170, nine (60%) were located in codon 12, four (27%) in codon 13, and two (13%) in codon 61 (**Figure 11**).

Figure 11. Distribution of *KRAS* variants detected in cfDNA of mCRC patients by TST170. A, Frequency of CRC patients with *KRAS* variants detected in cfDNA by TST170. **B,** Frequency of *KRAS* variants detected in cfDNA by TST170 according to codon location.



A high correlation between *KRAS* variants detected in tumor tissue and cfDNA of CRC patients has previously been reported [348,412]. **Table 4** summarizes, for each mCRC patient, the *KRAS* variant detected in tumor tissues by pyrosequencing and in cfDNA by BEAMing or TST170 assays. TST170 analysis of cfDNA was able to detect *KRAS* variants in 81% (13/16) of the 16 patients with available information on the genetic status of *KRAS* in their tumor tissue (**Table 4**). Importantly, 77% (10/13) of the *KRAS* variants

detected in cfDNA by TST170 were the same as those found in tumor tissue, showing high concordance between both analyses. In addition, considering the *KRAS* codon number altered, the concordance between cfDNA analysis by TST170 and the genetic status of tumor tissue increased to 92% (12/13) (**Table 4**). As expected, we obtained a similarly high correlation (15/16, 94%) when we compared the available *KRAS* variants in tumor tissue with the results of cfDNA analysis by BEAMing (Table 4), which is an extensively validated method for assessing *KRAS* variants in the cfDNA of mCRC patients [410,413].

3.5 Concordance of *KRAS* variants in cfDNA of mCRC patients by BEAMing and TST170

Since BEAMing is a reference assay for analysis of the status of *KRAS* in the cfDNA of CRC patients [410,413], we compared the *KRAS* variants obtained in cfDNA by this method with those obtained by TST170 (**Table 4**).

TST170 was able to detect *KRAS* variants in patients in whom the variant allele fractions (VAFs) obtained with BEAMing were ≥0.5%. Therefore, we used this value of VAF as the limit of detection (LOD) to perform comparative analysis between TST170 and BEAMing. Importantly, TST170 detected the *KRAS* variants in the same codon as BEAMing in 15 of the 16 patients analyzed, showing a high sensitivity (94%) to detect *KRAS* variants. In addition, linear regression analysis showed a high correlation ($R2 = 0.9366$, $y = -0.49 + 0.51x$) between *KRAS* VAFs obtained by BEAMing and TST170 (**Figure 12A**). To quantitate the agreement between both methods, we performed a Bland–Altman analysis, which showed a mean difference of 10.12% between BEAMing and TST170 (**Figure 12B**).

Table 4. *KRAS* variants detected in tumor tissues and cfDNA.

Sample ID	*KRAS* variants		
	Tumor tissue	cfDNA	
		BEAMing (VAF, %)	TST170 (VAF, %)
CRC032	p.Q61L	KR3Cdn61 (0.55)	p.Q61L (3.08)
CRC035	p.G12S	KR2Cdn12 (11.68)	p.G12S (23.72)
CRC062	p.G12V	KR2Cdn12 (23.68)	p.G12V (50.37)
CRC095	p.G12V	KR2Cdn12 (5.33)	p.G12V (8.87)
CRC100	p.G13D	KR2Cdn13 (6.96)	p.G13D (14.18)
CRC106	p.G12D	KR2Cdn12 (0.32)	ND
CRC112	NA	KR2Cdn12 (4.38)	p.G12D (9.6)
CRC116	p.G13D	KR2Cdn13 (30.12)	p.G13D (48.61)
CRC131	p.G12V	KR2Cdn12 (12.10)	p.G12V (34.09)
CRC133	NA	KR2Cdn13 (10.84)	p.G13D (19.19)
CRC137	p.G12D	KR2Cdn12 (0.21)	ND
CRC142	p.G13D	KR2Cdn13 (0.11)	ND
CRC145	NA	KR2Cdn12 (0.70)	ND
CRC152	p.G12D	KR2Cdn12 (3.35)	p.G12S (9.32) *
CRC154	p.Q61L	KR3Cdn61 (20.17)	p.Q61L (43.83)
CRC158	p.G12V	KR2Cdn12 (5.69)	p.G12V (12.53)
CRC160	p.G12D	KR2Cdn12 (3.59)	p.G12A (7.04) *
CRC164	WT	KR2Cdn13 (1.98)	p.G13D (4.48) *
CRC 168	p.G12A	KR2Cdn12 (3.16)	p.G12A (6.53)

VAF, variant allele fraction; NA, not available; ND, not detected; KR2, *KRAS* exon 2; KR3, *KRAS* exon 3; Cdn12, codon 12; Cdn13, codon 13; Cdn61, codon 61. * *KRAS* variant detected in cfDNA with TST170 showing a discrepancy with tumor tissue.

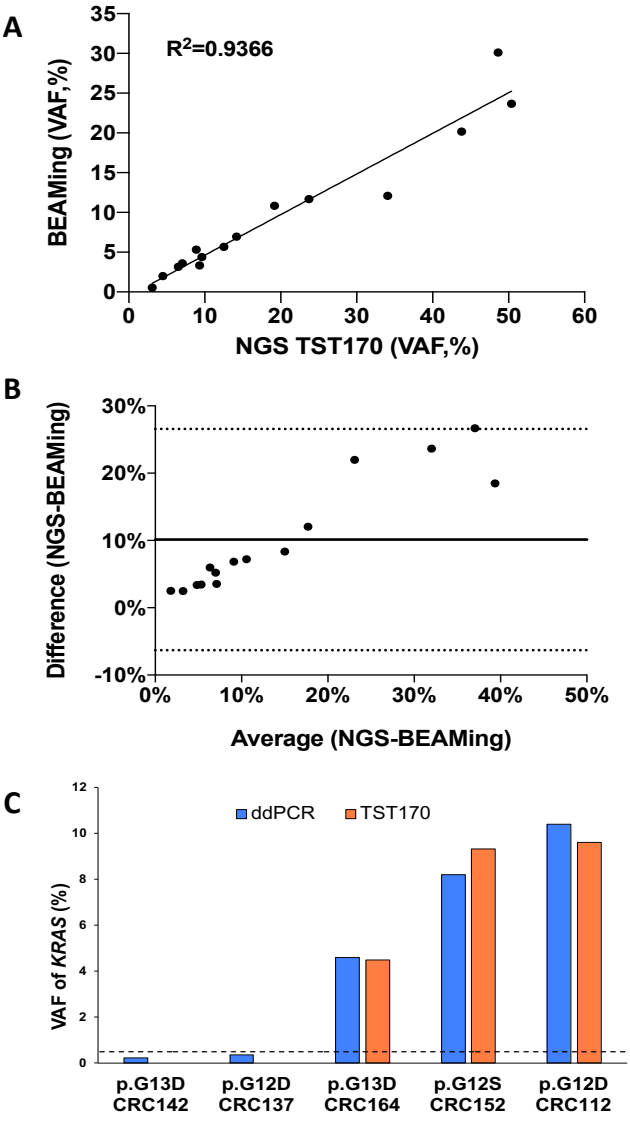


Figure 12. TST170 and digital PCR analysis for *KRAS* variants in cfDNA. A, Linear regression analysis of *KRAS* VAFs in cfDNA analyzed by TST170 and BEAMing. **B,** Bland–Altman plot of the *KRAS* VAFs between TST170 and BEAMing. **C,** Validation of the KRAS status by ddPCR in cfDNA samples with discordant results between TS170 analysis and BEAMing or tumor tissue status. The dotted line represents a VAF ≥ 0.5%.

In addition to evaluating the sensitivity of TST170 and its concordance with the BEAMing assay, we also decided to evaluate the specificity of TST170 for cfDNA analysis. Since all patients of our cohort were *KRAS* mutated in cfDNA by BEAMing, we evaluated the specificity of TST170 based on the status of NRAS, another relevant gene for CRC that was WT in cfDNA by BEAMing analysis for most of the patients of our cohort (**Table 3**). Thus, considering the LOD of TST170 previously observed in our work for cfDNA analysis (VAF ≥ 0.5%), TST170 detected the WT status of *NRAS* in the cfDNA of all the patients (15/145 of our cohort, showing a specificity for cfDNA analysis of 100% (**Supplementary Table 8**).

3.6 Description of *KRAS* variants in discordant samples detected in cfDNA by TST170

In some patients, the analysis of *KRAS* variants in cfDNA with TST170 showed some discordance with respect to the cfDNA analyzed by BEAMing or the status of tumor tissues. To verify whether these discordances were due to limitations of TST170, we performed a reanalysis of the available cfDNA samples using a *KRAS*-specific droplet digital PCR (ddPCR) assay. TST170 was unable to detect *KRAS* variants in three patients (CRC106, CRC137, CRC142) in whom these alterations were detected both by BEAMing and tissue analysis (**Table 4**). In these samples, *KRAS* showed a VAF < 0.5% by BEAMing, which is the LOD obtained in our cohort to detect *KRAS* variants by TST170. *KRAS* reanalysis of two of the cfDNA samples (CRC137 and CRC142) by ddPCR detected the expected *KRAS* variants with a VAF < 0.5% (**Figure 12C**), confirming the low allele fraction as the cause for the discordancy. In three patients (CRC152, CRC160, CRC164), tumor tissue showed variants in the same *KRAS* codon number as was seen in cfDNA analyzed by BEAMing and TST170; however, the specific variant identified was different between TST170 and tumor tissue (**Table 4**). Importantly, we

reanalyzed the cfDNA of two of these three patients (CRC152, CRC164) by ddPCR and identified the same *KRAS* variant as TST170 (**Figure 12C**). Furthermore, two patients (CRC112, CRC133) without *KRAS* genetic status available in tumor tissue showed the same codon of *KRAS* altered after the analysis with both BEAMing and TST170 (**Table 4**). Importantly, reanalysis of one patient by ddPCR (CRC112) confirmed the type of variant identified by TST170 (**Figure 12C**).

3.7 *KRAS* analysis in cfDNA according to clinical-pathological characteristics of the patients

Despite the limited size of the patient cohort for finding clear statistically significant associations, we explored the potential relationship of *KRAS* variants identified by TST170 and BEAMing with clinical–pathological characteristics of patients. Both TST170 and BEAMing showed lower *KRAS* VAFs in patients without liver metastasis than in those with this type of metastasis (**Supplementary Figure 2**). According to these results, none of the four patients with *KRAS* variants detected by BEAMing but undetected with TST170 showed liver metastasis. In particular, of these four cases, two of them had lung metastasis, and the other two cases showed only peritoneal affectation. In addition, both TST170 and BEAMing showed lower *KRAS* VAFs in patients with previous primary tumor resection than those without resection (**Supplementary Figure 2**). In fact, all BEAMing/TST170 discordant cases had previously undergone primary tumor resection.

4. Discussion

Approaches based on targeted NGS have been demonstrated to be useful for the detection of gene variants in tumor tissues and cfDNA of several tumor types, including CRC [405,407]. The use of new NGS assays in liquid biopsy is a very relevant approach for the non-invasive management of CRC patients, which can facilitate precision medicine strategies with clinical benefits for oncological practice [406]. In this study, we analyzed a cohort of mCRC patients with known *KRAS* variants in both tissue and plasma to investigate the performance of the targeted NGS panel TST170 in detecting gene variants in cfDNA. TST170 covers the coding regions of 170 cancer-related genes and has been successfully used in tumor tissues to characterize genetic alterations [408,409]. The use of this assay in cfDNA may provide a new non-invasive tool for the study of gene variants in cancer research or in a clinical setting without the need of a specific design for cfDNA. To our knowledge, this is the first study that evaluates the TST170 panel on cfDNA from cancer patients. The results obtained in this work demonstrate the feasibility of using TST170 to detect gene variants in cfDNA. Thus, using this NGS panel in cfDNA of mCRC patients and following ACMG and AMP guidelines, we were able to frequently identify cancer-associated variants with strong clinical significance in relevant genes, such as *KRAS* and PIK3CA. In addition, we also identified variants with potential clinical significance in another 27 cancer-related genes. Of note, the *KRAS* variants identified in cfDNA by TST170 showed high concordance with tumor tissue and cfDNA analyzed by BEAMing. This proof-of-principle study indicates that cfDNA can be assayed by TST170 to identify the presence of clinically relevant variants in mCRC patients, representing an alternative non-invasive approach that could be useful in cancer research and in the clinic, contributing to solving some of the limitations of tumor tissue biopsies [414,415].

In the present work, the TST170 assay was especially useful for identification of SNVs and indels in cfDNA, as these variants were detected in all CRC patients analyzed. Importantly, many of the variants detected were frameshift, inframe, missense, and stop gain variants. These types of variants are associated with the capability of producing clinically relevant effects in genes driving cancer progression [416]. Therefore, detection of these variants in our work supports the possibility of using the TST170 panel, not only in tissue samples, but also in cfDNA in a research or clinical setting. Of note, missense variants are among the most frequently observed alterations in CRC [417-419]. In accordance with this, missense variants were the most frequently detected type of alteration among all analyzed patients in our cohort. In contrast to the high frequency of SNVs and indels identified, analysis of cfDNA with TST170 only detected CNVs in one CRC patient. The low number of CNVs detected could be due to: (i) this panel's capacity to detect CNVs in a small subset of genes with respect to SNV/indel genes; (ii) the small size of the cohort analyzed; or (iii) the lower overall frequency of CNVs in the human genome, making their detection more technically challenging than SNVs and indels [420,421].

Importantly, as expected, the analysis of cfDNA by TST170 in our cohort was able to detect frequent *KRAS* variants with strong clinical significance. In addition, this assay also detected variants in other relevant genes associated with cancer pathways and/or with clinical implications for CRC patients, such as *PIK3CA*, *BRAF*, *EGFR*, *APC*, and *TP53*, among others [418,422,423]. The high frequency of variants observed in some of these genes, such as for *PIK3CA*, could be influenced by the small size of our cohort and the high frequency of patients with *KRAS* variants [424]. In addition, we observed a low frequency of *BRAF* variants, which is in line with previous studies showing that concomitant variants in BRAF and *KRAS* rarely occur in CRC [425].

Detection of variants in specific codons of the oncogene *KRAS* is of particular interest in mCRC patients for its value to predict response to anti-*EGFR* targeted therapies [396]. Therefore, it is especially relevant to have non-invasive approaches to detect this type of variant in the clinic. BEAMing is a highly sensitive digital PCR assay considered the gold standard method for the genetic analysis of *KRAS* in cfDNA of CRC patients [410,413]. In this work, TST170 was able to detect *KRAS* variants in patients who had a value of VAF obtained by BEAMing of ≥0.5%, indicating that TST170 can successfully identify variants with VAFs ≥ 0.5%. This value of VAF represents the LOD for *KRAS* variants in our cohort and is in the range of analytical sensitivity reported in other studies for targeted NGS assays to evaluate cfDNA in cancer patients [327,426].

Analysis of cfDNA by TST170 was not able to detect any of the expected *KRAS* variants in 4 out of 19 patients evaluated in our study. Of note, these four patients presented lung or peritoneal metastasis without liver affectation. The lack of expected variant detection in these patients could be explained by the location of metastasis, since it has been recently reported that in mCRC both lung and peritoneal lesions, compared with other metastatic sites such as the liver, have significantly lower maximum allele frequencies and a lower number of detected variants, suggesting lower levels of ctDNA release as compared with other metastatic sites like the liver [356,427]. In addition, the lack of expected variant detection in these four patients could be influenced by the amount of cfDNA used for the TST170 assay, which was in these four cases at the limit of the manufacturer´s recommendations.

Importantly, TST170 was able to detect *KRAS* variants in the cfDNA of most of the patients (77%) who also had *KRAS* alterations in their tumor tissue. This result is in agreement with previous studies in CRC patients that showed a high correlation between the *KRAS* variants detected in their tumor

tissues and cfDNA [348,412]. Relevantly, we obtained high concordance (94%) for detection of *KRAS* variants in cfDNA between TST170 and BEAMing. However, although there was a strong correlation between both methods, TST170 showed different values of *KRAS* VAFs than BEAMing. Similarly to this work, other authors found variations in *KRAS* VAF values by NGS and digital PCR approaches, but with a good correlation between both technologies [428]. Importantly, the few discordant cases observed between TST170 and BEAMing were patients without liver metastasis, and with previous primary tumor resection, for whom ctDNA shedding is well accepted to be low [356]. In these cases, TST170 could be more limited than BEAMing in detecting *KRAS* variants in cfDNA. However, TST170 was able to detect other gene variants in the discordant cases, reinforcing the interest of applying a more comprehensive assay to have a more global view of the disease. Besides, in all samples in which *KRAS* sequences showed discordance between tissue and TST170, reanalysis of cfDNA by ddPCR confirmed the results obtained by TST170, supporting the reliability of the data obtained with this NGS assay. The observed discordances in *KRAS* sequencing from tissue and TST170 could be explained by the heterogeneity and/or clonal evolution of tumors, which yield variable representation within the cfDNA of subclonal tumor cell populations [429]. The high correlation in the genetic status of *KRAS* obtained between cfDNA and matched tumor tissue suggests that the *KRAS* variants identified in the cfDNA of our patient cohort accurately represent the tumor tissue. However, the tumoral origin of the genetic variants in other genes found by our analyses of cfDNA should be interpreted with caution due to lack of data from matched tumor tissue or white blood cells (WBCs) [430].

This proof-of-principle study indicates that targeted NGS analysis of cfDNA with the TST170 panel could be useful for non-invasive detection of clinically relevant variants in liquid biopsy of mCRC patients. In future studies,

analysis of cfDNA samples with unknown genetic variant status (blind samples) would be useful to obtain additional information on the capabilities of TST170.

5. Conclusions

Taken together, our data indicate that targeted NGS analysis of cfDNA with the TST170 panel could be useful for non-invasive detection of gene variants in metastatic CRC patients, providing an assay that could be easily implemented for detecting somatic alterations in the clinic. These data support further investigation into applications of this NGS approach to non-invasively characterizing the genetic landscape of tumors.

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Conflicts of interest

R.L.-L. has received honoraria for participation in Advisory Boards from Roche, AstraZeneca, Merck, MSD, Bayer, BMS, Novartis, Janssen, Lilly, Pfizer, and Leo; travel, accommodations, and expenses from Pharmamar, Roche, BMS, and Pierre Fabre; research funding from Roche and Merck; and is co-founder and shareholder in Nasasbiotech, S.L., Mtrap Inc. The rest of the authors declare no potential conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Author contributions

Conceptualization, A.R.-C., A.B.-C., R.L.-L., L.M.-R. and A.D.-L.; resources, E.B.-V., Y.V.-I., F.V.R., S.C.F. and I.A.; Data curation, E.B.-V., Y.V.-I., F.V.R., S.C.F., I.F.-V., A.R.-C. and A.B.-C.; methodology, A.R.-C., A.B.-C. and R.M.L.-L.; formal analysis, A.R.-C., A.B.-C., N.C.-F., I.F.-V., L.M.-R. and A.D.-L.; investigation, A.R.-C., A.B.-C., L.M.-R., R.L.-L. and A.D.-L.; writing—original draft preparation, A.R.-C., A.B.-C., L.M.-R. and A.D.-L.; writing—review and editing, A.R.-C., A.B.-C., R.M.L.-L., E.B.-V., N.C.-F., I.F.-V., I.A., Y.V.-I., F.V.R., S.C.F., R.L.-L., L.M.-R. and A.D.-L.; supervision, A.D.-L.; funding acquisition, L.M.-R., R.L.-L. and A.D.-L. All authors have read and agreed to the published version of the manuscript.

Data availability

The data presented in this study are openly available in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI), reference number PRJNA761891.

CHAPTER II

Identification of novel cfDNA methylation biomarkers for the management of CRC

II.A. Non-invasive early detection of colorectal cancer by hypermethylation of the *LINC00473* promoter in plasma cell-free DNA.

II.B: A novel cfDNA methylation signature for the prediction of FOLFOX-based therapy response in advanced colorectal cancer.

CHAPTER II.A

Non-invasive early detection of colorectal cancer by hypermethylation of the *LINC00473* promoter in plasma cell-free DNA

Non-invasive early detection of colorectal cancer by hypermethylation of the *LINC00473* promoter in plasma cell-free DNA

This chapter has been adapted from: Juan Ruiz-Bañobre[1,2,†], **Aitor Rodriguez-Casanova**[3,4,5,†], Nicolas Costa-Fraga[3,5], Aida Bao-Caamano[3,5], Ana Alvarez-Castro[6], Francisco Luis Martín Carreras-Presas[6], Elena Brozos-Vazquez[1], Yolanda Vidal-Insua[1], Francisca Vazquez-Rivera[1], Sonia Candamio-Folgar[1,2], Manuel Mosquera-Presedo[3,5], Ramón M Lago-Lestón[7], Laura Muinelo-Romay[2,7], José Ángel Vázquez-Bueno[8], Rebeca Sanz-Pamplona[9,10,11], Víctor Moreno[9,10,11,12], Ajay Goel[13,14,15], Lourdes Castillo[16], Ana C Martin[16], Rocio Arroyo[16], Manel Esteller[2,17,18,19], Ana B Crujeiras[20,21], Rafael López-López[1,2,4], Angel Díaz-Lagares[2,3]. **Non-invasive early detection of colorectal cancer by hypermethylation of the *LINC00473* promoter in plasma cell-free DNA**. Clin Epigenetics. 2022 Jul 9;14(1):86. DOI: 10.1186/s13148-022-01302-x.

[1]Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), 15706 Santiago de Compostela, Spain.

[2]Centro de Investigación Biomédica en Red Cáncer (CIBERONC), ISCIII, 28029 Madrid, Spain.

[3]Cancer Epigenomics Laboratory, Epigenomics Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), 15706 Santiago de Compostela, Spain.

[4]Roche-Chus Joint Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), 15706 Santiago de Compostela, Spain.

[5]Universidade de Santiago de Compostela (USC), 15782 Santiago de Compostela, Spain

[6]Department of Gastroenterology and Hepatology, University Clinical Hospital of Santiago (CHUS/SERGAS), 15706 Santiago de Compostela, Spain.

[7]Liquid Biopsy Analysis Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), 15706 Santiago de Compostela, Spain.

[8]Department of Pathology, Complejo Hospitalario Universitario de Ferrol, 15405 Ferrol, Spain.

[9]Unit of Biomarkers and Susceptibility, Oncology Data Analytics Program, Catalan Institute of Oncology (ICO), 08907 Barcelona, Spain.

[10]Colorecal Cancer Group, Bellvitge Biomedical Research Institute (IDIBELL), 08907, Barcelona, Spain.

[11]Biomedical Research Centre Network for Epidemiology and Public Health (CIBERESP), 28029 Madrid, Spain.

[12]Department of Clinical Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, 08907 Barcelona, Spain.

[13]Center for Gastrointestinal Research, Center for Translational Genomics and Oncology, Baylor Scott & White Research Institute, Charles A Sammons Cancer Center, Baylor University Medical Center, Dallas, Texas, USA.

[14]Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute of City of Hope, Monrovia, California, USA.

[15]City of Hope Comprehensive Cancer Center, Duarte, California, USA.

[16]Advanced Marker Discovery (AMADIX), 47004 Valladolid, Spain.

[17]Josep Carreras Leukaemia Research Institute (IJC), Barcelona, Spain.

[18]Institucio Catalana de Reserca I Estudis Avançats (ICREA), Barcelona, Spain.

[19]Physiological Sciences Department, School of Medicine and Health Sciences, University of Barcelona (UB), Spain.

[20]Epigenomics in Endocrinology and Nutrition Group, Epigenomics Unit, Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), 15706 Santiago de Compostela, Spain.

[21]Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (CIBERobn), ISCIII, 28029 Madrid, Spain.

[†]These two authors share first authorship.

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Abstract

Background: Current non-invasive assays have limitations in the early detection of CRC. We evaluated the clinical utility of promoter methylation of the long noncoding RNA *LINC00473* as a non-invasive biomarker to detect colorectal cancer and precancerous lesions.

Methods: We evaluated the epigenetic regulation of *LINC00473* through promoter hypermethylation in CRC cell lines using bisulfite genomic sequencing and expression analyses. DNA methylation of *LINC00473* was analyzed in primary CRC using 450K arrays and RNAseq from The Cancer Genome Atlas (TCGA). Tissue-based findings were validated in several independent cohorts of CRC and advanced colorectal polyp patients by pyrosequencing. We explored the clinical utility of *LINC00473* methylation for the early detection of CRC in plasma cell-free DNA by quantitative methylation-specific PCR and droplet digital PCR.

Results: *LINC00473* showed transcriptionally silencing due to promoter hypermethylation in colorectal cancer cell lines and primary tumors. Methylation of the *LINC00473* promoter detected primary colorectal tumors in two independent clinical cohorts, with areas under the receiver operating characteristic curves (AUCs) of 0.94 and 0.89. This biomarker also identified advanced colorectal polyps from two other tissue-based clinical cohorts (AUCs of 0.99 and 0.78). Finally, methylation analysis of the *LINC00473* promoter in plasma cell-free DNA accurately identified patients with colorectal cancer and advanced colorectal polyps (AUCs of 0.88 and 0.84, respectively), which was confirmed in an independent cohort of patients.

Conclusions: *LINC00473* is regulated by DNA methylation in CRC. Hypermethylation of the *LINC00473* promoter is a new promising biomarker for non-invasive early detection of colorectal cancer and related precancerous lesions.

1. Introduction

Colorectal cancer (CRC) is the third most frequently detected cancer in both sexes worldwide and is expected to increase by 60% to more than 2.2 million new cases by 2030 [14]. CRC is usually diagnosed at an advanced stage of disease [431] and represents a leading cause of cancer mortality worldwide [9]. For CRC patients, the 5-year survival rate ranges from 90% to 14% depending on whether they are diagnosed at early or advanced stages, respectively [432]. The growing incidence and high mortality rates of CRC highlight the clinical need for novel strategies to improve early CRC detection and patient management [195].

Colorectal carcinogenesis is a multistep process involving genetic and epigenetic alterations [433]. The majority of CRCs (70%) originate from a common colorectal precursor lesion, adenomatous polyp or conventional adenomas, which can potentially become malignant through the “traditional” carcinogenesis pathway (adenoma-carcinoma sequence). In addition, another type of colorectal lesion, serrated polyps, has been recently recognized as a precursor of 30% of all CRCs through the “serrated” carcinogenesis pathway. Among these precancerous lesions, some are defined as advanced colorectal polyps (ACPs) and present a higher risk of cancer transformation [434]. There are several screening assays used to detect CRC at an early stage, including the fecal immunochemical test (FIT), which is a widely used non-invasive and cost-effective assay for detecting the presence of fecal hemoglobin [435]. However, this type of stool-based assay has shown some limitations, including the inability to reliably detect colorectal precancerous lesions, such as ACPs [436]. Although colonoscopy is considered the gold standard CRC diagnosis technique, it is an invasive procedure that, in addition to requiring tedious and time-consuming preparation, can potentially cause serious complications [437] and has low

patient adherence [438]. Therefore, novel non-invasive tests for early detection of CRC are urgently needed.

Long noncoding RNAs (lncRNAs) are an emerging group of heterogeneous noncoding transcripts longer than 200 nt involved in the regulation of many biological processes in normal cells [439]. Of note, the expression of lncRNAs can be disrupted in cancer by several mechanisms, such as hypermethylation of CpG islands (CGIs) in their promoters [231,440]. DNA methylation is a well-known epigenetic mechanism based on the incorporation of a methyl group (CH3) into the 5′ carbon of cytosines in cytosine-phosphate-guanine (CpG) dinucleotides that generates 5-methylcytosine (5mC). In carcinogenesis, hypermethylation of tumor suppressor genes (TSGs) represents an early event usually associated with their transcriptional silencing, which leads to tumor initiation and disease progression. This epigenetic mechanism can be detected in tumors but also in cell-free DNA (cfDNA) released into circulation by tumor cells, showing great potential as a dynamic and non-invasive tool for CRC diagnosis [195].

