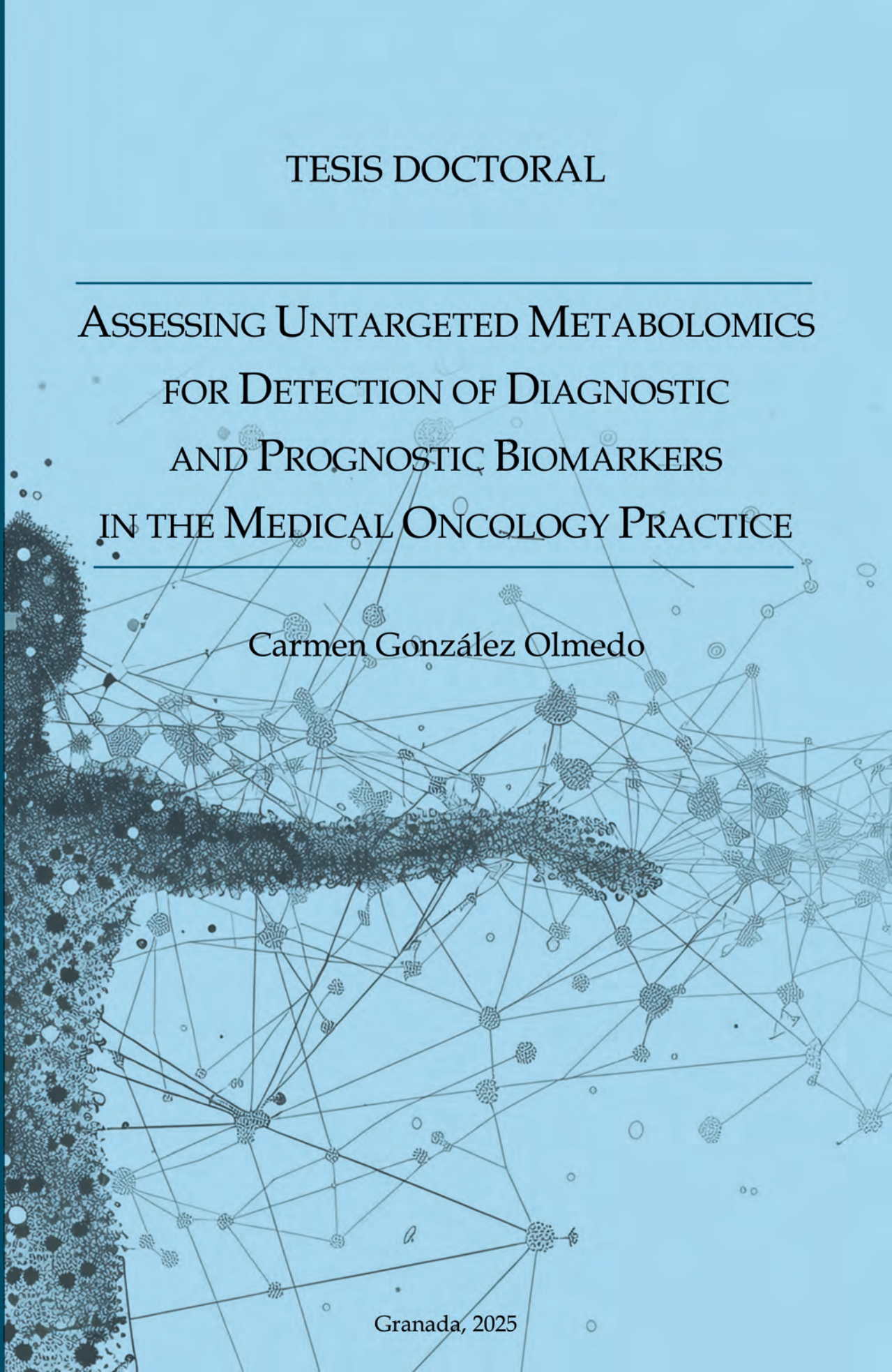
TESIS DOCTORAL

Assessing Untargeted Metabolomics for Detection of Diagnostic and Prognostic Biomarkers in the Medical Oncology Practice

Carmen González Olmedo



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| *Tesis Doctoral* |

ASSESSING UNTARGETED METABOLOMICS FOR DETECTION OF DIAGNOSTIC AND PROGNOSTIC BIOMARKERS IN THE MEDICAL ONCOLOGY PRACTICE

Carmen González Olmedo

Directores: Dra. Caridad Díaz Navarro

Dr. Juan Antonio Marchal Corrales

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A mi madre,

y a mi padre.

"Desde el tiempo de mi niñez, no he sido
como otros eran, no he visto
como otros veían, no pude sacar
mis pasiones desde una común primavera" (…)

Solo, Edgar Allan Poe.

(…)

"Y aquí estoy

jodida por este camino que escogí

pero vale la pena llegar hasta el fin

hay que sentir la magia del amanecer,

para crecer.

Pero sé

que aún me quedan lágrimas por derramar,

será el precio que pague por mi libertad,

quiero sentir que hice lo que yo

de verdad soñaba."

(…)

De momento, Los Aslándticos.

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"Solo un **exceso** es recomendable en el mundo: el **exceso de gratitud",** por lo que hoy, aquí, me voy a tomar la libertad de excederme.

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ABBREVIATIONS

Abbreviation	Meaning	Page
A.C	Ante Christum	17
AcN	acetonitrile	81
A.D	Anno Domini	17
AD	adjuvant	22
AhR	aryl hydrocarbon receptor	78
AI	artificial intelligence	42
AJCC	American Joint Committee on Cancer	23
ANOVA	analysis of variance	63
ASCA	ANOVA–simultaneous component analysis	63
ATP	adenosine triphosphate	47
AUC	area under the curve	49
BAs	bile acids	192
BC	breast cancer	9
BLVR/BVR	biliverdin reductase	79/191
BMI	body mass index	74
BR	bilirubin	80
BV	biliverdin	80
CA	cancer antigen	38
CCR	cáncer colorrectal	13
CEA	carcinoembryonic antigen	38
CIMP	CpG island methylator phenotype	31
CIN	chromosomal instability	31
CM	cáncer de mama	13
CMSs	consensus molecular subtypes	30
CoA	coenzyme A	47
CO_2	carbon dioxide	47
CRC	colorectal cancer	9
CRLM	colorectal liver metastasis	29
CSCs	cancer stem cells	18
CSCsT	cancer stem cells theory	19
ctDNA	circulating tumour deoxyribonucleic acid	40
ctRNA	circulating tumour ribonucleic acid	40
DA	deoxycholic acid	80
Da	Dalton	41
DC	deoxycholate	80
DHA	docosahexaenoic acid	137
DHAP	dihydroxyacetone-phosphate	47
DNA	deoxyribonucleic acid	29
ECM	extracellular matrix	18
ECOG	Eastern Cooperative Oncology Group	62
EDTA	ethylenediaminetetraacetic acid	62
ER	estrogen receptors	24

ESI	electrospray ionization	46
FA	fatty acid	79
FAO	fatty acid oxidation	195
FC	fold change	64
FDA	Food and Drug Administration	37
FDR	false discovery rate	64
FIBAO	Fundación para la Investigación Biomédica de Andalucía Oriental-Alejandro Otero	64
FISH	fluorescent in situ hybridization	126
FIT	fecal immunochemical test	29
FOBT	fecal occult blood test	29
FXR	farnesoid X receptor	193
g	relative centrifugal force	62
GA3P	glyceraldehyde-3-phosphate	47
GC	gas chromatography	44
GCDC	glycochenodeoxycholate	81
GDCA	glycodeoxycholic acid	138
GHCA	glycohyocholic acid	138
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GUDCA	glycoursodeoxycholic acid	76
HC	healthy control	74
HO-1	heme oxygenase-1	80
HER2+	human epidermal growth factor receptor-2 positive	23
HILIC	hydrophilic interaction liquid chromatography	46
HRMS	high resolution-mass spectrometry	9
ICH-GCP	International Conference on Harmonization-Good Clinical Practices	81
IDA	information dependent acquisition method	82
IDO1	indoleamine-2,3-dioxygenase	78
INE	Instituto Nacional de Estadística	17
Ki-67	marker of proliferation Kiel 67	23
Kyn	kynurenine	78
LA	luminal A	23
LB	luminal B	23
LC	liquid chromatography	9
M&P	Miller and Payne	63
m/z	mass-to-charge ratio	44
mCRC	metastatic colorectal cancer	30
MCT	monocarboxylate transporter	47
miR	micro ribonucleic acid	38
MPC	mitochondrial pyruvate carrier	47
mR	messenger ribonucleic acid	38
MRD	minimal residual disease	22
mRS	metabolomic risk score	172

MS	mass spectrometry	41
MSI	microsatellite instability	32
NA	neoadjuvant	22
N/A	not applicable	25
NACT	neoadjuvant chemotherapy	26
NAD+	oxidized nicotamide adenine dinucleotide	47
NADH/H+	reduced nicotinamide adenine dinucleotide	47
NAT	neoadjuvant treatment	80
Nmi	micrometastasis	25
NGS	next-generation sequencing	21
NMR	nuclear magnetic resonance	42
NR	nonresponders	129
OAA	oxaloacetate	47
OS	organic solvent	75
O2	oxygen	47
PCA	principal component analysis	65
PC	phosphatidylcholine	79
pCR	pathological complete response	63
PCR	polymerase Chain Reaction	37
PE	phosphatidylethanolamine	79
PL	phospholipids	136
PLS-DA	partial least squares discriminant analysis	64
PR	progesterone receptors	24
QC	quality control	75
Q-TOF	quadrupole-time-of-flight	63
R	responders	129
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REDECAN	Red Española de Registros de Cáncer	22
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ROC	receiver operating characteristic	49
ROS	reactive oxygen species	80
RP	reverse phase	46
RSD	relative standard deviation	131
SCNAs	somatic copy number alterations	31
SEOM	Sociedad Española de Oncología Médica	27
SMT	somatic mutation theory	18
Succ-CoA	succinyl-coenzyme A	47
SVM	support vector machine	133
TDO2	tryptophan-2,3-dioxygenase	78
TIC	total ion chromatogram	46
Tis	tumour *in situ*	25
TME	tumour microenvironment	21
TN	triple negative	23
TNM	tumour nodes metastasis	25
TOFT	tissue organization field theory	18
TR	translational research	34
Trp	tryptophan	78
t1	extraction time point 1	62

t2	extraction time point 2	62
t3	extraction time point 3	62
UHPLC	ultra-high performance liquid chromatography	43
UGT1A1	uridine diphosphate glucuronosyltransferase 1A1	191
USA	United States of America	28
UVA	univariate analysis	83
VIP	variable importance in projection	64
XOR	xanthine oxidoreductase	189
yo	years old	28
3PG	3-phosphoglycerate	47
α-KG	α-ketoglutarate	47
°C	degree Celsius	62

ABSTRACT

Cancer remains a major global health concern, responsible for over 19 million new cases and nearly 10 million deaths annually. Breast cancer (BC) is the most commonly diagnosed cancer in women, while colorectal cancer (CRC) ranks third worldwide. Despite substantial advancements in research, these diseases continue to pose significant challenges, due to their molecular heterogeneity and varied clinical outcomes.

Precision medicine has transformed cancer care by enabling more personalized therapies. However, there still exists an unmet need for enhanced diagnostic and prognostic tools, particularly for managing advanced disease, recurrence and treatment resistance. One promising solution is the identification of circulating biomarkers through metabolomics, which provides insights into disease mechanisms and offers new candidate biomarkers for early detection of residual disease and prediction to treatment response. This approach will enhance precision oncology and improve clinical decision-making.

This thesis aimed to analyse the metabolomic profiles of BC and CRC using untargeted metabolomics to discover metabolites in plasma that could be applied as potential biomarkers in clinical practice. To achieve this, we utilized liquid chromatography (LC) methods coupled to high-resolution mass spectrometry (HRMS). This high-resolution technique facilitated comprehensive metabolite detection, deepening our understanding of cancer biology.

Our research resulted in three original studies that demonstrated the ability of untargeted LC-HRMS-based metabolomics to identify potential cancer biomarkers in the following clinical contexts: 1) metabolomic differentiation of BC subtypes, 2) BC subtype-specific metabolomic changes in response to neoadjuvant chemotherapy, and 3) CRC metabolomics-based prediction of recurrence and survival following liver metastasis resection. These findings underscore the value of metabolomics in distinguishing cancer subtypes, predicting therapeutic outcomes, and improving post-surgical prognosis.

In conclusion, this work establishes untargeted metabolomics as a powerful tool for cancer biomarker discovery, laying the groundwork for integrating metabolomic data into future molecular models to guide personalized cancer treatment, making metabolomic profiling a valuable resource in medical oncology practice.

RESUMEN

El cáncer es una constante amenaza contra la salud global, con más de 19 millones de casos nuevos y casi 10 millones de muertes anuales. El cáncer de mama (CM) es el tipo más común entre las mujeres, mientras que el cáncer colorrectal (CCR) es el tercer tumor más prevalente a nivel mundial. A pesar de los avances en investigación, estas enfermedades presentan grandes desafíos debido a su heterogeneidad molecular y la diversidad en la respuesta terapéutica.

La medicina de precisión ha transformado la clínica del cáncer, permitiendo terapias más personalizadas; sin embargo, aún existe una necesidad insatisfecha de herramientas diagnósticas y pronósticas para manejar pacientes con enfermedad avanzada, con recurrencias y resistencia a los tratamientos. Una solución prometedora es la identificación de biomarcadores mediante metabolómica, la cual ofrece información sobre los mecanismos moleculares de la enfermedad, permitiendo la detección temprana de enfermedad residual y la predicción de la respuesta al tratamiento. Este enfoque mejorará la toma de decisiones clínicas en la práctica habitual.

El objetivo principal de esta tesis es el de analizar los perfiles metabolómicos del CM y el CCR, utilizando metabolómica no dirigida para descubrir metabolitos en plasma que puedan aplicarse como potenciales biomarcadores en la práctica clínica. Para ello, utilizamos métodos diferentes de cromatografía líquida acoplados a espectrometría de masas de alta resolución. Esta estrategia facilita la detección de una amplia gama de entidades moleculares, lo que permite profundizar en el entendimiento de la biología del cáncer.

Como resultado, presentamos tres estudios originales que demuestran la capacidad de la metabolómica no dirigida para identificar posibles biomarcadores de cáncer en los siguientes contextos clínicos: 1) diferenciación metabolómica de los subtipos del CM, 2) cambios metabolómicos en respuesta a la quimioterapia neoadyuvante en el CM, y 3) predicción, mediante metabolómica, de la recurrencia y supervivencia del CCR tras la resección de metástasis hepáticas.

En conclusión, se establece la metabolómica no dirigida como una herramienta valiosa para el descubrimiento de biomarcadores en cáncer, sentando las bases para la integración de los datos metabolómicos en futuros modelos moleculares que guíen el tratamiento personalizado, convirtiendo el perfilado metabolómico en un nuevo recurso para la oncología médica de precisión.

INTRODUCTION

1. INTRODUCTION

1.1 Cancer

Globally, cancer is a major public health and economic concern being the second deadliest and most pervasive malignancy in modern times, after cardiovascular diseases, affecting more than 30 million people in the world by 2050. However, in Spain, a paradigm shift occurred in 2023 as tumours became the leading cause of death for the first time, accounting for 26.6% of total deaths. According to the provisional data from the National Institute of Statistics (INE for its acronym in Spanish), this fact is not due to an increase in tumours, but to a significant decrease in cardiovascular diseases (INE 2024). Worldwide, 19.3 million new cases of cancer were diagnosed and almost 10 million cancer-related deaths occurred in 2020. By 2040 it is estimated that we will face a 47 % increase with 28.9 million new cancer cases and 16.2 million deaths a year (Siegel et al. 2023; H. Sung et al. 2021). Due to its insidious increase in the last decade, it may appear as a modern disease. However, this, is far from reality. The first case of human malignant neoplastic disease was found 1.7 million years ago in a South African bone (Odes et al. 2016). Indeed, cancer existed on Earth even before men appeared as evidenced by paleopathological findings in animal fossils (Ekhtiari et al. 2020). Nevertheless, very little is known about cancer in ancient times (Figure 1).

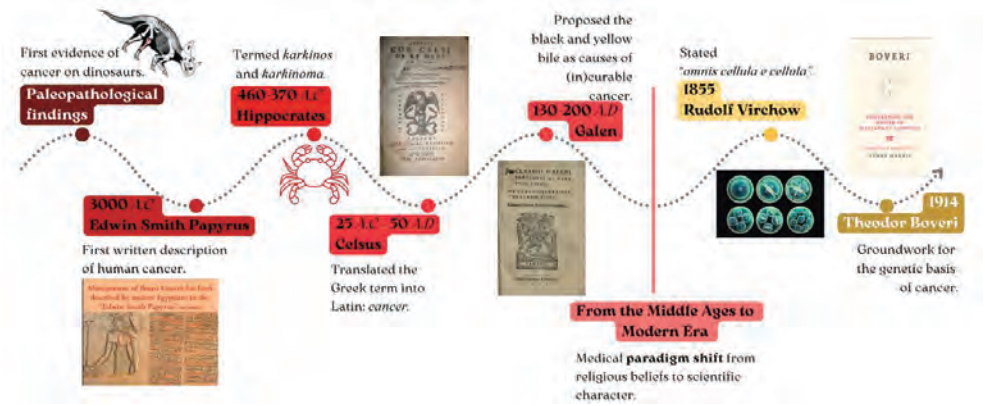


FIGURE 1. Timeline from Ancient to Modern cancer course and peculiarities on cancer etymology. The earliest mention known about cancer was found in the Edwin Smith Papyrus from ancient Egypt; Hippocrates was who first used the terms *karkinos* and *karkinoma* to refer to the similarity of the tumour to a crab; Celsus translated the Greek term into the Latin word for crab: *cancer;* Galen used the term *oncos* from the Greek term for swelling to refer to the disease, and implemented the humoral theory of Hippocrates (McAleer 2022; Hajdu 2011). In Occident, it

was at the end of the 19th, beginning of the 20th century, when Rudolf Virchow stated that cancer cells were the body's own cells (Virchow 1989) and Theodor Boveri laid the groundwork for modern cancer research and our understanding of the genetic basis of cancer (di Lonardo, Nasi, and Pulciani 2015; Boveri 1914). A.D: Anno Domini; A.C: Ante Christum. Figure created with *Canva.co,* adapted from the original books, cover pages, and publications of Ekhtiari et al. (2020).

Nowadays, cancer could be considered as a set of complex diseases which differ from the genotype aetiology to the vast complexity in their phenotype expression, being some of its distinctive marks the uncontrolled growth and the possibility of metastasis or spread of cancer cells to damage normal tissues and organs. Multiple factors such as genetic mutations, environmental agents or lifestyle factors, can interact to each other determining the risk, type and outcome of the disease. Therefore, for cancer prevention and treatment management it must be taken into account its multifactorial character (Stein and Colditz 2004). Regarding cancer onset, various theories of cancer origins have emerged (Figure 2) which emphasize the acquisition of different molecular alterations in a multistep process, and that have failed to show a unique initiator factor (Jassim et al. 2023): 1) the somatic mutation theory, 2) the tissue organization field theory, 3) the cancer stem cells (CSCs) theory, and 4) the ground state theory.