LINC00473 is a lncRNA downregulated in CRC that exerts tumor suppressor functions in this disease by promoting apoptotic protease-activating factor 1 (APAF1) IRES activity through competitively sponging miR574-5p and miR15b-5p in tumor initiation and pathogenesis [441]. Of note, Diaz-Lagares et al. recently identified that the CGI in the promoter of *LINC00473* is hypermethylated in CRC [231]. Therefore, in this study, we evaluated the epigenetic regulation of *LINC00473* by DNA methylation and its clinical impact for non-invasive early detection of CRC.

2. Materials and methods

2.1 Cancer cell lines and treatments

The human CRC cell line HCT-116 (American Type Culture Collection [ATCC]) were cultured in DMEM with GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco) at 37 °C and 5% CO2. To promote DNA demethylation, HCT-116 cells were treated with 5-aza-2′-deoxycytidine (AZA) (Sigma-Aldrich) at 5 μM for 72 h. Treatment was performed in triplicate, and data were compared with the corresponding non-treated cell line.

2.2 Study participants

In this retrospective study, methylation levels of *LINC00473* were analyzed in eight independent clinical cohorts of patients, including four cohorts of colorectal tissue samples (Cohorts 1 to 4) and four cohorts of plasma samples (Cohorts 5 to 8) (**Supplementary Table 9**). Polyps were categorized into advanced colorectal polyps (ACPs) and non-ACPs (N-ACPs). ACP was defined as adenomas of at least 10 mm in size, containing high-grade dysplasia or with tubulovillous or villous histology, or a serrated polyp of at least 10 mm in size or containing any grade of dysplasia. Advanced neoplasia (AN) was defined as CRC or ACP [442]. The main clinical characteristics of the respective cohorts are described in **Supplementary Table 10** and **Supplementary Table 11**. Inclusion and exclusion criteria are detailed in **Supplementary Methods**. The study was approved by the Galician Ethical Committee (2017/538) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. Patients signed the informed consent to participate.

2.3 Blood sample collection and plasma isolation

Blood samples were collected by phlebotomy into collection tubes with EDTA as an anticoagulant. Plasma was isolated within 2 h after collection by an initial centrifugation at 1,500-1,600 g for 10 min at 4 °C, followed by a second centrifugation at 15,000-16,000 g for 10 min at 4 °C. Isolated plasma was stored at -80 °C until analysis.

2.4 Isolation of nucleic acids from cell lines, tissues, and plasma samples

Genomic DNA (gDNA) and total RNA were isolated from cell lines using TRIzol (Invitrogen) according to the manufacturer's protocol. gDNA was also isolated from formalin-fixed paraffin embedded (FFPE) colorectal tissues with the AllPrep DNA/RNA FFPE Kit (Qiagen). cfDNA was isolated from 2-4 mL of plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) and the vacuum system QIAvac 24 Plus (Qiagen) following the manufacturer´s recommendations. The quality and quantity of gDNA and RNA were evaluated with a NanoDrop (Thermo Fisher), and cfDNA was quantified by the QuantiFluor® ONE dsDNA kit with the QuantusTM Fluorometer (Promega). DNA and RNA were stored at −80 °C until analysis.

2.5 Bisulfite genomic sequencing

Methyl Primer Express v1.0 software (Applied Biosystems) and Primer3 (v.0.4.0) was used to design primers for the methylation analysis. DNA (1 µg) was subjected to sodium bisulfite treatment using the EZ DNA Methylation-Gold kit (Zymo Research). A 490-bp fragment of the *LINC00473* promoter was amplified using 2 µL of bisulfite-converted DNA with ImmolaseTaq polymerase (Bioline) at 60 °C for 40 cycles. The resulting PCR product was gel-purified (2% agarose) with NucleoSpin® Gel and PCR Clean-

up (Macherey-Nagel) and then cloned into the pGEMT Easy Vector System (Promega) following the manufacturers' protocols. For all samples, 10 colonies were randomly chosen, and DNA was purified using NucleoSpin® 96 Plasmid (Macherey-Nagel) and sequenced with a 3730 DNA Analyzer (Applied Biosystems). Results were transformed into percentages of CpGs showing methylation.

2.6 Gene expression analysis

For reverse transcription-PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR), 1-2 µg of total RNA was reverse-transcribed using the ThermoScriptTM RT-PCR System (Invitrogen) according to the manufacturer's recommendations. RT-PCR was performed with 100 ng of cDNA and IMMOLASE™ DNA polymerase (Bioline). Reactions for qRT-PCR were performed in triplicate on an Applied Biosystems 7,900HT Fast Real-Time PCR using 25-50 ng cDNA and TaqMan gene expression assays (*LINC00473*: Hs03677577_m1; GAPDH: Hs02758991_g1) as previously described [231]. For both RT-PCR and qRT-PCR, GAPDH was used as an endogenous control, and water as a negative control. RNA-seq data of *LINC00473* from CRC cell lines and normal colon mucosa were obtained from the public database Gene Expression Omnibus (GEO) (GSE138734). RNA-seq data of *LINC00473* in CRC primary tumors and matched normal tissues were obtained from TCGA.

2.7 DNA methylation analysis of 450K array data

To analyze the DNA methylation levels of the *LINC00473* promoter in CRC cell lines we obtained 450K array data (β-values) from the public database GEO (GSE49143). For the methylation analysis of *LINC00473*

promoter in primary colorectal tumors and matched normal tissues, we obtained the β-values of the 450K array from TCGA.

2.8 Methylation analysis of the LINC00473 promoter in colorectal tissues by pyrosequencing

The methylation status of the *LINC00473* promoter was analyzed in primary colorectal tumors and matched normal tissues by pyrosequencing. Primers for PCR amplification and sequencing were designed using PyroMark Assay Design 2.0 software (Qiagen). 500 ng of DNA was bisulfite-converted with the EZ-96 DNA Methylation kit (Zymo Research) and used as a template for subsequent PCR. Before pyrosequencing, PCR products were observed on 2% agarose gels. Pyrosequencing and methylation quantification were performed in a PyroMark Q96 System (Qiagen) according to the manufacturer's instructions. CpG site methylation was quantified using Pyro Q-CpG 1.0.9 (Qiagen). Water was used as negative control.

2.9 Methylation analysis of the LINC00473 promoter in cfDNA by qMSP

The methylation levels of the *LINC00473* promoter in plasma cfDNA were determined by quantitative methylation-specific PCR (qMSP) in a StepOne Plus system (Applied Biosystems). cfDNA (15-50 ng) was bisulfite-converted with the EZ DNA Methylation-Lightning Kit (Zymo Research) according to the manufacturer's recommendations. Each reaction contained 2 µL of bisulfite-converted cfDNA as a template, 10 µl Power SYBR™ Green PCR Master Mix (Thermo Fisher) and 150 nM each forward and reverse primers in a total volume of 20 µl. Thermocycling conditions in the StepOne Plus system were as follows: 10 min at 95 °C, followed by 50 cycles of 94 °C for 15 s and 60 °C for 30 s. Water was included as a no-template control. HCT-

116 and normal leukocytes (NLs) were used as positive controls for methylation and unmethylation, respectively. All the samples and controls were analyzed in triplicate. The DNA methylation level was expressed as a percentage of methylation (%) according to the following formula [443]: Methylation (%) = 100/[1+2^(CTCG–CTTG)], where CTCG and CTTG represent, respectively, the threshold cycle (CT) of the methylation and unmethylation status of the *LINC00473* promoter.

2.10 Methylation analysis of the LINC00473 promoter in cfDNA by ddMSP

Methylation of the *LINC00473* promoter was analyzed by droplet digital PCR (ddPCR) in a QX200 system (Bio-Rad). cfDNA (30-50 ng) was bisulfite-converted with the EZ DNA Methylation-Lightning Kit (Zymo Research) according to the manufacturer's recommendations. A custom Bio-Rad assay to detect *LINC00473* methylation (*LINC00473*-M) or unmethylation (*LINC00473*-U) was designed. First, a multiplex preamplification reaction was performed using ~2 ng of bisulfite-converted DNA, 25 µl SsoAdvanced™ PreAmp Supermix (Bio-Rad), 0.5 µl of *LINC00473*-M and 0.5 µl of *LINC00473*-U in a total volume of 50 µl. PCR conditions were as follows: 3 min at 95 °C, 10 cycles of 95 °C for 15 s and 56.2 °C for 4 min, and a final hold step of 4 °C. Next, a multiplex reaction mix was prepared by combining 2 µL of the preamplification product, 11 µl ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 2.2 µl of *LINC00473*-M, and 2.2 µl of *LINC00473*-U in a total volume of 22 µl. The QX200™ Droplet Generator (Bio-Rad) was used to generate droplets. Thermocycling conditions were as follows: 10 min at 95 °C, 40 cycles of 95 °C for 15 s and 56.2 °C for 30 s; 98 °C for 10 min and a final hold step of 4 °C. The temperature ramp increment was 2.5 °C/s for all steps. Droplets were counted and analyzed using the QX200™ Droplet Reader (Bio-Rad) and QuantaSoft analysis (Bio-Rad) was performed to acquire data.

Water was included as a no-template control, and HCT-116 and NL as positive controls for methylation and unmethylation, respectively. Reactions were performed in triplicate. DNA methylation was expressed according to the following formula: Methylation (%) = [M/(U + M)] x 100, where M represents the copies/µl of methylated cfDNA, and U the copies/µl of unmethylated cfDNA.

2.11 Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate the normality of the distribution of the data. Subsequently, the nonparametric Mann-Whitney U test was used for the comparison of methylation data. Nonparametric regression with generalized additive models (GAMs) [444] was used to evaluate the effect of age on methylation levels. To assess the diagnostic accuracy, a receiver operating characteristic (ROC) curve was generated. To obtain the greatest combination of sensitivity and specificity, the Youden index (J) was used: J = sensitivity + specificity - 1 [445]. The positive predictive value (PPV) and negative predictive value (NPV) were calculated: PPV = true positive/(true positive + false positive); NPV = true negative/(true negative + false negative). GraphPad Prism 7.0 software was used for statistical analysis and graphic representation. All expressed p-values were calculated with two-tailed tests and were considered significant when the p-value < 0.05.

3. Results

3.1 Epigenetic regulation of LINC00473 by promoter methylation in colorectal cancer

CGI hypermethylation in the promoter of *LINC00473* has been recently described in the colorectal cancer cell line HCT-116 using the Infinium HumanMethylation450 (450K) microarray (Illumina) [231]. To confirm this epigenetic feature, we first analyzed the methylation status of *LINC00473* in HCT-116 cells in comparison to normal colon mucosa by bisulfite genomic sequencing of multiple clones (**Figure 13A**). Importantly, the CGI promoter region of *LINC00473* that we analyzed contains CpGs included in the 450K array. As expected, we observed hypermethylation of all the CpGs analyzed in HCT-116 cells compared to unmethylated normal colon mucosa. To assess the effect of the CGI hypermethylation of *LINC00473* on gene expression (**Figure 13B**), we analyzed by RT-PCR the expression levels of the two transcriptional variants of this lncRNA (NR_026860.1, NR_026861.1) annotated according to the human Refseq database at UCSC (GRCh37/hg19). This analysis showed the downregulation of the two transcriptional variants of *LINC00473* in HCT-116 cells with respect to unmethylated normal colon mucosa. Importantly, the use of the demethylating agent 5-aza-2'-deoxycytidine (AZA) in HCT-116 cells restored the expression of *LINC00473*, as analyzed by RT-PCR and qRT-PCR (**Figure 13B-C**). Thus, we extended the methylation and expression analysis to other common colorectal cancer cell lines (**Figure 13D**) using 450K array and RNA-seq datasets obtained from Gene Expression Omnibus (GEO): GSE49143 and GSE138734, respectively. These analyses confirmed CGI promoter hypermethylation and downregulation of *LINC00473* in all CRC cell lines analyzed (COLO-205, HCC-2998, HCT-116, HCT-15, HT-29, KM12, and SW-620) in comparison to normal colon mucosa.

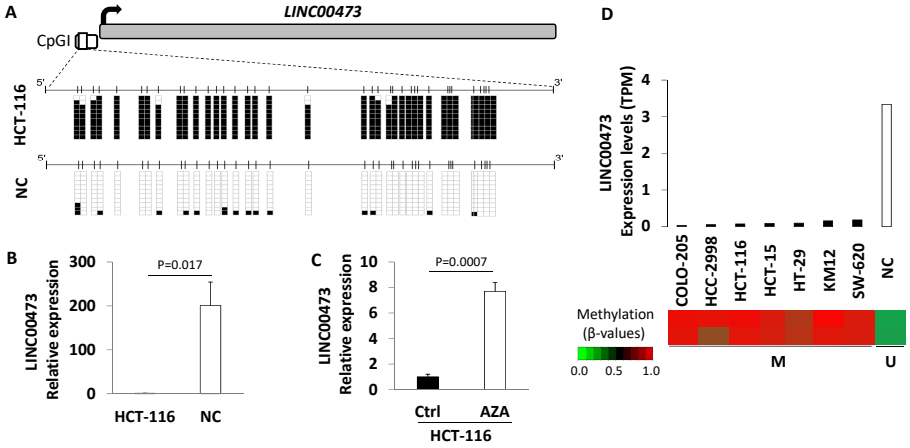

Figure 13. Epigenetic silencing of the *LINC00473* in colorectal cancer cells. A, Bisulfite genomic sequencing analysis of *LINC00473* promoter CpG island in the colorectal cancer cell line HCT-116 and normal tissue. Locations of CpG dinucleotides (vertical lines) and the TSS (long black arrow) are shown. Ten single clones are represented for each sample. The presence of unmethylated and methylated CpGs is indicated by white and black squares, respectively. **B**, DNA methylation-associated transcriptional silencing of *LINC00473* in the colorectal cancer cell line HCT-116. Expression levels of *LINC00473* were determined by RT-PCR in the methylated cancer cell line HCT-116 before and after treatment with the DNA demethylating agent 5-aza-2´-deoxycytidine (AZA), and in colorectal normal tissues (N=3). **C**, Restored *LINC00473* expression in the methylated cancer cell line HCT-116 after AZA treatment analyzed by qRT-PCR. Values were determined from triplicates and are expressed as the mean ± SEM. **D**, Methylation and expression analysis of *LINC00473* in common colorectal cancer cell lines and normal colon mucosa using 450K array and RNA-seq public datasets. TSS, transcription start site; NC; normal colon mucosa.

3.2 Methylation status of the LINC00473 promoter in colorectal tumors

After confirming the epigenetic regulation of *LINC00473* by promoter methylation in CRC cell lines, we decided to evaluate whether this epigenetic alteration was a general event in primary colorectal tumors. Thus, we evaluated the methylation status of the *LINC00473* promoter and the

expression level of the corresponding gene by using a 450K array and RNA-seq, respectively, from a TCGA dataset (Cohort 1) of primary colorectal tumors (from TNM stage I to IV) and matched normal tissues (controls). As expected from our results with CRC cell lines, this analysis revealed a significantly higher methylation level of *LINC00473* in colorectal tumors than in controls (**Figure 14A**), which was consistent across all TNM tumor stages (**Figure 14B**). In addition, *LINC00473* promoter hypermethylation was associated with a significant downregulation of its expression levels in primary colorectal tumors (**Supplementary Figure 3**).

Next, we generated receiver operating characteristic curves (ROCs) to evaluate the robustness of the methylation status of the *LINC00473* promoter for CRC diagnosis across all tumor stages. This analysis revealed a significantly high CRC detection accuracy with an area under the receiver operating characteristic curve (AUC) of 0.941 (95% CI: 0.915-0.966, $p < 0.0001$) (**Figure 14C**), a sensitivity of 91% (CI 95%: 87%-94%), a specificity of 100% (CI 95%: 91%-100%), a PPV of 100% and a NPV of 58%. Moreover, ROC curve analyses yielded a very high diagnostic accuracy across all tumor stages separately (**Supplementary Figure 4**). Subsequently, these results were confirmed in an independent cohort (Cohort 2) of primary colorectal tumors (from TNM stage I to IV) and matched non-tumor controls using bisulfite pyrosequencing (**Figure 14D-F**). This analysis also showed a significantly higher methylation level of the *LINC00473* promoter in primary colorectal tumors than in controls (**Figure 14D**), which was constant across all TNM tumor stages (**Figure 14E**). As expected, the *LINC00473* methylation level identified primary colorectal tumors with an AUC of 0.893 (95% CI: 0.851-0.935, <0.0001) (**Figure 14F**), a sensitivity of 78% (CI 95%: 71%-84%), a specificity of 98% (CI 95%: 92%-100%), a PPV of 99% and an NPV of 67%. The detection accuracy was constant across all CRC stages separately, as shown in **Supplementary Figure 5**.

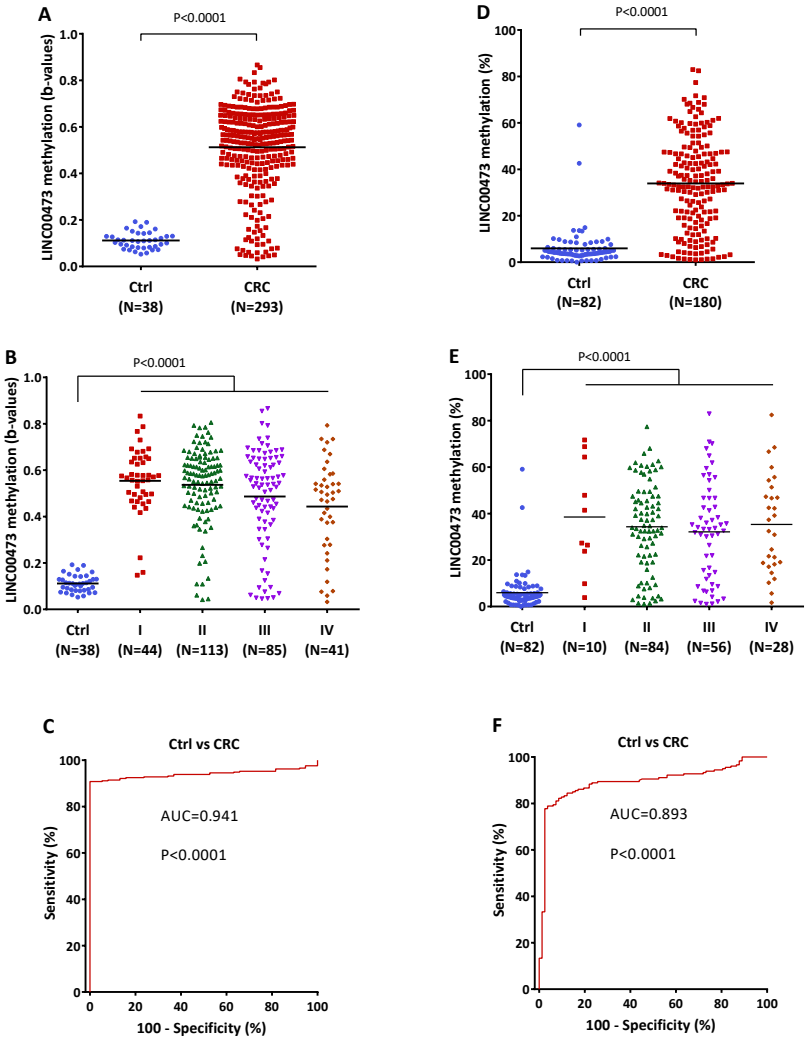

Figure 14. Methylation of *LINC00473* promoter in colorectal cancer tissues. **A-B**, Methylation status of *LINC00473* in CRC and across all the TNM tumor stages analyzed by 450K array (Cohort 1). **C**, ROC curve analysis evaluating the methylation of *LINC00473* for the detection of CRC in tissue samples in Cohort 1. **D-E**, Validation of the methylation status of *LINC00473* in CRC and across all the TNM tumor stages analyzed by pyrosequencing (Cohort 2). **F**, ROC curve analysis to validate the methylation of *LINC00473* for the detection of colorectal tumors in Cohort 2. Horizontal lines represent mean methylation levels. P, p-value analyzed by Mann-Whitney U test or ROC curve; AUC, area under the ROC curve; Ctrl, controls; CRC, colorectal cancer.

3.3 Methylation status of the LINC00473 promoter in tissue from precancerous colorectal lesions

DNA methylation is an epigenetic mechanism that can be deregulated in precancerous colorectal lesions [446]. To confirm this feature, we analyzed the methylation status of the *LINC00473* promoter by bisulfite pyrosequencing in tissues from premalignant colorectal polyps, CRC and matched normal colorectal mucosa (controls) (Cohort 3) (**Figure 15**). As expected, the methylation of *LINC00473* was significantly higher in polyps and in CRC than in healthy controls, while no differences were found between polyps and CRC (**Figure 15A**). Importantly, from a clinical viewpoint, we also found significant differences between *LINC00473* methylation levels in N-ACP compared to ACP (**Figure 15B**) and in N-ACP compared to CRC (**Figure 15C**) but not in controls compared to N-ACP or in ACP compared to CRC (**Supplementary Figure 6A-B**). Next, we generated ROC curves to evaluate the robustness of this methylation biomarker to detect CRC or premalignant colorectal polyps, and we observed a high capacity of *LINC00473* to differentiate controls from polyps (AUC=0.840, CI 95%: 0.657-1.00, p=0.0047; sensitivity=83%, CI 95%: 52%-98%; specificity=92%, CI 95%: 62%-100%; PPV=91%; NPV=85%) and controls from CRC (AUC=0.917, CI 95%: 0.794-1.00, p=0.0005; sensitivity=83%, CI 95%: 52%-98%; specificity=100%, CI 95%: 74%-100%; PPV=100%; NPV=86%) (**Supplementary Figure 6C-D**). More importantly, the methylation status of *LINC00473* was able to accurately detect ACP (AUC=0.992, CI 95%: 0.967-1, p=0.0001; sensitivity=100%, CI 95%: 63%-100%; specificity=94%, CI 95%: 70%-100%, PPV=89%; NPV=100%) (**Figure 15D**) and AN (AUC=0.947, CI 95%: 0.873-1.00, p < 0.0001; sensitivity=85%, CI 95%: 62%-97%; specificity=100%, CI 95%: 79%-100%, PPV=100%; NPV=84%) (**Figure 15E**).

To better appreciate the translational potential of our previous findings, we asked whether this methylation biomarker could be successfully validated using pyrosequencing in an additional cohort (cohort 4) composed of colorectal polyps and normal mucosa from non-cancer patients (controls). In addition to confirming methylation differences between controls and polyps (**Figure 16A**), with a larger number of N-ACPs, in this cohort, we were able to detect significantly higher methylation levels in controls than in N-ACPs (**Figure 16B**). Importantly from a clinical viewpoint, we confirmed significantly higher methylation levels of *LINC00473* in ACPs than in N-ACPs (**Figure 16C**). Furthermore, ROC curves demonstrated the high accuracy of the methylation status of *LINC00473* to detect polyps (AUC=0.831, CI 95%: 0.738-0923, p=0.0009; sensitivity=70%, CI 95%: 57%-81%; specificity=100%, CI 95%: 69%-100%, PPV=100%; NPV=36%) (**Figure 16D**) and, even more importantly, to detect ACPs (AUC=0.776, CI 95%: 0.665-0.888, $p < 0.0001$; sensitivity=71%, CI 95%: 54%-85%; specificity=79%, CI 95%: 62%-91%, PPV=78%; NPV=73%) (**Figure 16E**).

Additionally, we also assayed for a possible effect of age on *LINC00473* methylation levels in tissue samples; however, no significant effect was found ($p > 0.05$). Furthermore, we also did not find any significant difference ($p > 0.05$) in methylation levels according to the sex of the individuals.

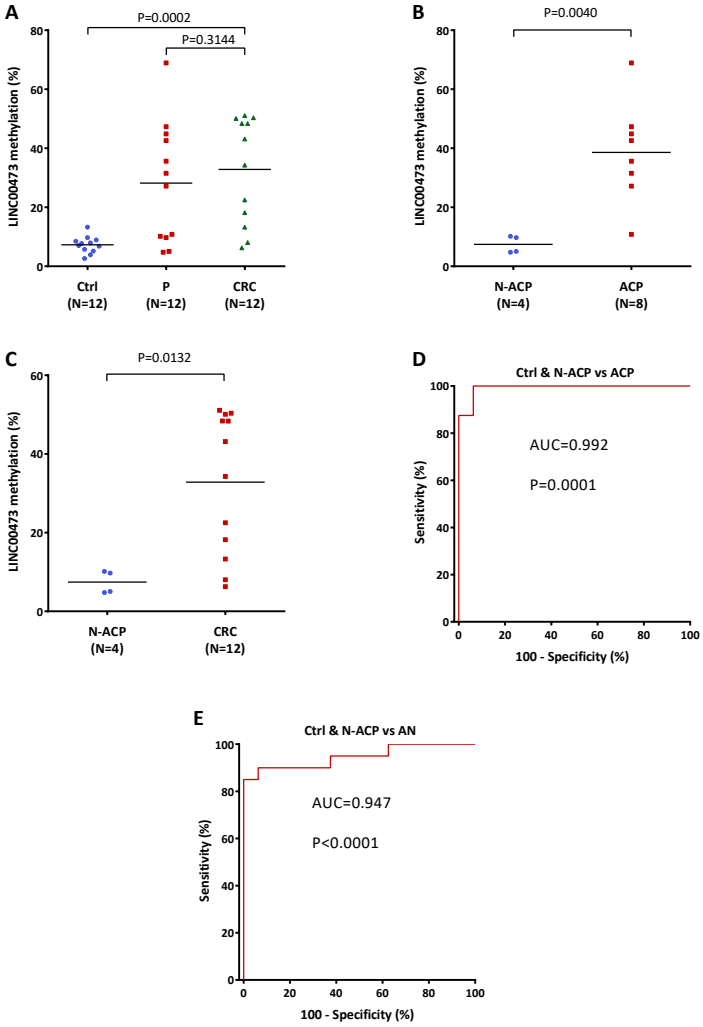

Figure 15. Methylation levels of *LINC00473* promoter in tissue precancerous colorectal lesions, CRC and controls by pyrosequencing (Cohort 3). A, Methylation levels in tissues from premalignant colorectal polyps, CRC and normal colorectal mucosa. **B-C**, Methylation levels in tissues from N-ACP, ACP and CRC. **D**, ROC curve analysis evaluating the methylation of *LINC00473* for the detection of ACP respect to the combination of controls and N-ACP. **E**, ROC curve to evaluate the methylation of *LINC00473* for the detection of AN respect to the combination of controls and N-ACP. Horizontal lines represent mean. P, p-value; AUC, area under the ROC curve; Ctrl, controls; P, polyps; CRC, colorectal cancer; N-ACP, non-advanced colorectal polyps; ACP, advanced colorectal polyps; AN, advanced neoplasia.

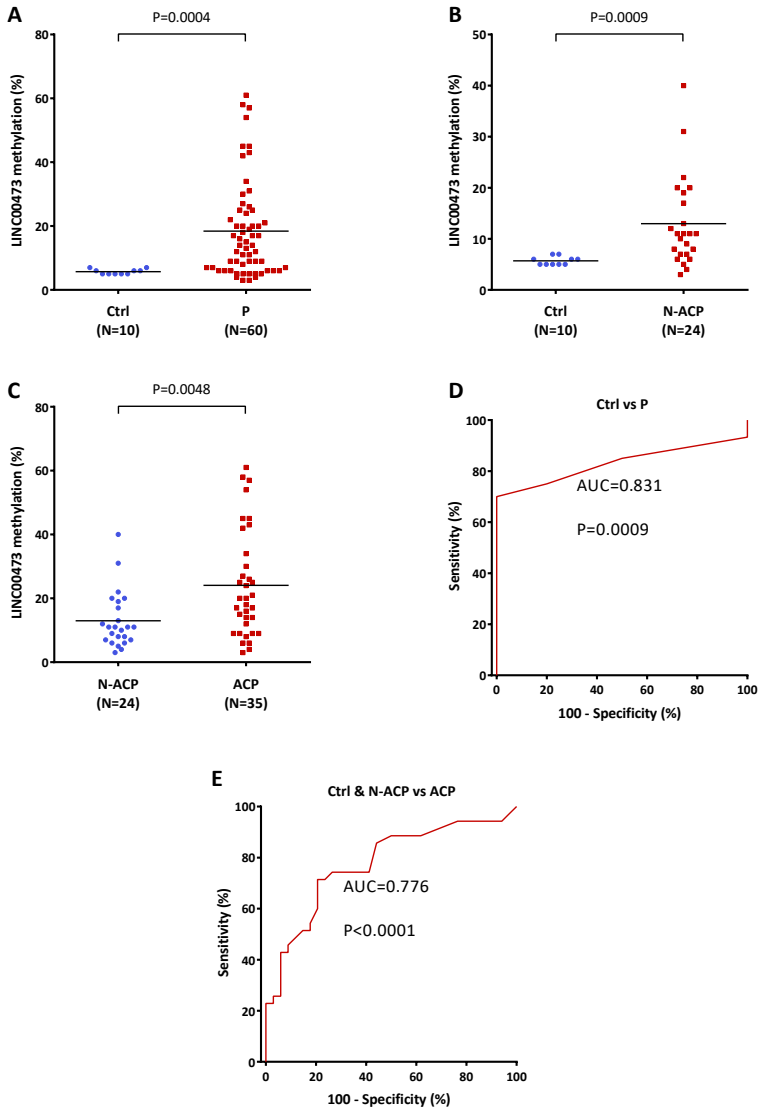

Figure 16. Validation of the methylation levels of *LINC00473 promoter* to detect tissue precancerous lesions (Cohort 4). A-C, Methylation levels of *LINC00473* in tissues from premalignant colorectal polyps and normal colorectal mucosa by pyrosequencing. **D-E**, ROC curve analysis evaluating the methylation of *LINC00473* for the detection of premalignant colorectal polyps. Horizontal lines represent mean. P, p-value; AUC, area under the ROC curve; Ctrl, controls; P, polyps; N-ACP, non-advanced colorectal polyps; ACP, advanced colorectal polyps.

3.4 Diagnostic potential of methylation of the LINC00473 promoter to detect colorectal cancer and precancerous lesions in plasma cell-free DNA

Beyond tissue samples, promoter hypermethylation of several genes in plasma cfDNA of patients with ACPs or CRC has also been described [195]. Based on this fact, we analyzed the methylation of the *LINC00473* promoter in plasma cfDNA of a cohort of self-declared healthy controls and CRC patients by qMSP (Cohort 5). The result of this analysis showed significantly higher methylation levels in CRC than in controls (**Figure 17A**). In addition, the methylation levels of *LINC00473* in cfDNA exhibited a very high diagnostic accuracy to detect CRC patients with an AUC of 0.881 (CI 95%: 0.776-0983, $p < 0.0001$), a sensitivity of 81% (95% CI: 61%-93%), a specificity of 100% (95% CI: 88%-100%), a PPV of 100% and an NPV of 85% (**Figure 17B**). Furthermore, we also analyzed the plasma cfDNA of a cohort of self-declared healthy controls and patients with ACPs presenting at least one polyp >10 mm previously confirmed by colonoscopy (Cohort 6). Of note, this assay revealed significantly higher methylation levels of *LINC00473* in cfDNA of ACPs than in controls (**Figure 17C**), showing a high diagnostic accuracy to detect ACPs (AUC = 0.836; 95% CI: 0.722-0.949, $p < 0.0001$) with a sensitivity of 79% (95% CI: 58%-93%), a specificity of 88% (95% CI: 73%-97%), a PPV of 83% and an NPV of 86% (**Figure 17D**).

To confirm the feasibility of using methylation of the *LINC00473* promoter for the non-invasive early detection of CRC, we retrospectively analyzed an independent cohort of plasma cfDNA samples (Cohort 7) obtained either prior to a scheduled colonoscopy as part of standard CRC screening or prior to colonic surgery for primary tumors (**Figure 17E-F**). Due to the need for very sensitive methodologies for the early detection of cancer in liquid biopsy [195], we used ultrasensitive droplet digital PCR (ddPCR) for

the methylation analysis of *LINC00473*. Consistent with our previous results in tissue and cfDNA, the methylation levels of *LINC00473* were significantly higher in ACP and CRC than in confirmed healthy controls (**Figure 17E**). In addition, no significant differences were found between the methylation status of *LINC00473* of ACP and CRC. Of note, methylation of the *LINC00473* promoter showed high accuracy for the detection of ACP, with an AUC of 0.721 (95% CI: 0.553-0.890, p=0.0235), a sensitivity of 76% (95% CI: 50%-93%), a specificity of 63% (95% CI: 38-84%), a PPV of 65% and an NPV of 75% (**Figure 17F**). Similarly, methylation of *LINC00473* was able to identify CRC patients with a high AUC of 0.833 (95% CI: 0.709-0.958, p=0.0003), a sensitivity of 90% (95% CI: 70%-99%), a specificity of 63% (95% CI: 38%-84%), a PPV of 73 and an NPV of 86. In addition, *LINC00473* showed the ability to detect AN with an AUC of 0.783 (95% CI: 0.662-0.904, p=0.0005), a sensitivity of 84% (95% CI: 69%-94%), a specificity of 63% (95% CI: 38%-84%), a PPV of 82%, and an NPV of 67%.

Similar to our previous analysis in tissue samples, we also found no significant effect of age ($p > 0.05$) on *LINC00473* methylation levels in cfDNA samples. In addition, no significant difference ($p > 0.05$) in methylation levels according to the sex of the individuals was found.

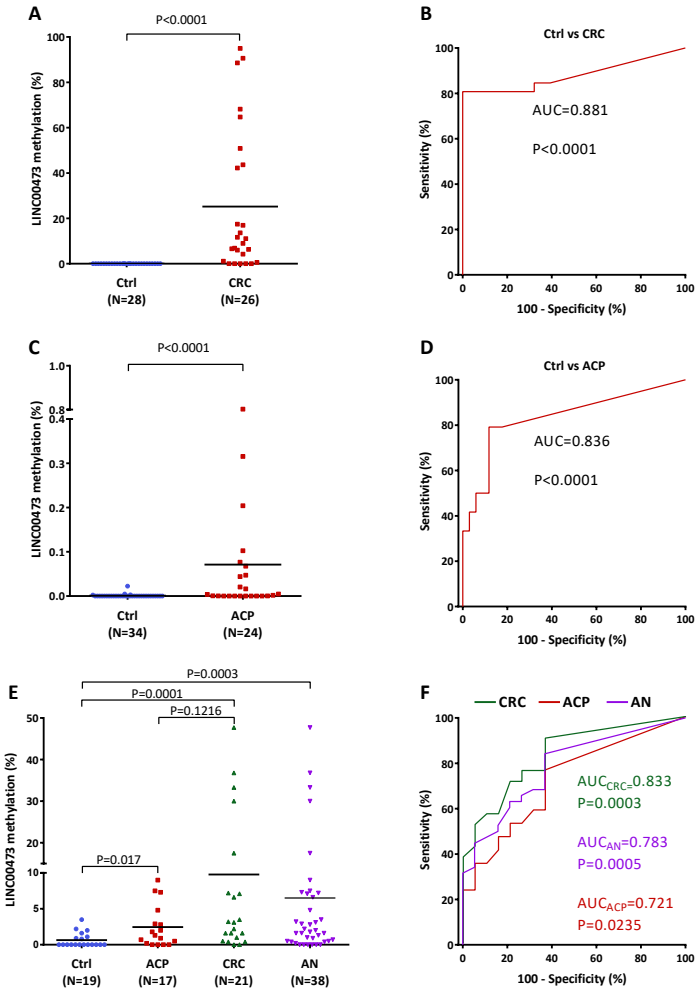

Figure 17. Methylation of *LINC00473* promoter in cell-free DNA from patients with advanced colorectal polyps and colorectal cancer. A, Methylation status of *LINC00473* in cfDNA of CRC patients analyzed by qMSP (Cohort 5). **B**, ROC curve analysis evaluating the methylation of *LINC00473* for the detection of CRC in cfDNA (Cohort 5). **C**, Methylation status of *LINC00473* in cfDNA of patients with ACP analyzed by qMSP (Cohort 6). **D**, ROC curve analysis evaluating the methylation of *LINC00473* for the detection of ACP in cfDNA (Cohort 6). **E**, Validation of the methylation status of *LINC00473* in cfDNA of patients with ACP by ddPCR analysis (Cohort 7). **F**, ROC curve analysis to validate the methylation of *LINC00473* for the detection of ACP and CRC in cfDNA (Cohort 7). Horizontal lines represent mean; P, p-value; AUC, area under the ROC curve; Ctrl, controls. ACP, advanced colorectal polyps; CRC, colorectal cancer.

3.5 Clinical utility of LINC00473 promoter methylation for the non-invasive detection of colorectal cancer during the follow-up of metastatic patients

After confirming its utility in the detection of precancerous lesions and CRC, we decided to evaluate whether the methylation status of the *LINC00473* promoter in plasma cfDNA could be useful to diagnose the presence of CRC in the palliative setting. For this purpose, we analyzed the methylation of the *LINC00473* promoter in plasma cfDNA by ddPCR at various clinically relevant time points from a cohort of six randomly selected mCRC patients (Cohort 8), whose disease evolution was evaluated according to a standard clinical practice (serial serum carcinoembryonic antigen (CEA) determinations and computed tomography scans). As shown in **Figure 18**, the plasma cfDNA methylation levels of *LINC00473* decreased with effective therapy and increased with disease progression. Notably, in some cases (e.g., cases #1, #4, and #5), *LINC00473* methylation preceded CEA in detecting the presence of CRC. Together, these six cases represent proof of concept of the utility of *LINC00473* methylation as a potential biomarker to detect the presence of CRC during the follow-up of metastatic patients.

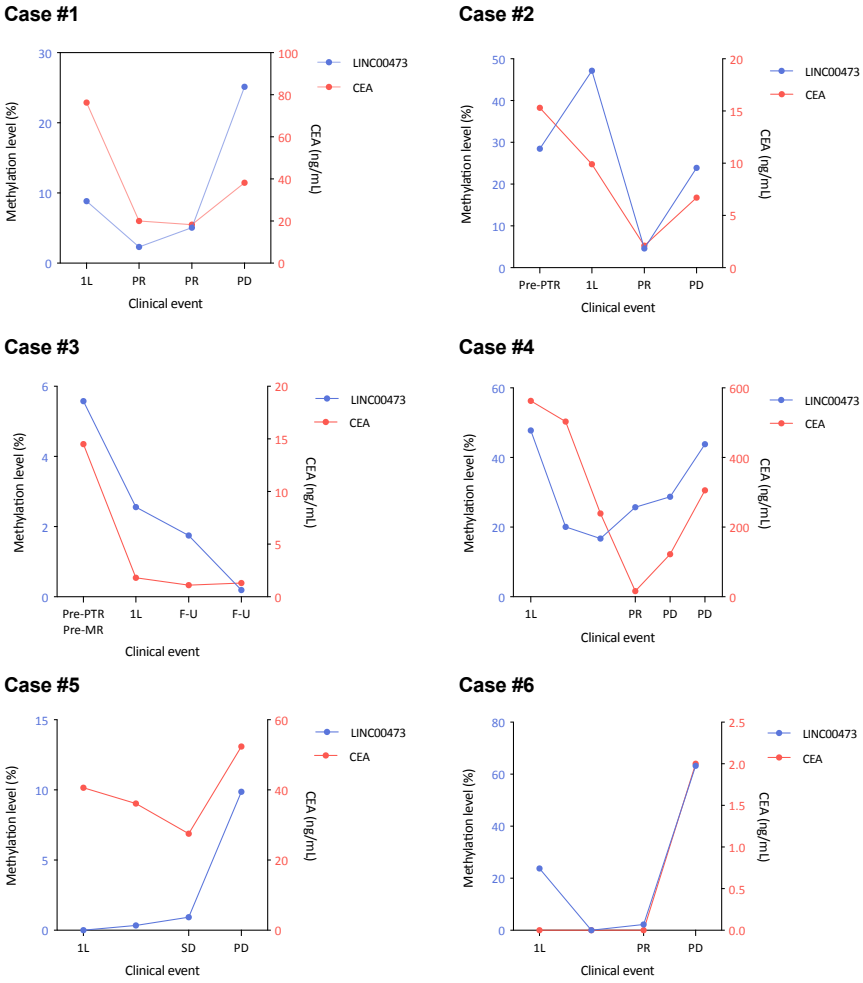

Figure 18. Clinical utility of *LINC00473* methylation for the non-invasive detection of colorectal cancer during the follow-up. Methylation levels of *LINC00473* promoter (blue color) were evaluated in serial plasma cfDNA samples at clinically relevant time points from 6 randomly selected metastatic CRC patients by ddPCR (Cohort 8). CEA (red color) was also analyzed in the same patients. ddPCR, droplet digital PCR; CEA, carcinoembryonic antigen; CRC, colorectal cancer; 1L, first-line therapy initiation; PD, progressive disease; PR partial response; Pre-PTR, pre-primary tumor resection; Pre-MR, pre-metastases resection; SD, stable disease; F-U, follow-up.

4. Discussion

Colorectal cancer (CRC) is one of the most common malignancies and is a major cause of cancer-related deaths worldwide [395]. The high incidence and mortality of CRC highlight the clinical need for novel strategies to improve early detection and personalize the management of patients with this type of tumor. Deregulation of epigenetic mechanisms, such as the promoter hypermethylation of lncRNAs, has relevant implications for CRC development and progression [231,447]. Importantly, DNA methylation can be detected both in tumor cells and in various components of liquid biopsy, such as cfDNA, and has shown clinical utility as a cancer biomarker [195]. Thus, in this work, we studied epigenetic regulation through DNA methylation of lncRNA *LINC00473* and evaluated the clinical utility of hypermethylation of its promoter for the detection of CRC and its precancerous lesions in both tissue and non-invasively collected patient samples.

LINC00473 is a lncRNA with pro-apoptotic tumor suppressor properties in CRC whose expression is downregulated in this tumor type. This lncRNA is able to sponge endogenous miR574-5p or miR15b-5p, inhibit cell proliferation and colony formation capacity, and induce cell apoptosis by activating the APAF1-CASP9-CASP3 pathway [441]. In addition, a genome-wide analysis of the CRC cell line HCT-116 recently identified promoter hypermethylation of *LINC00473* [231]. However, information is lacking on the methylation status of *LINC00473* in CRC and the regulation of this lncRNA by this epigenetic mechanism. Thus, in this work, we confirmed that *LINC00473* is hypermethylated in CRC and that this epigenetic modification is associated with the transcriptional silencing of this lncRNA. In line with this, previous works have described the aberrant hypermethylation of various lncRNAs in several tumor types, including CRC [231,448].

The deregulation of DNA methylation is an epigenetic alteration that takes place during colorectal carcinogenesis and can be detected from precancerous lesions, such as ACP, to advanced stages of CRC [195]. In this context, our study revealed that the promoter of *LINC00473* is hypermethylated in primary colorectal tumors and that this epigenetic deregulation is present from early to advanced stages of CRC. Importantly, *LINC00473* was also hypermethylated in ACP tissues, indicating that the hypermethylation of *LINC00473* is an early event in colorectal carcinogenesis that is present throughout all stages of tumor development. In addition, we found in primary colorectal tumors that the hypermethylation of *LINC00473* was associated with a decrease in the expression of this lncRNA. These results are in line with a previous work, which identified the downregulation of expression and tumor suppressor properties of *LINC00473* in association with the initiation and pathogenesis of CRC [441]. Similarly, other authors have found epigenetic deregulation of important TSGs, such as p16/CDKN2A and hMLH1, throughout the colorectal carcinogenesis process [449,450]. In addition to biological effects on gene expression, epigenetic alterations have shown clinical utility as tumor biomarkers at different stages of CRC disease [195]. In this regard, we found that the methylation status of *LINC00473* in tissue samples was able to accurately identify ACPs and the different stages of CRC from early to advanced disease, indicating the feasibility of using the methylation of *LINC00473* as a biomarker for the early detection of CRC.

There is a pressing need in the clinic to detect CRC at the beginning of the disease, since the majority of patients can be successfully treated if detected early [432]. Although colonoscopy is an effective diagnostic tool for the screening and early detection of CRC, it is an invasive method that limits its application [437]. Currently, FIT is a non-invasive stool-based assay widely used for the early detection of CRC in screening programs; however, it has a low sensitivity for the early detection of precancerous lesions [436]. To date,

several non-invasive biomarkers have been proposed for the early detection of CRC, including circulating epigenetic biomarkers [436]. Alterations in DNA methylation are promising candidates for early diagnosis, since they are covalent and stable marks that can be found early in carcinogenesis [451]. In line with this, the detection of *SEPT9* methylation in plasma cfDNA (EpiproColon), which was approved by the U.S. Food and Drug Administration (FDA) in 2016 and has been proposed as a non-invasive test for the early detection of CRC [315]. However, a large prospective study revealed that the detection of SEPT9 methylation has low diagnostic accuracy for CRC (sensitivity, 48.2%) and advanced precancerous lesions (sensitivity, 11.2%) [373], highlighting the need for new accurate, non-invasive tests for the early detection and management of CRC. In this work, we found that, similar to tissue samples, the promoter of *LINC00473* was hypermethylated in plasma cfDNA of CRC and ACP patients. Of note, the methylation of *LINC00473* in cfDNA allowed the identification of CRC and ACP with very good AUCs, indicating that it is an assay with high diagnostic accuracy for the very early detection of CRC [452]. In addition to showing interesting specificity and PPV, methylation of the LINC00473 promoter in cfDNA also showed high sensitivity and NPV in a CRC and precancerous lesions screening context. While high PPV is desired when the costs or risks of further testing are significant, NPV gains importance when the disease is serious and curable in its preclinical phase, as is the case of ACP and CRC [453]. Another advantage of analyzing the methylation of *LINC00473* in plasma would be the higher adherence that blood-based assays provide in comparison with widely used fecal tests, such as FIT [454].

While we only performed a small proof-of-concept study, the methylation levels of *LINC00473* in cfDNA were able to detect the presence of the disease during the follow-up of metastatic CRC patients, anticipating, in some cases, the response and progression compared with a standard

protocol based on serial CEA determinations and CT scans [455]. These results show the promising potential of *LINC00473* methylation in cfDNA to facilitate disease detection in the palliative setting of CRC patients.

Altogether, the results of this work indicate that the methylation status of *LINC00473* in cfDNA is a promising biomarker for the non-invasive early detection of CRC and its precancerous lesions that could be used in the clinic. Although methylation analysis of cfDNA was performed in a limited number of patients, our results support further validation to confirm the clinical applications of this novel epigenetic biomarker in large-scale prospective studies with asymptomatic screening participants and colorectal cancer patients. Because the combination of different types of biomarkers may increase the accuracy of diagnostic assays [451], future studies should also evaluate whether the combination of *LINC00473* with other circulating biomarkers may improve its high diagnostic accuracy for the early detection of CRC.

5. Conclusions

CRC is a leading cause of cancer mortality worldwide that is usually diagnosed at an advanced stage, highlighting the need for new early detection strategies. We evaluated the clinical utility of the promoter methylation of the long noncoding RNA LINC00473 in plasma cfDNA as a non-invasive biomarker for the detection of CRC. The methylation of *LINC00473* showed high diagnostic accuracy to detect CRC and associated precancerous lesions both in tissues and in cfDNA, indicating that the methylation of *LINC00473* has a huge potential as a biomarker for the non-invasive early detection of CRC and related precancerous lesions.

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Conflicts of interest

J.R.-B. has received honoraria for educational activities from Roche; honoraria for consultancies from Boehringer Ingelheim; institutional research funding from Roche; and travel, accommodations, expenses from Bristol-Myers Squibb, Merck Sharp & Dohme, Ipsen, PharmaMar, Merck, Pfizer, and Roche. E.B.-V. has received honoraria for consultancies from Leo, and Rovi; and travel, accommodations, expenses from Servier, Merck, Sanofi, Roche, Pierre Fabre, and Amgen. Y.V.-I. has received honoraria for consultancies from PharmaMar. F.V.-R. has received honoraria for consultancies from Roche, Eisai, and Servier; and travel, accommodations, expenses from Lilly, Merck, and Pierre Fabre. S.C.-F. has received honoraria for educational activities from Servier, and Pierre Fabre; and travel, accommodations, expenses from Lilly, and Merck. R.L.-L. has received honoraria for participation in Advisory Boards from Roche, AstraZeneca, Merck, Merck Sharp & Dohme, Bayer, Bristol-Myers Squibb, Novartis, Janssen, Lilly, Pfizer, and Leo; travel, accommodations, and expenses from PharmaMar, Roche, Bristol-Myers Squibb, and Pierre Fabre; research funding from Roche and Merck; and is co-founder and shareholder in Nasasbiotech, S.L., Mtrap Inc. A.R.-C., A.B.C., R.L.-L. and A.D.-L. are inventors in one patent application over these results licensed to Advanced Marker Discovery, S.L. (Amadix). L.C., A.C.M. and R.A. are employees of Advanced Marker Discovery, S.L. (Amadix). The rest of the authors declare no potential conflicts of interest. All authors have read the journal's policy on disclosure of potential conflicts of interest. All authors have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

Author contributions

Conceptualization, J.R.-B., A.R.-C., R.L.-L., and A.D.-L.; resources, J.R.-B., A.A.-C., F.L.M.C.-P., L.M.-R., J.A.V.-B., E.B.-V., Y.V.-I., F.V.-R., S.C.-F., R.S.-P., V.M., A.G., L.C., A.C.M., R.A., and M.E.; Data curation, A.R.-C., A.A.-C., J.R.-B., E.B.-V., and R.S.-P.; methodology, A.R.-C., J.R.-B., N.C.F, A.B.C., M.M.-P., R.M.L.-L., and A.D.L.; formal analysis, J.R.-B., A.R.-C., N.C.-F., A.B.-C., and A.D.-L.; writing—original draft preparation, J.R.-B., A.R.-C., and A.D.-L.; writing—review and editing, J.R.-B., A.R.-C., A.C.M., A.B.-C, R.L.-L., and A.D.-L.; supervision, A.D.-L. All authors have read and agreed to the published version of the manuscript. All authors have read the journal's authorship statement.

Data availability

The methylation and expression data obtained from the 450K array and RNA-seq used in this study are publicly available in TCGA and GEO (GSE49143 and GSE138734).

CHAPTER II.B

A novel cfDNA methylation signature for the prediction of FOLFOX-based therapy response in advanced colorectal cancer.

Abstract

Background: The first-line treatment of mCRC is usually based on fluoropyrimidine-based chemotherapies, where the combined use of 5-FU, leucovorin and oxaliplatin (FOLFOX) is the most common treatment. However, there are patients that do not respond to this therapy, highlighting the urgent unmet clinical need to discover novel biomarkers in order to identify the patients who will benefit from this treatment. The presence of DNA methylation in biological fluids represents a promising non-invasive biomarker. Therefore, the aim of this study was to discover novel non-invasive methylation biomarkers in cfDNA to predict FOLFOX-based therapy response of mCRC patients.

Methods: A retrospective cohort of 20 mCRC patients before starting first-line treatment with FOLFOX was selected. Following RECIST 1.1 criteria, therapy response was evaluated at 3-6 months of starting FOLFOX. Using the Infinium MethylationEpic (EPIC) array v2.0, we analyzed the cfDNA methylome of the plasma samples of 8 patients with progressive disease (PD) and 12 patients with non-progressive disease (nPD). Bioinformatic analysis of methylation data was carried out using RnBeads 2.0.

Results: A total of 1,174 differentially methylated CpGs (DMCpGs) were identified in cfDNA between patients with nPD and PD. These DMCpGs were widely distributed throughout all the chromosomes of the genome. Interestingly, patients with nPD showed more hypomethylated CpGs than those with PD. Of note, we were able to identify a cfDNA methylation signature (episignature) comprising 406 DMCpGs that clearly differentiated nPD and PD. Furthermore, the genes of this episignature were involved in relevant cancer pathways related to chemotherapy resistance, such as PI3K/AKT, Wnt and Cadherin signaling.

Conclusions: In this study, using a genome-wide non-invasive methylation approach, we discovered a novel episignature of cfDNA able to predict the response to FOLFOX therapy in mCRC patients. This cfDNA methylation signature represents a promising non-invasive tool for precision oncology of CRC. We also identified the epigenetic deregulation of relevant genes and cancer pathways involved in the response to FOLFOX therapy, opening new avenues to overcome the resistance to this treatment.

1. Introduction

First-line therapies for the common treatment of mCRC often include 5-fluorouracil (5-FU) in combination with leucovorin and oxaliplatin (FOLFOX) [456,457]. However, there are patients that are unable to response to this therapy (primary resistance), resulting in low response rates [458,459]. Identifying patients who will benefit from a particular therapy prior to treatment initiation remains a great challenge. Consequently, there is the clinical need to identify biomarkers that can predict response to therapy in patients with mCRC [64,460].

Understanding resistance mechanisms is crucial to predict therapy response and identify potential biomarkers. In this sense, epigenetic modifications, including DNA methylation, have shown to be involved in the development of therapy resistance [459,461,462]. Multiple resistance mechanisms may coexist within a single patient and conventional biopsy analysis of a single lesion may not be enough to identify clinically significant molecular alterations responsible for treatment failure [463]. Liquid biopsy, particularly cfDNA, has the potential to non-invasively detect these resistance mechanisms while comprehensively analyzing all tumor heterogeneity [415,463,464].

In this context, using a genome-wide non-invasive approach, we explored the methylation profile of cfDNA in relation to FOLFOX therapy response of mCRC patients, with the aim to identify a predictive cfDNA methylation signature (episignature) for this treatment.

2. Materials and methods

2.1 Study participants

In this retrospective study, mCRC patients before starting first-line treatment with FOLFOX were recruited between April 2019 and June 2021 at the Medical Oncology Departments at the University Clinical Hospital of Santiago de Compostela and the Ramon y Cajal Hospital of Madrid. The study was approved by the Galician Ethical Committee (reference number 2019/017) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All participants included in the study signed the informed consent to participate. Inclusion and exclusion criteria are collected in **Supplementary Table 12**. The response to treatment of patients was evaluated with computed tomography (CT) scan during follow-up. Patients were classified according to their best response to treatment, as determined by the result of the first and second CT scans, ~3 and ~6 months respectively, after starting therapy. Classification of response was performed according to RECIST 1.1 criteria, allowing to differentiate patients with non-progressive disease (nPD: stable disease or partial response) and patients with progressive disease (PD).

2.2 Blood sample collection and plasma isolation

Blood samples were collected by venipuncture into collection tubes with EDTA as an anticoagulant at the time of diagnosis of metastasis and before starting the treatment. Plasma was isolated within 2 h after collection by an initial centrifugation at 1,600 g for 10 min at 4 °C, followed by a second centrifugation at 16,000 g for 10 min at 4 °C. Isolated plasma was stored at -80 °C until analysis.

2.3 Isolation of nucleic acids from plasma samples

CfDNA was isolated from 2-5 mL of plasma using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) and the vacuum system QIAvac 24 Plus (Qiagen) following the manufacturer´s recommendations. cfDNA was quantified using the Qubit 1× dsDNA High-Sensitivity Assay Kit and a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). The cfDNA isolated from plasma was stored at −80 °C until analysis.