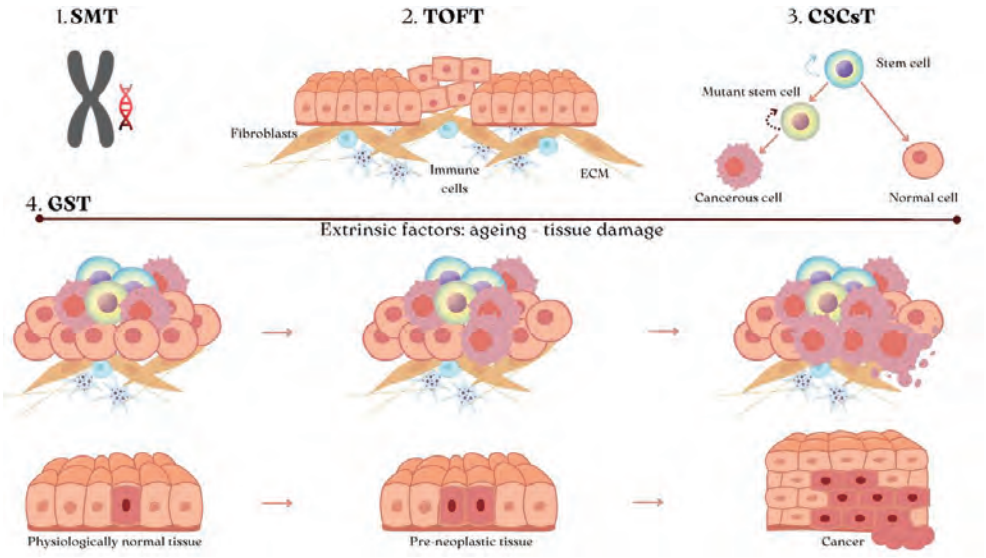


FIGURE 2. Theories of cancer origin. 1) Universally accepted *somatic mutation theory* (SMT), defines that tumours' initiation is a cell problem due to gene alterations and independent of external environmental factors. 2) The *tissue organization field theory* (TOFT) enunciates that the interactions of tissue components such as fibroblasts, extracellular matrix (ECM) or immune cells

drives carcinogenesis. 3) The *cancer stem cells theory* (CSCsT) proposes that cancer cells come from a mutated progenitor stem-like cell which has the unique ability to promote tumour growth and progression. 4) Finally, the *ground state theory* (GST) integrates all contributions from the previous theories to explain cancer risk. Herein, tissue exposure to ageing or other extrinsic factors can result in: normal tissue tolerating mutations (left bottom corner); abnormal growth like pre-cancerous lesions (middle); or full malignant transformation into cancer (right bottom corner). Figure created with *Canva.com* and *BioRender.com,* adapted from Jassim et al. (2023).

To simplify the understanding of cancer cells transformation, the *hallmarks of cancer* describe the common capabilities that healthy human cells acquire through the neoplastic process, while the *enabling capabilities* are those involved in activating these traits. At the beginning of the 21st century, one main activating feature and six cancer hallmarks were identified by Hanahan and Weinberg (Hanahan and Weinberg 2000). Since 2011, two more hallmarks have been sufficiently validated to be part of the set, as well as a second enable characteristic (Hanahan 2022; Hanahan and Weinberg 2011). Thus, during the last two decades, the set of cancer-essential characteristics have grown as research itself which denotes the complexity of the disease behaviour (Figure 3).

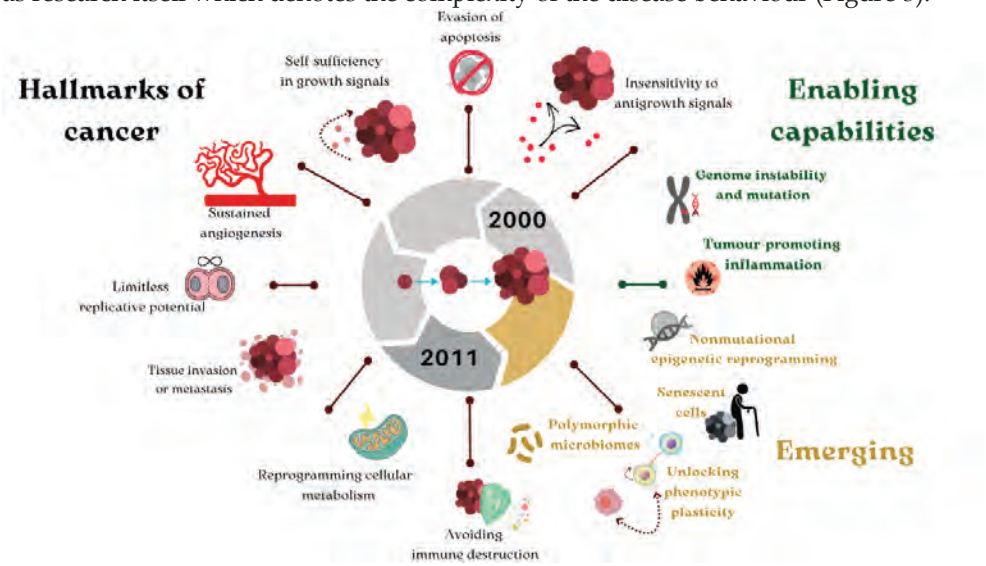


FIGURE 3. Hallmarks of cancer. Characterization of cancer acquired capabilities through the neoplastic process has evolved during the last decades. Initially, one activating feature and six hallmark were identified. Currently, two more hallmarks and a second enable characteristics have been well-evidenced to be part of the set. Beyond these characteristics, emerging hallmarks have been recently proposed for further investigation. Figure created with *Canva.com* and *BioRender.com* based on Hanahan and Weinberg (2000, 2011, 2022).

Importantly, investigations on the molecular mechanisms underlying tumorigenesis have evidenced genotypic and phenotypic variations between the same type of cancer from different patients what it is called inter-tumour heterogeneity, and within the own tumour or intra-tumour heterogeneity. Hence, intra-tumour heterogeneity may exhibit an uneven distribution of genetically diverse malignant cells occupying different sites of the tumour, as well as genetic and morphological transformations over time. These spatial and temporal changes can be explained by two principal models: 1) the clonal evolution, and 2) the CSC models (Figure 4a).

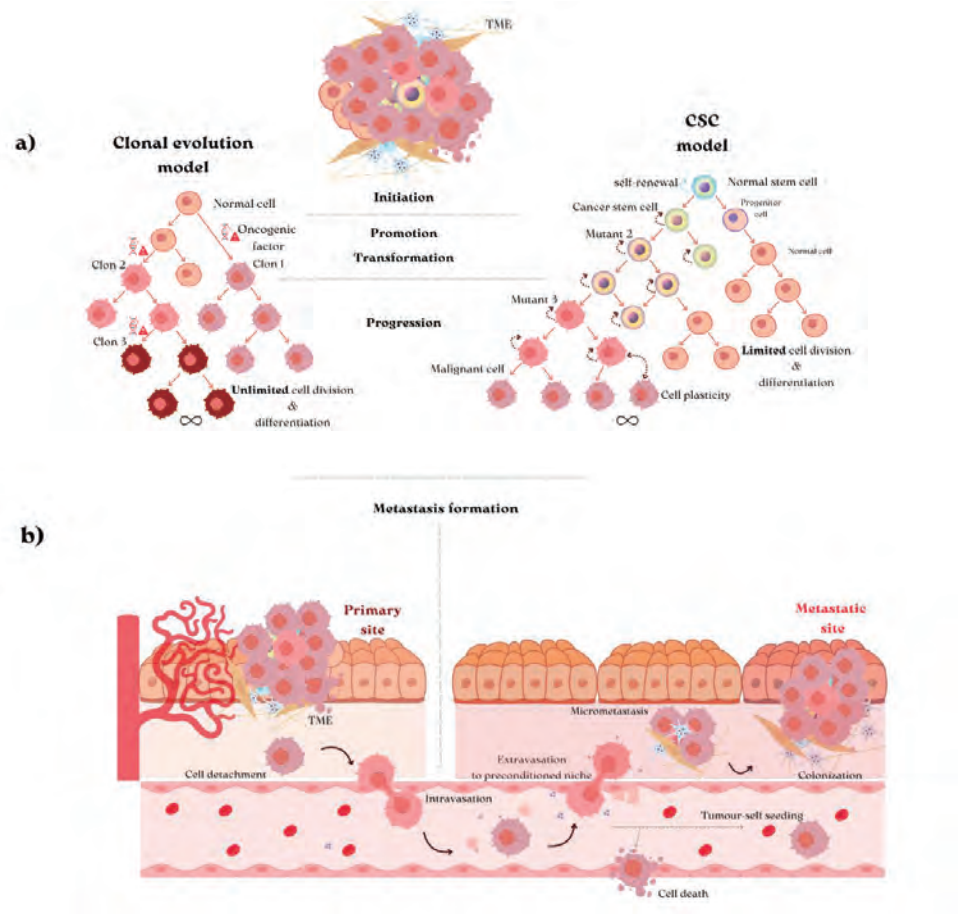


FIGURE 4. Biological and metabolic heterogeneity during carcinogenesis. a) Different theoretical models explain intra-tumour heterogeneity based on clonal evolution and cancer stem cells (CSCs). According to the clonal evolution model, any cell under the influence of an oncogenic factor may acquire stochastic mutations, becoming a cancer cell with unlimited division and differentiation abilities. On the contrary, the CSC model limits the division and differentiation abilities to a group of cells with self-renewal properties. Both models seem to converge into a

more flexible one, in which the tumour microenvironment (TME) facilitates unlocking phenotypic cells plasticity for epithelial-mesenchymal transition and *viceversa*. b) The metastatic process exemplifies how cancer cells adapt metabolically to enhance invasiveness. First, epithelial-mesenchymal transition stimulates angiogenesis and extracellular matrix remodelling for cellular detachment. Second, *anoikis*-resistant cells enter bloodstream and adjust theirmetabolism to survive migration. Third, platelets protect metastatic cells and aid preconditioning the niche. Fourth, malignant cells that evade immune surveillance, enter the pre-metastatic niche and form micrometastasis. Finally, mesenchymal-epithelial transition favors the metastatic outgrowth and colonization. Figure created with *Canva.com* and *BioRender.com*.

Consequence of this biological heterogeneity, cancer metabolism should not be studied as a homogenous process (Danhier et al. 2017). The diversity in cancer cells activities occurring in each area of the tumour, and along the process of carcinogenesis and dissemination, will require them to adapt their metabolism to their necessities in a dynamic spatio-temporal way. This flexibility results in both intertumoral and intratumoral metabolic heterogeneity. Herein, mutational dynamics and epigenetic plasticity are key hallmarks in remodelling the niche during carcinogenesis, which define the importance for further investigations exploring metabolic reconfiguration with profound implications for the progression to metastasis (Figure 4b) that seriously affects therapy response and patients' prognosis (Jinesh and Brohl 2022; Nascentes Melo et al. 2022; Zhu et al. 2021; Prasetyanti and Medema 2017).

In relation to cancer diagnosis, lately with the emergence of genomics, there has been a substantial change in the traditional tumour stratification, which it was based on its histology and the site of origin: bladder cancer, breast cancer (BC), colorectal cancer (CRC), kidney cancer, lung cancer, lymphoma, melanoma, oral and oropharyngeal cancers, ovarian cancer, pancreatic cancer, prostate cancer, stomach cancer, thyroid and uterine cancers. However, the use of next-generation sequencing (NGS) of the tissue, has provided with a more precise molecular classification at a single-person level for each type of cancer as well as with genetic targets for a more personalized anticancer drug development (Nakagawa and Fujita 2018; Tobin et al. 2015; Roukos and Ku 2012). Furthermore, a great and recent advance in high-throughput methodologies such as multiplexed imaging, single-cell metabolomics or spatial -omics allows to profile the molecular expression of the phenotypic cells' heterogeneity preserving tissue architecture. This novel approach represents an outstanding method for cancer

characterization in a more comprehensive and high-dimensional manner (Wan et al. 2024; S. Lee et al. 2024).

Thus, in the era of precision medicine, cancer therapy research has paved the way for more effective, accurate and minimally invasive treatments when comparing with the conventional approaches (Kaur, Bhardwaj, and Gupta 2023). Nevertheless, chemotherapy and radiotherapy still play pivotal roles in the current clinical practice along with surgery, being the cornerstone therapies in cancer management. In this regard, two main therapy settings may be defined according to the time point in which they are carried out: 1) the regimen of treatment used before the surgery or neoadjuvant (NA), and 2) the regimen of treatment used after the surgery or adjuvant (AD). Moving forward, the combination of conventional with novel targeted and immunotherapeutic approaches has led to synergistic effects that enhance treatment outcomes in various types of cancers (Qi et al. 2021; Dailah et al. 2024). Continued research and innovation will be essential for further refining novel therapies such as: stem cell therapy, gene therapy, nanoparticle-based therapy, natural products' therapy and exosome-based therapy, with the aim to overcome the traditional drawbacks of cancer therapy and to usher in a new period of personalized medicine (A. X. Wang et al. 2023; Pezzani et al. 2019; Yong et al. 2019; Y. Li, Atkinson, and Zhang 2017).

Despite the burgeoning comprehension of the fundamental biology of cancer, and the improvements achieved in the realization of a better precision oncology, there still exists relatively limited efficacy of the current treatments and frequently associated side effects (Lustberg et al. 2023; Anand et al. 2023). In this regard, the early detection of minimal residual disease (MRD) could define the risk to relapse and the choice for further therapeutic strategies after a curative procedure. Thus, improving programs for early detection of cancer is essential since it would increase the likelihood of cure, as the disease can be treated with less aggressive and invasive therapies, reducing side effects and improving the patient's quality of life. Additionally, early diagnosis can significantly reduce the personal, social and financial impact of this devastating disease, thereby enhancing the overall efficiency of the healthcare system through better resource allocation.

1.1.1 Breast cancer

According to the Spanish Network of Cancer Registries (REDECAN for its acronym in Spanish) it is estimated that BC is the second most incident type of tumour in the

general population while the most frequently detected in Spanish women (REDECAN 2024). In 2020, BC was the most diagnosed type of cancer around the world with 2.3 million of new cases, exceeding lung cancer rates. The rising incidence of BC in developing and developed countries is the result of the convergence of drastic changes in lifestyle such as unbalanced diet intake, alcohol and smoke consumption, poor physical activity, obesity, the late age for first childbirth and menopause (Amato, Guarneri, and Girardi 2023). Although mortality rates have decreased during the last years due to the improvements in early detection by screening programs and targeted therapies, BC ranks as the second most common cause of cancer-related deaths in women worldwide and the first in Spain (Siegel, Giaquinto, and Jemal 2024; H. Sung et al. 2021).

Nowadays, early detection BC programs consist on breast self-examination and clinical breast examination, which are often inconclusive and controversial for the detection of the disease Moreover, any of these techniques provide with tissue malignancy, nor discrimination between BC subtypes, whereas the bases to define the molecular and genetic information for the BC taxonomy tend to be time-consuming, invasive or unspecific (Alcaide Lucena et al. 2021). Thus, breast imaging by mammography screening is the only effective method for the detection of the disease decreasing mortality rates by 41%. However, it also has disadvantages as the cost-expensive resources, exposition to radiation, the breast compression and the final biopsy of the tissue (Ginsburg et al. 2020). So that, it urges to define a less invasive method for early detection and monitoring of BC behaviour.

It is noteworthy that improvements in molecular biology have provided with a more precise stratification of this heterogeneous type of cancer. Indeed, BC prognosis and the treatment of choice vary according to the combination of the histopathological classification with three main molecular factors: the expression of hormone receptors (progesterone and estrogens), the overexpression of the human epidermal growth factor receptor-2 (HER2) gene and the proliferation marker Kiel 67 (Ki-67). Figure 5 summarizes the BC morphological and molecular classification, while Table 1 shows the eighth edition of cancer's staging system for BC (Giuliano, Edge, and Hortobagyi 2018), standardized by the American Joint Committee on Cancer (AJCC) in 1959. Considering these criteria, in clinical practice, BC is stratified in Luminal (A or B), HER2+ or triple negative (TN):

1) Luminal: it is the most common BC molecular subtype, characterized by the expression of estrogens and / or progesterone receptors (ER/ PR). Within this group it can be found two subtypes, luminal A (LA) and luminal B (LB) which mainly differ from each other in the percentage expression of hormonal receptors, frequency and prognosis.
2) HER2+: it accounts for ~ 15-25% of BC cases and it is characterized by the overexpression of the receptor tyrosine-protein kinase erbB-2, due to *ERBB2* amplification or somatic mutations (Oh and Bang 2020; Yarden and Sliwkowski 2001). They have worse prognosis in comparison with luminal tumours and higher response rates to chemotherapy. HER2+ tumours are effectively treated with targeted therapies in combination with chemotherapy, such as: trastuzumab, pertuzumab or lapatinib. It is the molecular subtype most frequently affected by metastasis in the central nervous system.
3) TN: it is the most aggressive subtype of BC and it affects about 20% of the BC cases. It may express < 1% hormone receptors and the HER2 non-amplified. It normally affects a younger population of women. Chemotherapy is the only available treatment following the scheme of antrathiclines plus taxanes. TN management is an actual challenge for precision oncology. Although response rates to chemotherapy are high, early therapy resistance occurs. Recent studies have demonstrated that within the TN phenotype can be observed very heterogeneous lesions that might be subclassified for a much-personalized management (Lehmann et al. 2011).

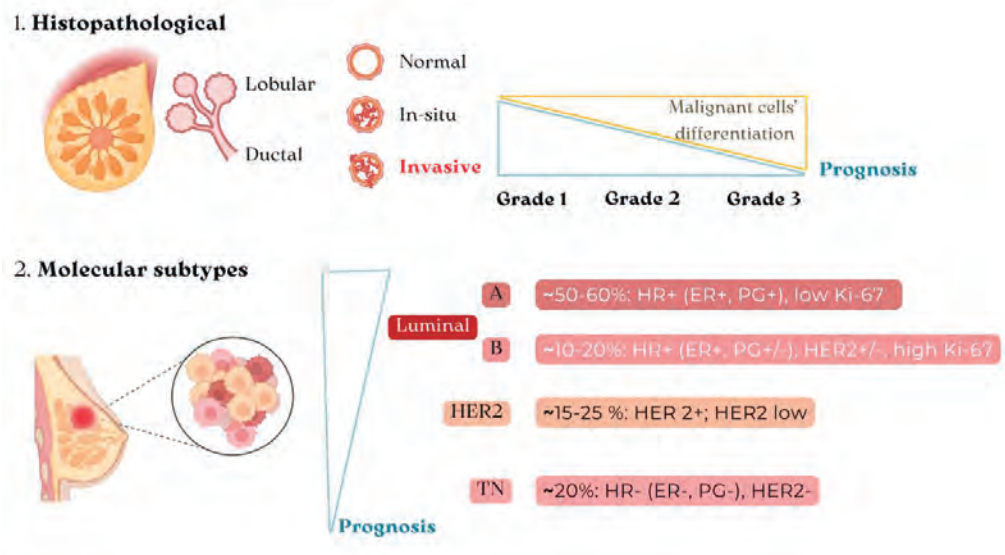


FIGURE 5. Summary of breast cancer (BC) classification according to their histopathological affection (1), and their molecular characteristics (2). 1) Morphological categorization of BC is mainly based on the normal breast structures, ducts and lobules, that may be partially or completely filled with tumour cells. Depending on the aggressiveness, we find *in situ* BC tumours or *invasive*. The histologic grade is an independent prognostic factor of the invasive carcinomas and it is determined by malignant cells' differentiation. 2) BC molecular subtypes are stratified based on the expression of: hormone receptors - estrogen (ER) or progesterone (PR) -, the proliferative marker Ki-67, and the human epidermal growth factor receptor-2 amplification (HER2+). TN phenotype is considered the most aggressive molecular subtype with less tailored therapeutical options and lower survival rates. Figure created with *Canva.com* and *BioRender.com* based on BC classification published elsewhere.

TABLE 1. Key points of the AJCC eighth BC staging edition and examples of stage migration.

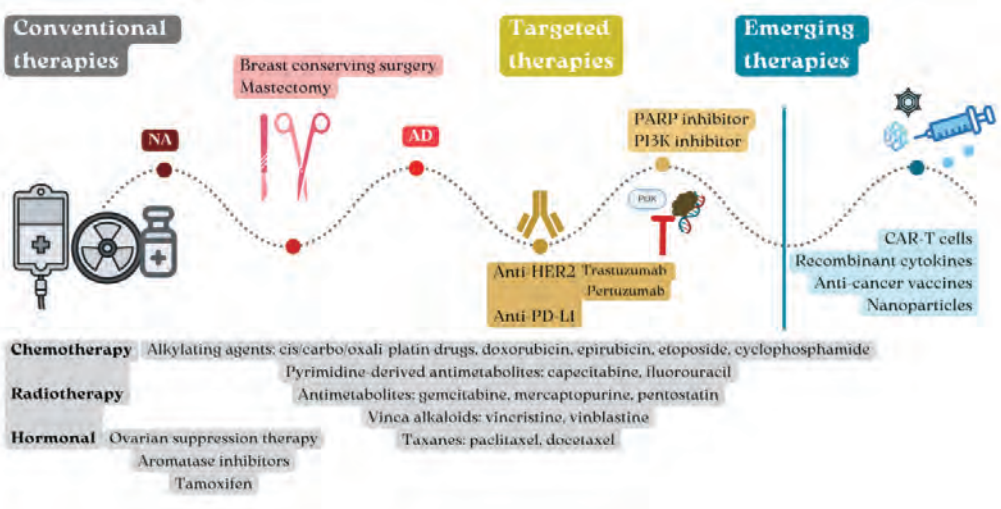
TNM	ANATOMIC STAGE (a)	HISTOLOGIC GRADE	BIOMARKERS	ONCOTYPE SCORE	PROGNOSTIC STAGE (b)
Tis N0 M0	**0**				
T1 N0 M0	**IA**	1-3	ER-, PR-, HER2–	N/A	**IB**
T0 N1mi M0 T1 N1mi M0	**IB**	1-3	ER-, PR-, HER2–	N/A	**IIA**
T0 N1 M0 T1 N1 M0	**IIA**	1-3	ER-, PR-, HER2–	N/A	**IIB**
T2 N0 M0		1-3	ER+, PR+/-, HER2-	< 11	**IA**
T2 N1 M0	**IIB**	1-3	ER+, PR+, HER2+	N/A	**IB**
T3 N0 M0		2-3	ER-, PR-, HER2–		**IIIB**
T0 N2 M0 T1 N2 M0 T2 N2 M0	**IIIA**	1-2	ER+, PR+, HER2+	N/A	**IIA**
T3 N1 M0 T3 N2 M0		1-3	ER-, PR-, HER2-	N/A	**IIIB**
T4 N0 M0 T4 N1 M0	**IIIB**	1-2	ER+, PR+, HER2+	NA	**IIB**
T4 N2 M0		1-3	ER-, PR-, HER2-	NA	**IIIC**
Any T N3 M0	**IIIC**	1-2	ER+, PR+, HER2+/-	NA	**IIIB**
		1-3	ER-, PR-, HER2-	NA	**IV**
Any T or N M1	**IV**				

Since the *AJCC Cancer Staging Manual* creation, cancer stages have been determined by cells' progression or spread in the body and relied on anatomic methods, using population-based

survival data to predict clinical outcomes. Up-to-date manual edition has been modified according to the precision medicine era, including two staging systems: (a) the traditional anatomic stage -primarily based on the size of the tumour (T), nodal status (N), and distant metastasis (M)- to be used when biomarker analysis is not available; and the prognostic stage (b), which considers tumour grade, hormone receptor -estrogen (ER) or progesterone (PR)-, the human epidermal growth factor receptor-2 (HER2) overexpression and multigene panel testing. As a result of the 8th edition, anatomic stages are "upstaged" or "downstaged" depending on the tumor biology: triple negative tumours tend to have upstaging prognosis due to the survival rates comparable with patients with disease one anatomic stage higher, whereas HER2 overexpression is generally a downstaging factor given the success of anti—HER2 targeted therapy. Tis: Tumour *in situ*; Nmi: micrometastasis; N/A: not applicable.

In BC, understanding each molecular profile of the disease is crucial for tailoring to a particular, more accurate and personalized treatment. In this regard, the treatment regimen in BC is driven by combination of (Figure 6):

- Surgery: it is the initial treatment in case of an AD chemotherapy setting. It may involve breast-conserving surgery (lumpectomy) or mastectomy; lymph node removal can also be performed to assess the sentinel lymph node or axillary lymph nodes.
- Radiotherapy: it may be used before surgery in a NA regimen or recommended following surgery (AD) to destroy any remaining cancer cells in the breast or nearby the lymph nodes to reduce the risk of recurrence.
- Chemotherapy: it is classified according to chemical drugs nature, molecular target, mechanism/mode of action, or effectiveness. NA chemotherapy (NACT) it is used in order to make operable those tumours that might be inoperable, increasing the breast-conserving surgery rate, and allowing determine the short-term efficacy. AD chemotherapy is used to prolong survival in those potential cases of latent micro-metastases.
- Hormonal therapy: it is used in case of hormone-receptor positive BC to block the effects of estrogen or progesterone on cancer cells.
- Targeted therapy: it is used in the HER2+tumours to specifically target cancer cells which overexpress the HER2 protein.
- Immunotherapy: it is typically used in combination with other chemotherapy drugs for advanced or metastatic BC, stimulating the body's immune system to recognize and attack cancer cells (Debien et al. 2023).



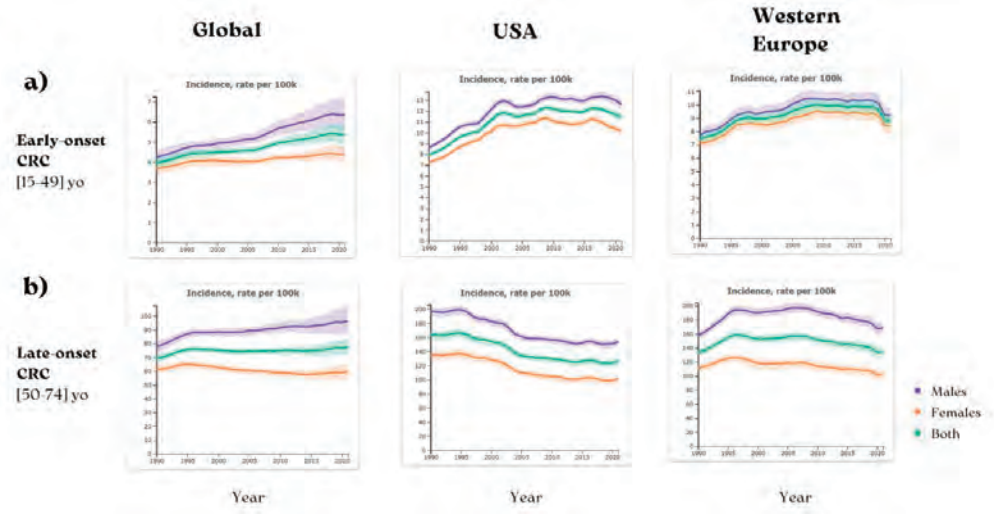
F I G U R E 6. Conventional, targeted and emerging therapies in the breast cancer setting. Chemotherapy, radiotherapy and hormonal therapy can be administered before the surgery in the neoadjuvant (NA) regimen or after the surgery in the adjuvant regimen (AD). Targeted therapies are approved as AD therapies or in combination with NA chemotherapy. Figure created with *Canva.com*.

While multiple advances achieved in diagnosis, surgical and radio-chemotherapeutic strategies have improved BC survival rates, this malignancy remains a significant clinical challenge when relapse occurs (Amato, Guarneri, and Girardi 2023). Metastasis cause the dissemination from the primary site to a secondary organ, implying main vital organ disfunctions and subsequent cancer-related deaths (Dillekås, Rogers, and Straume 2019). In BC, 5-20% of patients at early or late stage will have metastatic disease and 20-30% of early BC patients still die from metastasis (Courtney et al. 2022; Riggio, Varley, and Welm 2021). Therefore, there is an urgent need to define more personalized-based strategies to discriminate response to treatment according to each BC molecular subtype which may ultimately lead to a more accurate diagnosis, prognosis and therapy selection for a tailored follow-up.

1.1.2 Colorectal cancer

Colorectal cancer was the most frequently diagnosed type of cancer in Spain in 2022, followed by breast and lung cancers (SEOM 2022). In 2020, CRC was the third most commonly diagnosed cancer, with more than 1.9 million of new cases per year and the second leading cause of cancer-related deaths, with almost 1 million deaths worldwide

(H. Sung et al. 2021). About 55% of CRC cases are diagnosed in global developed areas and the mortality rate is considerably higher in men than in women. Although age remains the major unchangeable risk factor of colon cancer onset, by 2030 it is expected a 90% increase of colon cancer and 124.2% for rectal cancers in Americans aged 20-34 years of age (Spaander et al. 2023). As shown in Figure 7, the increase in this young onset CRC represents a drastic change to be taken into account in the behaviour of the disease (Siegel, Giaquinto, and Jemal 2024; Saad et al. 2020). Interestingly, it is estimated that 80% of CRC cases in Western countries are caused by dietary factors (Sawicki, Ruszkowska, and Danielewicz 2021; Vernia et al. 2021), whereas it is well-established that combination of a healthy diet along with avoiding smoking, overweight, being



sedentary and alcohol consumption, might decrease CRC risk (Botteri et al. 2023).

FIGURE 7. Temporal patterns of (a) early-onset colorectal cancer (CRC) burden, and (b) late-onset CRC worldwide (left), in the United States of America (USA, middle) and Western Europe (right), 1990-2020. Males experienced higher increases in CRC incidence than females all over the world. Furthermore, a disturbing increase in early-onset CRC from 15 to 49 years old (yo) differs from the stabilized-decreased tendency of late-onset CRC incidence in the last three decades. Data source: Global Burden of Diseases, Injuries, and Risk Factors Study 2019.

The initial phase of the standard preventive protocol for CRC involves immunochemistry to detect fecal occult blood. This first stage is characterized by a significant percentage of false positive results due to its limited sensitivity and leads to unwarranted patients' selection for subsequent screening by colonoscopy (Figure 8). In response to this issue, recent investigations propose the inclusion of additional biomarkers in the first stage of screening, such as molecular risk signatures coupled with

pertinent clinical-demographic parameters of the patients (Arnau-Collell et al. 2022). Nevertheless, to date, there is a dearth of validated tools enabling the tracking of markers associated with tumour progression or the presence of MRD in CRC. In this sense, serum levels evaluation and monitoring of the carcinoembryonic antigen is highly recommended before surgery and during post-operative follow-up for an early detection of metastasis (Konishi et al. 2018; Duffy et al. 2003).

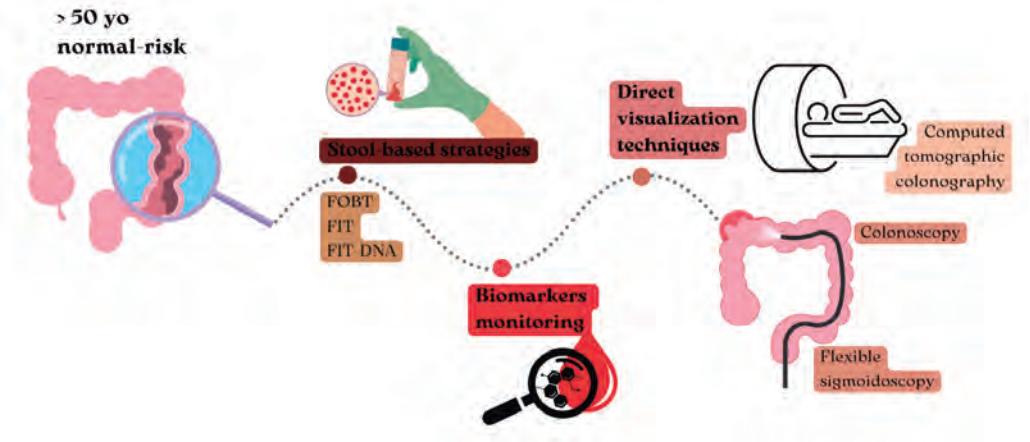


FIGURE 8. Colorectal cancer (CRC) typical screening. Normal-risk people should start CRC screening at age 50 by fecal immunochemical test (FIT) or fecal occult blood test (FOBT) every year. The FIT-DNA (Cologuard test) needs to be taken once every 3 years. When stool-based results are negative, normal-risk people may have a sigmoidoscopy or computed tomographic colonography every 5 years or colonoscopy every 10 years. Further monitoring of blood biomarkers remains controversial in clinical practice due to the low sensitivity and specificity. Nevertheless, carcinoembryonic antigen is the most frequently examined marker to predict early recurrence in post-operative patients. DNA: deoxyribonucleic acid. Figure created with *Canva.com*.

The liver stands as the primary site of metastatic dissemination in this malignancy (Biller and Schrag 2021). Approximately two-thirds of CRC cases manifest metastatic spread to various organs, while roughly 30% of CRC patients exhibit exclusive liver metastasis (CRLM) (Xi and Xu 2021). Despite achieving a five-year survival rate as high as 90% through early detection by the standard protocol, advanced CRC stages correlate with a survival rate less than 10% and a pronounced escalation in associated economic burdens (Kamel et al. 2022; Gornick et al. 2022). In the CRLM scenario, only one-third of the patients are eligible for surgery or ablative treatments but, still, disease recurrence occurs in more than a half of the cases (H. Sung et al. 2021; Kawaguchi et al. 2019; Creasy et al. 2018). For that reason, it would be essential the identification of prognostic and

predictive biomarkers by a simple blood extraction during the monitoring of the disease for risk stratification of these patients.

The management of CRC has been pioneer moving forward to precision oncology due to the application of molecular profiling, biomarkers' identification and the use of targeted therapies or immunotherapy in clinical practice (Ahluwalia et al. 2024). In this regard, main clinicopathological criteria for CRC assessment is based on staging by TNM (O'Sullivan et al. 2017) and the status of the mismatch repair, microsatellite instability, as well as the gene mutations in *RAS* or *BRAF* in the metastatic colorectal cancer (mCRC). Further validation is pending for other realizations of personalized medicine such as testing mutations in *PIK3CA*, *ERBB2*, *ALK*, *ROS1* or *NTRK,* which are yet only recommended in clinical trials (Cervantes et al. 2023). Despite these mainstay factors, the molecular biology of CRC is especially complex and heterogeneous which results in remarkable differences in disease progression and treatment response (Punt, Koopman, and Vermeulen 2017). So, a taxonomy of CRC was developed in 2015 basically based on tumour heterogeneity at the gene-expression level: the consensus molecular subtypes (CMSs) (Guinney et al. 2015), which could be used to guide drug development, to estimate patient survival and for tailoring therapy options (Hoorn et al. 2022). With the main aim to define a standard method for CRC subtyping and translate it into the clinic, this classification system coalesced two dominant genomic alterations (microsatellite and chromosomal instability) into four CMSs with their own (epi)genomic distinguishing features (Table 2):

1) Microsatellite instability immune or CMS1:it is characterized by the infiltration of tumour microenvironment and immune cells, mainly differentiated by the overexpression of DNA damage repair proteins/defective DNA mismatch repair, subsequent pattern of hypermutation and CpG island hypermethylation, it shows distinctive microsatellite instability, enriched of *BRAF* mutations and low somatic copy number alterations.
2) Canonical or CMS2: it has epithelial characteristics with high chromosomal instability, strong upregulation of *WNT* and *MYC* signalling; it shows the most frequently gained copy number in oncogenes and losses in tumour suppressor genes.

3) Metabolic or CMS3: it has the distinction of the metabolic reprogramming, although it also has an epithelial character, enriched for *KRAS*– activating mutations.
4) Mesenchymal or CMS4: it shows a clear upregulation of pathways related to the epithelial-to-mesenchymal transition and the activation of transforming growth factor – β signalling, extracellular matrix remodelling and the complement-mediated inflammatory system.

TABLE 2. Colorectal cancer (CRC) consensus molecular subtypes (CMSs)

	SUBTYPE	FREQUENCY AND LOCATION	MAIN HALLMARKS	PROGNOSIS*	TAILORED THERAPY
CMS1	Immune	14% Proximal colon	Immune infiltration: CD8+ cytotoxic T lymphocytes, CD4+ T helper 1 and natural killer Distinctive MSI, CIN and CIMP *BRAF* enriched SCNAs low	Worst survival in mCRC	Immunotherapy in mCRC Second-line regimen of chemotherapy combined with bevacizumab
CMS2	Canonical	37% Distal colon and rectum	Epithelial characteristics CIN high Upregulation WNT and MYC	Most favorable prognosis in both local and mCRC	AD chemotherapy Cetuximab benefits *KRASwt* mCRC
CMS3	Metabolic	13% Without predominance	Metabolic reprogramming *KRAS* activation	-	AD chemotherapy
CMS4	Mesenchymal	23% Distal colon and rectum	Epithelial-mesenchymal transition *TGFβ* activation	Worst prognostic value in local disease than CMS1 and CMS2	Irinotecan-based first-line regimen in mCRC also in combination with cetuximab (*KRASwt* tumours) and bevacizumab (*KRAS*)

After colonoscopy and biopsy, colorectal cancer (CRC) diagnosis undergoes subsequent stratification by tumor-node-metastasis (TNM) staging system and further molecular characterization by genome sequencing to detect proto-oncogene mutations, microsatellite instability (MSI), chromosomal instability (CIN), CpG island methylator phenotype (CIMP) and somatic copy number alterations (SCNAs). *The consensus molecular subtypes (CMSs) can be used to evaluate prognosis as demonstrated by Hoorn et al. (2022).