2.4 Genome-wide cfDNA methylation analysis

For the methylome analysis, 20–50 ng of cfDNA was bisulfite-converted using the EZ-96 DNA Methylation kit (Zymo Research), following the manufacturer's recommendations for the EPIC array (Illumina). Subsequently, the bisulfite-modified cfDNA was then treated with the Infinium HD FFPE Restore Kit (Illumina) according to the manufacturer's instructions. This kit effectively repairs the converted DNA prior to whole genome amplification. Following cfDNA restoration, the samples were hybridized to the Illumina Infinium MethylationEPIC v2.0 BeadChip Kit (900K EPIC v2, Illumina) according to the manufacturer´s protocol. This new microarray assay is a genome-wide methylation screening tool, targeting over 935,000 CpG sites within the most biologically significant regions of the human methylome at a single-nucleotide resolution [465]. After the hybridization, the BeadChips were analyzed using an iScan system (Illumina).

Next, the methylation microarray data (IDAT files) were processed within the R statistical environment, with packages available in Bioconductor [466], which provide tools for high-throughput analysis of genomic data. Quality control and data preprocessing was performed with the *minfi* package [467]. Subsequent differential methylation analysis between groups was conducted using the *limma* package [468]. The methylation score of each

CpG was expressed as β-values, having undergone prior normalization for colour bias and background level adjustment. Additionally, quantile normalization was applied across BeadChips. Samples with a mean detection p-value < 0.01 were considered valid for the analysis. The resulting β-values, ranging from 0 (no methylation) to 1 (complete methylation), were further subjected to a two-step filtering process. This process involved the exclusion of SNPs, probes located on the sex chromosomes and unreliable β-values with a detection p-value > 0.01. After this filtering step, the remaining CpGs were considered valid for the study. Significance was determined at a threshold of p-value < 0.05, indicating statistical differentiation between groups. Unsupervised hierarchical clustering heatmaps with the *ComplexHeatmap* package [469] and circos plot with the circlize package [470], were carried out using R environment. Furthermore, the gene ontology (GO) enrichment analysis for the methylation profiles was conducted using GENECODIS [471], and the protein-protein interactions with STRING.

3. Results

3.1 Patient characteristics

Clinicopathological characteristics of mCRC patients selected for the study are described in **Table 5**. Seven patients were female (35%) and 13 males (65%). The average age was 66 ± 10 years. Most of the patients had tumors with adenocarcinoma histology (95%) and had liver metastases (85%). Regarding response to treatment, 8 patients (40%) were classified with PD and 12 with nPD (8 with partial response and 4 with stable disease).

Table 5. Clinical characteristics of mCRC patients included in the study.

Characteristics	Patients (N=20)
Age (years)	
<65	7 (35%)
65-75	10 (50%)
>75	3 (15%)
Gender	
Female	7 (35%)
Male	13 (65%)
Histology	
Adenocarcinoma	19 (95%)
Mucinous	1 (5%)
Primary tumor location	
Right colon	6 (30%)
Left colon	6 (30%)
Rectum	7 (35%)
Rectosigmoid	1 (5%)
Metastatic sites	
1	7 (35%)
≥2	13 (65%)
Metastatic location	
Liver	17 (85%)
Lung	6 (30%)
Peritoneum	4 (20%)
Lymph nodes	9 (45%)
First-line therapy	
FOLFOX	20 (100%)
Therapy response	
PD	8 (40%)
nPD	12 (60%)

PD, progressive disease; nPD, non-progressive disease.

3.2 Genome-wide cfDNA methylation analysis of metastatic colorectal cancer patients

We performed a genome-wide cfDNA methylation analysis for profiling the methylome of 12 patients with nPD and 8 with PD in the cohort of our study. After quality control, all the samples, except 1 PD, showed a mean detection p-value < 0.01 and were considered valid for the study. Then, we

compared the methylation status of cfDNA in nPD and PD, which revealed 1,174 differentially methylated CpGs (DMCpGs) ($p < 0.05$) between both groups(**Figure 19A**). These DMCpGs showed a wide distribution throughout all chromosomes of the genome (**Figure 19B**). Out of these DMCpGs, 67% (784 CpGs) were hypomethylated, and 33% (390 CpGs) were hypermethylated in patients with nPD respect to those with PD (**Figure 19C**).

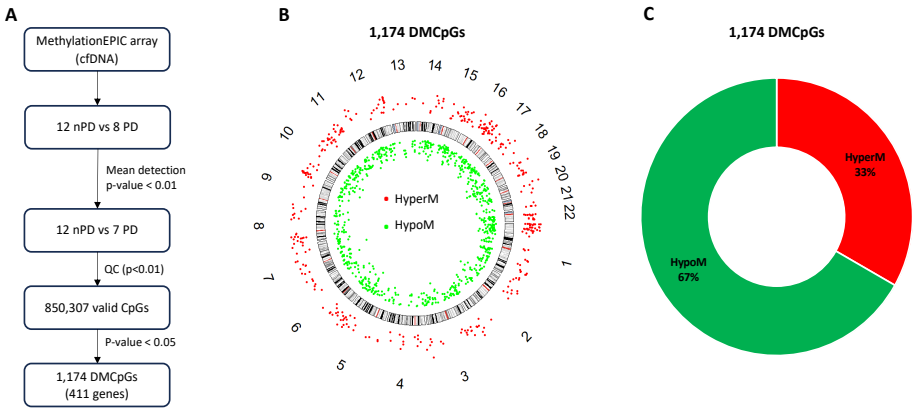

Figure 19. Methylation landscape of cfDNA in mCRC patients. A, Flowchart of the cfDNA methylation analysis. **B–C**, Distribution of the 1,174 differentially methylated CpGs (DMCpGs) found in cfDNA of mCRC patients according to (B) chromosome location and (C) methylation status. cfDNA, cell-free DNA; PD, progressive disease; nPD, non-progressive disease; QC, quality control; HypoM, hypomethylated; HyperM, hypermethylated.

In general, most of the hyper- and hypomethylated CpGs observed in nPD respect to PD showed a similar distribution according to their gene region location and CpG context. In the case of CpG context (**Figure 20A**), most of these DMCpGs were distributed in regions with low CpG density (open sea). Regarding gene region (**Figure 20B**), both hyper- and hypomethylated CpGs showed a similar distribution, being around 40% in gene promoters, 32% in

intergenic regions and about 20% in gene body. A slightly higher presence of hypomethylated CpGs in 3' UTR regions (9%) was observed respect to the hypermethylated ones (5%).

We also observed that a small percentage of DMCpGs were located within CTCF-binding sites (**Figure 20C**), with a slightly higher percentage in hypermethylated CpGs (5%) compared to hypomethylated (2%). Furthermore, enhancer regions also showed a small proportion of DMCpGs. Interestingly, in these enhancer regions we observed a higher proportion of hypomethylated (10%) than hypermethylated CpGs (**Figure 20D**).

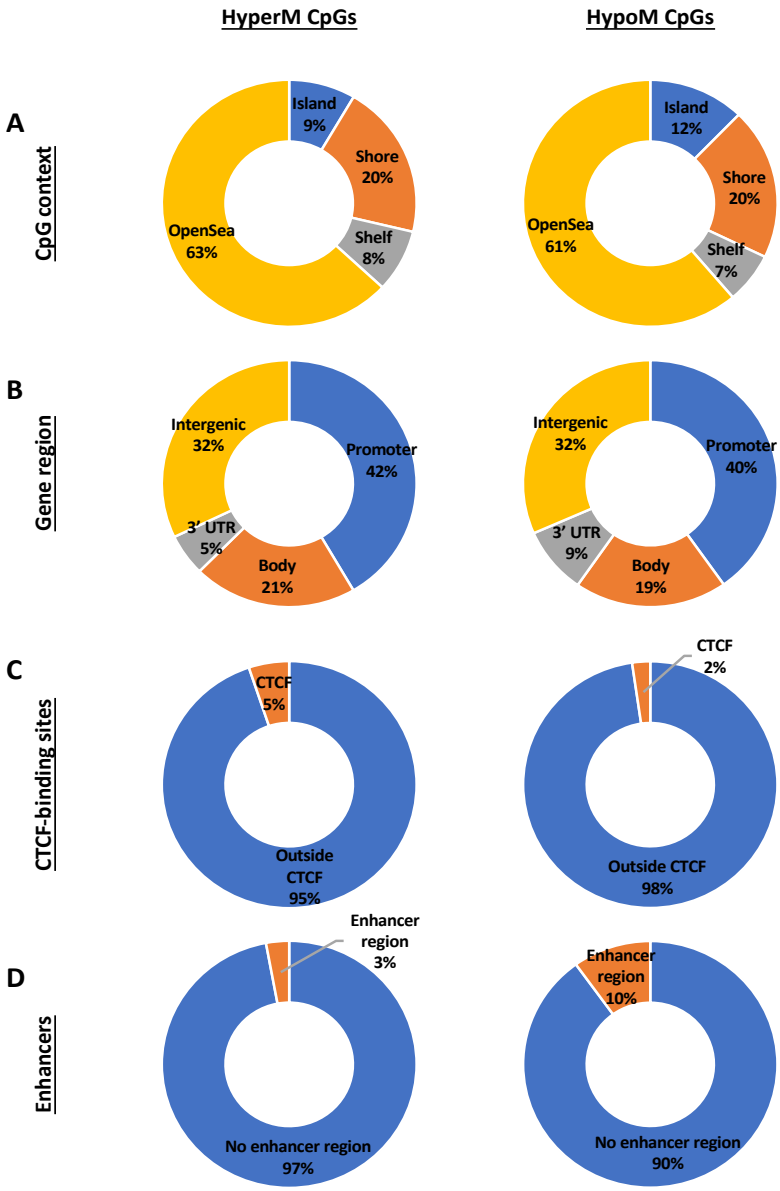

Figure 20. Distribution of the 1,174 DMCpGs found in the cfDNA of patients with nPD respect to PD. A, CpG context. **B,** gene location. **C,** CTCF-binding sites. **D,** enhancer region. 3' UTR, 3' untranslated regions; CTCF, CCCTC-Binding Factor.

3.3 Identification of a cfDNA episignature to predict response to FOLFOX in mCRC patients

Among the 1,174 DMCpGs previously described, we selected the CpGs with a difference in methylation (Δβ value) higher than 0.10 (Δβ value > |0.10|), leading to a cfDNA methylation signature (episignature) of 406 CpGs. By means of a hierarchical clustering analysis, this cfDNA episignature was able to clearly differentiate between patients with nPD and with PD (**Figure 21**). **Supplementary Table 13** shows the 20 most DMCpGs of the episignature found between nPD and PD.

Importantly, the 406 DMCpGs of the episignature identified were located in 133 different genes. After performing a GO analysis (**Figure 22**), we observed that these genes were involved in relevant processes related to cancer, including regulation of transcription, cell proliferation and apoptosis. These genes were also involved in several relevant pathways, such as PI3K/AKT, Wnt and Cadherin signaling. Interestingly, we also performed a protein-protein interaction analysis by STRING, revealing that the genes of these 406 DMCpGs are related among them and belong to a network significantly enriched in protein interactions ($p < 0.01$) (**Supplementary Figure 7**).

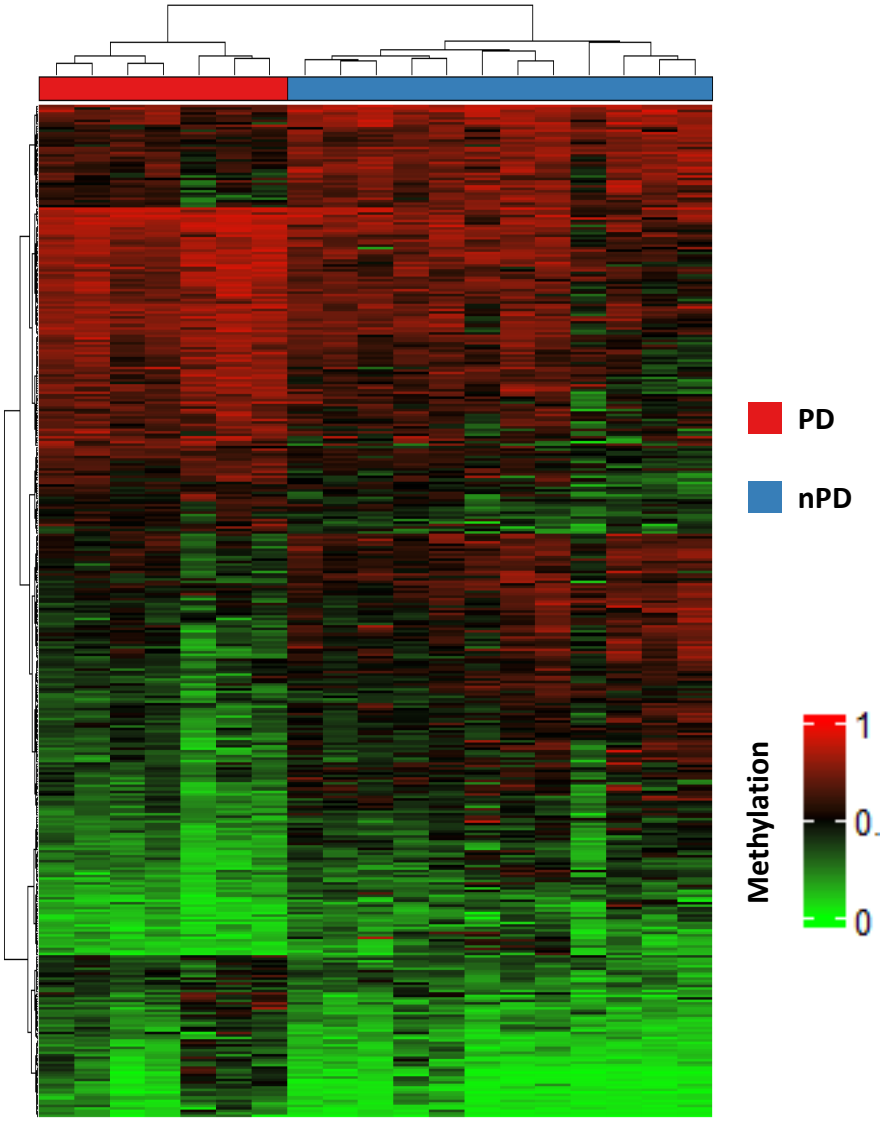

Figure 21. CfDNA episignature predictive of response to FOLFOX in mCRC patients. Unsupervised hierarchical clustering heatmap of the episignature (406 DMCpGs) obtained in cfDNA that differentiates patients with nPD patients from those with nPD. PD, progressive disease; nPD, non-progressive disease.

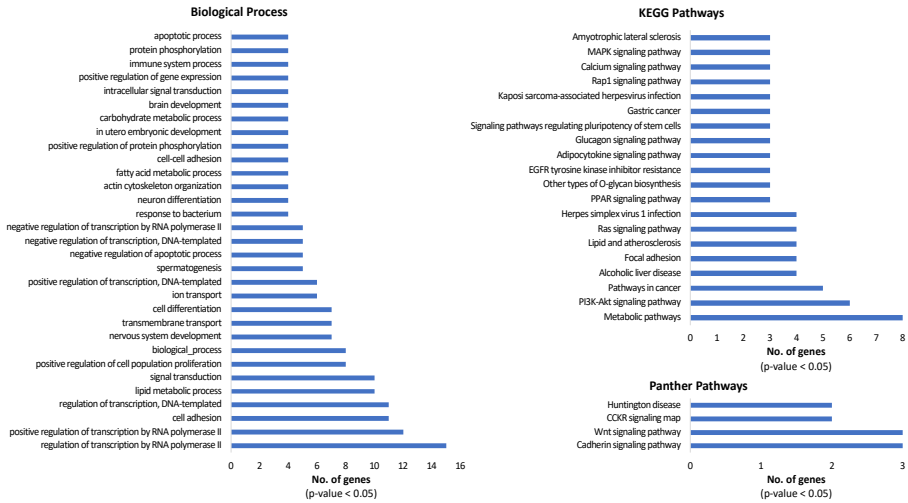

Figure 22. Gene Ontology (GO) enrichment analysis of the 133 genes involved in the cfDNA episiganture predictive of therapy response to FOLFOX in mCRC patients. GO analysis representing some of the most relevant biological processes, KEGG and Panther pathways of the genes involved in the cfDNA episignature.

4. Discussion

Although the survival rates of mCRC patients have improved in recent years due to advances in treatments, the majority of cases remain incurable [53]. Five-year survival is less than 15%, mainly due to the presence of therapy resistance [472], being urgent to discover novel biomarkers that identify the patients who will benefit from a particular therapy before starting its administration. This is the case of FOLFOX-based therapy, which nowadays lacks predictive biomarkers to guide its clinical use [473]. Thus, in this study, using a genome-wide DNA methylation approach based on EPIC arrays, we analyzed the methylome of cfDNA in mCRC patients to discover novel non-invasive predictive biomarkers of response to FOLFOX-based therapy.

Although EPIC arrays have been designed for the methylation analysis of tissue and cell samples, we [474] and others [475] were able to adapt this technology for the profiling of cfDNA methylation in liquid biopsy samples, such as plasma, indicating that this a robust strategy that could be widely used in multiple studies for the discovery of epigenetic biomarkers by liquid biopsy.

Epigenetic modifications are responsible for the emergence of resistances to various therapies in mCRC patients [461,476]. In particular, some studies have demonstrated that the alteration of DNA methylation in colorectal tumor cells is related with the development of resistance to 5-FU-based treatments [477,478]. Similarly, epigenetic alterations of colorectal tumors have been explored as potential biomarkers of response to FOLFOX [479]. In this context, our study successfully identified a substantial number of CpGs differentially methylated in cfDNA of plasma samples of mCRC patients that contribute to explain the response to FOLFOX therapy. These CpGs were widely distributed throughout the entire genome, suggesting a generalized alteration of the methylome in relation to therapy response, which could modify the expression of many genes and alter different molecular pathways [197]. Interestingly, the methylation profile at baseline of the patients that will not progress after receiving FOLFOX is more hypomethylated than those that will progress, indicating that the patients with lack of response to FOLFOX are characterized by a hypermethylated profile. In this regard, previous studies have identified some hypermethylated genes in colon tumor cells associated with the lack of response to 5-FU and oxaliplatin, such as *hMLH1* and *SRBC*, respectively [477,480].

In this work we have identified, as far as we know, the first non-invasive cfDNA methylation signature able to predict response to FOLFOX therapy in mCRC patients. In line with this, other studies have proposed non-invasive biomarkers of chemotherapy response in CRC based on other

epigenetic mechanisms, including the expression of ncRNAs [481,482], but these studies still need to be expanded and validated.

The episignature identified was characterized by presenting genes involved in very relevant cancer pathways, including PI3K/AKT, Wnt and cadherin signaling. In this sense, it has been reported that the activation of PI3K/AKT pathways is critical to induce anti-apoptosis activity and increase the aggressiveness of colon cancer cells resistant to 5-fluorouracile based therapy [483]. Similarly, the deregulation of Wnt signaling is important to confer chemoresistance by maintaining the cancer stem cell population, favoring transcriptional plasticity, improving DNA damage repair, or inducing immune evasion [484]. In addition, it has been proposed that the alteration of cadherin signaling is able to induce cancer stem cells properties such as enhancement of chemoresistance to 5-FU and oxaliplatin [485]. The fact that several genes of this episignature are involved in pathways related to chemotherapy resistance reinforces its role as an efficient tool to predict the response to FOLFOX therapy in mCRC patients. Previous studies have demonstrated a high concordance between the molecular alterations found in cfDNA and colorectal tumors [486], suggesting that the epigenetically deregulated genes that we have identified in cfDNA could be also present in colorectal tumors, representing potential therapeutic targets that could be modulated to overcome FOLFOX resistance in mCRC patients.

Despite the promising results of the predictive cfDNA episignature discovered in this work, we should keep in mind that this study was conducted in a small cohort of patients, so a validation in larger cohorts of mCRC patients will be essential to establish the performance of this predictive epigenetic biomarker. If validated in future studies, this non-invasive episignature could serve as a valuable tool for pre-treatment stratification of mCRC patients, helping to identify the patients who would benefit from a frequently used

chemotherapy, which will have important implications for precision medicine of mCRC patients.

5. Conclusions

In conclusion, this study represents an important step forward in the search for predictive biomarkers in the context of mCRC treatment with FOLFOX-based therapy. The non-invasive cfDNA episignature identified represents a very promising biomarker to stratify the mCRC patients according to the response to FOLFOX. This work also reinforces the possibility of using EPIC arrays in cfDNA to discover novel non-invasive epigenetic biomarkers for the management of cancer patients. In addition, the epigenetically deregulated genes and pathways identified in this work in association with therapy response represent potential therapeutic targets to overcome the resistance to FOLFOX treatment in CRC.

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CHAPTER III

A genome-wide cell-free DNA methylation analysis identifies an episignature associated with metastatic luminal B breast cancer

A genome-wide cell-free DNA methylation analysis identifies an episignature associated with metastatic luminal B breast cancer

This chapter has been adapted from: **Rodriguez-Casanova A[1,2,3,†]**, Costa-Fraga N[1,3,†], Castro-Carballeira C[4], González-Conde M[2,5], Abuin C[2], Bao-Caamano A[1,3], García-Caballero T[6], Brozos-Vazquez E[7], Rodriguez-López C[7], Cebey V[7], Palacios P[7], Cueva JF[5,7], López-López R[2,5,7], Costa C[2,5], Díaz-Lagares A[1,5]. **A genome-wide cell-free DNA methylation analysis identifies an episignature associated with metastatic luminal B breast cancer**. Front Cell Dev Biol. 2022 Oct 25;10:1016955. DOI: 10.3389/fcell.2022.1016955..

[1]Epigenomics Unit, Cancer Epigenomics, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), Santiago de Compostela, Spain.

[2]Roche-Chus Joint Unit, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago (IDIS), Santiago de Compostela, Spain.

[3]Universidade de Santiago de Compostela (USC), Santiago de Compostela, Spain.

[4]Department of Oncology, Marqués de Valdecilla University Hospital, Santander, Spain.

[5]Centro de Investigación Biomédica en Red Cáncer (CIBERONC), ISCIII, Madrid, Spain.

[6]Department of Morphological Sciences, University of Santiago de Compostela and Xerencia de Xestión Integrada de Santiago (XXIS/SERGAS), Santiago de Compostela, Spain.

[7]Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), Santiago de Compostela, Spain.

[†]These two authors share first authorship.

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Abstract

Background: Breast cancers of the luminal B subtype are frequent tumors with high proliferation and poor prognosis. Epigenetic alterations have been found in breast tumors and in biological fluids. We aimed to profile the cfDNA methylome of metastatic luminal B breast cancer (LBBC) patients using an epigenomic approach to discover potential non-invasive biomarkers.

Methods: Plasma cfDNA was analyzed using the Infinium MethylationEpic array in a cohort of 14 women, including metastatic LBBC patients and healthy controls. The methylation levels of cfDNA and tissue samples were validated with droplet digital PCR. The methylation and gene expression data of 582 primary luminal breast tumors and 79 non-tumor tissues were obtained from The Cancer Genome Atlas (TCGA).

Results: We found an episignature of 1,467 differentially methylated CpGs that clearly identified patients with LBBC. Among the genes identified, the promoter hypermethylation of *WNT1* was validated in cfDNA, showing an area under the ROC curve (AUC) of 0.86 for the non-invasive detection of metastatic LBBC. Both paired cfDNA and primary/metastatic breast tumor samples showed hypermethylation of *WNT1*. TCGA analysis revealed significant *WNT1* hypermethylation in the primary tumors of luminal breast cancer patients, with a negative association between *WNT1* methylation and gene expression.

Conclusions: In this proof-of-principle study, we discovered an episignature associated with metastatic LBBC using a genome-wide cfDNA methylation approach. We also identified the promoter hypermethylation of *WNT1* in cfDNA as a potential non-invasive biomarker for luminal breast cancer. Our results support the use of EPIC arrays to identify new epigenetic non-invasive biomarkers in breast cancer.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide, with 2.3 million new cases (11.7% of all cancer cases) in 2020, representing the leading cause of cancer death in women [9]. BC is a heterogeneous disease with several distinct clinical characteristics that, according to a gene expression profile, can be divided into four molecular subtypes: luminal, HER2-enriched, basal-like, and normal breast-like [487]. In addition, luminal tumors can be divided into the luminal A and B subtypes according to the expression profile of the estrogen receptor (ER), progesterone (PR), HER2, and proliferation tumor status [488]. The luminal B subtype is a common BC subtype characterized by high proliferation, resistance to standard therapies, risk of early relapse, and poor prognosis [489-491]. In addition, this tumor subtype is more likely to exhibit local recurrence and single bone metastases than nonluminal BC. However, recent studies have not investigated this tumor subtype as thoroughly as other subtypes [490]. Notably, the incidence of luminal B tumors has increased in recent years in many racial/ethnic and age groups [492].

Cancer metastasis is characterized by highly variable clinical manifestations and is responsible for over 90% of cancer-related deaths [110,493]. However, despite recent advances, the clinical need to identify biomarkers in metastatic BC disease remains unmet [10]. In recent years, liquid biopsy has emerged as a good opportunity to address this clinical need. This non-invasive approach allows for the characterization of the molecular landscape of circulating tumor elements in body fluids, such as epigenetic modifications of cell-free DNA (cfDNA), to obtain biomarkers for the management of cancer patients [343].

The most well-known epigenetic modification is DNA methylation, which is an important regulator of gene expression originating from the addition of a methyl group (CH3) to the 5' carbon of cytosines in cytosine–phosphate–guanine (CpG) dinucleotides [183]. The deregulation of this epigenetic mechanism in breast tumor cells has major implications for cancer development, progression, and therapy response [494-497]. Notably, the analysis of DNA methylation in liquid biopsy has shown utility as a potential clinical biomarker for BC patients [283].

Recently published studies of other tumor types have shown that epigenomic approaches based on the Infinium MethylationEPIC array (EPIC array) technology, which covers over 850,000 CpG sites along the human genome, could be useful to profile the methylation of cfDNA in biological fluids [341,342]. Therefore, this proof-of-principle study aimed to profile the cfDNA methylome of luminal B breast cancer (LBBC) patients using an EPIC array approach to discover new non-invasive biomarkers. In this study, we identified an epigenetic signature (episignature) based on the methylation of cfDNA associated with metastatic LBBC. Among the genes of this episignature, we confirmed the hypermethylation of *WNT1* in cfDNA and tumor tissues (primary and metastatic) as a potential new biomarker for LBBC patients. The results of our work support the application of the EPIC array technology as a non-invasive tool to identify new biomarkers in breast cancer.

2. Materials and methods

2.1 Study participants

In this retrospective study, 9 luminal B metastatic breast cancer patients and 5 healthy donors (non-tumor controls) were recruited between 2016 and 2018 at the Medical Oncology Department at the University Clinical Hospital of Santiago de Compostela (Spain). Most of the metastatic patients

of this study (7 out of 9) had been diagnosed in the past at M0 stage. Two patients of our cohort had metastases at the time of primary tumor diagnosis. The study was approved by the Galician Ethical Committee (reference number 2015/772) and conducted in accordance with the guidelines for Good Clinical Practice and the Declaration of Helsinki. All participants included in the study signed the informed consent to participate.

2.2 Blood and tissue samples

Blood sample was obtained from all the patients at the time of diagnosis of metastasis and before starting the treatment. Blood samples were collected by phlebotomy into collection tubes containing K2EDTA as an anticoagulant. Plasma was isolated within 2 h of collection by initial centrifugation at 1,700 × g for 10 min at room temperature (RT), followed by a second centrifugation at 15,000 × g for 10 min at RT. Isolated plasma was stored at -80 °C until analysis. All tumor tissues used were obtained according to standard-of-care (SOC) procedures. We used formalin-fixed and paraffin-embedded (FFPE) primary and/or metastatic tumor and matched non-tumor tissue samples available from 4 patients included in the study. Whole slide FFPE tissue sections of 10 μm were obtained.