Despite major discoveries of (epi)genetic influence on CRC behaviour, these findings have limitations in clinical practice, as they vary with patients' race and ethnicity (Ping et al. 2022). In fact, the most effective risk model, QCancer-10, relies on non-genetic factors (Hippisley-Cox and Coupland 2015). Polygenic risk scores may fall short due to factors beyond DNA or epigenetic changes contributing to CRC pathogenesis. In this regard, recent studies emphasize the importance of combining intrinsic and extrinsic factors for better risk prediction (Briggs et al. 2022; Kachuri et al. 2020). Hence, integration of multiple -omics data provides deeper insights into CRC molecular heterogeneity, potentially overcoming the limitations of single-omics approaches and offering clinical utility for developing new diagnostic and prognostic tools (Díez-Villanueva et al. 2022; Sardo et al. 2022).

1.2 Precision Medicine

In medical oncology practice, the evolution of medicine is a constant process, driven by the increasing understanding of the complex health-disease status of a patient. Concerning this, the timeline towards the conquest of personalized medicine illustrates how the medical perception of health and disease has evolved, albeit practicians have always sought to personalize therapy to their patients. Specifically, Western medicine has recently transitioned from a "one-size-fits-all approach" based on population averages to a more individualized scenario. This medical paradigm can be traced back to thousands of years (Figure 9).

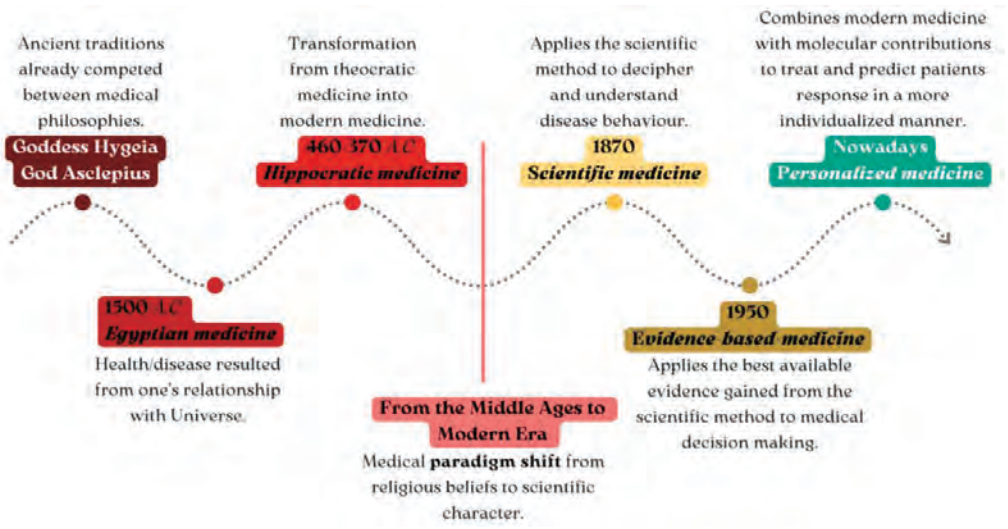


FIGURE 9. Timeline of Western medicine evolution rooted in ancient traditions such as those of the Greek gods *Asclepius* and the goddess *Hygeia* (Tannock 2006; Pizzorno 2018), evolving from *Egyptian medicine* to *Hippocratic medicine* (Jouanna 2012), *scientific medicine*, *evidence-based medicine* and reaching its latest iteration of *personalized medicine* (Madden and Bhandari 2020). Figure created with *Canva.com*.

Then, at the present we are witnessing a transition to personalized or precision medicine, defined by the individualization of the treatment for each patient, prioritizing their unique epidemiological, clinical and molecular characteristics. This philosophy is grounded on principles of personalization, prediction, prevention and patient participation, with the aim to enhance early disease detection, facilitate treatment selection with optimal dosing, mitigate adverse effects, improve therapeutic adherence for a better quality of life, and to reduce overall healthcare costs (Visvikis-Siest et al. 2020). Over the past two decades the paradigm shift towards a patient-centric approach in medical practice has been driven by advancements in targeted therapies which results from technological progress in biomedical research. As previously mentioned, genomics has played a pivotal role in the evolution of precision medicine, particularly in the field of medical oncology, with significant applications in cancer therapy. Consequently, significant milestones have been achieved (di Lonardo, Nasi, and Pulciani 2015), culminating in the emergence of *precision cancer medicine* or *precision oncology* (Figure 10).

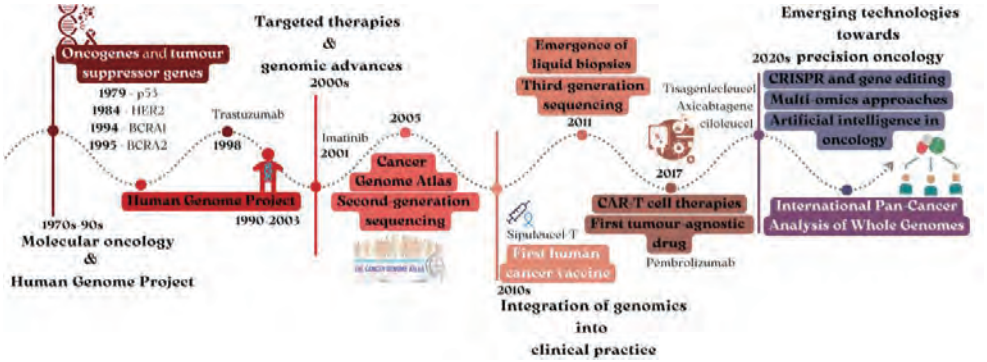


FIGURE 10. Timeline with remarkable strides made over the past three decades to achieve precision oncology. These key milestones have collectively transformed oncology from a one-size-fits-all approach to a more personalized, data-driven field, improving outcomes for countless cancer patients. Figure created with *Canva.com*.

Contemporary medicine has undergone a conceptual change to conceive pathology as individual-specific molecular alterations that interact with biological systems and the environment. Understanding each person's molecular idiosyncrasy, based on their (epi)genetics, proteomics, transcriptomics, metabolomics and microbiome enables a comprehensive assessment of their biological status. In this sense, the rise of -omics sciences alongside high-throughput analytical platforms, has clarified cancer's molecular aberrations, improving prognostic and predictive insights into tumour subgroups, identifying biomarkers, and uncovering therapeutic targets (Putignani, Gasbarrini, and Dallapiccola 2019; Carneiro et al. 2020).

Precision medicine contributes to a more holistic view of cancer biology by emphasizing the intricate interactions of small molecules in modulating cellular organelles, organs, and organismal functions. This underscores the necessity of integrative biomedical approaches to fully comprehend complex multi-factorial conditions such as cancer. In this context, it is fundamental to elucidate the individual components of biological systems and the specific functions of biomolecules through what is commonly known as basic research. This first stage of research is essential, as it explores the underlying mechanisms of health and disease, to form the basis for hypothesis generation and validation. Lately, biomedical advances accelerate the translation of basic discoveries to improve clinical outcomes across a range of pathologies. Thus, basic research serves as the cornerstone of personalized medicine and the process to implement results from the "bench-to-bedside" is facilitated by translational research (TR).

1.2.1 Translational research

TR is a relative new concept in the medical framework. Indeed, when we have a look into the bibliography, it can be observed the exponential growth of peer-reviewed articles related to TR from the first decade of the 21st century (Figure 11).

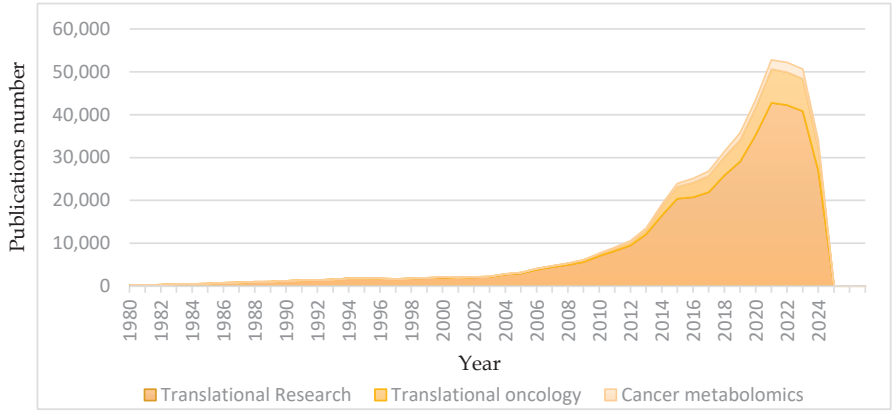


FIGURE 11. Changing trends in translational research, translational oncology and cancer metabolomics literature on PubMed database from the last three decades.

In the healthcare system, TR commonly refers to human studies aimed at translating laboratory findings into clinical applications, improving public health outcomes with new targets, drugs, devices or biomarkers. To achieve this, the Institute of Medicine's Clinical Research Roundtable defined two main "translational blocks" at the beginning of the century (described by N. S. Sung et al. 2003): T1 for translating new disease mechanism insights into diagnosis, therapy, and prevention by their initial human testing; and T2 for applying clinical research into routine practice and healthcare decisions. To address these challenges, the National Institutes of Health launched the Clinical and Translational Science Award program, facilitating research translation into clinical practice. However, over the years, the scope, goals, and methodologies of TR models have evolved, leading to inconsistent terminologies across the literature (Rajan et al. 2012). Thus, the need to redefine these translational blocks became evident (Woolf 2008), and gradual development of TR has ended up in the following key elements: phases, data transfer and gaps (Figure 12).

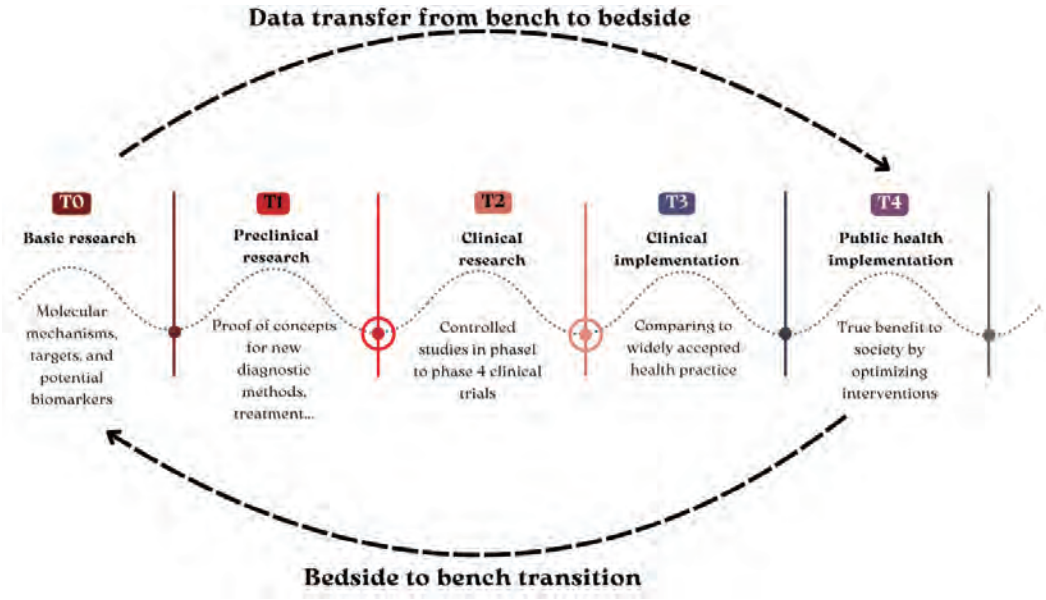


FIGURE 12. Key elements of translational research are broadly established as phases, scientific content transition between phases, and challenges found during the translational research phases due to issues related to ethical data confidentiality, resources or financial conflicts to data transfer. Figure created with *Canva.com*.

In this regard, clinical research provides evidence-based medicines, and TR produces evidence-based biomarkers which are increasingly considered in the design of a clinical trial for improving patient management and essential for achieving real personalized medicine. However, a main issue is faced in this "knowledge transition": the wide period from discovery, development and implementation. In order to manage this, we still lack a common and well-established massive TR infrastructure to lead the integration and good communication between the multi-disciplinary stakeholders that may interplay such as: the clinical specialists, biobanks, basic researchers, bioinformatics, statistics, data analysis, publication or reporting, project and resource coordinators, healthcare administrations.

Specifically in the medical oncology scenario, the emergence of high-throughput methods that enable the molecular characterization of tumours yielded to the implementation of TR for achieving the long-awaited precision cancer medicine. In this sense, beyond the outstanding (epi)genetic cancer-driven factors, different onco-omics have demonstrated that the integration of the biomolecular interplay is crucial for the understanding of the disease. The study of the disease from a more comprehensive perspective would facilitate the development of new high-resolution, effective and low-

cost diagnostic and prognostic tools that are potentially useful for implementation in the clinical setting (Lu and Zhan 2018). In this sense, the goal to bring the gap between basic sciences and clinical care through a practical application of TR might be reached by the development of molecular risk scores rather than (epi)genetic scores alone.

1.2.2 Omics and biomarkers' discovery

TR has significantly advanced precision oncology by enabling the identification of genes, proteins and metabolites that serve as biomarkers for disease initiation, prognosis, and monitoring cancer recurrence or prevention. According to the US Food and Drug Administration (FDA), biomarkers are measurable indicators used for patients' stratification (Atkinson et al. 2001; https://www.fda.gov/drugs/biomarker-qualification-program/about-biomarkers-and-qualification). While traditionally early cancer biomarker identification relied on empirical observations and histopathological methods, personalized medicine has surpassed the limitations of these strategies (Henry and Hayes 2012; Sarhadi and Armengol 2022). To date, molecular biomarker discovery leverages high-throughput technologies including:

- Genomics. NGS detects germline and somatic variants, as well as mutations involving single nucleotide variants or small nucleotides insertions and deletions. Table 3 lists common genetic variants associated to BC and CRC.
- Epigenomics. Techniques such as methylation-specific polymerase chain reaction (PCR), methylation-sensitive high-resolution melting or pyrosequencing, DNA methylation analysis or histone protein modifications.
- Transcriptomics. Reverse transcription PCR, microarray or sequencing are used to study both coding and non-coding ribonucleic acid (RNA). Known transcripts variants are detailed in Table 3.
- Proteomics. It is the field responsible for the study of cancer-related protein alterations which may not always correlate with the gene expression. Proteomic biomarkers were among the initial utilized in cancer diagnostics. Table 3 highlights key protein biomarkers in BC and CRC.
- Metabolomics. It provides with potential biomarkers at the metabolome level, identifying candidate metabolites linked to the presence of tumour or treatment response. Table 3 summarizes notable candidate metabolites identified in the context of BC and CRC.

TABLE 3. Molecular alterations in breast cancer (BC) and colorectal cancer (CRC) evidenced by multi-omics high-throughput technologies.

	BC	**CRC**
GENOMICS	*TP53, PIK3CA, CDH1, MUC16, GATA3, KMT2C, MAP3K1, PTEN, NCOR1, FAT3, CSMD3, MAP2K4, TEKT4, BRCA1/2*	*APC, TP53, KRAS, MUC16, FAT4, PIK3CA, CSMD3, FAT3, LRP1B, FBXW7, KMT2D, ARID1A, NBEA, BRAF*
TRANSCRIPTOMICS	miR-124, 125b, 127, 132, 1307-3p, 940, 340-3p, hsa-miR-503, 1307, 212, and hsa-miR-592	miR-21, miR-31, miR-143, miR-145, LGR5mR
PROTEOMICS	HER2, ER/PR, CA27.29, CA15-3, CEA, Ki-67	CEA, KRAS, BRAF, MMR, fibrin/fibrinogen degradation product (DR-70), human hemoglobin
METABOLOMICS	Tryptophan, histidine, taurine, docosahexaenoate, propionylcarnitine	3-hydroxybutyric acid, L-valine, L-threonine, 1-deoxyglucose, glycine, hypoxanthine

Some of these variants have not yet been extensively applied as biomarkers in the clinic due to the lack of reproducibility. Hence, it is essential to seek more specific biomarkers to improve the accuracy of their diagnosis. The most frequently mutated genes per type of cancer are publicly available on "the Cancer Genome Atlas Program": https://www.cancer.gov/ccg/research/genome-sequencing/tcga. miRNA: micro ribonucleic acid; mRNA: messenger ribonucleic acid; HER2: human epidermal growth factor receptor 2; ER: estrogen receptor; PR: progesterone receptor; CA: cancer antigen; CEA: carcinoembryonic antigen breast cancer; CRC: colorectal cancer; Ki-67: marker of proliferation Kiel 67.

Biomarkers are classified based on their applications, characteristics, genetics or molecular biology. These categories often overlap, reflecting the diverse uses of biomarkers in different contexts. In the clinical setting, we can broadly classify them into three primary categories:

- Diagnostic biomarkers are used for screening, early detection or confirmation of the presence of a disease or condition.
- Prognostic biomarkers provide information about likely course and outcome of a disease, risk assessment or patient prognosis.
- Predictive biomarkers allow prediction of the likely response to a particular treatment, monitoring the therapy plan.

However, the translation of biomarkers from the bench to bedside is a current drawback in precision oncology, since biomarker qualification requires the adherence to

guidelines laid by the National Cancer Institute (Srivastava and Wagner 2020; Purkayastha et al. 2022). Hence, the main phases for systemic discovery and evaluation of biomarkers are:

I. Exploratory or pre-clinical.
II. Establishment of a clinical validation assay.
III. Assessment of retrospective longitudinal specimens of subjects before the onset.
IV. Evaluation of sensitivity and specificity in a prospective screening.
V. Evaluation of overall benefits and risks in a cancer control study.

Finally, the ideal biomarker should be quantifiable, robust, have a significant over/down expression in the condition under study, correlate with the outcome progression, be consistent across gender and ethnic groups and be cost-effective (Purkayastha et al. 2022). In addition, the identification, validation and clinical translation must involve a multidisciplinary strategy to obtain a reliable integrative model capable of stratifying patients at risk to develop the disease or guiding clinical decision-making to improve patient outcomes in a comprehensive manner.

1.2.3 Liquid biopsy and minimal residual disease

In clinical practice, one of the major accomplishments of TR advances in precision oncology is the increase in survival rates and improvements in quality of life. In this sense, early detection of cancer is the most important factor for an optimal treatment of the disease, as it substantially influences the patient's likelihood of survival. Likewise, prior knowledge of treatment response is another key factor to consider when planning and administering the anti-tumour therapeutic regimen. In some cases, when the disease is asymptomatic, there is a delay in diagnosis that results in treatment initiation when criteria for radical therapy is no longer met.

Other major milestone in this revolutionary era is the advent of liquid biopsy for assessing cancer status, which has overcome some limitations of the traditional methods such as tissue biopsies and imaging techniques (Figure 13). Within the advantages of liquid biopsy (Pantel and Alix-Panabières 2019) it is worth noting the detection of MRD or micrometastases, which refers to the presence of small number of cancer cells remaining after the treatment which do not cause clinical symptoms. Thus, the set of tumour-derived biomarkers that are currently assessed in the context of MRD are

basically circulating tumour cells, cell-free DNA, circulating tumour DNA (ctDNA), exosomes and ctRNA. For the analysis of these cancer cells components, there exist various liquid biopsy techniques such as NGS, digital PCR, and multiplexed assays (Bayle et al. 2023; Pascual et al. 2022). Advances in these technologies and biomarkers' discovery hold promise to enhance the clinical utility of liquid biopsy in cancer management. It should be noted that, future research will require to integrate multiple biomarkers and complementary technologies to enhance the sensitivity and specificity of MRD detection. So that, integration of other -omics such as metabolomics and epigenomics with the collaborative efforts among researchers, clinicians and industry partners are essential to accelerate the translation of liquid biopsy into routine clinical practice for an earlier and more accurate assessment of residual disease and personalized cancer management (Johnston et al. 2023; Ge et al. 2022).

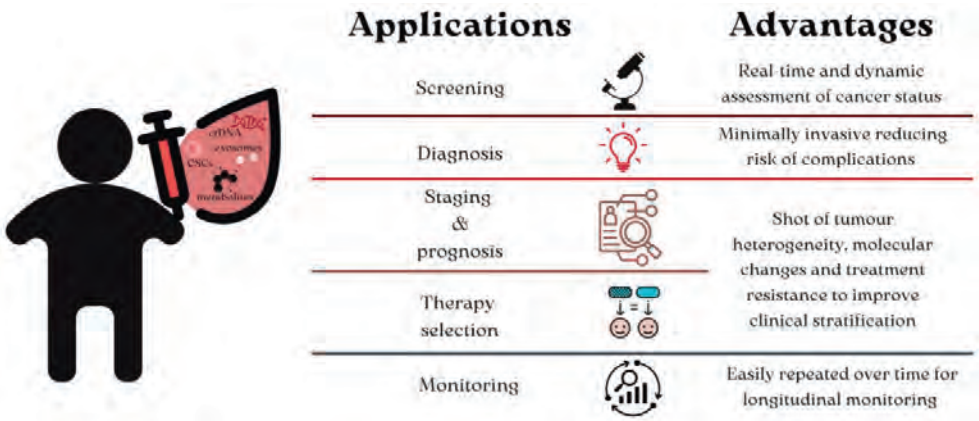


FIGURE 13. Advantages and applications of liquid biopsy in medical oncology practice: 1) liquid biopsy offers a real-time and dynamic assessment for screening of cancer status; 2) this technique is minimally invasive, reducing patient discomfort and the risk of complications associated with tissue biopsies at diagnosis; 3) liquid biopsy enables the sampling of tumour heterogeneity, capturing genetic and molecular changes in cancer cells that may not be represented in a single tissue biopsy or by imaging, improving clinical stratification of prognosis or therapy selection; 4) it can be easily repeated over time, facilitating longitudinal monitoring of disease progression and treatment response. ctDNA: circulating tumour deoxyribonucleic acid; CSCs: cancer stem cells. Figure created with *Canva.com,* adapted from Kulasinghe et al. (2018).

Although MRD detection is widely established in haematological malignancies, liquid biopsy faces several limitations in patients with solid tumours. One of the major challenges is the low abundance of tumour-derived biomarkers in circulation, requiring sensitive and specific detection methods. Additionally, standardization of sample

collection, processing and analysis protocols is essential to ensure the reproducibility and reliability of the results. Furthermore, the accessibility of liquid biopsy assays in clinical settings needs to be addressed to enable widespread adoption. Last, to ensure clinical relevance and accuracy, the results obtained from liquid biopsy must be validated and correlated to the traditional diagnostic methods (Lone et al. 2022). By overcoming these drawbacks, the full potential of this encouraging technique could be realized, allowing to identify and monitor patients with poorer prognosis using a minimally invasive and cost-effective tool that may even guide selection of the most appropriate treatment, thereby avoiding unnecessary toxicities, costs and loss of survival opportunities for patients (Pich et al. 2022).

1.3 Metabolomics

Metabolomics is a rapidly expanding -omics science that aims to detect and identify the global set of small molecules or metabolites that may be related to a biological status or system. Metabolites, the byproducts of metabolism, are low to medium molecular weight compounds (<1500 Da) that participate in cellular processes and reveal how metabolism works in living organisms. Recent discoveries made through metabolomics yielded new insights into the influence that metabolites may have on both normal physiology and disease pathophysiology (Qiu et al. 2023; Wishart 2019). Given that metabolites are the ultimate product of the -omics cascade, changes and interactions among gene expression, proteins and the environment are directly reflected in the metabolome. For that reason, metabolomics emerges as a powerful tool for biomarker discovery with advantages over other -omics since it is able to represent the genotype-phenotype correlation. In consequence, it appears as the burgeoning science that best represents the molecular phenotype of health and disease (Aderemi et al. 2021).

According to the desired level of detail or the ability to detect a certain number of metabolites, the methodologies in metabolomics might be categorized into two distinctive groups (Cajka and Fiehn 2016): 1) untargeted metabolomics, which aims to conduct a comprehensive analysis using scan mode of all measurable analytes in a sample, including unknown chemicals; and 2) targeted metabolomics, which focuses on measuring well-defined groups of biochemically characterized metabolites. In this dissertation, untargeted metabolomics is evaluated as a potential tool for biomarker discovery in cancer. Although compound identification remains a bottleneck in this approach, the steadily increasing structural information available in databases, along

with the growing availability of new analytical standards, and the advent of artificial intelligence (AI) is mitigating this challenge (Table 4).

TABLE 4. Characteristics of the two main metabolomic methodologies.

CHARACTERISTICS	UNTARGETED	TARGETED
Objective	Profiling all detectable metabolites in a sample.	Quantifying specific, pre-selected metabolites.
Scope	Broad, comprehensive coverage of the metabolome.	Focused on a defined set of metabolites.
Sensitivity	Variable. It may miss low-abundance metabolites.	High for the selected metabolites.
Reproducibility	Lower since depends on experimental and analytical conditions.	High due to standardized methods.
Quantification	Relative.	Absolute.
Data analysis	Complex. Requires extensive data processing and statistical analysis.	Simpler and easier interpretation with predefined targets.
Time and cost	Time-consuming and expensive due to the broad analysis.	Quicker and less expensive due to the focused analysis.
Biological insight	Potential for discovering unknown metabolites and related pathways.	Limited to known metabolites and specific pathways.
Applicability	Hypothesis generation, comprehensive profiling.	Hypothesis-driven research, biomarker validation.

The platforms utilized for metabolomic applications encompass mass spectrometry (MS) and nuclear magnetic resonance (NMR), each possessing their strengths and weaknesses depending on the type of analysis needed in metabolomics. MS is generally preferred for its high sensitivity and ability to detect a wide range of metabolites at low concentrations. On the other hand, NMR is valued for its high reproducibility and ability to provide structural information without extensive sample preparation (Table 5).

TABLE 5. Advantages and disadvantages of mass spectrometry (MS) and nuclear magnetic resonance (NMR) for metabolomics.

CHARACTERISTICS	MS	NMR
Sensitivity	High. Detects metabolites at very low concentrations.	Low. Requires relatively high concentrations of metabolites.
Selectivity	Very high. Identifies and quantifies specific compounds.	High. Provides detailed information about the molecular structure, including arrangement of atoms.
Metabolite coverage	Broad. Detects a wide variety of metabolites, including low molecular weight compounds.	Limited. Detects mainly more abundant and high molecular weight metabolites.
Absolute quantification	Difficult. Requires internal standards and can be affected by differential ionization.	Good. Allows direct quantification of concentrations without the need for internal standards.
Reproducibility	Good, though it can be affected by variability in ionization and instrumental environment.	Very high, due to the stability of the magnetic field.
Structural identification	Good. Allows identification based on fragmentation analysis but it struggles between isomers.	Very high. Distinguishes between different compounds with similar masses or related structures.
Sample requirements	Low sample amount required.	Larger sample amount required in comparison to MS.
Sample preparation	More complex, with multiple steps: extraction, derivatization…	Generally simple, with few preparation steps.
Equipment cost	High. Especially if instruments like UHPLC or GC are included.	Very high, due to installation and maintenance costs.
Analysis time	Relatively fast, depending on the spectrometer.	Slow compared to MS, especially for complex analyses.
Applications in metabolomics	Ideal for both targeted and untargeted metabolomics studies.	More commonly used for untargeted metabolomics studies and profiling.

Quantitative analysis	Strong in relative quantification, but challenging when absolute quantification.	Strong in absolute quantification, though limited by sensitivity.
Compatibility with complex matrices	Good, but the matrix may influence ionization and accuracy.	Very good and less affected by complex matrices of the sample.
Resolution and accuracy	Both techniques offer high accuracy and resolution in their respective domains: MS in mass determination and NMR in the chemical environment of atoms within a molecule.	Resolution and accuracy.

UHPLC: ultra-high performance liquid chromatography; GC: gas chromatography; MS: mass spectrometry; NMR: nuclear mass resonance.

Typically, MS is coupled with chromatography to facilitate prior separation of the compounds present in a sample. Then, metabolites' ionization is followed by compounds' detection according to their ionic mass-to-charge (*m/z*) ratio. The selection of analytical technique for a metabolomics study is contingent upon both the nature of the sample type and the study approach (Alseekh et al. 2021). Liquid biopsies such as plasma, urine or saliva are particularly suited for cancer metabolomics due to be promising sources for the detection of high number of metabolites reflective of the cancerous metabolic dysregulation. However, the diverse origins of samples pose a challenge in metabolomics, since it is necessary the adherence to specific protocols tailored to each sample type.

In addition, it is essential to choose the appropriate technique to identify the maximum number of metabolites in a biological sample and their level correlation to the behaviour of the disease. To this end, the chromatography-based and coupled techniques most frequently used in biomedical cancer research are the gas chromatography (GC)-MS, liquid chromatography (LC)-MS, and to a less extent the ion chromatography, affinity chromatography and capillary electrophoresis (Kałuzna-Czaplińska and Jóźwik 2014). Notably, GC and LC coupled to MS are employed in metabolomics due to their advantages when analyzing body fluids. Both chromatography-based approaches are characterized by minimal sample handling requirements, and large dynamic range of molecular characterization achieving high sensitivity and selectivity. Nevertheless, some methodological differences between these

separative techniques have driven LC-MS as the most suitable methodology in cancer metabolomics (Table 6) (Asensio et al. 2021; Jacob et al. 2019).

TABLE 6. Differences between liquid chromatography-based mass spectrometry (LC-MS) and gas chromatography.

DIFFERENCES	LC-MS	GC-MS
Sample compatibility	Non-volatile. Low to high weight compounds.	Volatile. Small molecular weight compounds.
Sample preparation	Proteins' precipitation to handle wide range of polarities and sizes.	Samples' derivatization to obtain volatile compounds.
Separation principle	Based on interaction with the stationary and mobile phases in LC.	Based on volatility and interaction with stationary phase.
Chromatographic columns	Reversed-phase, ion exchange, hydrophilic interaction…	Long capillary columns.
Ionization methods	Electrospray ionization, atmospheric pressure chemical ionization.	Electron ionization, chemical ionization.
Sensitivity	Highly sensitive, for polar and high molecular weight compounds.	Highly sensitive for volatile and semi-volatile compounds.
Specificity	High, enhanced with tandem MS.	High, due to extensive fragmentation and GC separation.
Typical applications	Pharmaceuticals, metabolomics, proteomics, complex mixtures.	Environmental pollutants, pesticides, food flavors.
Advantages	Broad applications range, adaptable to various chemical properties, suitable for large molecules.	Oldest technique in targeted metabolomics, with large number of mass spectral databases/libraries to identify metabolites.
Disadvantages	Requires liquid-phase compatibility and more complex instrumentation.	Limited to volatile, thermally stable compounds.

LC-MS: liquid chromatography mass spectrometry; GC-MS: gas chromatography mass spectrometry.

On the other hand, ionization sources and chromatographic columns are also diverse and must be chosen according to the metabolomics platform or technique used in order to improve the ionization efficiency and separation among different types of metabolites (Figure 14) (González Olmedo et al. 2024; Nordström et al. 2008). Lately, the electrospray ionization (ESI) technique has been widely used in combination with liquid chromatography high-resolution mass spectrometry (LC-HRMS) providing with the detection of polar to medium polar molecules (Ghosson et al. 2021).

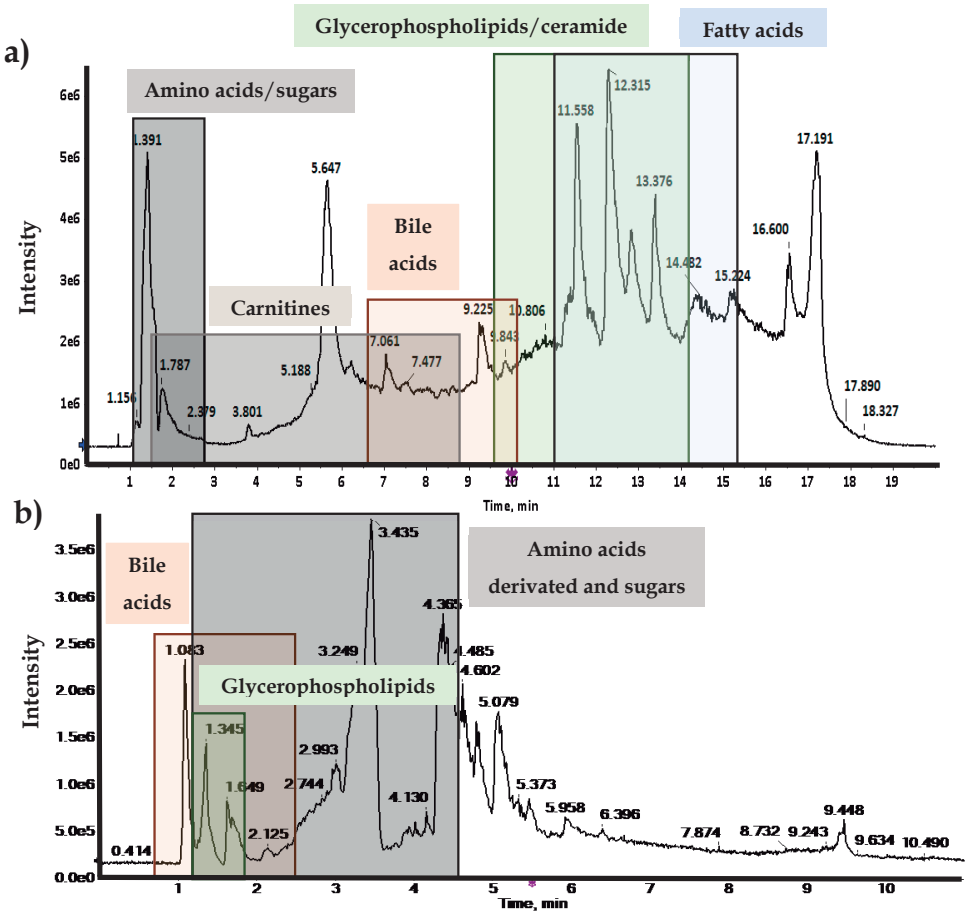


FIGURE 14. Total ion chromatograms (TICs) obtained from plasma samples analysed using two different liquid-chromatographic (LC) modes: a) reverse-phase (RP)LC with positive electrospray ionization (ESI+); and b) hydrophilic interaction liquid chromatography with negative ionization mode (HILIC, ESI-). TICs above show the different molecular families that can be retained in each chromatographic column and how differ the elution time ranges between these modes according to the polarity molecular characteristics.

1.3.1 Cancer metabolomics

Over the last decade, research into cancer as a metabolic disease has expanded significantly, leading to the emergence of a new field of study known as *cancer metabolomics* (Schmidt et al. 2021). Cancer metabolomics is grounded in the hypothesis that differences in central carbon metabolism exist between cancerous and normal cells (Figure 15), as initially demonstrated by Otto Warburg in the 1920s (DeBerardinis and Chandel 2020; Warburg 1956). The alteration in cancer metabolism causes that certain metabolites change their serum concentrations, defining a distinct metabolic characteristic of the disease state. This molecular pattern, often referred to as a metabolomic signature, holds potential as biomarker for early disease detection and analysis in liquid biopsies.

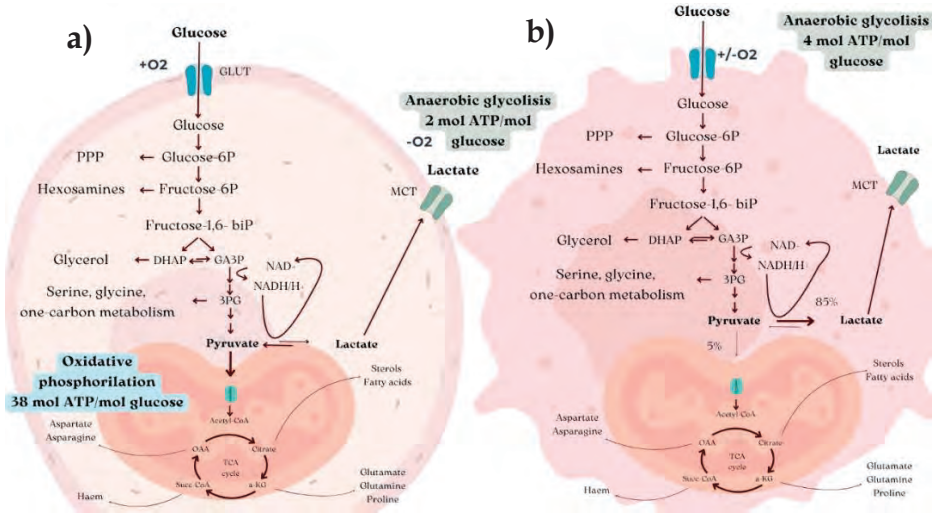


FIGURE 15. Differences in carbon metabolism between normal and cancer cells. a) Whereas normal cells prefer to perform oxidative phosphorylation which produces 38 molecules of ATP per molecule of glucose in the presence of oxygen (O_2), b) Warburg effect is grounded on the fact that tumour cells take up glucose and converted it to lactate even when there is sufficient oxygen to convert glucose to carbon dioxide (CO_2). This process is similar to the anaerobic glycolysis of normal cells where 2 molecules of ATP are produced per molecule of glucose. acetyl-CoA, acetyl coenzyme-A; ATP: adenosine triphosphate; DHAP: dihydroxyacetone-phosphate; GA3P: glyceraldehyde-3-phosphate; GLUT: glucose transporter; MCT: monocarboxylate transporter; MPC: mitochondrial pyruvate carrier; NAD+: oxidized nicotinamide adenine dinucleotide; NADH/H+: reduced nicotinamide adenine dinucleotide; OAA: oxaloacetate; Succ-CoA: succinyl-coenzyme A; 3PG: 3-phosphoglycerate; α-KG: α-ketoglutarate. Figure created with *Canva.com* and *BioRender.com*, adapted from DeBerardinis and Chandel (2020).