2.3 Isolation of DNA from plasma and tissue samples

We used the QIAamp® Circulating Nucleic Acid Kit (Qiagen) and the vacuum system QIAvac 24 Plus (Qiagen) following the manufacturer´s recommendations to isolate cfDNA from 2 mL of plasma. DNA was also isolated from 10-μm FFPE tissue sections using the AllPrep DNA/RNA FFPE Kit (Qiagen) following the manufacturer's protocol. The quality and quantity of DNA from FFPE tissue sections were evaluated with a NanoDrop (Thermo Fisher), and cfDNA was quantified using the Qubit 1× dsDNA High-Sensitivity

Assay Kit and a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). The DNA from FFPE tissue sections and cfDNA from plasma were stored at −80 °C until analysis.

2.4 Genome-wide cell-free DNA methylation analysis

Fifteen nanograms of each individual sample of plasma cfDNA was bisulfite-converted using the EZ DNA Methylation Lightning Kit (Zymo Research) following the manufacturer's recommendations. Subsequently, the bisulfite-modified cfDNA was then subjected to whole genome amplification (WGA) using the EpiTect Whole Bisulfitome Kit (Qiagen) according to the manufacturer's protocol. Briefly, the bisulfite-modified cfDNA of each individual sample was amplified with a reaction buffer containing REPLI-g Midi DNA Polymerase (Qiagen) at 28 °C for 8 h, which was subsequently inactivated at 95 °C for 5 min. After the WGA of cfDNA, the Illumina Infinium HD methylation protocol was followed using MethylationEPIC BeadChips that were analyzed in a HiScan (Illumina). Samples with a mean detection p-value <0.01 were considered valid for the analysis. The methylation data were processed in the R statistical environment using RnBeads 2.0 [498]. Raw intensity data files (IDATs) were imported into RnBeads 2.0 for quality control and preprocessing. First, a greedycut algorithm was used to filter out low-quality probes. Probes overlapping with SNPs and probes whose sequences mapped to multiple genomic locations (cross-reactive) were removed. IDATs obtained in the array were normalized using the beta-mixture quantile (BMIQ) method. Hierarchical linear models were used to obtain the methylation differences between groups. P-values were corrected for multiple testing using the Benjamini–Hochberg method, and a false discovery rate (FDR) <10% was selected for significance. The DNA methylation level was represented as the average β-value, which was calculated as the ratio of the fluorescent signal intensity of the methylated probe to those of total

(methylated and unmethylated) probes. Average β-values were used to calculate the mean methylation difference between groups as the Δβ-value (β-value Luminal B – β-value Control). An unsupervised hierarchical clustering heatmap of β-values was generated using the ComplexHeatmap package. Gene ontology (GO) enrichment analysis of biological pathways from the PANTHER database was performed using GENECODIS [471].

2.5 Methylation and expression analysis from The Cancer Genome Atlas

The DNA methylation (β-values) and expression data of *WNT1* in luminal primary breast tumors and non-tumor controls were obtained from the public datasets of The Cancer Genome Atlas (TCGA) [499]. The breast cancer subtype of patients was obtained from the clinical information available at TCGA and the classification of these TCGA patients based on PAM50 assay performed by Netanely et al [500].

2.6 Methylation analysis of the *WNT1* promoter in cell-free DNA by droplet digital PCR

The methylation of the *WNT1* promoter was analyzed via droplet digital PCR (ddPCR) in a QX200 system (Bio-Rad). Twenty nanograms of plasma cfDNA and 30 ng of DNA from FFPE tissue samples were bisulfite converted using the EZ DNA Methylation Lightning Kit (Zymo Research) following the manufacturer's recommendations. A custom Bio-Rad assay to detect the methylation status of *WNT1* (cg27196808) was designed: *WNT1*-M for methylation and *WNT1*-U for unmethylation (**Supplementary Table 14**). A multiplex preamplification reaction was performed with ~2 ng of bisulfite-converted DNA using SsoAdvanced™ PreAmp Supermix (Bio-Rad), *WNT1*-M, and *WNT1*-U. The PCR conditions were as follows: 3 min at 95 °C, 10

cycles of 95 °C for 15 s and 50.6 °C for 4 min, and a final hold step at 4 °C. Next, a multiplex reaction mix was prepared with 2 µL of the preamplification product using ddPCR Supermix for Probes (No dUTP) (Bio-Rad), *WNT1*-M, and *WNT1*-U. The QX200™ Droplet Generator (Bio-Rad) was used to generate droplets. The thermocycling conditions were as follows: 10 min at 95 °C, 40 cycles of 95 °C for 15 s and 50.6 °C for 30 s, 98 °C for 10 min, and a final hold step at 4 °C. The temperature ramp increment was 2.5 °C/s for all steps. Droplets were counted and analyzed using the QX200™ Droplet Reader system (Bio-Rad), and the QuantaSoft analysis software (Bio-Rad) was used to acquire data. Water was included as a no-template control, and the Human Methylated and Non-Methylated DNA set (Zymo Research) was used as a positive control for methylation and unmethylation. Reactions were performed in triplicate. DNA methylation was expressed according to the following formula: Methylation (%) = [M/(U + M)] x 100, where M represents the copies/µl of methylated cfDNA, and U the copies/µl of unmethylated cfDNA.

2.7 Statistical analysis

The Kolmogorov–Smirnov test was used to evaluate the normality of the distribution of the data. The nonparametric Mann–Whitney U test was used to compare methylation data according to its normality. To assess the diagnostic accuracy, a receiver operating characteristic (ROC) curve was generated. The greatest combination of sensitivity and specificity was obtained using the Youden index (J): J = sensitivity + specificity - 1. The association between DNA methylation and gene expression was evaluated with a Spearman correlation. The GraphPad Prism 6.0 software (GraphPad Software) and the R statistical environment (version 4.2.0) were used for statistical analysis and graphical representation. All expressed p-values were

calculated with two-tailed tests and were considered significant when the p-value < 0.05.

3. Results

3.1 Clinical characteristics of patients

A retrospective cohort of 14 women was included in this study: 9 patients with LBBC at the time of metastatic disease diagnosis and 5 healthy controls. The mean age of the patients was 66 ± 16 years, whereas the control group had a mean age of 53 ± 10 years. The main clinical characteristics of the analyzed cohort are described in **Supplementary Table 15**. All patients had metastases and invasive ductal carcinoma with a high Ki-67 proliferative index (≥20%), and they were positive for estrogen receptors (ER+). Eight out of nine patients were positive for progesterone receptors (PR+), and two patients had HER2 overexpression (**Supplementary Table 15**). Six out of the 9 patients (66%) included in the study had lung metastasis, 4 patients (44%) showed bone lesions, and 3 patients (33%) had liver affectation. In addition, 5 of the patients (55%) had multiple metastatic locations.

3.2 Genome-wide cell-free DNA methylation analysis of metastatic patients with luminal B breast cancer

The analysis of DNA methylome with the EPIC array methodology usually needs a high amount of DNA, which is difficult to obtain in the clinic from individual plasma samples. As a novelty in our study, to overcome this limitation, we have used small amounts of cfDNA from individual plasma samples, which were genome-wide amplified after bisulfite modification and then analyzed using EPIC arrays. Thus, using this approach we performed a genome-wide cell-free DNA methylation analysis in our cohort of 9 LBBC

patients and 5 non-tumor controls (**Figure 23A**). After hybridizing the samples in the EPIC array, 2 LBBC samples showed a mean detection p-value > 0.01 and were not considered valid for the analysis. Therefore, we ultimately compared the methylation status of cfDNA in 7 LBBC patients and 5 non-tumor controls, leading to 28,799 differentially methylated CpGs (DMCpGs) ($p < 0.05$; FDR<10%) between LBBC and non-tumor controls. These DMCpGs showed a wide distribution throughout all chromosomes of the genome (**Figure 23B**). Of these DMCpGs, 92% (26,486) were hypomethylated and 8% (2,313) were hypermethylated in LBBC patients with respect to non-tumor controls (**Figure 23C**). Most of the hypomethylated CpGs were distributed in regions with low CpG density (open sea) (**Figure 23D**) and outside promoter regions (**Figure 23E**), whereas hypermethylated CpGs were mainly located in CpG islands (CGIs) (**Figure 23F**) and promoters (**Figure 23G**).

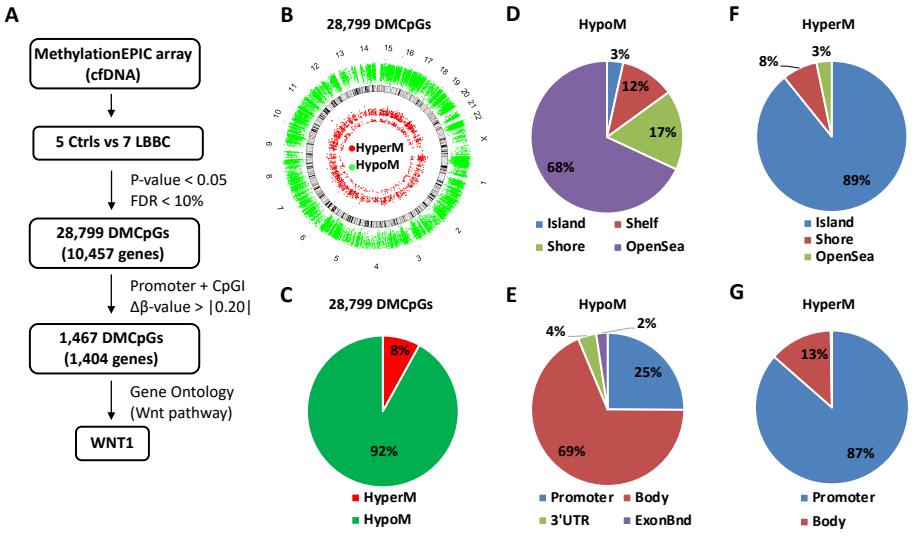

Figure 23. Methylation landscape of cell-free DNA in metastatic patients with luminal B breast cancer. A, Flowchart of the cfDNA methylation analysis. Individual cfDNA samples from controls and luminal B breast cancer patients were analyzed with EPIC array. **B–G**, Description of the 28,799 differentially methylated CpGs (DMCpGs) found in cfDNA of luminal B breast cancer patients according to (B) chromosome location, (C) methylation status, (D, F) CpG context, and (E, G) gene location. cfDNA, cell-free DNA; Ctrls, controls; LBBC, luminal B breast cancer; FDR, false discovery rate; HypoM, hypomethylated; HyperM, hypermethylated.

3.3 Identification of cell-free DNA episignature in metastatic patients with luminal B breast cancer

The aberrant hypermethylation of CGI promoters is a very relevant feature that usually occurs in tumor cells [501]. Therefore, we focused our study on analyzing the methylation profile of cfDNA at the CGIs of promoters. In these regions of cfDNA, we identified 1,467 DMCpGs ($p < 0.05$; FDR<10%) with a difference in methylation (Δβ-value) higher than 0.20 (Δβ-value > |0.20|) (**Figure 23A**). Notably, this epigenetic signature (episignature) of 1,467 DMCpGs was able to clearly differentiate LBBC patients from non-tumor

controls (**Figure 24A**). Next, to obtain information related to the functional pathways involved in the identified episignature, we performed a gene ontology (GO) enrichment analysis based on the PANTHER database. This analysis revealed that methylation differences in the cfDNA of LBBC patients and non-tumor controls were mainly associated with genes related to the Wnt signaling pathway (**Figure 24B**). **Table 6** shows the 34 DMCpGs (corresponding to 24 genes) of the episignature of cfDNA that are associated with the Wnt signaling pathway. Relevantly, the genes of these 34 DMCpGs that are associated with Wnt signaling belonged to a network significantly enriched in protein interactions ($p < 0.001$) according to a STRING analysis (**Supplementary Figure 8**).

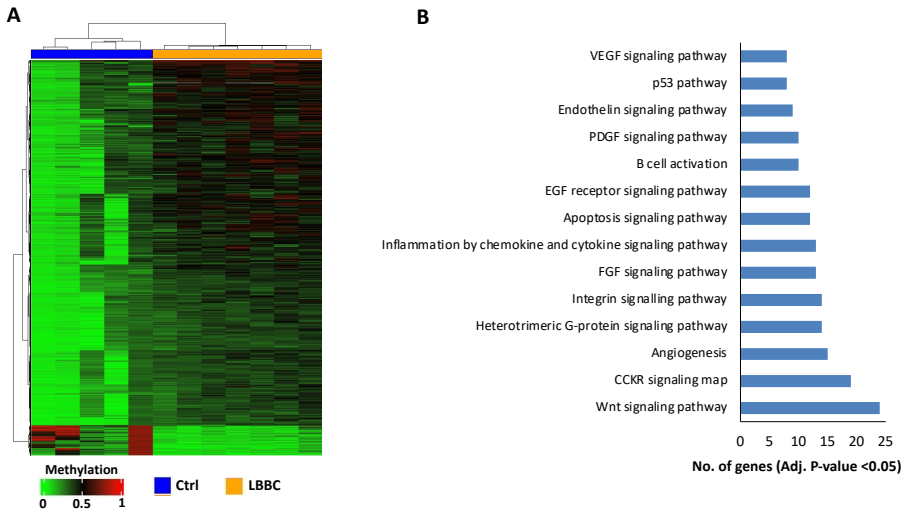

Figure 24. Episignature of cell-free DNA in metastatic patients with luminal B breast cancer. A, Unsupervised hierarchical clustering heatmap of the episignature (1,467 DMCpGs) obtained in cfDNA that differentiates LBBC patients (n=7) from non-tumor controls (n=5). **B**, Gene Ontology enrichment analysis by the PANTHER database, showing the most representative pathways associated with the episignature of cfDNA in luminal B breast cancer patients. Ctrls, controls; LBBC, luminal B breast cancer; DMCpGs, differentially methylated CpGs; cfDNA, cell-free DNA.

Table 6. The 34 CpGs of cfDNA episignature found in metastatic patients with luminal B breast cancer associated with the Wnt signaling pathway.

TargetID[1]	Chr	Position	Gene name	Gene region[2]	Δβ[3]	P-value
cg26821418	9	2016890	*SMARCA2*	5'UTR	0.35	0.0028
cg27201625	10	6622279	*PRKCQ*	TSS200	0.33	0.0008
cg04351665	10	6622297	*PRKCQ*	TSS200	0.21	0.0057
cg03306374	16	23847325	*PRKCB*	1stExon;5'UTR	0.28	0.0024
cg06931245	8	28351501	*FZD3*	TSS1500	0.26	0.0023
cg18463655	8	28351544	*FZD3*	TSS200	0.23	0.0041
cg26631144	8	30670260	*PPP2CB*	5'UTR;1stExon	0.28	0.0014
cg02478409	6	33589019	*ITPR3*	TSS200	0.32	0.0020
cg16490096	1	40367661	*MYCL1*	1stExon;5'UTR	0.23	0.0024
cg20462899	1	40367831	*MYCL1*	TSS200	0.29	0.0056
cg02771661	**12**	**49372162**	***WNT1***	**TSS200**	**0.21**	**0.0047**
cg27196808	**12**	**49372281**	***WNT1***	**1stExon;5'UTR**	**0.22**	**0.0066**
cg13469346	3	53195186	*PRKCD*	TSS200	0.24	0.0026
cg21950287	19	54385441	*PRKCG*	TSS200	0.23	0.0051
cg13885159	11	62473858	*GNG3*	TSS1500	0.23	0.0064
cg03922588	11	62473871	*GNG3*	TSS1500	0.27	0.0021
cg25220961	17	64298782	*PRKCA*	TSS200	0.27	0.0023
cg11676500	17	64298789	*PRKCA*	TSS200	0.23	0.0026
cg08221093	16	68119222	*NFATC3*	TSS200	0.25	0.0067
cg21367137	16	68119381	*NFATC3*	5'UTR;1stExon	0.26	0.0046
cg22722737	9	82187628	*TLE4*	5'UTR;1stExon	0.22	0.0050
cg26753733	4	102268824	*PPP3CA*	TSS200	0.24	0.0039
cg08764167	10	103113933	*BTRC*	5'UTR;1stExon	0.21	0.0050
cg20359285	2	119603969	*EN1*	1stExon	0.21	0.0043
cg00557469	5	133562427	*PPP2CA*	TSS1500	0.25	0.0049
cg18671773	5	141016477	*HDAC3*	TSS200	0.27	0.0060
cg16248329	4	187644739	*FAT1*	5'UTR	0.23	0.0026
cg02968914	19	1955395	*CSNK1G2*	5'UTR	-0.32	0.0042
cg01895482	19	2556145	*GNG7*	5'UTR	-0.35	0.0015
cg07223632	22	46930499	*CELSR1*	1stExon	-0.36	0.0028
cg00875636	22	46931138	*CELSR1*	1stExon	-0.28	0.0030
cg27334938	18	77167042	*NFATC1*	5'UTR	-0.30	0.0051
cg02113385	18	77203443	*NFATC1*	5'UTR	-0.35	0.0060
cg27475132	4	187645120	*FAT1*	TSS200	-0.23	0.0053

[1]CpGs located in CGIs of promoters (TSS1500, TSS200, 5´UTR, 1st exon); [2]Chromosome; [3]Δβ-values (β-value Luminal B - β-value Control). CpGs of gene *WNT1* are indicated in bold.

3.4 Hypermethylation of the *WNT1* promoter in the cfDNA and tumor samples of patients with luminal B breast cancer

Among the DMCpGs in the episignature obtained from the cfDNA of LBBC patients that were associated with the Wnt signaling pathway (**Table 6**), we found 2 CpGs (cg27196808 and cg02771661) located in *WNT1* that were hypermethylated in the cfDNA of LBBC patients with respect to non-tumor controls. To confirm this aberrant methylation, we selected the most DMCpG of *WNT1* (cg27196808), and we analyzed its methylation status in the cfDNA of our cohort using ddPCR. As expected, the methylation of *WNT1* was significantly higher in LBBC patients than in non-tumor controls (**Figure 25A**). In addition, using a ROC curve analysis, the methylation status of the *WNT1* promoter analyzed by ddPCR accurately differentiated LBBC patients from non-tumor controls, with an area under the ROC curve (AUC) of 0.86 (95% CI: 0.65 - 1.00, p=0.045) (**Figure 25B**), a sensitivity of 78% (CI 95%: 40% - 98%), and a specificity of 100% (CI 95%: 40% - 100%).

To verify that the hypermethylation of *WNT1* found in cfDNA (cg27196808) was also present in the tumor tissues of patients with LBBC, we assessed its methylation status by ddPCR in the available matched primary and/or metastatic tumor tissue samples (n=4) of our cohort. This assay revealed that *WNT1* hypermethylation was present not only in the cfDNA but also in the paired primary and/or metastatic tumor samples of the LBBC patients analyzed (**Figure 25C**). Next, we took advantage of public DNA methylation array data from The Cancer Genome Atlas (TCGA) to evaluate whether the hypermethylation of the *WNT1* promoter was a frequent event in LBBC. This analysis showed that the methylation of the *WNT1* promoter (cg27196808) was significantly higher in luminal B primary tumors (stages I-IV) than in non-tumor controls (**Figure 25D**), and this observation was consistent across all TNM tumor stages (**Figure 25E**). However, the

methylation status of *WNT1* did not differ among the tumor stages of LBBC analyzed (**Figure 25E**). An ROC curve analysis showed that *WNT1* methylation differentiated luminal B primary tumors (stages I-IV) from non-tumor controls with high diagnostic accuracy, with an AUC of 0.87 (95% CI: 0.82 - 0.92, $p < 0.0001$) (**Figure 25F**). In addition, we also analyzed the *WNT1* expression data (RNAseq) available from LBBC patients and non-tumor controls included in TCGA, revealing that *WNT1* was significantly downregulated in this BC subtype (**Supplementary Figure 9**).

Finally, we also evaluated in breast primary tumors from TCGA whether the hypermethylation of *WNT1* was a specific event of LBBC patients. The methylation levels of *WNT1* were significantly higher in LBBC than in the other breast tumor subtypes (**Figure 26**). Interestingly, we observed that *WNT1* was also significantly hypermethylated in other breast cancer subtypes (Luminal A, triple negative and HER2+) in comparison with non-tumor controls.

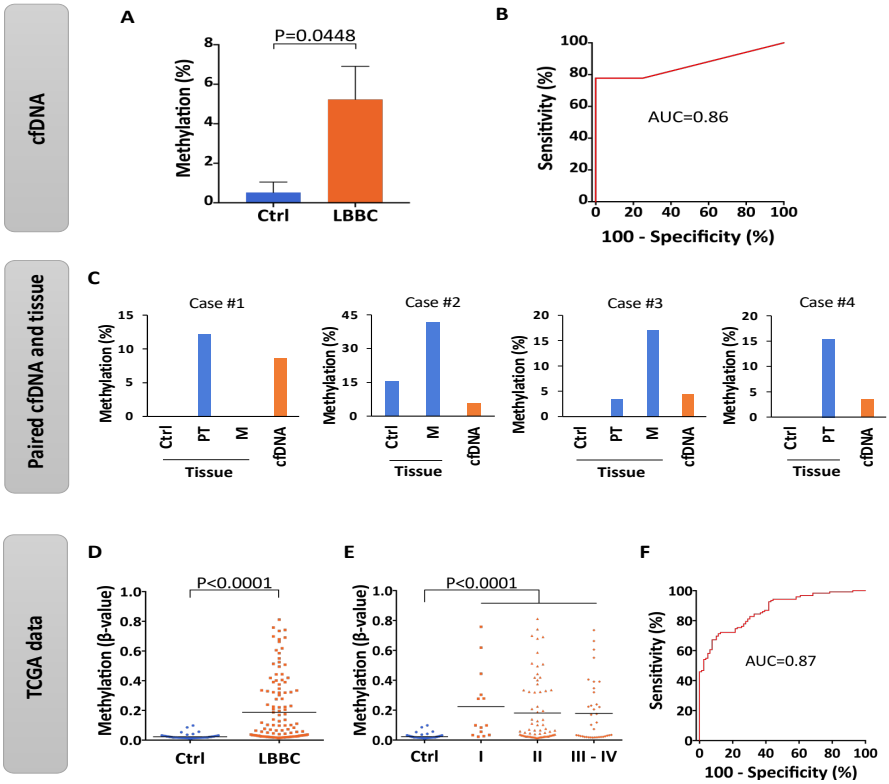

Figure 25. Methylation status of the *WNT1* promoter in the cfDNA and tumor samples of patients with luminal B breast cancer. A, Methylation levels of the *WNT1* promoter (cg27196808) in cell-free DNA (cfDNA) of luminal B breast cancer patients (n=9) and non-tumor controls (n=4) analyzed by droplet digital PCR (ddPCR). Methylation levels are represented as the mean ± SEM. **B**, Diagnostic accuracy of the methylation of the *WNT1* promoter using ddPCR in cfDNA for the detection of metastatic luminal B breast cancer. **C**, Methylation levels of *WNT1* in cfDNA and paired breast primary and/or metastatic tumor samples of 4 luminal B breast cancer patients analyzed by ddPCR. Non-tumor tissues from the same patients were used as controls. **D-E**, Methylation status of *WNT1* in primary tumors of luminal B breast cancer patients (n=122) and non-tumor controls (n=79) analyzed by 450K methylation array and obtained from TCGA considering (D) all TNM stages together or (E) separated (I, n=14; II, n=70; III-IV, n=37). The horizontal line represents mean methylation levels. **F**, ROC curve evaluating the methylation of *WNT1* to detect primary tumors of luminal B breast cancer patients (stages I-IV, n=122) with respect to non-tumor controls (n=79) from TCGA. Ctrl, control; P, p-value; AUC, area under the ROC curve; ROC curve, receiver operating characteristic curve. PT, primary tumor; M, metastasis.

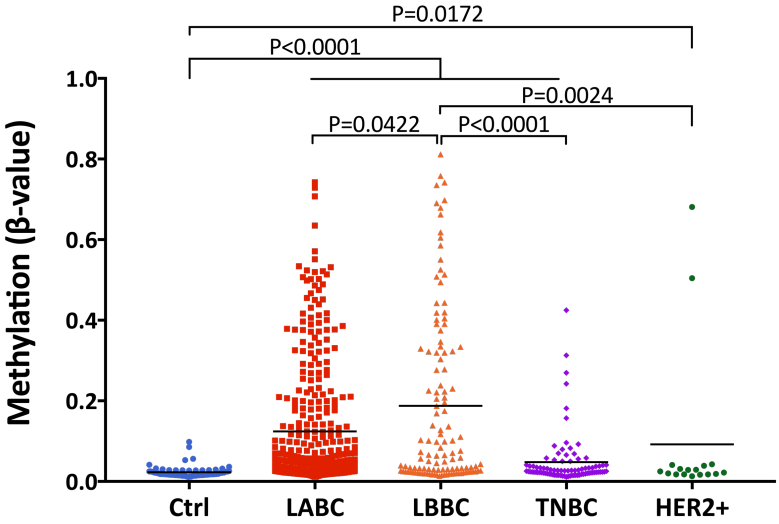

Figure 26. Methylation status of *WNT1* in primary tumors of breast cancer subtypes (LABC, n=358; LBBC, n=122; TNBC, n=87; HER2+, n=17) and non-tumor controls (n=79) from TCGA. Methylation data have been obtained from EPIC array analysis considering all TNM stages together (stages I-IV). Ctrl, control; P, p-value. LABC, luminal A breast cancer; LBBC, Luminal B breast cancer; TNBC, triple negative breast cancer; HER2+, HER2-positive.

4. Discussion

Alterations in epigenetic mechanisms, such as aberrant DNA methylation, are implicated in the development, progression, and therapy response of BC [494,495,502]. In recent years, the methylation analysis of liquid biopsy samples in BC patients has shown clinical utility as a biomarker for the detection, prognosis, and monitoring of the disease [283,503,504]. However, a clinical need to find new non-invasive biomarkers associated with metastatic BC subtypes persists [10]. Herein, we focused our study on patients with advanced LBBC, since it represents a frequent, aggressive and poor prognosis BC subtype [505]. The characterization of liquid biopsy samples using epigenomic tools for genome-wide methylation analyses has

been recently proposed as a good approach to discover new non-invasive biomarkers [506]. Thus, we used a genome-wide DNA methylation approach based on the EPIC array methodology to profile the methylome of cfDNA and discover novel non-invasive biomarkers in metastatic LBBC patients.

Our work revealed that the cfDNA of patients with metastatic LBBC is characterized by the hypomethylation of regions with a low density of CpGs and the site-specific hypermethylation of CGIs in promoter regions. Importantly, this pattern is similar to the deregulation of DNA methylation that has been previously described in cancer cells [507], suggesting that the methylation profile in the cfDNA of the patients in our cohort mirrors the epigenetic alterations of BC cells.

Specific genes, such as *RASSF1A* and *BRCA1*, have been previously described to exhibit aberrant hypermethylation of their promoter CGIs in BC [271,508]. Accordingly, we focused our study on promoter CGIs and were able to identify a novel non-invasive episignature in cfDNA based on 1,467 CpGs that was associated with LBBC patients. We found that the genes of this episignature were related to relevant biological pathways, mainly Wnt signalling pathway. Among these genes, we focused on *WNT1*, which is involved in the canonical Wnt signaling pathway (also known as Wnt/β-Catenin) in cancer cells [509,510]. Thus, we confirmed that the promoter CGI of *WNT1* was hypermethylated in the cfDNA of patients with metastatic LBBC and that this aberrant methylation showed a high diagnostic accuracy to detect this BC subtype, suggesting that the hypermethylation of *WNT1* could be a suitable biomarker for cancer detection and monitoring of metastatic patients. In line with this, it has been recently shown that methylation biomarkers of cfDNA with high diagnostic accuracy are useful not only for diagnosis but also for monitoring tumor burden dynamics under different therapeutic regimens in advanced disease [384]. Importantly, evaluating prognosis and monitoring tumor response in real time during treatment

continues to be an unmet clinical need in advanced BC [10]. Wnt signaling is a very relevant pathway in BC whose molecular alterations have clinical implications to establish the prognosis of the disease [511] and has been associated to breast cancer therapy response [512]. Therefore, it would be interesting to evaluate in future studies whether the hypermethylation of *WNT1* could be useful for the selection of patients susceptible to systemic therapies (CDK inhibitors for example) in the BC metastatic setting.