The heightened metabolic demands of malignant cells, driven by their accelerated cell proliferation, leads to a profound metabolic reprogramming characterized by modifications in biosynthetic and bioenergetic pathways to fulfill cancer cells requirements, exceeding those of normal proliferating cells (Yang et al. 2024). This metabolic shift represents an adaptation to support cell survival, tumour growth, tissue remodelling and cancer metastasis. Some evidence suggests that this metabolic rearrangement is regulated by a genetic program and influence by the tumour microenvironment while, under certain circumstances, the metabolic alteration may play a primary role in oncogenesis (Hirschey et al. 2015; Ge et al. 2022). Furthermore, metabolism can also dictate the course of the cancer process or even trigger and adverse response to medication. Therefore, investigating metabolism in cancer patients represents a fundamental approach to identify specific metabolic dependencies of tumour cells to be exploited as therapeutic targets (Cheung et al. 2019).

The characterization of molecular changes associated with the imbalance between growth, apoptosis and differentiation in tumours is crucial for enhancing early detection of cancer (Jové et al. 2017). Cancer metabolomics focuses on two main objectives in the search for biomarkers: establishing the metabolic profile of cancer samples for prognosis and diagnosis across different disease stages; and identifying early biomarkers through liquid biopsy for clinical application (S. Huang et al. 2021). These biomarkers are valuable for early detection, monitoring treatment response, and predicting metastatic potential (Díaz-Beltrán et al. 2021; Diaz et al. 2022; González-Olmedo et al. 2024).

Thus, metabolomics offers a minimally-invasive and cost-expensive approach, providing circulating biomarkers when other diagnostic tests are inconclusive. As a result, it facilitates more efficient use of the current therapeutic arsenal including standard chemotherapy, biological agents and targeted treatments (Debik et al. 2019; Ghini et al. 2020). Overall, the greatest advantage of metabolomics lies in its ability to provide clear insights into a patient's health status, supporting risk factor detection, accurate diagnosis, and enhanced cancer treatment strategies.

1.3.2 Metabolic biomarkers and oncometabolites

Assessment of classical metabolic-associated biomarkers in clinical practice is widely utilized for several disorders such as diabetes, cardiovascular disease, hypercholesterolemia, chronic kidney disease, uremia, and metabolic syndrome. Aberrant levels of circulating glucose, hemoglobin A1c, insulin, cholesterol, creatinine,

and urea are well-established indicators for diagnosis, patient monitoring, and risk prediction of those pathological conditions (Califf 2018; X. H. Chen, Huang, and Kerr 2011). In this context, advances in clinical metabolomics have yielded critical understanding about novel candidate metabolites through biomarker discovery (Wishart 2019). For instance, untargeted metabolomics has revealed promising biomarkers like Trimethylamine N-oxide for cardiovascular risk, although such discoveries require time to reach sufficient maturity to be integrated into routine clinical practice (Tang et al. 2013; Z. Wang et al. 2011).

In cancer, it is foreseen that metabolomics-based biomarkers may offer a faster, cheaper, and more comprehensive method to identify novel cancer-associated metabolites or *oncometabolites* (L. Dang et al. 2009). This term refers to the molecular intermediates of essential metabolic pathways which aberrant concentrations may lead to pro-oncogenic mechanisms. To date, key oncometabolites identified by metabolomics include fumarate, succinate, sarcosine and the well-established 2-hyroxyglutarate, as well as other emerging candidates such as secondary bile acids, glutamine, glutamate, glucose and lactate (Luo, Brooks, and Wicha 2018; Sreekumar et al. 2009; L. Dang et al. 2009).

To leverage metabolomics for cancer biomarker discovery, statistical tools like the area under the receiver-operating characteristic (AUROC) curves are used to evaluate the diagnostic accuracy of candidate metabolites (Pang et al. 2022). Specifically, the performance of the AUROC to discriminate between the experimental groups ranges from 0.5 (random chance) to 1 (excellent diagnostic performance) (Çorbacıoğlu and Aksel 2023). After identifying potential biomarkers, validation in an independent cohort is critical to ensure model's robustness and generalizability across populations, reducing the risk of overfitting, and enhancing clinical reliability. Given the complexity and multifactorial character of cancer, it is crucial to use panel of metabolites as biomarkers since relying on individual compounds may lack diagnostic accuracy (Firpo et al. 2023). Hence, in cancer metabolomics, key individual metabolites are selected based on their area under the curve (AUC) performance and combined into ensemble models for improved classification. This strategy allowed us to assess the diagnostic ability of several candidate metabolites identified by untargeted LC-MS in the current dissertation.

HYPOTHESIS

2. HYPOTHESIS

Metabolomics has revealed the existence of alterations in the normal metabolic processes that occur in cancer cells and do not occur in healthy cells. This leads to changes in normal blood concentrations of certain metabolites that can be detected and analysed from liquid biopsy. Metabolites represent the final expression of biochemical processes from an organism and, thus, could be used to classify cancer patients during the course of their disease. The emergence of precision medicine in the oncological practice to provide a better personalized cancer treatment, raises the question of characterizing the metabolome of these patients.

Thereby, the hypothesis of this thesis proposal is that the set of circulating metabolites altered in patients with a specific type of cancer will constitute the representative "signature" of the presence of the tumour that would provide the most specific prognosis for each patient, aiding to optimize the selection of a personalized therapy and a tailored follow-up.

OBJECTIVES

3. OBJECTIVES

The main objective of this doctoral thesis is to analyse the metabolomic profile of various types of cancer to enhance the understanding of specific predictive or prognostic biomarkers. The goal is to identify new candidate metabolites that could be integrated into clinical practice as part of a predictive model for treatment response or the detection of residual disease.

The following secondary objectives are also proposed:

1. To analyse the differential metabolic signature of the BC disease according to the molecular subtype.
2. To determine the predictive value of response to neoadjuvant chemotherapy of an identified metabolomic model depending on the BC phenotype.
3. To assess the metabolomic profile of recurrence in CRC after surgery of liver metastasis.

METHODOLOGY

4. METHODOLOGY

4.1 Inclusion and exclusion criteria

Two different types of cancer were analysed in the present doctoral thesis. Therefore, the inclusion and exclusion criteria were specific to each clinical scenario.

4.1.1 Chapters 1 and 2: *breast cancer*

For the recruitment of BC patients, the project protocols followed were the "PI-0455-2016" approved by the Research Ethical Committee of the University Hospital of Jaén the 27th of October, 2016; and the protocol code "ONCO-CHJ-UNICAJA-P1" approved the 25th of November, 2021.

Inclusion criteria

- Signature of the informed consent form prior to perform any study-specific procedure.
- Female sex and age ≥ 18 years.
- Histologically confirmed invasive breast carcinoma with the following characteristics: primary tumour larger than 2 cm in diameter (T1-T4), measured by clinical examination and mammography or ultrasound, with any lymph node involvement (N0-N2), with no evidence of metastasis (M0).
- BC suitable for primary surgery after neoadjuvant therapy.
- Availability of tumour tissue obtained by biopsy of the breast tumour.
- Eastern Cooperative Oncology Group (ECOG) functional status of 0 or 1.
- Absence of any psychological, family, sociological or geographical problems that may prevent compliance with the study protocol and follow-up schedule.
- Ability and willingness to comply with study visits and testing, at the investigator's discretion.

Exclusion criteria

- Any prior treatment for primary invasive BC.
- Bilateral invasive BC.
- Patients requiring immediate surgical intervention.
- Inability to comply with study procedures and follow-up.
- History of other malignancy within 5 years prior to screening, except adequately treated carcinoma in situ of the cervix, non-melanoma skin carcinoma or stage I uterine cancer.

4.1.2 Chapter 3: *metastatic colorectal cancer*

Liver metastatic CRC patients met the recruitment criteria of the protocol code: "6.2.05.2017", approved by the Research Ethical Board of the University Hospital of Jaén in May, 2017.

Inclusion criteria

- Subjects of both sexes.
- Age over 18 years old.
- Diagnosis of CRC and metastasis limited to liver suitable for radical hepatic resection.

Exclusion criteria

- Patients with different histology from adenocarcinoma.
- Unresectable liver metastases.
- No data from post-surgery follow-up.

4.2 Sample collection

The sample chose to the untargeted metabolomics analyses carried out in the present dissertation was plasma. All samples were obtained from patients under fasting conditions using standard venipuncture processes and collected in EDTA tubes. Plasma was obtained by centrifugation at 1400 x *g* for 10 min at 4 °C. All samples were kept at -80 °C until the metabolomic analysis.

4.2.1 Chapters 1 and 2: *breast cancer*

The BC patients recruited in these studies required treatment prior to surgery with NACT. So, according to the specific goal of each study, plasma samples were collected and compared in several timepoints:

1. Chapter 1. Blood samples were obtained at basal time before the patients received the first therapy cure with anthracyclines.
2. Chapter 2. Blood samples were obtained at three different timepoints: t1 or basal, pre-treatment; t2 or pre-surgery, once they received taxol, and t3 or post-surgery.

The tissue sample obtained during the surgical procedure after the NACT was analysed following the protocol from the Anatomical Pathology Unit of the UHJ for the

evaluation of the response by using the Miller & Payne (M&P) grading system (Ogston et al. 2003):

- M&P1. It is the grade that indicates minimal or no response to NACT. There is no significant change in size or structure of tumour cells in comparison with pre-treatment biopsy. There may be minimal tumour cell necrosis.
- M&P2. This grade indicates partial response. There exits reduction in tumour size or cellular changes. Tumour cells may show evidence of degeneration, necrosis or decrease in cellularity.
- M&P3. It indicates significant response with marked reduction in tumour size or extensive changes in morphology. Tumour cells often exhibit extensive degeneration, necrosis and decrease in cellularity.
- M&P4. It is the category related as a complete response with no evidence of residual invasive tumour cells, although some residual ductal in situ carcinoma may be present.
- M&P5. It represents the most favorable outcome: the pathological complete response (pCR) with no residual tumour cells neither invasive nor ductal.

4.2.2 Chapter 3: *colorectal cancer*

Paired plasma samples were obtained one before liver metastasis resection, at the time of the surgery, and the second 48-72 hours post-surgery.

4.3 Metabolomics processing

Untargeted metabolomics analyses were performed as detailed elsewhere (Díaz C. and González-Olmedo C. 2022), following the next steps:

1) Metabolite extraction and LC-HRMS analysis.
   1.1 Metabolite extraction.
   1.2 Separation of molecules performed on an Agilent UHPLC 1290 system, coupled to a quadrupole-time-of-flight 5600 mass spectrometer (Q-TOF-MS/MS) in positive and negative ESI modes.
   1.3 Identification of metabolites and molecular signatures with clinical prognostic and predictive potential.
   1.4 Structural validation of the significant signature.

2) Statistical analysis

Statistics on the metabolomic data were based on the following main approaches

2.1 A statistically significant p-value < 0.05 (corrected by false discovery rate - FDR) and fold change (FC) value > 1.3 between the groups under study.

2.2 Variable importance in projection (VIP) from the partial least square discriminant analysis (PLS-DA) set at > 1 (Akarachantachote, Chadcham, and Saithanu 2014; I. G. Chong and Jun 2005).

2.3 AUROC curve > 0.75 for the reliability of the detected metabolites as potential prognostic biomarkers, allowing the selection of the optimal diagnostic or predictive models for the study.

2.4 For temporal metabolomic analysis, the analysis of variance (ANOVA)-simultaneous component analysis (ASCA) was performed as previously detailed (Smilde et al. 2005; Thiel, Féraud, and Govaerts 2017; Madssen et al. 2020; Camacho, Díaz, and Sánchez-Rovira 2022).

2.5 Survival analysis was performed using the Kaplan-Meier curves with the support of the expert technician in Research Methodology and Biostatistics from the Foundation for Biomedical Research of Eastern Andalucía – Alejandro Otero (FIBAO).

3) Bioinformatic analysis of the data.

For the bioinformatic analyses, the following software were used:

- MarkerView software (version 1.2.1, AB SCIEX, Concord, ON, Canada): for LC-HRMS raw data processing; it performs peak detection, alignment and data filtering, providing a data matrix where the *m/z*, retention time (R.T) and intensities of the metabolomic features are measured.
- PeakView (version 1.2 with the add-on Formula Finder version 1.1, AB SCIEX, Concord, ON): to predict the elemental formula of selected candidates based on the accurate mass, isotopic clustering and fragmentation patterns.
- CEU Mass Mediator, MassBank, NIST2014 (version 2.2): for structural identification of the molecular formula by comparing the experimental fragmentation spectra with the spectra provided by these databases.
- Metaboanalyst 4.0 and 5.0 (Web Server software):

i) Module "Statistical Analysis" for univariate and multivariate statistical analyses including t-tests, ANOVA, principal component analysis (PCA) or PLS-DA.

ii) Module "Biomarker Analysis" to perform univariate or multivariate ROC curves of a single or multiple biomarkers.Module "Pathway Analysis" to determine the biological related pathways of the candidate biomarkers.

RESULTS

Chapter 1

5. RESULTS

Chapter 1: Original Research Article.

Human Plasma Metabolomics for Biomarker Discovery: Targeting the Molecular Subtypes in Breast Cancer

Leticia Díaz-Beltrán, **Carmen González-Olmedo**, Natalia Luque-Caro, Caridad Díaz, Ariadna Martín-Blázquez, Mónica Fernández-Navarro, Ana Laura Ortega-Granados, Fernando Gálvez-Montosa, Francisca Vicente, José Pérez del Palacio and Pedro Sánchez-Rovira

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Simple Summary. Breast cancer is the leading cause of female cancer-related deaths worldwide. New technologies with enhanced sensitivity and specificity for early diagnosis and tailored monitoring are in critical demand. Thus, metabolomics appears to be a growing tool in order to detect molecular differences between distinct groups. In this case, an untargeted analytical approach was used to identify metabolomics differences between molecular subtypes of breast cancer in comparison with healthy matched controls. Footprints for each breast cancer subtype provided diagnostic capacities with an area under the receiver-operating characteristic curve above 0.85, which suggests that our results may represent a major advance towards the improvement of personalized medicine and precise targeted therapies for the various breast cancer phenotypes. To validate these molecular profiling as potential therapeutic strategies for the different breast cancer subtypes, further analysis and larger cohorts would be necessary in the near future.

Abstract

Purpose: The aim of this study is to identify differential metabolomic signatures in plasma samples of distinct subtypes of breast cancer patients that could be used in clinical practice as diagnostic biomarkers for these molecular phenotypes and to provide a more individualized and accurate therapeutic procedure. *Methods*: Untargeted LC-HRMS metabolomics approach in positive and negative electrospray ionization mode was used to analyse plasma samples from LA, LB, HER2+ and TN breast cancer patients and healthy controls in order to determine specific metabolomic profiles through univariate and multivariate statistical data analysis. *Results*: We tentatively identified altered metabolites displaying concentration variations among the four breast cancer molecular subtypes. We found a biomarker panel of 5 candidates in LA, 7 in LB, 5 in HER2 and 3 in TN that were able to discriminate each breast cancer subtype with a false discovery range corrected p-value < 0.05 and a fold-change cutoff value > 1.3. The model clinical value was evaluated with the AUROC, providing diagnostic capacities above 0.85. *Conclusion*: Our study identifies metabolic profiling differences in molecular phenotypes of breast cancer. This may represent a key step towards therapy improvement in personalized medicine and prioritization of tailored therapeutic intervention strategies.

Keywords: human plasma metabolomics; breast cancer; molecular subtypes; metabolic profiling; personalized medicine

1. Introduction

Breast cancer (BC) is currently the most common malignancy in women, both in developed and less developed countries, and the leading cause of cancer-related deaths among women worldwide, with a high incidence rate [1,2]. Every breast cancer subtype is characterized by intrinsic molecular features and metastatic lesions, and their natural het- erogeneity leads to a high diversity in prognosis and clinical responses to available medical treatments, even for patients with similar diagnosis, histology and stage of disease [3–9]. Therefore, determining the molecular subtypes of breast cancer becomes crucial for personalized treatment. In fact, there is evidence reporting that patients receiving molecular-matched therapy have an increased overall response rate, longer period of time to treatment failure and a longer survival rate in comparison to patients with non-matched therapy [3,9]. Successive biopsy procedures and subsequent histopathological analysis are normally used to study molecular and genetic information from tumour cells in order to diagnose and classify BC into a subtype. This analytical technique is invasive and time consuming [3]. Thus, non-invasive, fast, sensible and precise analytical methods for distinction of different BC subtypes are in critical demand [10,11]. In this sense, metabolomics has quickly arisen as a novel approach in the cancer biomarker field to overcome the current limitations of standard diagnostic techniques [12]. This expanding research area provides a dynamic portrait of an individual overall metabolic status, assessing the final products of the myriad of intrinsic molecular processes and intercellular pathways that may be altered in response to genetic, pathological and/or environmental factors [3,13]. Hence, the end products of the diverse biological processes known as metabolites can be analysed from high-throughput screening technologies such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) enabling the discovery of altered pathways that may give us new insights into dysregulated metabolism in tumour development and progression. Therefore, the altered metabolites reflecting these pathophysiological changes might be considered as potential new therapeutic targets for breast cancer diagnosis, prognosis, early recurrence and drug efficacy [14–16]. Several studies have already been conducted to explore the possibility of using metabolite panels as biomarkers for early diagnosis, tumour characterization and clinical outcome

prediction [3,14–20]. Human body fluids such as saliva, urine, serum and plasma have been re-discovered as a great source of potential biological markers, and hence analysed in the search of a metabolic profile that may be representative of systemic metabolic dys- regulation in breast cancer patients [19–23]. However, up to today, efforts on proving highly accurate markers or proven targets for tailored therapeutic treatments have not yet delivered the expected results [24–28] due to the high heterogeneity displayed by breast cancer, from histology to prognosis, early recurrence, risk of metastatic progression or response to treatment and survival rates [29]. With this aim in view, we explore whether metabolomics is able to provide an accurate pathological diagnosis, phenotypic classification and a tailored follow-up of individuals with this malignancy. A high-throughput untargeted metabolic approach was used to identify the capacity of different metabolic profiles to predict various BC subtypes. Based on a liquid chromatography-mass spectrometry (HPLC/Q-TOF 5600) platform-based metabolomics study, we propose and test the notion that a differential metabolic signature representative of the distinct breast cancer subtypes exists, and it can be ultimately detected in plasma of individuals with this disease.

2. Results

2.1. *Patients' Characteristics*

To avoid the effect of potential confounding variables like age and Body Mass Index (BMI), the homogeneity of BC group and its corresponding HC subjects was evaluated. Normality's distribution was checked with a Shapiro-Wilk normality test and the equality of variances of both study groups was studied with the Levene´s test when corresponded. Finally, the appropriated t test was applied without significant differences observed in any case.

2.2. *LC-HRMS Analysis*

Four different liquid chromatography-high resolution mass spectrometry (LC-HRMS) analyses were carried out for each ionization mode, in order to determine the molecular differences between the major subtypes of breast cancer (luminal A (LA), luminal B (LB), triple negative (TN) and human growth factor receptor 2 positive (HER2) and the healthy control (HC) groups. The reverse phase (RP) column is recommended for the separation of medium-polar metabolites (such as phospholipids, lysophospholipids or steroids) and non-polar metabolites.

Total ion chromatograms (TICs) in positive electrospray ionization mode (ESI+) are shown in Figure 1, where clear differences are observed between BC subtypes and HC groups corresponding to the most significant discriminatory features detected: very polar metabolites eluted in the first 3 min (Figure 1a, c); medium-polar metabolites were found to elute from 8.5 to 12.5 min (Figure 1a,b,d); non-polar metabolites were not found in our work to be discriminatory after all the statistical analysis.

2.3. *Chemometric Analysis*

Different data matrices were obtained depending on the ionization mode and the set of BC molecular subtype analysed. Retention time (RT) windows and mass tolerances were determined for each analysed set based on the data of selected chromatographic peaks. After monoisotopic selection, contaminants were removed based on the organic solvent (OS) filtration and several features presented in the quality control (QC) samples were excluded for unacceptable variability (relative standard deviation > 30%). Remaining variables were evaluated by multivariate statistical analysis (Table S1). The close clustering of the QC samples in Figure 2 indicates that the separation observed between the corresponding study groups was mainly due to biological reasons in ESI−. The authors found that PC1 and PC2 explained 54.6%, 47.9%, 40.5% and 39% of the total of variance in LA, HER2, TN, LB in the ESI− mode analysis, respectively. The variance obtained with PC1 and PC2 was 42.5%, 40.5%, 44.8% and 43% in LA, HER2, TN, LB in the ESI+ mode analysis, respectively. Unsupervised principal component analysis (PCA) score plots obtained by ESI+ are shown in Figure S1. Score plots of the partial least squares-discriminant analysis (PLS-DA) models illustrated a marked separation between the HC group and BC molecular subtypes by both ESI modes (Figure 3 and Figure S2); the "goodness" of the PLS-DA model, measured by R2 and Q2, showed that no over-fitting was observed and, consequently, these models are acknowledged for successful discernment between HC patients and the LA, LB, TN and HER2 BC molecular subtypes [30] (Table S1). Signals with false discovery range (FDR) corrected p-values < 0.05 were selected as altered metabolites; those with a fold-change (FC) value of at least 1.3 between the study groups were selected as potential biomarkers (BM) to identify.

2.4. *Differential Metabolomic Profiling*

A tentative identification of the final

candidates was achieved as it was previously reported by the Schymansky classification. All identified metabolites were classified at level 1 and 2, therefore, their identities or probable structures are confirmed [31,32]. Hence, 5 metabolites were defined for the LA phenotype, 7 for LB, 5 for HER2 and 3 for TN (Table 1). The rest of metabolites (Table S2) met the criteria established for potential biomarkers of BC, although they could not be identified due to their MS/MS pattern, which did not match any of the queries of the compound databases searched (Metlin, Human Metabolome Database, Lipid Maps, PubChem, MassBank and NIST) or commercial standards used. This is likely to happen since the major part of the identity queries belonged to a similar molecular family whose virtual MS/MS spectra differences needed to be clarified, or because some of the signals have not been discovered yet. Thus, RT and MS/MS spectra of L-Tryptophan and Glycoursodeoxycholic acid (GUDCA) could be compared with their commercial standards under the same analytical conditions (Figure 4a, b and Figure S3). The experimental pattern of these metabolites matched with their standards so that the tentative identity could be confirmed.

2.5. *Biomarker Evaluation and Model Creation*

The diagnostic ability of the final tentatively identified candidates was evaluated with a multivariate receiver-operating characteristic (ROC) analysis. In this regard, we applied a PLS-DA model to combine our set of biomarkers to obtain the area under curve (AUC), which is a measure of how well a parameter can distinguish between two diagnostic groups. The AUC values obtained for each set of metabolites (Table 1) to discriminate between healthy patients and subtypes of breast cancer were 0.870, 0.919, 0.961 and 0.954 in LA, HER2+, TN and LB respectively. The performance of this biomarker model was evaluated using a balanced Monte Carlo cross-validation procedure. Although the model might improve when adding more of the potential biomarkers proposed in our work (Table S2), these features did not have a reliable structure ID since they could be only identified by their m/z and RT. Therefore, we preferred to use those metabolites based on the FDR corrected p value < 0.05, FC value > 1.3 and a tentative identification with a level classification of 1 or 2 by Schymansky (Table 1). The outcomes obtained for diagnostic potential of the selected biomarkers are summarized in

Figure 5 and Table 2. MetaboAnalyst 4.0 Web Server software (Wishart Research Group at the University of Alberta, Alberta, Canada) provided an average of predicted class probabilities of each sample in the 100 cross-validations. Confusion matrix in LA_BC revealed 14 BC and 16 HC samples correctly classified. Concurrently, 26 HER2_BC samples were correctly classified, whereas 28 samples were correctly distributed in the HC group. In TN_BC samples 13 BC and 11 HC samples were correctly classified; while 50 BC and 54 HC samples were properly assigned in LB_BC molecular subtype.

2.6. *Pathway Analysis*

We have found a set of biomarkers, which were able to discriminate each breast cancer subtype significantly. These first finding to distinguish at molecular level using untargeted metabolomics may improve the treatment of breast cancer and move towards to the priority of personalized medicine and customized therapeutic intervention strategies. According to the deregulated metabolites tentatively identified in each BC molecular subtype by ESI+ and ESI−, we determined the major altered pathways implicated in the four different subtypes. The outcomes were obtained by analyzing results in ESI+ and ESI−, differentiating by phenotypes. Thus, pathway analysis revealed that porphyrin and chlorophyll metabolism, glycerophospholipid metabolism, tryptophan metabolism and aminoacyl-tRNA biosynthesis appeared to be altered (Table S3). Statistically significant pathways ($p < 0.05$) are shown in Table 3.

3. Discussion

The advent of the –omics techniques is substantially accelerating predictive, preventing and personalized medicine. Next-generation sequencing (NGS), genomics and transcriptomics provide a better understanding of the genomic architecture of cancer and allow the discovery of differentially expressed genes that drive and maintain tumourigenesis. Genomic profiling has yielded potential biomarkers clinically relevant for early diagnosis of breast cancer, but these analytical platforms have some disadvantages, like shorter read lengths that challenges genome alignment and assemble, how to navigate through mega-datasets and, additionally, their cost is still high in comparison with other techniques. In contrast with the gene panels discovered by other techniques, metabolites are closer to the phenotype of the organism than genes and proteins, so the metabolome can be a point of convergence for genetic variation

influencing complex traits, and can efficiently elucidate the mechanisms underlying phenotypic variation. Thus, metabolomics profiling is considered as a relatively more rapid, accurate and non-invasive method to discover diagnostic and prognostic biomarkers. In this work, we applied an untargeted high-throughput metabolomics approach to compare the plasma metabolic profiling changes associated with the distinct BC molecular subtypes (LA, LB, TN and HER2) versus healthy controls. By using RPLC-HRMS in ESI+ and ESI− modes, we were able to detect statistically significant differences in certain metabolites with high diagnostic capacity in the four different BC phenotypes, which are involved in relevant biological cancer-related pathways such as: glycerophospholipid metabolism, porphyrin and chlorophyll metabolism, tryptophan metabolism and aminoacyl-tRNA biosynthesis. Otto Warbug described in great detail how cancer cells increase their glucose consumption as a fuel source to support the anabolic processes that promote their uncontrolled proliferation. Not only have Warburg's findings been confirmed, but other catabolic pathways have demonstrated their fundamental role in cancer progression [33,34]. Our findings go in accordance with the essential necessity of upregulating the energy supply in breast cancer cell growth and proliferation. Interestingly, a significant decreased concentration of L-Tryptophan (Trp) was observed in plasma of LA, TN and HER2 molecular subtypes of BC in comparison with healthy controls (FDR corrected p value < 0.05, FC < 0.6). Decreased tryptophan in plasma and serum of BC patients has also been reported in several studies [35–38]. Although the role of Trp catabolism in tumour proliferation is still unclear, it has been discovered to indirectly promote the degradation of the extracellular matrix and invasion on cancer cells [39]. Two main enzymes catalyze tryptophan into metabolites of the kynurenine (Kyn) pathway: tryptophan-degrading dioxygenases indoleamine-2,3- dioxygenase (IDO1) and tryptophan-2,3-dioxygenase (TDO2) [40,41]. Kyn activates the aryl hydrocarbon receptor (AhR) which contributes to cancer immune escape since it promotes an immunosuppressive tumour microenvironment by an increase of IL-10, Treg cells and suppressing immune activation cells [42]. Therefore, in cancer with an overexpression of IDO1/TDO2, increased Trp catabolism could lead to the depletion of its serum concentration and the accumulation of Kyn

metabolites, which enhanced cancer scenario [43–46]. Nevertheless, up to date there are no IDO1/TDO2 inhibitors currently approved by the US Food and Drug Administration. The most recent clinical trial publishing the effect of an IDO1/TDO2 inhibitor, Indoximod (D-1MT/NLG-8189), did not show a clinical benefit in metastatic BC patients when combined with taxane chemotherapy [47]. In fact, a lot more research is needed in order to warrant the efficacy of these inhibitors in clinical practice [48]. The reprogramming of lipid metabolism is a hallmark of many cancers, including breast cancer. Several lipoids were identified to be differentially altered in LA, LB, TN and HER2 molecular subtypes when comparing with healthy controls, which emphasize the importance of investigating the lipid metabolism differences in breast cancer. Phospholipids are a main component of cell membranes, they play a major role in cell signaling and cycle regulation and are a source of fatty acids (FA) which oxidative metabolism and ATP production is critical, not only in normal cells but also in cancer function [49]. In particular, a decreased plasma concentration of phosphoethanolamines (LysoPE (16:0), (18:1), (18:2) FDR < 0.05 and fold change < 0.6) and phosphocholines (LysoPC (14:0), (16:0), (20:3) FDR < 0.05 and fold change < 0.7) was observed. Our findings are in line with the already suggested distinction in membrane dynamics across molecular subtypes of breast cancer, where the acyl-chain constituents of PC and PE is remodeled by the action of phospholi- pases and lysohpospholipid acyltransferases with the delivery of fatty acid molecules for structural, signaling, and energy-producing purposes of breast cancer cells [50]. However, in accordance with other studies, breast cancer cells adapt to metabolic stress under given experimental conditions (glutamine deprivation or serum deficiency), by changing PE and DAG homeostasis. In both cases, an accumulation of phosphoethanolamine (PEtn) was observed in breast cancer cells with reduced expression of PCYT2, suggesting tumour progression in response to glutamine deprivation [51,52]. Moreover, in conformity with a recent prospective study where 1624 first primary incident invasive breast cancer cases were compared by their molecular phenotypes with 1624 matched controls, a phosphatidylcholine (LysoPC (20:3)) was found to have a negative association with risk of breast cancer as we found in

our analysis [53]. These biomarkers might open the possibility of identifying an early poor prognosis as well as detecting residual disease after neoadjuvant treatment (NAT). Furthermore, only two non-related metabolites were found to be differently expressed under our experimental conditions in luminal A, luminal B and HER2 molecular subtypes: biliverdin and glycoursodeoxycholic acid. High levels of biliverdin (FDR < 0.05 and FC > 1.5) were detected in plasma of luminal B and HER2 cancer patients. Although both biliverdin (BV) and its catabolite bilirubin (BR) are non-toxic molecules that, under most conditions, act as anti-oxidants by scavenging or neutralizing reactive oxygen species (ROS) [54], they are also endogenous activators of aromatic hydrocarbon receptors [aryl hydrocarbon receptor (AhR)] [55]. So, the increment of BV in plasma of LB and HER2 cancer patients would suggest its implication in signaling and gene expression related to cell growth and cancer progression either by its increased plasma concentration, an up-regulation of the heme oxygenase-1 (HO-1) or a dysregulation of its catabolic enzyme biliverdin reductase (BLVR-A or BLVR-B) [56–58]. Moreover, not many studies have had an impact on our understanding on how the bile acid pattern differs in BC subtypes until now. Although an influence of bile acids on the development of breast cancer cells and the estrogen receptor function had been suggested [59], both pro and anti-proliferative effects of bile acids in different breast cancer cell models have been determined. Plasma deoxycholic acid (DA) concentrations were found to be higher in breast cancer patients than in controls without considering the BC molecular differences [60], while deoxycholate (DC) inhibited human luminal A breast cancer cell lines proliferation and glycochenodeoxycholate (GCDC) enhanced patient survival in another study [61]. In this aspect, our results show low levels of GUDCA in plasma of 21 luminal A cancer patients when compared with 21 healthy controls (FDR < 0.05 and FC < 0.06), which makes it interesting for further study in order to clarify its function in breast cancer development. Finally, this study demonstrated that the four major BC subtypes could be discriminated using an untargeted metabolomics approach. Precise classification of these phenotypes has important implications in breast cancer personalized treatment, tailored follow up and detection of early recurrence.

4. Materials and Methods

4.1. *Participants and Ethics*

A total of 131 breast cancer patients and 134 healthy control subjects were recruited over 12 months at the Medical Oncology Unit of the University Hospital of Jaén (Spain). The study was approved by the Institutional Review Board of the Clinical Research Ethics Committee of Jaén and all clinical investigations were conducted under the Helsinki Declaration guidelines and the International Conference on Harmonization-Good Clinical Practices (ICH-GCP) guidelines. Every patient provided written informed consent for participation prior to blood sample extraction. The patient selection protocol was set as follows: female subjects being at least 18 years old with histological confirmation of BC, no detectable macro metastases and no previous anticancer treatment. Demographic details and clinical diagnosis of studied subjects are summarized in Table 4. The cancer stage was classified according to the 2002 Tumour Nodes Metastasis (TNM) system. Particularly, those BC patients diagnosed with HER2- and ER+ with Ki67 > 20% were defined as luminal B group and patients diagnosed with HER2- and ER+ with Ki67 < 20% were categorized as luminal A. As for non-luminal subtypes, all BC patients who neither expressed hormone receptors (PR-, ER-) nor overexpressed human epidermal growth factor 2 (HER2-) were considered as triple negative breast cancer patients; and finally, patients overexpressing human epidermal growth factor 2 were diagnosed as HER2+ breast cancer patients.

4.2. *Plasma Sample Preparation*

Samples were collected in EDTA tubes after at least 8 h fasting using standard venipuncture procedures. Blood was then centrifuged at 1400× g for 10 min at 4 ◦C and the supernatant was carefully aspirated and transferred into new vials, and immediately stored at −80 ◦C until the analysis.

4.3. *Metabolite Extraction*

An aliquot of 600 µL of acetonitrile (AcN) was added to 75 µL of plasma and the mixture was shaken for 2 min. Then, samples were centrifuged at 15,200× g for 10 min at 4 ◦C. The supernatant was collected in HPLC analytical vials. After that, it was evaporated in a GeneVac HT-8 evaporator (Savant, Holbrook, NY, USA) and kept frozen at −80 ◦C till the analysis. Finally, dry residues were reconstituted in 210 µL of water:AcN (50:50) with 0.1% formic acid and 250 ppb of L-leucine (1–13C, 99%), Roxithromycin, Caffeine-d3, Creatine

(methyl-d3) monohydrate, L-abrine (methyl-d3) monohydrate and Bisphenol A-d16 as internal standards.

4.4. *LC-HRMS Analysis*

Samples were analysed using an Agilent 1290 LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight 5600 mass spectrometer (AB SCIEX Q-TOF 5600, Concord, ON, Canada) in positive and negative electrospray ionization modes (ESI+, ESI−). A high performance liquid chromatography (HPLC) mode separation in ESI+ was carried out using an Atlantis T3 HPLC C18 column (2.1 mm × 150 mm, 3 µm; Waters Corporation, Milford, MA, USA) kept at 25 ◦C. Organic Solvent (OS) consisted of water:AcN (90:10) with 0.1% formic acid (eluent A) and AcN:water (90:10) with 0.1% formic acid (eluent B). The column was eluted with the following gradient: 0–0.5 min 1% eluent B; 0.5–11 min 99% eluent B; 11–15.50 min 99% eluent B; 15.50–15.60 min 1% eluent B and 15.60–20 min 1% eluent B. The elution flow rate was set at 300 µL/min [62]. Then, chromato- graphic separation was performed using a Gemini HPLC C18 column (100 mm × 2 mm, 3 µm; Phenomenex, CA, USA) kept at 25 ◦C in ESI− mode. The flow rate was 300 µL/min with mobile phases A (90% water: 10% AcN) and B (10% water: 90% AcN), both containing 0.1% ammonia at 20%. The gradient consisted of 0–0.3 min 1% eluent B; 0.3–7.3 min 99% eluent B, 7.3–10.3 min 99% eluent B and 10.3–13.3 min 1% eluent B. The TOF method operated with the Q-TOF 5600 allowed mass selection (80–1600 Da) with high resolution, in combination with an information dependent acquisition (IDA) method, which enabled the fragmentation of the eight most intense ions, to collect full-scan HRMS and MS/MS information simultaneously.

The exact mass calibration was automatically performed for every 10 injections of 5 µL of randomly injected plasma samples. Organic solvent samples were analysed along the sequence for every 30 injections; quality control samples were analysed for every 10 injections. The analysis of OS samples provided high impurity identification on either organic solvents or extraction procedure, and allowed discarding of carryover contamination. System stability and performance are evaluated by QC samples—a pool of equal volume of all plasma samples used in the study.

4.5. *Data Processing*

MarkerView software (version 1.2.1, AB SCIEX, Concord, ON, Canada) was used for LC-HRMS raw data processing. This tool performs peak detection, alignment

and data filtering, providing a data matrix where the measured mass-to-charge ratio (m/z), retention time (RT) and intensities are defined for each sample. Afterwards, to minimize mass redundancy and enhance the true molecular feature selection, only monoisotopic peaks were considered. Background and contaminants were removed from the OS by applying an additional filtering procedure with fold change (<1.5) and a t test ($p > 0.05$) between OS samples and study samples. Finally, according to FDA criteria for untargeted metabolomics, features with relative standard deviation higher than 30% were discarded because of their unacceptable variability in the QC samples [63]. The next steps were carried out using MetaboAnalyst 4.0 Web Server software (Wishart Research Group at the University of Alberta, Alberta, Canada) [64].

4.6. *Normalization and Analytical Validation*

Prior to the statistical analysis, normalization by a QC reference sample (probabilistic quotient normalization), transformation and scaling were applied to convert data set into a more Gaussian-type distribution [65,66]. Then, the PCA was used to assess the quality of the analytical system performance [67]. Analytical system stability was validated by QC samples presentation on a PCA plot. In parallel, the PLS-DA score plot showed possible outliers. Parameters R2 and Q2, which estimate goodness of fit and goodness of prediction respectively, were calculated to evaluate the statistical quality model description.