Of note, we also found that the promoter hypermethylation of *WNT1* was present not only in cfDNA of LBBC patients but also in their primary and/or metastatic tumors. This finding is in accordance with our previous work and that of other authors showing that the molecular landscape present in liquid biopsy may also be detected in the corresponding tumor tissue of patients [486,513]. In addition, when we extended our study to the analysis of breast primary tumors using the public TCGA database, we were able to confirm that the hypermethylation of *WNT1* is a frequent event in early and advanced LBBC. Accordingly, the aberrant methylation of other genes involved in the Wnt signaling pathway (e.g., *WNT5A* and *WNT7A*) has previously been described in tumor cells from the BC luminal subtype [514] and in other tumor types, such as gastric cancer or chronic lymphocytic leukemia [515,516].

Several studies have shown that aberrant promoter hypermethylation is a relevant mechanism that is able to repress the expression of key genes in breast tumor cells [495,517]. Accordingly, the analysis of luminal breast tumors from the TCGA database also revealed that *WNT1* promoter hypermethylation was associated with a downregulation of its gene expression in primary tumors, suggesting that *WNT1* is epigenetically regulated in luminal BC. The downregulation of *WNT1* observed in particular BC subtypes corroborates work by Koval and Katanaev [518], who reported low expression of this gene in primary tumors of nontriple-negative BC patients (ER+/PR+ and HER+). Indeed, it has been reported that the

deregulation of some Wnt signaling components depends on the BC subtype, with many being downregulated in the luminal B subtype [519].

One limitation of our study is that the epigenomic profiling of cfDNA performed is based on a retrospective cohort of patients with a small sample size. Although the results obtained in this work should be taken with caution, they provide the basis for further large, prospective and independent studies that validate the clinical utility of the potential epigenetic biomarkers identified herein. In addition, it would be interesting to evaluate in future works the implications of the epigenetic deregulation of *WNT1* in the development of metastasis.

In summary, in this proof-of-principle study, we discovered an episignature associated with patients with advanced LBBC using a genome-wide cfDNA methylation approach. We also identified the promoter hypermethylation of *WNT1* in cfDNA as a potential non-invasive biomarker for luminal BC. Our results support the use of EPIC array technology to identify new non-invasive biomarkers in BC.

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Conflicts of interest

R.L.-L. has received honoraria for participation in Advisory Boards from Roche, AstraZeneca, Merck, Merck Sharp & Dohme, Bayer, Bristol-Myers Squibb, Novartis, Janssen, Lilly, Pfizer, and Leo; travel, accommodations, and expenses from PharmaMar, Roche, Bristol-Myers Squibb, and Pierre Fabre; research funding from Roche and Merck; and is co-founder and shareholder in Nasasbiotech, S.L., Mtrap Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

Conceptualization, C.C, R.L.-L., and A.D.-L.; resources, T.G.-C., E.B.-V., C. R.-L., V.C., P.P., J.F.C., and R.L.-L.; Data curation, A.R.-C., N.C-F., C.A.; methodology, A.R.-C., N.C.F, C.C-C, M.G.-C., C.A., A.B.C., M.A. J. and T.G.-C; formal analysis, A.R.-C., N.C.-F., C.C, and A.D.-L.; writing—original draft

preparation, A.R.-C., N.C.-F., C.C and A.D.-L.; writing—review and editing, A.R.-C., N.C-F, J.F.C., R.L.-L., C.C., and A.D.-L.; supervision, C.C., R.L.L., A.D.-L. All authors have read and agreed to the published version of the manuscript.

Data availability

The cfDNA methylation data analyzed in this study with EPIC array (5 non-tumor controls and 7 LBBC patients) are available at the NCBI GEO repository with accession number GSE214344.

OVERALL DISCUSSION

OVERALL DISCUSSION

Cancer encompasses a large group of different diseases characterized by uncontrolled cell proliferation, the invasion of nearby tissues and the appearance of new tumor foci in distant parts of the body, called metastasis [520]. Notably, CRC and BC are among the most frequently diagnosed types of tumors and contribute significantly to the mortality associated with this disease [9,11]. To improve the survival and quality of life of cancer patients, it is imperative to discover novel biomarkers and tools for the early detection and management of this disease.

In recent years, precision oncology has gained considerable importance, due to the possibility of personalizing the medical care based on the molecular characteristics of each cancer patient [521]. This approach takes into account the genetic and epigenetic heterogeneity of tumors [522]. The improvement of genome-wide technologies (like NGS and microarrays) in terms of sensitivity and cost reduction, has allowed their application for the identification of new tumor biomarkers with clinical utility [523]. The new information provided by these methodologies helps improve the diagnostic accuracy, refine therapy selection and identify mechanisms related to drug resistance and relapse, contributing to improve survival rates and overall outcomes of cancer patients [524]. Although tissue biopsies remain the gold standard for analyzing tumor biomarkers, liquid biopsy offers great advantages that make this approach as a non-invasive alternative very useful in the clinic [525]. The molecular profiling of blood samples or other fluids provides relevant information of the status of the primary and metastatic tumors at different time points of the disease [281].

Nowadays, several genetic tests based on liquid biopsy have obtained CE-IVD and FDA-approved status, such as the OncoBEAM RAS CRC assay for the detection of gene variants of *KRAS* and *NRAS* in CRC [356]; or NGS pancancer panels, including FoundationOne Liquid CDx and Guardant360 CDx [316]. However, it is important to have the possibility of using different types of NGS panels in liquid biopsy, increasing the variety of genes that can be analyzed in cancer research and in different clinical contexts. In this regard, in this work we have evaluated for the first time the performance of an NGS panel that cover 170 genes, called TST170, demonstrating its utility for the non-invasive detection of somatic variants at very low frequencies. The high concordance obtained between the gene variants detected by TST170 in cfDNA and those detected in tumor tissues reinforces the high performance of this panel to be used in liquid biopsy. Thus, TST170 allows the non-invasive analysis of a group of genes different from those available in other panels, representing a new tool of precision oncology for mCRC.

In addition to gene variants, emerging research highlights the role of epigenetic mechanisms, including DNA methylation, in tumor development and evolution [526,527]. Although the analysis of gene variants is essential in precision medicine, it is also imperative to delve into the study of epigenetic alterations. Changes in DNA methylation have shown utility as biomarkers for diagnosis, prognosis and selection of the most appropriate therapy [528-530], providing new insights for precision oncology. Importantly, this type of epigenetic alterations can be detected not only in tissues but also in liquid biopsy samples, such as blood [195,531], opening new avenues to identify potential non-invasive epigenetic biomarkers. Recently, the use of epigenomic tools for performing genome-wide DNA methylation analyses in liquid biopsy samples has been suggested as a promising method for identifying novel non-invasive biomarkers [532,533].

Due to the clinical need for the non-invasive early detection of CRC, several studies have proposed the use of epigenetic biomarkers in liquid biopsy as a promising approach [534,535]. Until now, these assays, such as the methylation of *SEPT9* in cfDNA, have shown limited sensitivity, particularly in the identification of advanced precancerous lesions [373,536]. Therefore, there is still the need to explore new biomarkers that allow the non-invasive early detection of CRC and their precancerous lesions. In line with this, in this work, we have analyzed the epigenetic regulation of the lncRNA *LINC00473* through the methylation of its promoter. Additionally, we have assessed the clinical utility of its hypermethylation for the detection of CRC and its precancerous lesions in both tissue and cfDNA samples, revealing that this hypermethylation occurs early in colorectal carcinogenesis and continues throughout the different stages of the disease. Furthermore, we demonstrate that hypermethylation of *LINC00473* identifies patients with CRC and precancerous lesions with high sensitivity and specificity, showing high diagnostic accuracy for early detection of the disease. The methylation status of *LINC00473* in cfDNA was also able to detect the response and progression of the disease in mCRC, in some cases even before classical biomarkers as CEA, showing a promising potential for the follow-up of these patients.

In addition to early detection and follow-up, it is very important to improve the management of commonly used chemotherapies in mCRC, such as FOLFOX. Importantly, nowadays there is a lack of predictive biomarkers to guide the clinical use of this treatment [473]. To improve this situation, using a genome-wide cfDNA methylation approach based on EPIC arrays, we identified in this work an episignature able to predict the response to FOLFOX, which represents a promising non-invasive predictive biomarker to personalize the use of this therapy in patients with mCRC. Similarly, other studies have proposed non-invasive biomarkers of chemotherapy response

in CRC based on other epigenetic mechanisms, such as the expression of ncRNAs [481,482], but these studies still need to be expanded and validated.

Altogether, the epigenetic alterations of CRC patients discovered in this work could serve as robust biomarkers for clinical use, enabling the early detection, the personalization of chemotherapy, and the follow-up of CRC patients, which could improve the overall clinical management of these patients.

Similarly to CRC, epigenetic mechanisms are also deregulated in BC [537], and the analysis of DNA methylation in liquid biopsy has also shown utility as a biomarker for the detection, prognosis and monitoring of this disease [538,539]. Currently there is a clinical need to search for new biomarkers associated with different molecular subtypes of BC [10], including LBBC, which is a common and aggressive subtype [540]. Thus, in this work, we performed a genome-wide cfDNA methylation analysis that identified an epigenetic signature that define the LBBC patients. Among the genes involved in this signature, *WNT1* promoter was found to be hypermethylated in both cfDNA and primary and metastatic tumors of LBBC patients. Of note, this hypermethylation was able to detect LBBC patients with high diagnostic accuracy, providing a potential non-invasive biomarker for the diagnosis of this BC subtype. The epigenetic alteration of *WNT1* also represents a potential therapeutic target, opening avenues for the study of epidrugs that can restore the activity of this gene. In agreement with our results, the aberrant methylation of other genes involved in the Wnt signaling pathway, such as *WNT5A* and *WNT7A*, has previously been described in tumor cells from the BC luminal subtype [514].

In this work we have used a genome-wide methylation approach based on EPIC arrays to profile the cfDNA of cancer patients. EPIC arrays offer the possibility to analyze the methylation of thousands of CpGs

distributed throughout the genome, however, this methodology has been designed for the methylation analysis of tissue and cell samples [541]. Of note, we were able to adapt this technology for the methylation profiling of cfDNA, suggesting that this is a robust approach that could be widely used in multiple studies for the discovery of epigenetic biomarkers by liquid biopsy.

In summary, in this thesis we have performed the genetic and epigenetic profiling of cfDNA in patients with colorectal and breast cancer, revealing novel information that contributes to the scientific comprehension of these tumors, and providing novel non-invasive biomarkers and tools to improve and personalize the clinical management of these patients.

CONCLUSIONS

CONCLUSIONS

The main conclusions derived from the research presented in this doctoral thesis are summarized below:

1. The TST170 panel demonstrated utility for the non-invasive detection of clinically relevant gene variants in cfDNA of mCRC patients. This assay represents a valuable non-invasive tool that could be easily implemented in cancer research and in the clinic.

2. The TST170 panel is able to detect variants with a low frequency (VAF ≥ 0.5%) in genes involved in relevant cancer pathways and with clinical implications for CRC patients.

3. The hypermethylation of the *LINC00473* promoter showed high diagnostic accuracy for the detection of CRC and their precancerous lesions both in tissues and in cfDNA, providing a promising biomarker for the non-invasive early detection of CRC.

4. The methylation status of *LINC00473* in cfDNA of mCRC patients was able to detect tumor changes related with the treatment and progression of the disease, showing a promising potential for the follow-up of these patients.

5. A genome-wide methylation approach based on EPIC arrays represents a useful tool to profile the methylome of cfDNA and discover novel non-invasive epigenetic biomarkers for precision oncology.

6. The methylation profiling of cfDNA in mCRC patients before starting therapy identified an episignature able to predict response to

FOLFOX, representing a promising non-invasive predictive biomarker.

7. LBBC patients are characterized by presenting a unique non-invasive episignature in cfDNA different from healthy individuals, which is related with relevant cancer pathways such as Wnt signaling.

8. LBBC patients showed hypermethylation of *WNT1* promoter in both plasma and primary/metastatic tumors. This hypermethylation was able to detect LBBC patients with high diagnostic accuracy, providing a potential non-invasive biomarker for this disease.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Characteristics of the *KRAS* ddPCR assays used by ddPCR.

Gene	Assay ID	COSMIC ID	Expected variant	
			AA mutation	CDS mutation
KRAS	dHsaMDV2010001	COSM521	p.G12D	c.35G>A
	dHsaMDV2510588	COSM517	p.G12S	c.34G>A
	dHsaMDV2510598	COSM532	p.G13D	c.38G>A

AA, amino acid; CDS, coding sequence.

Supplementary Table 2. List of genes analyzed by the TST170 panel.

SNVs and Indels						
AKT1	*CCNE1*	*ERCC1*	*FGFR1*	*MAP2K1*	*NOTCH1*	*RAD51D*
AKT2	*CD79A*	*ERCC2*	*FGFR2*	*MAP2K2*	*NOTCH2*	*RAD54L*
AKT3	*CD79B*	*ERG*	*FGFR3*	*MCL1*	*NOTCH3*	*RB1*
ALK	*CDH1*	*ESR1*	*FLT1*	*MDM2*	*NPM1*	*RET*
APC	*CDK12*	*EZH2*	*FLT3*	*MDM4*	*NRAS*	*RICTOR*
AR	*CDK4*	*FAM175A*	*FOXL2*	*MET*	*NRG1*	*ROS1*
ARID1A	*CDK6*	*FANCI*	*GEN1*	*MLH1*	*PALB2*	*RPS6KB1*
ATM	*CDKN2A*	*FANCL*	*GNA11*	*MLLT3*	*PDGFRA*	*SLX4*
ATR	*CEBPA*	*FBXW7*	*GNAQ*	*MPL*	*PDGFRB*	*SMAD4*
BAP1	*CHEK1*	*FGF1*	*GNAS*	*MRE11A*	*PIK3CA*	*SMARCB1*
BARD1	*CHEK2*	*FGF10*	*HNF1A*	*MSH2*	*PIK3CB*	*SMO*
BCL2	*CREBBP*	*FGF14*	*HRAS*	*MSH3*	*PIK3CG*	*SRC*
BCL6	*CSF1R*	*FGF2*	*IDH1*	*MSH6*	*PIK3R1*	*STK11*
BRAF	*CTNNB1*	*FGF23*	*IDH2*	*MTOR*	*PMS2*	*TERT*
BRCA1	*DDR2*	*FGF3*	*INPP4B*	*MUTYH*	*PPP2R2A*	*TET2*
BRCA2	*DNMT3A*	*FGF4*	*JAK2*	*MYC*	*PTCH1*	*TP53*
BRIP1	*EGFR*	*FGF5*	*JAK3*	*MYCL1*	*PTEN*	*TSC1*
BTK	*EP300*	*FGF6*	*KDR*	*MYCN*	*PTPN11*	*TSC2*
CARD11	*ERBB2*	*FGF7*	*KIT*	*MYD88*	*RAD51*	*VHL*
CCND1	*ERBB3*	*FGF8*	*KMT2A*	*NBN*	*RAD51B*	*XRCC2*
CCND2	*ERBB4*	*FGF9*	*KRAS*	*NF1*	*RAD51C*	
CNVs						
AKT2	*CCNE1*	*ERCC2*	*FGF4*	*FGFR4*	*MYCL1*	*RAF1*
ALK	*CDK4*	*ESR1*	*FGF5*	*JAK2*	*MYCN*	*RET*
AR	*CDK6*	*FGF1*	*FGF6*	*KIT*	*NRAS*	*RICTOR*
ATM	*CHEK1*	*FGF10*	*FGF7*	*KRAS*	*NRG1*	*RPS6KB1*
BRAF	*CHEK2*	*FGF14*	*FGF8*	*LAMP1*	*PDGFRA*	*TFRC*
BRCA1	*EGFR*	*FGF19*	*FGF9*	*MDM2*	*PDGFRB*	
BRCA2	*ERBB2*	*FGF2*	*FGFR1*	*MDM4*	*PIK3CA*	
CCND1	*ERBB3*	*FGF23*	*FGFR2*	*MET*	*PIK3CB*	
CCND3	*ERCC1*	*FGF3*	*FGFR3*	*MYC*	*PTEN*	

SNVs, single nucleotide variants; indels, insertion/deletion; CNVs, copy number variants.

Supplementary Table 3. Quantity of cfDNA used for TST170 assay.

Sample ID	cfDNA (ng)
CRC032	40
CRC035	50
CRC062	100
CRC095	35
CRC100	40
CRC106	35
CRC112	100
CRC116	100
CRC131	100
CRC133	100
CRC137	40
CRC142	40
CRC145	35
CRC152	40
CRC154	40
CRC158	40
CRC160	40
CRC164	40
CRC168	40

Ng, nanograms.

Supplementary Table 4. Tumor location of mCRC patients.

Sample ID	Primary tumor location	Metastasis location				Tissue biopsy location
		Liver	Lung	Peritoneal	Lymph node	
CRC032	Right Colon	Yes	No	No	No	Primary
CRC035	Right Colon	No	No	Yes	No	Primary
CRC062	Left Colon/rectum	Yes	Yes	No	No	Primary
CRC095	Left Colon/rectum	Yes	Yes	No	No	Primary
CRC100	Right Colon	No	Yes	Yes	Yes	Metastasis (lymph node)
CRC106	Right Colon	No	Yes	No	Yes	Primary
CRC112	Right Colon	Yes	No	No	No	Unknown
CRC116	Left Colon/rectum	Yes	No	No	No	Primary
CRC131	Left Colon/rectum	Yes	Yes	No	No	Primary
CRC133	Right Colon	Yes	No	No	No	Unknown
CRC137	Left Colon/rectum	No	No	Yes	No	Primary
CRC142	Left Colon/rectum	No	Yes	No	Yes	Metastasis (lung)
CRC145	Left Colon/rectum	No	No	Yes	No	Unknown
CRC152	Left Colon/rectum	No	Yes	No	Yes	Primary
CRC154	Left Colon/rectum	Yes	Yes	No	No	Primary
CRC158	Left Colon/rectum	Yes	Yes	No	No	Primary
CRC160	Left Colon/rectum	No	No	No	Yes	Primary
CRC164	Right Colon	No	Yes	Yes	Yes	Primary
CRC168	Right Colon	Yes	No	No	Yes	Primary

Supplementary Table 5. Analysis of variants in the reference standard cfDNA by TST170.

Gene	Expected variant	Expected VAF (%)	Detected variant		Detected VAF (%)	
			40 ng DNA	100 ng DNA	40 ng DNA	100 ng DNA
GNA11	p.Q209L	5.6	p.Q209L		5.5	4.6
AKT1	p.E17K	5	p.E17K		4.2	3.6
PIK3CA	p.E545K	5.6	p.E545K		5	4.3
EGFR	p.V769_D770ins	5.6	p.V769_D770ins		2.9	2.8
EGFR	p.E746_A750	5.3	p.E746_A750		4.4	4.1
MET	Amplification	4.5*	Amplification		1.8*	1.8*
MYCN	Amplification	9.5*	Amplification		4*	3.6*

*Fold change; SNV, single nucleotide variant; Indel, insertion/deletion; CNV, copy number variant; VAF, variant allele fraction; ng, nanograms.

Supplementary Table 6. List of genes with variants (frameshift, inframe, missense, stop gain) detected in cfDNA by TST170.

CRC032	CRC035	CRC062	CRC095	CRC100	CRC106	CRC112
ALK	*APC*	*ALK*	*APC*	*ALK*	*APC*	*APC*
APC	*ATM*	*APC*	*ATM*	*APC*	*ATM*	*ARID1A*
ATM	*ATR*	*ATM*	*ATR*	*ATM*	*ATR*	*ATM*
ATR	*BARD1*	*ATR*	*BARD1*	*ATR*	*BARD1*	*ATR*
BARD1	*BRCA1*	*BARD1*	*BRCA1*	*BARD1*	*BRCA1*	*BARD1*
BRCA1	*BRCA2*	*BCL6*	*BRCA2*	*BRCA2*	*BRCA2*	*BCL6*
BRCA2	*BRIP1*	*BRCA2*	*BRIP1*	*BRIP1*	*BRIP1*	*BRCA1*
BRIP1	*CHEK1*	*CHEK1*	*CHEK1*	*CARD11*	*CARD11*	*BRCA2*
CARD11	*CSF1R*	*CREBBP*	*EP300*	*CHEK1*	*CDKN2A*	*BRIP1*
CCNE1	*ERBB2*	*EGFR*	*ERBB2*	*EGFR*	*CHEK1*	*CCND1*
CHEK1	*ERCC2*	*EP300*	*ERBB4*	*EP300*	*ALK*	*CDKN2A*
EGFR	*EZH2*	*ERBB2*	*ERCC2*	*ERBB2*	*CSF1R*	*CHEK1*
EP300	*FANCI*	*ERCC2*	*FGF6*	*FGF23*	*DNMT3A*	*EP300*
ERBB2	*FGFR4*	*FANCL*	*FGFR4*	*FGFR2*	*ERBB2*	*ERBB2*
ERCC2	*FLT3*	*FGF23*	*FLT1*	*FGFR4*	*ERCC2*	*FAM175A*
FAM175A	*ALK*	*FAM175A*	*ALK*	*FAM175A*	*EZH2*	*ALK*
FANCI	*GEN1*	*FLT3*	*FLT3*	*FLT3*	*FANCI*	*FANCI*
FGFR4	*GNAS*	*GEN1*	*GEN1*	*GEN1*	*FGF5*	*FGF6*
FLT3	*HNF1A*	*HNF1A*	*IDH1*	*HNF1A*	*FGFR4*	*FGFR3*
GEN1	*KRAS*	*JAK2*	*KRAS*	*IDH1*	*GEN1*	*FGFR4*
JAK3	*MLH1*	*JAK3*	*MLLT3*	*KMT2A*	*HNF1A*	*FLT3*
KDR	*MSH3*	*KDR*	*MSH3*	*KRAS*	*KDR*	*FOXL2*
KRAS	*MSH6*	*KRAS*	*MUTYH*	*MLH1*	*KIT*	*GEN1*
MLH1	*MYCL1*	*MLH1*	*MYC*	*MSH3*	*MLLT3*	*HNF1A*
MSH3	*NOTCH3*	*MSH2*	*MYCL1*	*MUTYH*	*MSH2*	*KDR*
MUTYH	*PTCH1*	*MSH3*	*NBN*	*MYC*	*MSH3*	*KRAS*
MYCL1	*PTEN*	*MUTYH*	*NOTCH3*	*MYCL1*	*MYC*	*MLH1*
NBN	*RET*	*MYCL1*	*PIK3CA*	*NBN*	*MYCL1*	*MLLT3*
NOTCH2	*ROS1*	*NOTCH2*	*PTCH1*	*NOTCH1*	*NRG1*	*MSH3*
NOTCH3	*TET2*	*NOTCH3*	*PTEN*	*NOTCH2*	*PALB2*	*MYC*
NRG1	*TP53*	*PIK3R1*	*RAD51B*	*NOTCH3*	*PDGFRB*	*MYCL1*
PIK3CD	*TSC2*	*PTCH1*	*RAD51D*	*PALB2*	*PIK3R1*	*NBN*
PIK3CG		*PTEN*	*ROS1*	*PDGFRA*	*RAD51B*	*NOTCH3*
PTCH1		*RAD51B*	*TP53*	*PTCH1*	*RICTOR*	*NRG1*
RAD51D		*RAD51D*	*TSC2*	*PTEN*	*ROS1*	*PIK3R1*
RB1		*RICTOR*	*XRCC2*	*RAD51D*	*SLX4*	*PTEN*
RET		*SLX4*		*RET*	*SMO*	*PTPN11*
RICTOR		*TET2*		*RICTOR*	*TP53*	*RET*
ROS1		*TP53*		*SLX4*		*RICTOR*
TET2				*SRC*		*ROS1*
TP53				*TET2*		*TET2*
				TP53		*TP53*
						TSC1
						TSC2

Supplementary Table 6. Continuation.

CRC116	CRC131	CRC133		CRC137	CRC142	CRC145
ALK	*ALK*	*AKT1*	*GEN1*	*APC*	*ALK*	*APC*
APC	*APC*	*ALK*	*HNF1A*	*AR*	*APC*	*ALK*
ATM	*ATM*	*APC*	*JAK3*	*ALK*	*ATM*	*AR*
BARD1	*ATR*	*ARID1A*	*KDR*	*ATM*	*ATR*	*ARID1A*
BCL6	*BARD1*	*ATM*	*KRAS*	*ATR*	*BARD1*	*ATM*
BRCA1	*BRCA1*	*ATR*	*MDM4*	*BARD1*	*BCL6*	*ATR*
BRCA2	*BRCA2*	*BAP1*	*MET*	*BRCA1*	*BRCA1*	*BARD1*
BRIP1	*BRIP1*	*BARD1*	*MLH1*	*BRCA2*	*BRCA2*	*BRCA1*
CHEK1	*CHEK1*	*BRAF*	*MRE11A*	*CCNE1*	*BRIP1*	*BRCA2*
CREBBP	*CTNNB1*	*BRCA2*	*MSH3*	*CHEK1*	*CHEK1*	*BRIP1*
CSF1R	*ERBB2*	*BRIP1*	*MTOR*	*DNMT3A*	*DNMT3A*	*CARD11*
EP300	*ERCC2*	*CDK12*	*MUTYH*	*ERBB2*	*ERBB2*	*CHEK1*
EZH2	*FANCI*	*CHEK1*	*MYCL1*	*ERCC2*	*ERCC2*	*DDR2*
FANCI	*FGFR4*	*CREBBP*	*NBN*	*FANCI*	*FANCI*	*EP300*
FGF23	*GEN1*	*CSF1R*	*NOTCH1*	*FGF6*	*FGF2*	*ERBB2*
FAM175A	*FAM175A*	*FAM175A*	*NOTCH3*	*FAM175A*	*FAM175A*	*FAM175A*
FGFR4	*HNF1A*	*CTNNB1*	*NRG1*	*FGFR4*	*FGFR4*	*FANCI*
FLT3	*KDR*	*DDR2*	*PALB2*	*FLT3*	*FLT3*	*FGF6*
GEN1	*KIT*	*EP300*	*PIK3CA*	*GEN1*	*GEN1*	*FGFR4*
HNF1A	*KRAS*	*ERBB2*	*PIK3CG*	*KMT2A*	*HNF1A*	*FLT3*
KDR	*MLH1*	*ERBB3*	*PTCH1*	*MET*	*JAK3*	*GEN1*
KRAS	*MSH3*	*ERBB4*	*PTEN*	*MLLT3*	*KDR*	*KDR*
MLLT3	*MUTYH*	*ESR1*	*RET*	*MSH3*	*KIT*	*MAP2K2*
MSH3	*MYCL1*	*FANCI*	*RICTOR*	*MUTYH*	*MLH1*	*MLH1*
MSH6	*NOTCH2*	*FGF23*	*ROS1*	*MYCL1*	*MSH3*	*MLLT3*
MUTYH	*NOTCH3*	*FGFR1*	*SMAD4*	*NRG1*	*MUTYH*	*MSH2*
MYCL1	*PTEN*	*FGFR2*	*TET2*	*RAD51B*	*MYCL1*	*MSH3*
NBN	*RET*	*FGFR4*	*TP53*	*RAD51D*	*NOTCH3*	*MUTYH*
NOTCH3	*RICTOR*	*FLT1*	*TSC2*	*RAD54L*	*NRG1*	*MYCL1*
NRG1	*ROS1*	*FLT3*		*RET*	*PALB2*	*NOTCH3*
PDGFRB	*TET2*			*RICTOR*	*PIK3CG*	*PIK3CG*
PIK3CA	*TP53*			*ROS1*	*PTCH1*	*PIK3R1*
PTEN				*TET2*	*RAD51B*	*PTCH1*
RICTOR				*TP53*	*RAD51D*	*RAD51B*
TET2				*XRCC2*	*RET*	*RAD51D*
TP53					*RICTOR*	*TET2*
					ROS1	*TP53*
					TET2	*TSC1*
					TP53	
					TSC1	
					XRCC2	

Supplementary Table 6. Continuation.

CRC152	CRC154	CRC158	CRC160	CRC164	CRC168
ALK	*ALK*	*APC*	*APC*	*ALK*	*AKT2*
APC	*APC*	*ARID1A*	*ATM*	*APC*	*ALK*
ARID1A	*ATM*	*ATM*	*ATR*	*AR*	*APC*
ATM	*ATR*	*ATR*	*BARD1*	*ATM*	*AR*
ATR	*BARD1*	*BARD1*	*BRCA1*	*ATR*	*ATM*
BARD1	*BCL6*	*BRCA2*	*BRCA2*	*BARD1*	*ATR*
BRCA1	*BRCA1*	*BRIP1*	*BRIP1*	*BRCA2*	*BARD1*
BRCA2	*BRCA2*	*CCNE1*	*CDKN2A*	*CHEK1*	*BRCA1*
BRIP1	*BRIP1*	*CD79A*	*CHEK1*	*EP300*	*BRCA2*
CHEK1	*CARD11*	*CDK12*	*EP300*	*ERBB2*	*CEBPA*
EP300	*CEBPA*	*CHEK1*	*ERBB2*	*ERBB3*	*CHEK1*
ERBB2	*CHEK1*	*CSF1R*	*FANCI*	*ERCC2*	*CSF1R*
FANCI	*CSF1R*	*DDR2*	*FGFR4*	*FANCI*	*EGFR*
FGF23	*EGFR*	*DNMT3A*	*FLT3*	*FGF23*	*ERBB2*
FGFR4	*ERBB2*	*EP300*	*FOXL2*	*FGFR4*	*FGF1*
FAM175A	*FAM175A*	*ALK*	*ALK*	*FAM175A*	*FAM175A*
FLT3	*ERBB4*	*ERBB2*	*GEN1*	*FLT3*	*FGF23*
GEN1	*ERCC2*	*ERCC2*	*HNF1A*	*GEN1*	*FGF6*
GNAS	*FANCI*	*EZH2*	*KDR*	*GNAS*	*FGFR4*
HNF1A	*FGF6*	*FANCI*	*KRAS*	*HNF1A*	*FLT3*
KDR	*FGFR4*	*FGF6*	*MLLT3*	*JAK2*	*GEN1*
KIT	*FLT3*	*FGFR4*	*MSH2*	*KDR*	*GNAS*
KRAS	*GEN1*	*FLT3*	*MSH3*	*KIT*	*HNF1A*
MLLT3	*HNF1A*	*GEN1*	*MSH6*	*KRAS*	*KDR*
MSH2	*KDR*	*HNF1A*	*MUTYH*	*MLH1*	*KIT*
MSH3	*KIT*	*IDH1*	*MYCL1*	*MLLT3*	*KRAS*
MSH6	*KMT2A*	*KRAS*	*NBN*	*MSH3*	*MLLT3*
MYCL1	*KRAS*	*MLH1*	*NF1*	*MSH6*	*MRE11A*
NBN	*MLLT3*	*MRE11A*	*NOTCH3*	*MYC*	*MSH3*
NF1	*MRE11A*	*MSH3*	*NRG1*	*MYCL1*	*MSH6*
NOTCH1	*MSH3*	*MYCL1*	*PALB2*	*NBN*	*MUTYH*
NOTCH2	*MSH6*	*NBN*	*PDGFRB*	*NOTCH2*	*MYCL1*
NOTCH3	*MUTYH*	*NF1*	*PIK3CA*	*NOTCH3*	*NBN*
NRG1	*MYCL1*	*NOTCH3*	*PIK3R1*	*NRG1*	*NOTCH1*
PTCH1	*NBN*	*NRG1*	*PTEN*	*PTCH1*	*NOTCH3*
PTEN	*NOTCH3*	*PDGFRB*	*RET*	*PTEN*	*NRG1*
RAD51D	*NRG1*	*PIK3CA*	*RICTOR*	*RET*	*PIK3CA*
RET	*PALB2*	*PIK3R1*	*ROS1*	*RICTOR*	*PTCH1*
RICTOR	*PIK3CG*	*PTCH1*	*TET2*	*ROS1*	*PTEN*
SMAD4	*PTCH1*	*PTEN*	*TP53*	*TET2*	*RET*
TET2	*PTEN*	*RAD51D*	*XRCC2*	*TP53*	*RICTOR*
TP53	*RICTOR*	*RET*		*TSC1*	*ROS1*
VHL	*ROS1*	*RICTOR*			*SMAD4*
	TET2	*SLX4*			*TP53*
	TP53	*TET2*			
	XRCC2	*TP53*			

Supplementary Table 7. CNVs detected in cfDNA of patient CRC100 by TST170.

Gene	Variant type	Alteration	FC
FGF6	CNV	Amplification	4.59
FGF23	CNV	Amplification	4.93

FC, fold change; CNV, copy number variant.

Supplementary Table 8. *NRAS* status in cfDNA of mCRC patients analyzed by BEAMIng and TST170.

Sample ID	*NRAS*	
	BEAMing	TST170
CRC032	WT	WT
CRC035	NA	WT
CRC062	NA	WT
CRC095	WT	WT
CRC100	WT	WT
CRC106	WT	WT
CRC112	NA	WT
CRC116	WT	WT
CRC131	WT	WT
CRC133	WT	WT
CRC137	WT	WT
CRC142	WT	WT
CRC145	WT	WT
CRC152	WT	WT
CRC154	WT	WT
CRC158	WT	WT
CRC160	WT	WT
CRC164	M (VAF: 0.12%)	ND
CRC168	WT	WT

WT, wild type; M, Mutated; VAF, variant allele fraction; NA, not available.

Supplementary Table 9. General description of the patient cohorts included in the study.

Cohort ID	Description	Institution/Hospital
1	Primary colorectal tumors and matched normal colorectal mucosa.	The Cancer Genome Atlas (TCGA), NCBI, USA.
2		Bellvitge Biomedical Research Institute (IDIBELL), Spain.
3	Primary colorectal tumors and matched normal colorectal mucosa and colorectal polyps.	Department of Molecular Diagnostics and Experimental Therapeutics (MDET) of the City of Hope National Medical Center, USA.
4	Polyps and normal mucosa from non-cancer patients.	Complejo Hospitalario Universitario de Ferrol, Spain.
5	Plasma samples from self-declared healthy controls and CRC patients.	Complejo Hospitalario Universitario de Compostela, Spain.
6	Plasma samples from self-declared healthy controls and patients with ACPs presenting at least one polyp >10 mm previously confirmed by colonoscopy.	
7	Plasma samples obtained either prior to a scheduled colonoscopy as part of standard CRC screening or prior to colonic surgery for primary tumor.	Complejo Hospitalario Universitario de Vigo, Instituto Valenciano de Oncología, Hospital Universitario de Burgos, Hospital General Universitario de Alicante, Complejo Hospitalario Universitario de Ourense and Hospital Clínico Universitario Lozano Blesa de Zaragoza, Spain.
8	Serial plasma samples collected at different clinically relevant time points from six randomly selected metastatic CRC (mCRC) patients under first-line standard chemotherapy.	Complejo Hospitalario Universitario de Santiago de Compostela, Spain.

Supplementary Table 10. Demographic and clinical characteristics of the patient cohorts included in the study.

	Colorectal tissue (FFPE)									Plasma (cfDNA)						
	Cohort 1 (N=331)		Cohort 2 (N=262)		Cohort 3 (N=36)			Cohort 4 (N=70)		Cohort 5 (N=54)		Cohort 6 (N=58)		Cohort 7 (N=57)		
Characteristics	Ctrl (N=38)	CRC (N=293)	Ctrl (N=82)	CRC (N=180)	Ctrl (N=12)	Polyps (N=12)	CRC (N=12)	Ctrl (N=10)	Polyps (N=60)	Ctrl (N=28)	CRC (N=26)	Ctrl (N=34)	ACP (N=24)	Ctrl (N=19)	ACP (N=17)	CRC (N=21)
Mean age, years (range)	69 (42-90)	65 (31-90)	66 (31-90)	67 (31-91)	71 (49-89)	66 (47-87)	64	64 (62-82)	65 (50-88)	51	67	29	69	53 (21-88)	66 (54-83)	71 (50-88)
Gender																
Male	21 (55%)	157 (54%)	46 (56%)	114 (63%)	7	11 (91.7%)	3 (25%)	6 (60%)	42 (70%)	12 (42.9%)	17 (65.4%)	17 (50.0%)	14 (58.3%)	6 (31.6%)	14 (82.4%)	11 (52.3%)
Female	17 (45%)	136 (46%)	36 (44%)	66 (37%)	5	1 (8.3%)	9 (75%)	4 (40%)	18 (30%)	16 (57.1%)	9 (34.6%)	17 (50.0%)	10 (41.7%)	13 (68.4%)	3 (17.6%)	9 (42.9%)
Unknown	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1 (4.8)
CRC Stage																
I	-	44 (15.0%)	-	10 (5.5%)	-	-	7 (58.3%)	-	-	-	-	-	-	-	-	2 (9.5%)
II	-	113 (38.6%)	-	84 (46.7%)	-	-	2 (16.7%)	-	-	-	-	-	-	-	-	2 (9.5%)
III	-	85 (29.0%)	-	56 (31.1%)	-	-	-	-	-	-	4 (15.4%)	-	-	-	-	3 (14.3%)
IV	-	41 (14.0%)	-	28 (15.6%)	-	-	3 (25%)	-	-	-	22 (84.6%)	-	-	-	-	8 (38.1%)
Unknown	-	10 (3.4%)	-	2 (1.1%)	-	-	-	-	-	-	-	-	-	-	-	6 (28.6%)
Type of polyp																
Hyperplastic	-	-	-	-	-	-	-	-	10 (16.7%)	-	-	-	-	-	-	-
Adenomatous	-	-	-	-	-	11 (91.7%)	-	-	20 (33.3%)	-	-	-	23 (95.8%)	-	17 (100%)	-
Serrated	-	-	-	-	-	1 (8.3%)	-	-	30 (50%)	-	-	-	1 (4.2%)	-	0 (0%)	-
Unknown	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Polyp status																
N-ACP	-	-	-	-	-	4 (33.3%)	-	-	24 (40%)	-	-	-	-	-	-	-
ACP	-	-	-	-	-	8 (66.7%)	-	-	35 (58.3%)	-	-	-	24 (100%)	-	17 (100%)	-
Unknown	-	-	-	-	-	-	-	-	1 (1.7%)	-	-	-	-	-	-	-

Ctrl, control; CRC, colorectal, N-ACP, non-advanced colorectal polyp; ACP, advanced colorectal polyp.

Supplementary Table 11. Demographic and clinical characteristics of metastatic colorectal cancer patients from cohort 8.

Characteristics	Patients (N=6)
Mean age (range; years)	61(47- 81)
Sex	
Female	3 (50%)
Male	3 (50%)
ECOG-PS	
1	5 (83%)
2	1 (17%)
Primary tumor site	
Transverse colon	1 (17%)
Sigmoid colon	2 (33%)
Rectosigmoid junction	2 (33%)
Rectum	1 (17%)
KRAS status	
Mutated	3 (50%)
No mutated	3 (50%)
BRAF status	
No mutated	6 (100%)
MMR status	
Preserved	5 (83%)
MLH1/PMS2 deficient	1 (17%)
Stage at diagnosis	
I	1 (17%)
IV	5 (83%)
Site of metastases	
Liver	5 (83%)
Lung	3 (50%)
Peritoneum	1 (17%)
No. metastatic sites	
1	3 (50%)
2	3 (50%)

ECOG-PS, eastern cooperative oncology group performance status; MMR, mismatch repair.

Supplementary Table 11. Continuation.

Characteristics	Patients (N=6)
Primary tumor resection	
Yes	4 (67%)
No	2 (33%)
CEA	
≤5 ng/mL	1 (17%)
>5 ng/mL	5 (83%)
Chemotherapy regimen	
mFOLFOX6	4 (67%)
FOLFIRI	1 (17%)
5-FU/LV	1 (17%)
Antibody (Anti-EGFR)	
Cetuximab	2 (33%)
Panitumumab	1 (17%)

CEA, Carcinoembryonic antigen.

Supplementary Table 12. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
≥ 18 years.	
Presence of measurable metastatic disease by computed tomography (CT) scan before initiation first-line therapy.	Diagnosis of other tumors except basal cell carcinoma of the skin or in situ of cervix.
ECOG-PS ≤ 2.	
Not having received chemotherapy for metastatic disease and, in case of having received adjuvant treatment, it should have been completed within the previous 6 months.	Psychiatric conditions of the patient that make it difficult to understand informed consent.
Adequate organ function and blood cell counts.	

ECOG-PS, eastern cooperative oncology group performance status.

Supplementary Table 13. Top 20 CpGs of the cfDNA episignature predictive of response to FOLFOX in mCRC patients.

TargetID[1]	Chr[2]	Position	Gene	Gene region	CpG context	Δβ[3]
cg17330251	7	95324644	*PON1*	TSS200	Island	-0.340
cg19195069	3	187889780	-	-	OpenSea	0.271
cg10400937	19	36838428	*ZNF790*	TSS1500; TSS200	Island	-0.270
cg09047573	5	138139216	-	-	OpenSea	-0.268
cg23302673	20	24951040	-	-	OpenSea	0.260
cg21170682	11	122334674	-	-	OpenSea	0.259
cg16781647	2	72924466	*EMX1*	Exon 2	Island	-0.256
cg00457913	6	48068867	*PTCHD4*	TSS200	Island	-0.253
cg24476033	19	6710647	*C3*	Exon 13	Shelf	0.246
cg02414224	3	133783779	*SRPR; TF; TF; TF; TF; TF; TF*	TSS1500; Exon 17; 3'UTR; Exon 23; 3'UTR; Exon 16; 3'UTR	Island	-0.245
cg19290181	6	84547590	-	-	OpenSea	-0.243
cg17445666	19	37692354	*ZNF781*	TSS200	Island	-0.243
cg12976883	2	230225661	*SP140*; *SP110*	TSS200; TSS200	OpenSea	0.242
cg22379207	19	36838372	*ZNF790*	5'UTR; Exon 1; TSS1500	Island	-0.242
cg23809300	18	79435874	-	-	Shore	-0.241
cg00872984	6	32096214	*TNXB*	Exon 3	Island	-0.240
cg08100069	9	136122863	*LOC107987142*	Exon 2	Island	-0.239
cg00548429	18	11179361	-	-	OpenSea	0.236
cg01895504	11	133021818	-	-	OpenSea	0.235
cg21358466	7	39085710	-	-	OpenSea	-0.232

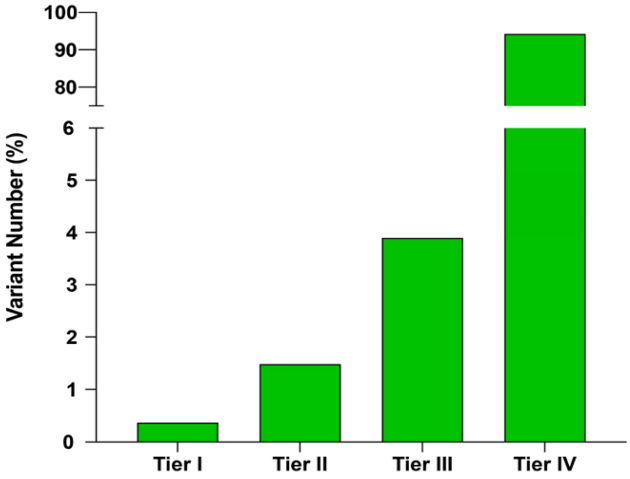
[1]Identification of the CpG according to EPIC array; [2]Chromosome; [3]Δβ-values (β-value nPD group - β-value PD group). TSS200, Transcription Start Site 200; TSS1500, Transcription Start Site 1500; 3'UTR, 3' untranslated regions; 5'UTR, 5' untranslated regions. CpGs in the table are arranged according to their absolute Δβ-value.

Supplementary Table 14. List of primers used for the methylation analysis of *WNT1* by ddPCR.

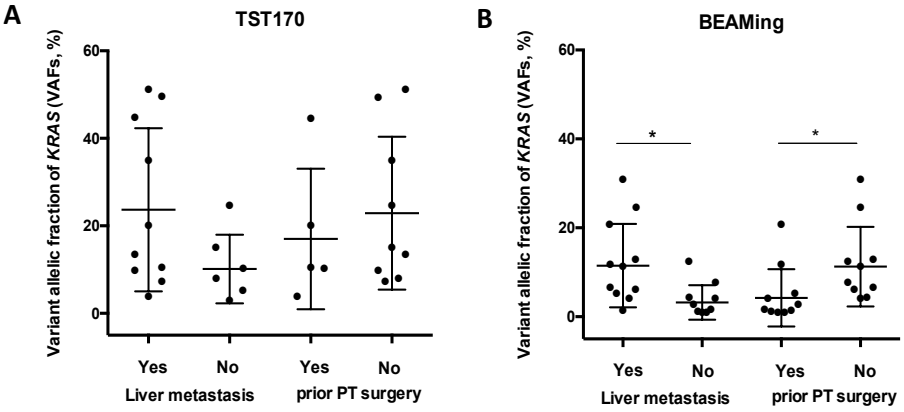
Assay	Primers/Probe	5'-Sequence-3'
***WNT1* - M**	Forward	GAGGGGTAGTTTTTTTT
	Reverse	TCTAACTTTAACAACCCTAAA
	Probe (FAM)	GATCGCGAGTTATGTTGT
***WNT1* - U**	Forward	GAGGGGTAGTTTTTTTT
	Reverse	TCTAACTTTAACAACCCTAAA
	Probe (HEX)	GGATTGTGAGTTATGTTGTT

Supplementary Table 15. Clinical characteristics of metastatic patients with luminal B breast cancer included in the study.

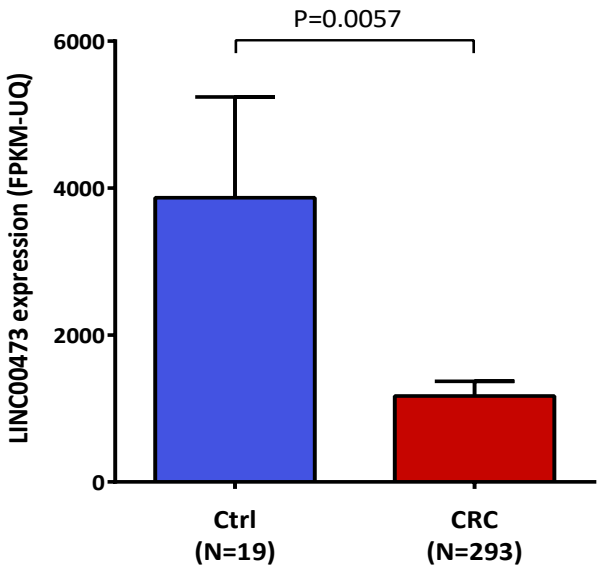
Characteristics	Patients (N=9)
Age (years)	
<65	3 (33%)
65-75	3 (33%)
>75	3 (33%)
Immunohistochemistry	
PR+	8 (89%)
ER+	9 (100%)
HER2+	2 (22%)
Ki67 ≥20% colon	9 (100%)
Histology	
Ductal	9 (100%)
Stage	
IV	9 (100%)
Metastatic sites	
1	4 (44%)
2	2 (22%)
3	3 (33%)
Metastatic location	
Visceral	6 (66%)
Bone	4 (4%)



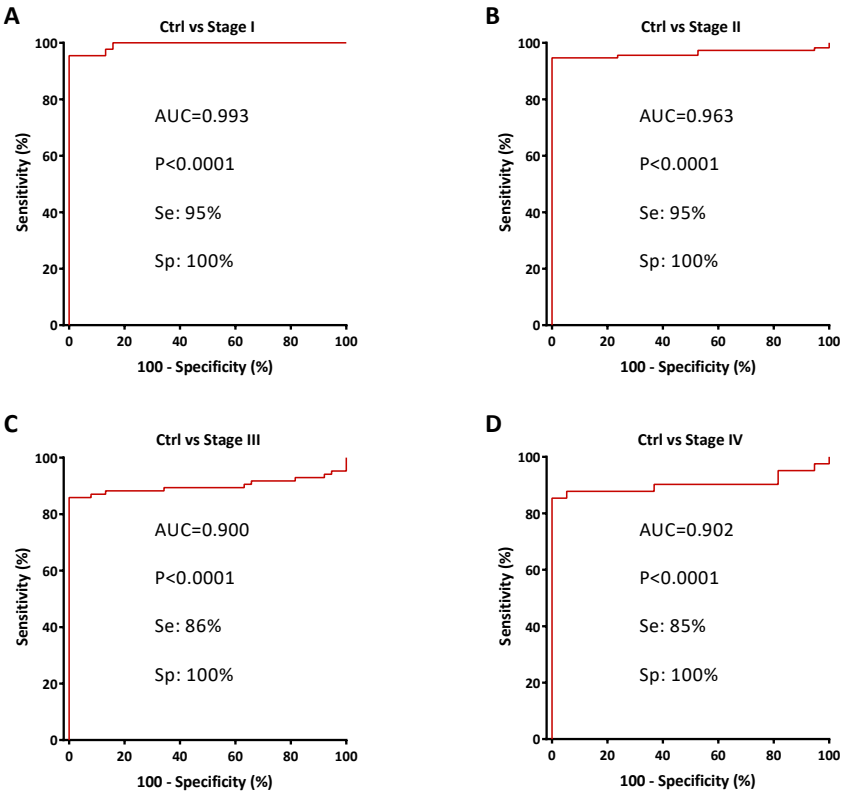
Supplementary Figure 1. Distribution of variants detected by TST170 in cfDNA of mCRC patients according to their clinical impact. Tier I, variants with strong clinical significance; tier II, variants with potential clinical significance; tier III, variants with unknown clinical significance; and tier IV, benign or likely benign variants.



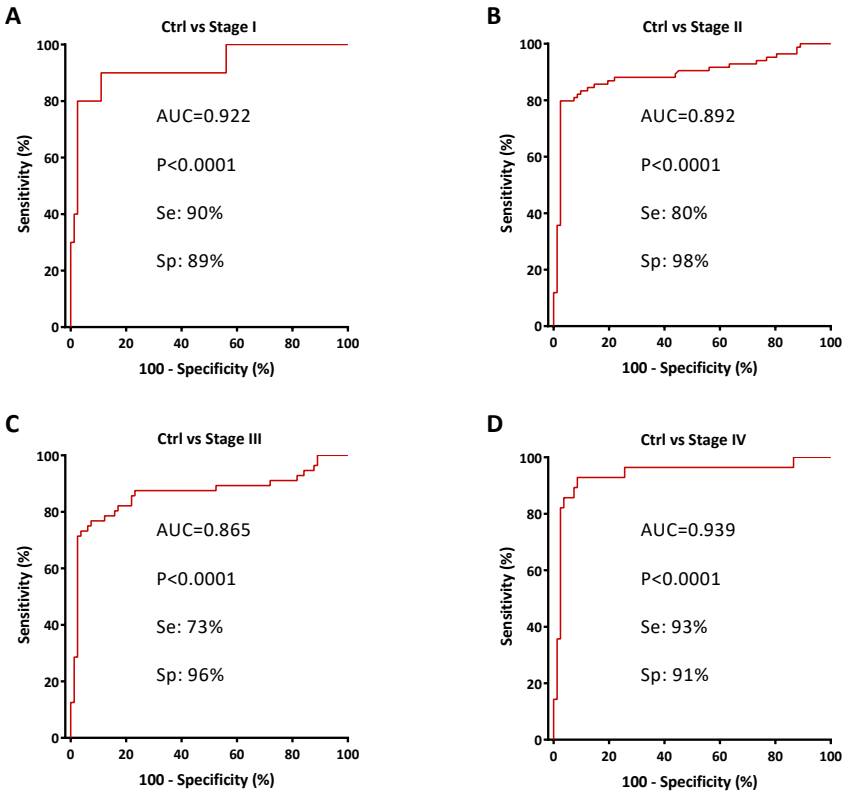
Supplementary Figure 2. Impact of *KRAS* VAFs detected by TST170 and BEAMing on patient clinical-pathological characteristics. A, Levels of *KRAS* VAFs obtained with TST170 according to metastasis location and previous surgery of primary tumor (PT). **B,** *KRAS* VAFs obtained by BEAMing according to metastasis location and previous surgery for primary tumor (PT). P-values were calculated using Student´s t-test. *p<0.05.



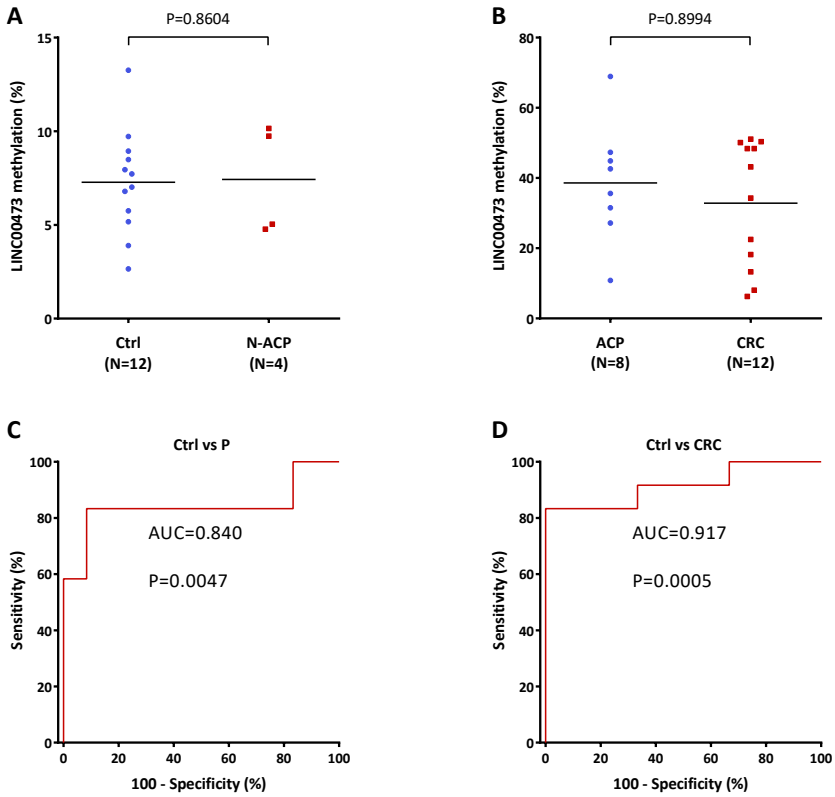
Supplementary Figure 3. Expression levels of LINC00473 in colorectal cancer tissues. Expression levels of LINC00473 were determined in tissues from primary colorectal cancer and matched normal colorectal mucosa (controls) by RNA-seq data obtained from The Cancer Genome Atlas (TCGA). P, p-value analyzed by Mann–Whitney U test. Ctrl, controls; CRC, colorectal cancer.



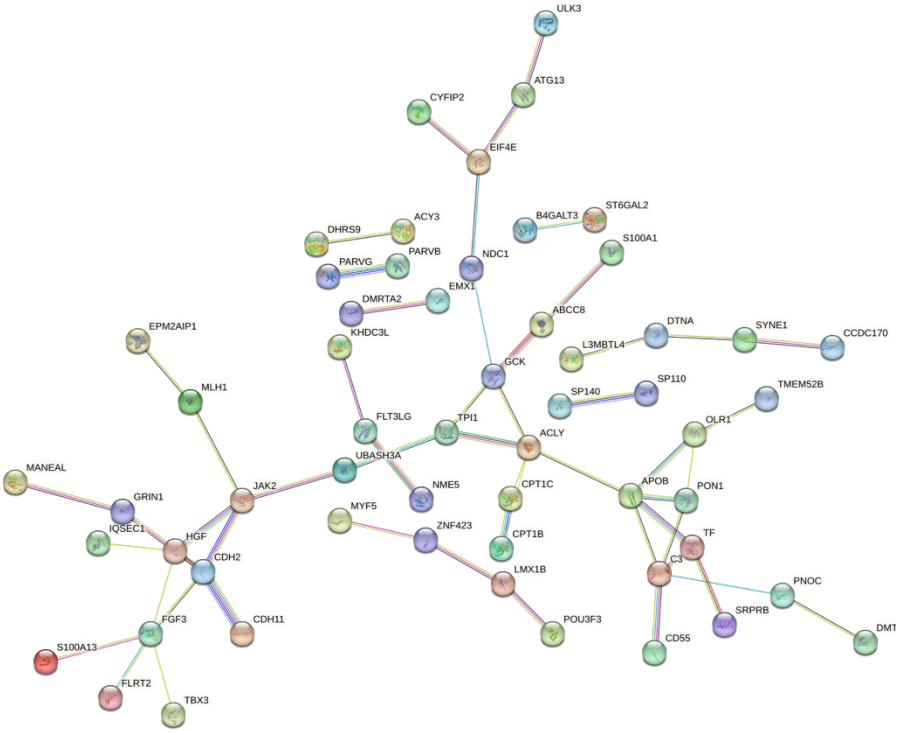
Supplementary Figure 4. Evaluation of the diagnostic accuracy of the methylation of LINC00473 to detect colorectal cancer stages. ROC curve analysis evaluating the methylation of LINC00473 for the detection of CRC at stage I (**A**), II (**B**), III (**C**) and IV (**D**), in tissue samples from primary colorectal cancer and matched normal colorectal mucosa (controls) by 450K array (Cohort 1). P, p-value analyzed by ROC curve; AUC, area under the ROC curve; Ctrl, controls; CRC, colorectal cancer; Se, sensitivity; Sp, specificity.



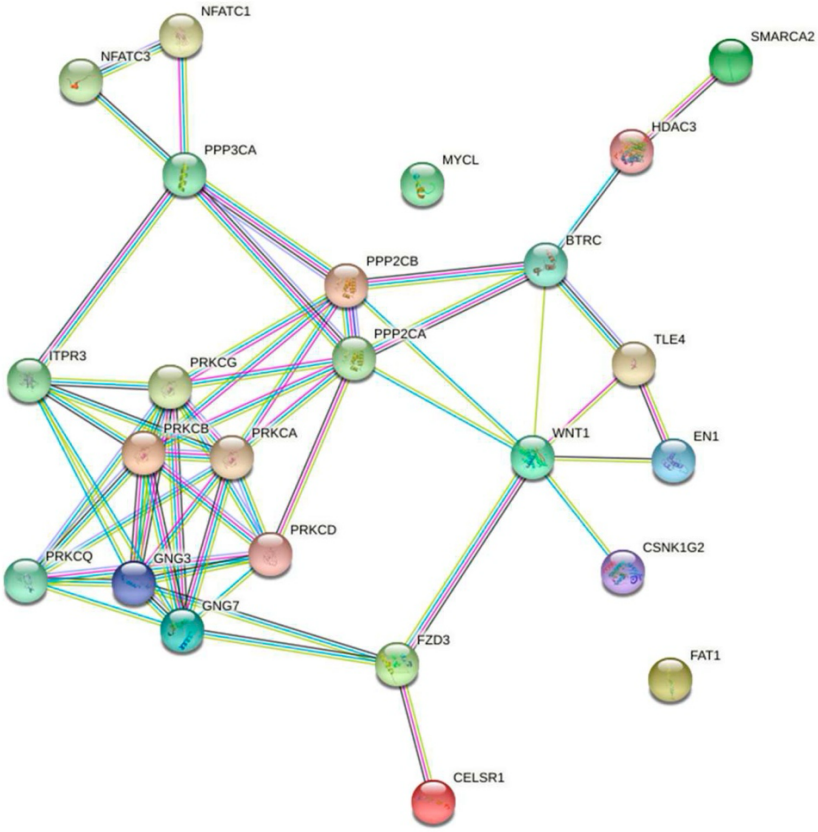
Supplementary Figure 5. Validation of the diagnostic accuracy of the methylation of LINC00473 to detect colorectal cancer stages. ROC curve analysis evaluating the methylation of LINC00473 for the detection of CRC at stage I (**A**), II (B), III (**C**) and IV (**D**), in tissue samples from primary colorectal cancer and matched normal colorectal mucosa (controls) by pyrosequencing (Cohort 2). P, p-value analyzed by ROC curve; AUC, area under the ROC curve; Ctrl, controls; CRC, colorectal cancer; Se, sensitivity; Sp, specificity.



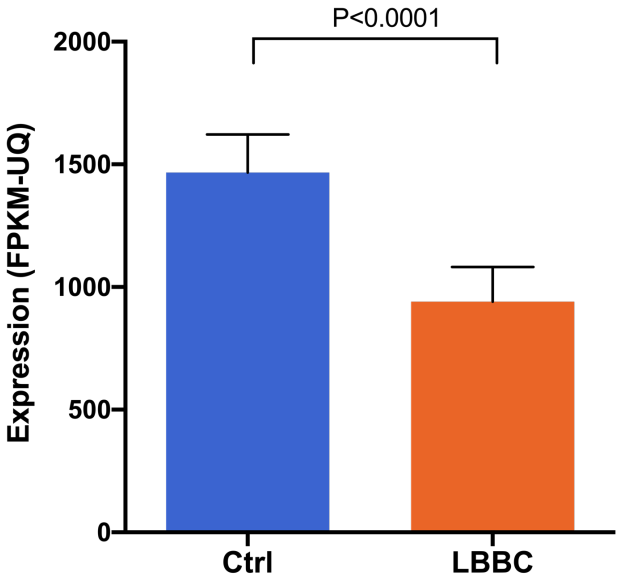
Supplementary Figure 6. Methylation levels of LINC00473 in tissue colorectal polyps and colorectal cancer. A-B, Comparison of methylation of LINC00473 between tissues from colorectal polyps, N-ACP, ACP and CRC and matched normal colorectal mucosa (controls) analyzed by pyrosequencing (Cohort 3). **C-D**, ROC curve analysis evaluating the methylation of LINC00473 for the detection of colorectal polyps and colorectal cancer with respect to controls (Cohort 3). Horizontal lines represent mean methylation levels of LINC00473. P, p-value analyzed by Mann–Whitney U test or ROC curve; AUC, area under the ROC curve; Ctrl, controls; P, polyps; CRC, colorectal cancer; N-ACP, non-advanced colorectal polyps; ACP, advanced colorectal polyps.



Supplementary Figure 7. Protein-protein association analysis by STRING. The genes involved in the cfDNA episignature predictive of response to FOLFOX belong to a functional network significantly enriched in protein interactions (P<0.01).



Supplementary Figure 8. The genes of the 34 DMCpGs related to the Wnt signaling pathway belong to a functional network significantly enriched in protein interactions (P<0.001).



Supplementary Figure 9. Expression of *WNT1* in primary luminal B breast tumors. Gene expression levels of *WNT1* available in primary tumors of luminal B breast cancer patients (n=122) and non-tumor controls (n=67) obtained by RNA-seq from TCGA. Expression levels are represented as the mean ± SEM.

Supplementary Methods: Inclusion and exclusion criteria

Tissue cohorts:

Inclusion criteria are those already described in the Description section of Supplementary Table 9. Exclusion criteria included samples from patients who have previously received chemotherapy or radiotherapy.

Plasma cohorts:

Cohort 5 and **cohort 6**: Inclusion criteria included either subjects self-declared healthy (without confirmatory colonoscopy), or subjects histologically diagnosed of advanced colorectal polyps (ACPs) who were referred to colonoscopy in the context of a colorectal cancer (CRC) screening program, or subjects diagnosed with CRC scheduled for surgery.

Cohort 7: Inclusion criteria included subjects who were referred to colonoscopy in the context of a CRC screening program and after colonoscopy they have been classified as healthy controls, or CRC or ACP based on histological diagnosis. In the case of CRC, subjects histologically diagnosed with CRC scheduled for surgery were also permitted.

Common exclusion criteria for cohorts 5 to 7 included patients (or controls) who have developed any another type of cancer in the previous 5 years, except curatively treated basal or squamous cell skin cancer, prostate intraepithelial neoplasm, carcinoma in-situ of the cervix, Bowen's disease or very low/low risk prostate cancer; those who have previously received chemotherapy or radiotherapy; subjects previously diagnosed with any hereditary polyposis syndrome, Lynch syndrome, inflammatory bowel disease, or those with a first degree relative previously diagnosed with CRC.

Cohort 8: Inclusion criteria are those already described in the Description section of Supplementary Table 9. Exclusion criteria included samples from patients who have previously received chemotherapy or radiotherapy in the advanced CRC setting, and those who have developed any another type of cancer in the previous 5 years, except curatively treated basal or squamous cell skin cancer, prostate intraepithelial neoplasia, carcinoma in- situ of the cervix, Bowen's disease or very low/low risk prostate cancer. None of the 6 cases included in cohort 8 were diagnosed with any hereditary polyposis syndrome, Lynch syndrome, or inflammatory bowel disease.

AGRADECIMIENTOS

AGRADECIMIENTOS

Después de 5 años pongo fin a una etapa apasionante, llena de retos y duro trabajo, pero también de recuerdos y personas que se quedarán conmigo toda la vida.

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muy bien y siempre estás presente. Un rato contigo pone de buen humor a cualquiera. **Gloria**, gracias por ser como eres. Por hacer que trabajar contigo sea tan sencillo, pero sobre todo, por saber escuchar y aconsejar. Y por qué no decirlo, porque una fiesta no es lo mismo sin ti. **Miriam**, **Celso** y **Cristóbal**, con vosotros he coincidido menos tiempo, pero solo tengo buenas palabras para vosotros.

A todas las *nanos*, Soraya, María, Emma, Laura, Jen, pero especialmente a las **Sandras** (Díez y Alijas) y a **Sai**, por estar siempre dispuestas a todo, a resolver un problema, a echar una mano o echar unas risas.

Gracias infinitas a todos los que formáis parte del Laboratorio 17 (y ahora también del 18). **Laura**, **Lore**, **Alba**, **Jorge** y **Ana**, gracias por vuestro trabajo, implicación y estar siempre disponibles para dar buenos consejos e ideas. A *Saliva team*, **Mercedes**, **Oscar** y **Ana**, siempre con una sonrisa y buenas palabras por delante. Anita, Dr. Oscarín, gracias por todos los buenos ratos, por las palabras de ánimo y porque teneros cerca es garantía de buenos momentos. A los últimos en llegar, **Andrea, Aroa**, **Manu**, **Mimí**, **Sonia**, y **Yoel**, ya sois parte fundamental del grupo y también de mí. Gracias porque estar con vosotros es sinónimo de buen rollo y de alegría. Mimí y Andrea, dejadme deciros que no cambiéis nunca, vuestra forma de ser es muy necesaria para todos. **Raquel**, lo único malo que tengo que decir de ti es que tardases tanto en llegar. Gracias por los innumerables buenos momentos que me has dado en este tiempo, por escuchar y saber quitarle peso a lo que no lo debe de tener y por toda la ayuda en el laboratorio y fuera de él, por la complicidad.

Vamos ahora con *los clásicos*. **Alicia**, sin tu trabajo y tu ayuda mi tesis sería mucho más delgadita, gracias por estar dispuesta siempre, por estar pendiente de todo y por hacernos adictos a tus caramelos. **Ramón**, te

has convertido en parte fundamental de mi día a día. Gracias por tu sabiduría, tu forma de ser, tu bondad. Por ayudar siempre, aunque no te lo pida, por resolver tantos y tantos problemas, por estar disponible cuando sea y para lo que sea. Gracias.

Patri, **Carlos** y **Carol**, ¿qué os puedo decir? Los tres sois parte inseparable de lo que ha sido esta etapa, mis compañeros y mis amigos. Gracias por estar ahí, por ser, por todo. Por la ayuda en el laboratorio, pero sobre todo fuera de él. Por tantas sesiones de desahogo y terapia conjunta, por los desayunos, comidas y cenas, por las escapadas, por todos los momentos vividos. Estoy convencido que no hubiese podido llegar hasta aquí sin vosotros. La vida nos irá llevando por caminos distintos, pero siempre sacaremos tiempo para *la caña de los martes*.

Gracias como no puede ser de otra forma a todos mis compañeros de la **Unidad de Epigenómica**, también a los que forman parte de Endocrino, **Ana**, **Jesús**, **Paula** y especialmente **Andrea**, por su ayuda a lo largo de estos años. **José**, mi portugués favorito, solo llevas unos meses con nosotros, pero has encajado enseguida como uno más del grupo. Obrigado por tu plena disposición, siempre con buenas ideas que aportar y por tu buen humor. **Nico**, mil gracias por tu generosidad, por echar una mano siempre que hace falta y por estar siempre disponible, por ser nuestro bioinformático favorito, el que nos resuelve los problemas tecleando. Nos quedan aún muchas cenas que compartir.

Aida, la amiga que se sentaba a mi lado. Si miro para atrás no me imagino haber recorrido este camino sin ti. Sería totalmente imposible. Parafraseándote, que bien hemos dado. Han sido tantos los momentos vividos desde aquel primer cumpleaños tuyo que pasamos juntos, trabajando en el labo hasta casi la medianoche, hasta el día de hoy. Aunque ya no te sientas conmigo, seguimos creando recuerdos juntos. Creo que nunca podré

agradecerte lo suficiente todo lo que has hecho por mí. Todos los buenos momentos (que han sido muchos) y también alguno malo, se quedarán siempre para nosotros.

Gracias también a mis amigos de fuera del laboratorio, que me han acompañado en este proceso y que han tenido que compartir los buenos y malos momentos. Especialmente quiero dar las gracias a **Paula**, **Iago**, **Aitor**, **Laura**, **Antía** y **Ari**, por darme siempre un lugar en el que apoyarme cuando lo necesitaba.

Por último, quiero agradecer a esta Tesis haber puesto a una gran persona en mi camino. **Manuel**, quizás si no fuese por la tesis que hoy termino de escribir, nunca nos hubiésemos conocido. Gracias por tu apoyo, tu comprensión y tu paciencia infinita. Por ser tú y dejarme ser yo mismo.

En definitiva, nadie podría tener la suerte que he tenido yo al encontrar a unos compañeros como vosotros y vosotras que me han acompañado y ayudado a lo largo de esta aventura. ¡¡Gracias!!

ANNEXES

ANNEX I

List of publications and patents

List of publications presented in this thesis:

Chapter I:

Evaluation of a Targeted Next-Generation Sequencing Panel for the Non-Invasive Detection of Variants in Circulating DNA of Colorectal Cancer.

Rodriguez-Casanova A†, Bao-Caamano A†, Lago-Lestón RM, Brozos-Vázquez E, Costa-Fraga N, Ferreirós-Vidal I, Abdulkader I, Vidal-Insua Y, Rivera FV, Candamio Folgar S, López-López R, Muinelo-Romay L, Diaz-Lagares A. †These two authors share first authorship.

Journal of clinical medicine. 2021 Sep 29;10(19):4487.

Full-text available: https://doi.org/10.3390/jcm10194487

EISSN: 2077-0383

Impact Factor: 4.964 (JCR 2021)

Quartile-Category: Q2 / Medicine, General & Internal (55/172)

Contribution to this work: I, Aitor Rodríguez Casanova, have been involved in the Conceptualization, Data curation, Methodology, Formal analysis, Investigation, Writing – original draft preparation, Writing – review and editing.

Chapter II.A:

Non-invasive early detection of colorectal cancer by hypermethylation of the *LINC00473* promoter in plasma cell-free DNA

Ruiz-Bañobre J†, **Rodriguez-Casanova A**†, Costa-Fraga N, Bao-Caamano A, Alvarez-Castro A, Martín Carreras-Presas FL, Brozos-Vazquez E, Vidal-Insua Y, Vazquez-Rivera F, Candamio-Folgar S, Mosquera-Presedo M, Lago-Lestón RM, Muinelo-Romay L, Vázquez-Bueno JA, Sanz-Pamplona R, Moreno V, Goel A, Castillo L, Martin AC, Arroyo R, Esteller M, Crujeiras AB, López-López R, Díaz-Lagares A. †These two authors share first authorship.

Clinical Epigenetics. 2022 Jul 9;14(1):86.

Full-text available: https://doi.org/10.1186/s13148-022-01302-x

ISSN: 1868-7075

Impact Factor: 7.280 (JCR 2021)

Quartile-Category: Q1 / Oncology (53/245)

Contribution to this work: I, Aitor Rodríguez Casanova, have been involved in the Conceptualization, Data curation, Methodology, Formal analysis, Writing – original draft preparation, Writing – review and editing.

Chapter III:

A genome-wide cell-free DNA methylation analysis identifies an episignature associated with metastatic luminal B breast cancer.

Rodriguez-Casanova A†, Costa-Fraga N†, Castro-Carballeira C, González-Conde M, Abuin C, Bao-Caamano A, García-Caballero T, Brozos-Vazquez E, Rodriguez-López C, Cebey V, Palacios P, Cueva JF, López-López R, Costa C, Díaz-Lagares A. †These two authors share first authorship.

Frontiers in cell and developmental biology. 2022 Oct 25;10:1016955.

Full-text available: https://doi.org/10.3389/fcell.2022.1016955

ISSN: 2296-634X

Impact Factor: 6.081 (JCR 2021)

Quartile-Category: Q1 / Developmental Biology (6/39)

Contribution to this work: I, Aitor Rodríguez Casanova, have been involved in the Methodology, Formal analysis, Writing – original draft preparation, Writing – review and editing.

Patents derived from this thesis:

In vitro method for the diagnosis or prognosis of colorectal cancer or a pre-cancerous stage thereof.

Rodríguez Casanova A; Crujeiras Martínez AB; López López R; Díaz Lagares A.

EP No. 19382290.5 (15/04/2019)

ANNEX II

Ethical considerations and attached permissions

Use of images:

Unless otherwise indicated, all images presented in this thesis have been created by the author.

Furthermore, BioRender.com software was employed to create the images presented in the Introduction of this doctoral thesis.

Cell Culture:

The cancer lines used in this work were cultured under recommended conditions and were only used for the research purposes specifically described in this thesis.

Patient's samples:

Blood samples were collected following the guidelines of Good Clinical Practice and the Declaration of Helsinki and in accordance with the guidelines and protocols approved by the Galician Ethical Committee:

Chapter I: Ref. 2015/746
Chapter II.A: Ref. 2017/538
Chapter II.B: Ref. 2019/017
Chapter III: Ref. 2015/772

All included participants signed the informed consent to participate.

Attached permissions:

Chapter I: Ref. 2015/746

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15781 SANTIAGO DE COMPOSTELA
Tlf: 881 546425 Fax: 881 541804
ceic@sergas.es

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

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

CERTIFICA:

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

Título: Valor de la Tecnología BEAMing para el análisis de mutaciones del gen RAS en biopsia líquida
Promotor: Rafael López López
Tipo de estudio: Outros
Version: Versión 4 de diciembre 2015
Código del Promotor: RAS-Bliq-001
Código de Registro: 2015/746

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y el la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del Comité.

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

Centros	Investigadores Principales
C.H. Universitario de Santiago	Laura Muinelo Romay, Rafael López López

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

En Santiago de Compostela, a
El/La Presidente/a 22/12/2015

NOMBRE RODRIGUEZ MORENO CARLOS - NIF 05614327G

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Fecha: 2015.12.22 13:55:58 +01'00'

Chapter II.A: Ref. 2017/538

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Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Tel: 881546425. Correo-e: oeic@sergas.es

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

Guillermo José Prada Ramallal, Secretario del Comité de Ética de la Investigación de Santiago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 21 de diciembre de 2017 el estudio:

Título: Identificación de marcadores de diagnóstico, pronóstico y seguimiento mediante el análisis de Biopsia Líquida en pacientes con cáncer
Promotor: Rafael López López
Tipo de estudio: Outros
Versión: Versión 1 de 31 de Octubre de 2017
Código del Promotor:
Código de Registro: 2017/538

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humana, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Postautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07/2004, de investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos da Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del Comité.

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

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C.H. Universitario de Santiago	Rafael López López, Laura Muinelo Romay, Roberto Díaz Peña

En Santiago de Compostela, a 28 de diciembre 2017.

El Secretario del Comité Territorial de Ética de la Investigación de Santiago Lugo,

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guillermo.jose.prada.ramallal@sergas.es
2017.12.29 13:33:30 +02'00'

Guillermo José Prada Ramallal

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CONSELLERÍA DE SANIDADE
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Comité Autonómico de Ética da Investigación de Galicia
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Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Tel: 881546425. Correo-e: ceic@sergas.es

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Guillermo José Prada Ramallal, Secretario del Comité de Ética de la Investigación de Santiago-Lugo,

HACE CONSTAR QUE:

1.- El Comité Territorial de Ética de la Investigación de Santiago-Lugo cumple tanto en su composición como en sus PNTs los requisitos legales vigentes (RD 1090/2015 de ensayos clínicos, y la Ley 14/2007 de Investigación Biomédica).

2.- La composición actual del Comité Territorial de Ética de la Investigación de Santiago-Lugo es:

- **Juan Manuel Vázquez Lago (Presidente).** Médico especialista en Medicina Preventiva y Salud Pública. Área de Gestión Integrada de Santiago.
- **Pilar Rodríguez Ledo (Vicepresidenta).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Guillermo José Prada Ramallal (Secretario).** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago. Fundación Ramón Domínguez.
- **Lorenzo Armenteros del Olmo (Vicesecretario).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Francisco Campos Pérez.** Biólogo. Instituto de Investigación Sanitaria de Santiago de Compostela.
- **Rosana Castelo Domínguez.** Farmacéutica de Atención Primaria. Área de Gestión Integrada de Santiago.
- **Ricardo García Martínez.** Licenciado en Derecho. Área de Gestión Integrada de Lugo.
- **Jaime Gulín Dávila.** Farmacéutico especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Lugo.
- **Víctor Herrán Carreira.** Paciente. ADIL-Asociación de Diabéticos Lucense.
- **María Jesús Lamas Díaz.** Farmacéutica especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Santiago.
- **Carlos Rodríguez Moreno.** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago.
- **Rafael Carlos Vidal Pérez.** Médico especialista en Cardiología. Área de Gestión Integrada de Lugo.
- **María Jesús Wandosell Picatoste.** Enfermera. Área de Gestión Integrada de Santiago.

Para que conste donde proceda, y a petición del promotor/investigador, en Santiago de Compostela, a 28 de diciembre de 2017.

El Secretario del Comité Territorial de Ética de la Investigación de Santiago Lugo,

XUNTA DE GALICIA SECRETARÍA XERAL TÉCNICA COMITÉS DE ÉTICA DE INVESTIGACIÓN CONSELLERÍA DE SANIDADE

guillermo.jose.prada.ramallal@sergas.es
2017.12.29 13:33:34 +02'00'

Chapter II.B: Ref. 2019/017

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CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Teléfono: 881546425
ceic@sergas.es

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DICTAMEN DEL Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

Paula M. López Vázquez, Secretaria del Comité de ética de la investigación con medicamentos de Galicia (CEIm-G)

CERTIFICA:

Que este Comité evaluó en su reunión del día 28/02/19 :

Título: Desarrollo de una firma epigenética en biopsia líquida para predecir la respuesta terapéutica en cáncer colorrectal metastásico
Promotor*:* Ángel Díaz Lagares
Tipo de estudio: EPA OD
Version: v02 de 09/12/2019 y HIP/CI de la misma fecha
Código del Promotor:ADL-FOL-2019-01
Código de Registro: 2019/017

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIm-G de Galicia

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Centros	Investigadores Principales
C.H. Universitario de Santiago	Ángel Díaz Lagares

Xacobeo 2021

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Teléfono: 881546425
ceic@sergas.es

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Y HACE CONSTAR QUE:

1. El comité cumple los requisitos legales vigentes aplicables a los Comités de ética de investigación.
2. El comité tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3. La composición actual del comité es:

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Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Belen Pérez Marcos (Vicesecretaria). Farmacéutica de Atención Especializada
Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
Juan Casariego Rosón. Médico Especialista en Cardiología.
Nuria Carballeda Feijóo. Miembro lego. Representante de los intereses de los pacientes.
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José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
José Luis Fernández Trisac. Médico Especialista en Pediatría.
Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Diego Santos García. Médico Especialista en Neurología.
Juan Vázquez Lago. Médico Especialista en Medicina Preventiva y Salud Pública.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela,

Xacobeo 2021

Chapter III: Ref. 2015/772

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CONSELLERÍA DE SANIDADE
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Edificio Administrativo San Lázaro
15703 SANTIAGO DE COMPOSTELA
Teléfono: 881546425
ceic@sergas.es

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DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonómico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 22/12/15 :

Título: Biopsia líquida para oncología de precisión
Promotor*:* Rafael López López
Tipo de estudio:EPA-SP
Version: versión 30 de noviembre de 2015 y HIP/CI (cultivo, control biopsia, y biopsia líquida) de la misma fecha
Código del Promotor:RLL-BL-2015_01
Código de Registro: 2015/772

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

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

Centros	Investigadores Principales
C.H. Universitario de Santiago	Clotilde Costa Nogueira

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Teléfono: 881546425
ceic@sergas.es

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Y HACE CONSTAR QUE:

1 El CAEIG cumple los requisitos legales vigentes (R.D 223/2004 por el que se regulan los ensayos clínicos con medicamentos, y la Ley 14/2007 de Investigación Biomédica).
2 El CAEIG tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
3 La composición actual del CAEIG es:

Manuel Portela Romero. (Presidente). Médico Especialista en Medicina Familiar y Comunitaria.
Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.
Paula Mª López Vázquez, (Secretaria). Médico Especialista en Farmacología Clínica.
Juan Vázquez Lago (Secretario Suplente). Médico Especialista en Medicina Preventiva y Salud Pública.
Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.
Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.
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José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.
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Mª José Ferreira Díaz. Diplomada Universitaria de Enfermería
Pablo Nimo Ríos. Licenciado en Derecho. Miembro externo
Pilar Gayoso Diz. Médico Especialista en Medicina Familiar y Comunitaria.
Agustín Pía Morandeira. Farmacéutico de Atención Primaria
Salvador Pita Fernández. Médico Especialista en Medicina Familiar y Comunitaria.
Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.
Susana María Romero Yuste. Médico Especialista en Reumatología.
Mª Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 05 de enero de 2015

Firmado digitalmente por LOPEZ VAZQUEZ PAULA MARIA - DNI 46900339G
Nombre de reconocimiento (DN): c=ES, o=XUNTA DE GALICIA, ou=certificado electrónico de empleado público, serialNumber=46900339G, sn=LOPEZ VAZQUEZ, givenName=PAULA MARIA, cn=LOPEZ VAZQUEZ PAULA MARIA - DNI 46900339G
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Colorectal and breast cancer are among the most frequent types of cancer. The identification of novel non-invasive genetic and epigenetic biomarkers is crucial to improve the management of this disease. This thesis evaluated the performance of a novel NGS targeted panel for the non-invasive detection of clinically relevant gene variants in cell-free DNA (cfDNA). Furthermore, this work characterized the cfDNA methylation profile of colorectal and breast cancer patients, providing non-invasive epigenetic biomarkers and signatures useful for the early detection of the disease and therapy selection. Therefore, the results obtained in this study will contribute to improve the management of colorectal and breast cancer, facilitating the advance towards precision oncology.