4.7. *Statistical Analysis*

Univariate analysis (UVA) was carried out using the non-parametric Wilcoxon rank- sum test to evaluate differences between BC patients and HC subjects. Benjamini-Hochberg false discovery rate (FDR) correction was performed afterwards to minimize the expected proportion of false positives (Type I errors) [68]. In this regard, a p value of 0.05 (corrected by FDR) for the t test is generally used in metabolomics as a cutoff threshold. Signals selected as potential candidates for a final discriminatory model were selected also based on their fold change ($FC > 1.3$). Eventually, a multivariate analysis was applied to identify features responsible for discriminating both study groups [30,69].

4.8. *Metabolite Identification*

PeakView software (version 1.0 with Formula Finder plug-in version 1.0, AB SCIEX, Concord, ON, Canada) was used to predict the elemental formula of

selected candidates from accurate mass, isotopic clustering and fragmentation patterns. The assignment of a tentative identification for each selected metabolite was possible by searching different compound databases (Metlin, Human Metabolome Database, Lipid Maps, Pub- Chem [70–73]) for accurate mass values. Structural identification of the molecular formula was achieved comparing the experimental fragmentation spectra against spectral databases (MassBank [74], NIST2014: version 2.2, Scientific Instrument Services, Inc, Ringoes, NJ, USA).

4.9. *Biomarker Evaluation*

Clinical relevance of the candidate metabolites was evaluated with the area under the receiver-operating characteristic curves (AUROC). In order to check the classifier performance of the biomarkers proposed for the diagnostic model, a multivariate ROC analysis was performed.

4.10. *Pathway Analysis*

MetaboAnalyst 4.0 Web Server software was used for the identification of altered metabolic pathways [64]. The metabolite ID matching was performed with Human Metabolome Database and KEGG database [71,75]. The analysis was adjusted by a hyper- geometric test and the impact on pathway topology was based on relative-betweenness centrality.

5. Conclusions

Here we present an untargeted LC-HRMS metabolomics approach as a non-invasive technique to identify differential metabolomics signatures for BC subgroups. We found distinct molecular profiles representative for LA, LB, HER2 and TN BC phenotypes, which may act as crucial biomarkers for accurate diagnosis, phenotypic discrimination and personalized therapeutic intervention. It is worth highlighting the importance of a deep understanding of the molecular differences among BC subtypes within the realm of personalized medicine to avoid unnecessary side effects or inadequate target engagement. The metabolomics profiles discovered could be used as a powerful tool in clinical practice, not only to determine the existence of residual disease after neoadjuvant therapy and, thereby, contribute to the identification of patients who will absolutely benefit from additional treatment, but also to enlighten the development of new therapeutic strategies for each BC molecular subtype and tailored follow-up. Finally, our findings reinforce a foundation to identify new biological targets in key metabolic pathways that

may help to identify early subsequent relapses in the different BC phenotypes. Further analyses in larger prospective cohort of patients would be necessary to validate the prognostic/diagnostic capability of the different metabolomics profiles found among the four major BC subtypes.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/1/147/s1. Figure S1: 2D score plots of the unsupervised PCA of HC group (green) and LA_BC (light blue) (a), HER2_BC (orange) (b), TN_BC (yellow) (c) and LB_BC (pink) (d) patients by ESI+ showed that the separation observed between the groups was due to biological reasons according to the close clustering of the QC samples (dark blue). Figure S2: 2D score plots of the supervised PLS-DA of HC group (green) and LA_BC (light blue) (a), HER2_BC (orange) (b), TN_BC (yellow) (c) and LB_BC (pink) (d) patients by ESI− determined a notably separation between BC molecular subtypes and matched controls. Figure S3: Characteristic MS/MS spectra of m/z 448.3066 in a biological sample (green) (a) and the glycoursodeoxycholic acid (GUDCA) standard (blue) (b) at 3.24 min. MS/MS spectra revealed the characteristic fragmentation pattern of GUDCA in ESI−. Table S1: Extracted peaks from RPLC ESI+ and ESI− HRMS, significant altered metabolites and quality model description. Table S2: Features identified by accurate mass (m/z) and retention time (RT). Table S3: Altered non-significant pathways associated with BC molecular subtypes by ESI+ and ESI−.

Author Contributions: Conceptualization, J.P.d.P. and P.S.-R.; methodology, A.M.-B. and C.G.-O.; formal analysis, J.P.d.P., C.G.-O., C.D. and L.D.-B.; investigation, M.F.-N. and L.D.B.; resources, M.F.-N., N.L.-C., A.L.O.-G., F.G.-M., L.D.-B., C.D. and C.G.-O.; data curation, A.M.-B. and L.D.-B; writing—original draft preparation, L.D.-B. and C.G.-O.; writing—review and editing, C.D., P.S.-R. and L.D.-B.; visualization, L.D.-B. and C.G.-O.; supervision, P.S.-R. and F.V.; project administration, P.S.-R.; funding acquisition, P.S.-R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and the International Conference on Harmonization-Good Clinical Practices (ICH-GCP), and approved by the Institutional Review Board of the Clinical Research Ethics Committee of Jaén (protocol code: PI-0455-2016 and date of approval: 27 October 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analysed during this study are included in this published article and its supplementary materials. Raw data are not publicly available due to ethical restrictions, since they contain information that could compromise the privacy of research participants, but are available from the corresponding author on reasonable request.

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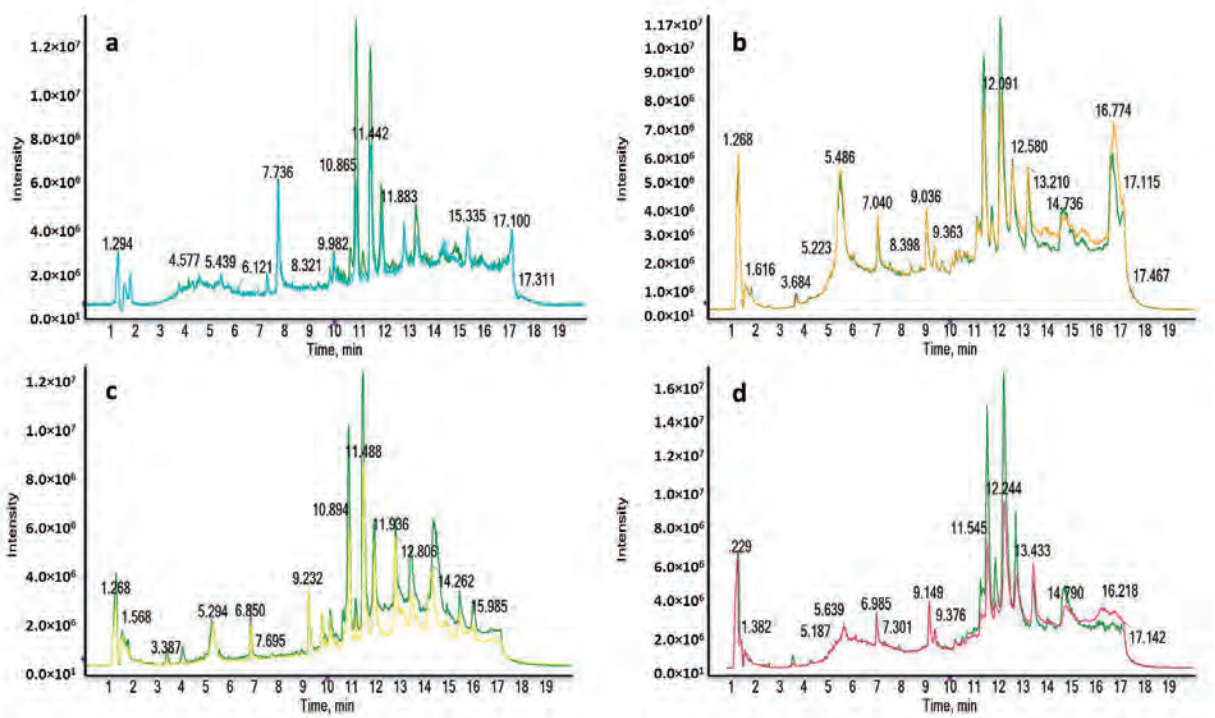


F I G U R E 1. Representative RPLC-ESI+-HRMS TICs of a LA_BC (light blue) (a), HER2_BC (orange) (b), TN_BC (yellow) (c) and LB_BC (pink) (d) sample compared to a HC sample (green). Remarkable differences were observed between BC and HC samples.

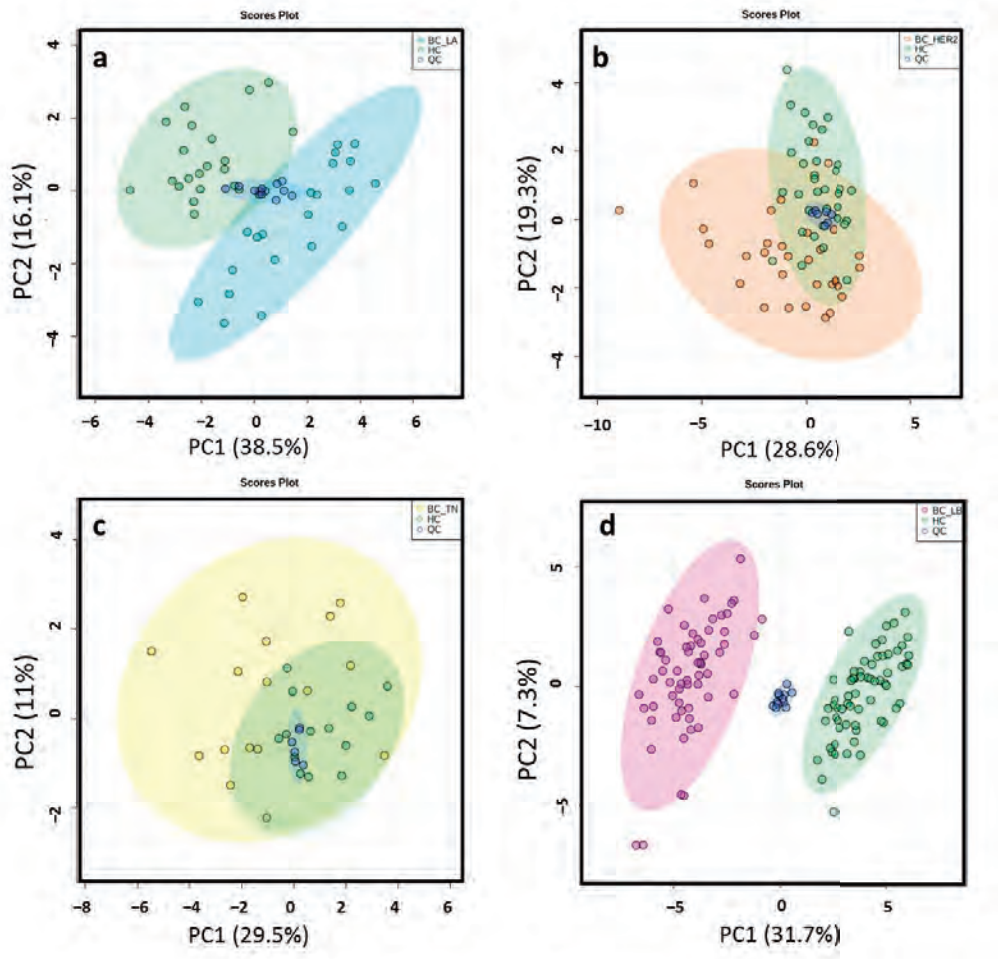


FIGURE 2. 2D score plots of the unsupervised PCA of HC group (green) and LA_BC (light blue) (a), HER2_BC (orange) (b), TN_BC (yellow) (c) and LB_BC (pink) (d) patients by ESI− showed that the separation observed between the groups was due to biological reasons according to the close clustering of the QC samples (dark blue).

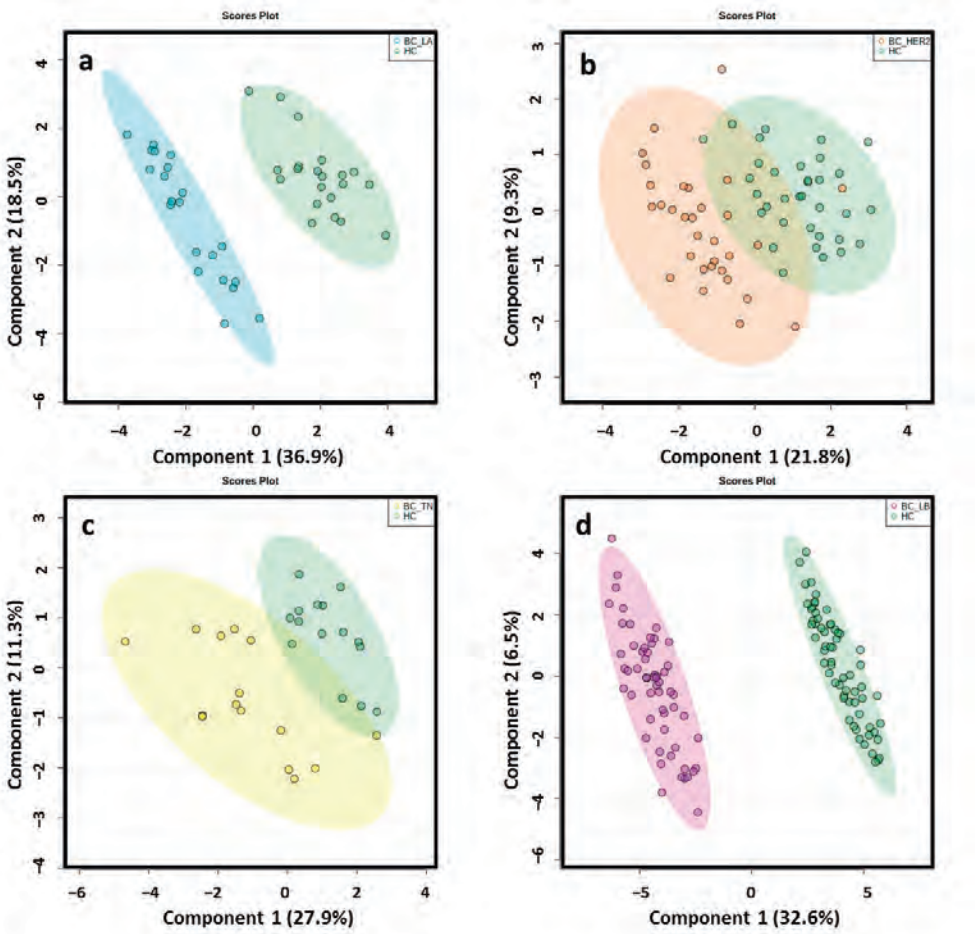
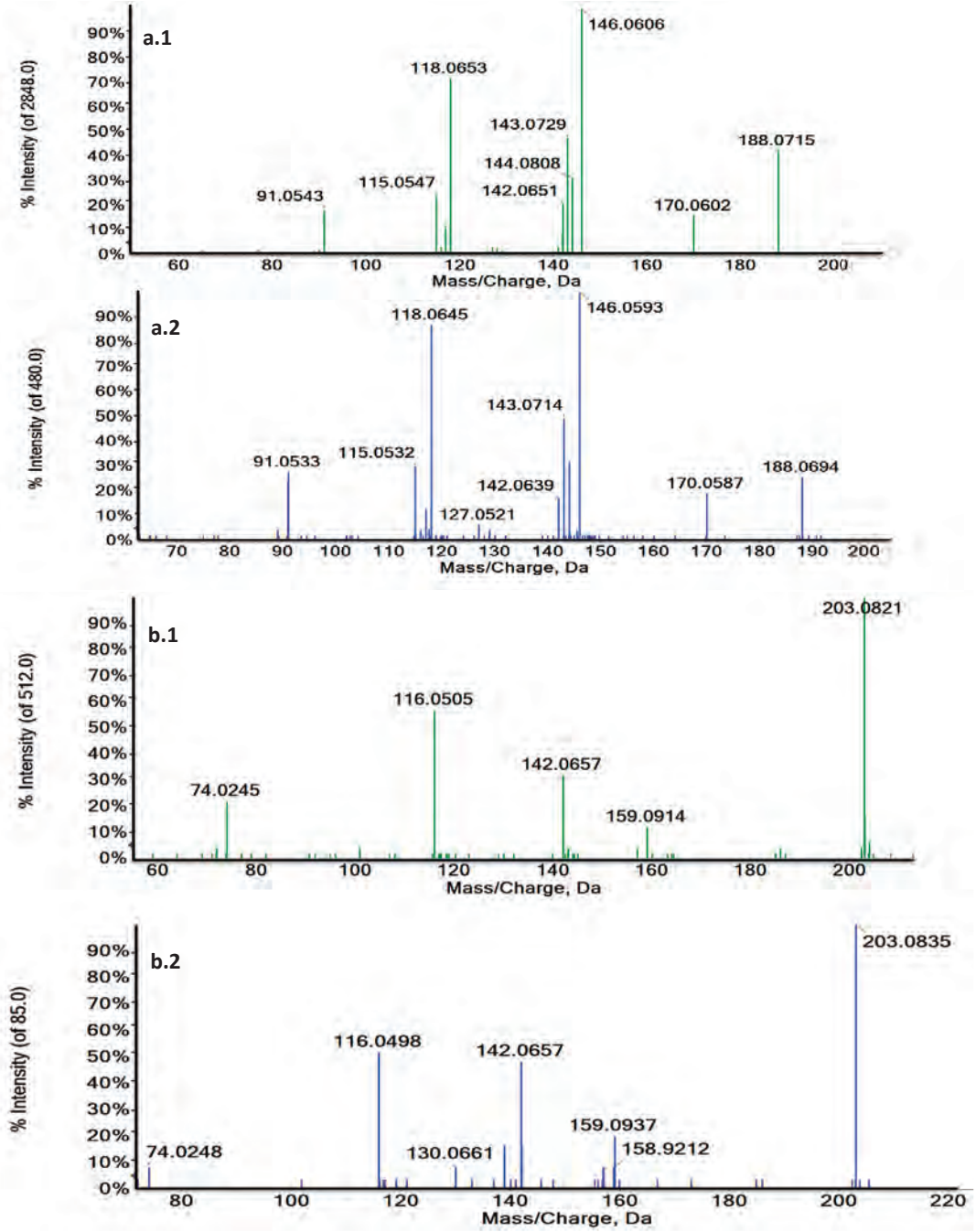


FIGURE 3. 2D score plots of the supervised PLS-DA of HC group (green) and LA_BC (light blue) (a), HER2_BC (orange) (b), TN_BC (yellow) (c) and LB_BC (pink) (d) patients by ESI−determined a notably separation between BC molecular subtypes and matched controls.



IGURE 4. (a.1) and the L-Tryptophan standard at 3.73 min in ESI + (blue) (a.2) and m/z 203.0824 at 1.27 min in a biological sample (green) (b.1) and the L-Tryptophan standard in ESI− (blue) (b.2). MS/MS spectra revealed the characteristic fragmentation pattern of L-Tryptophan both in ESI+ and ESI−.

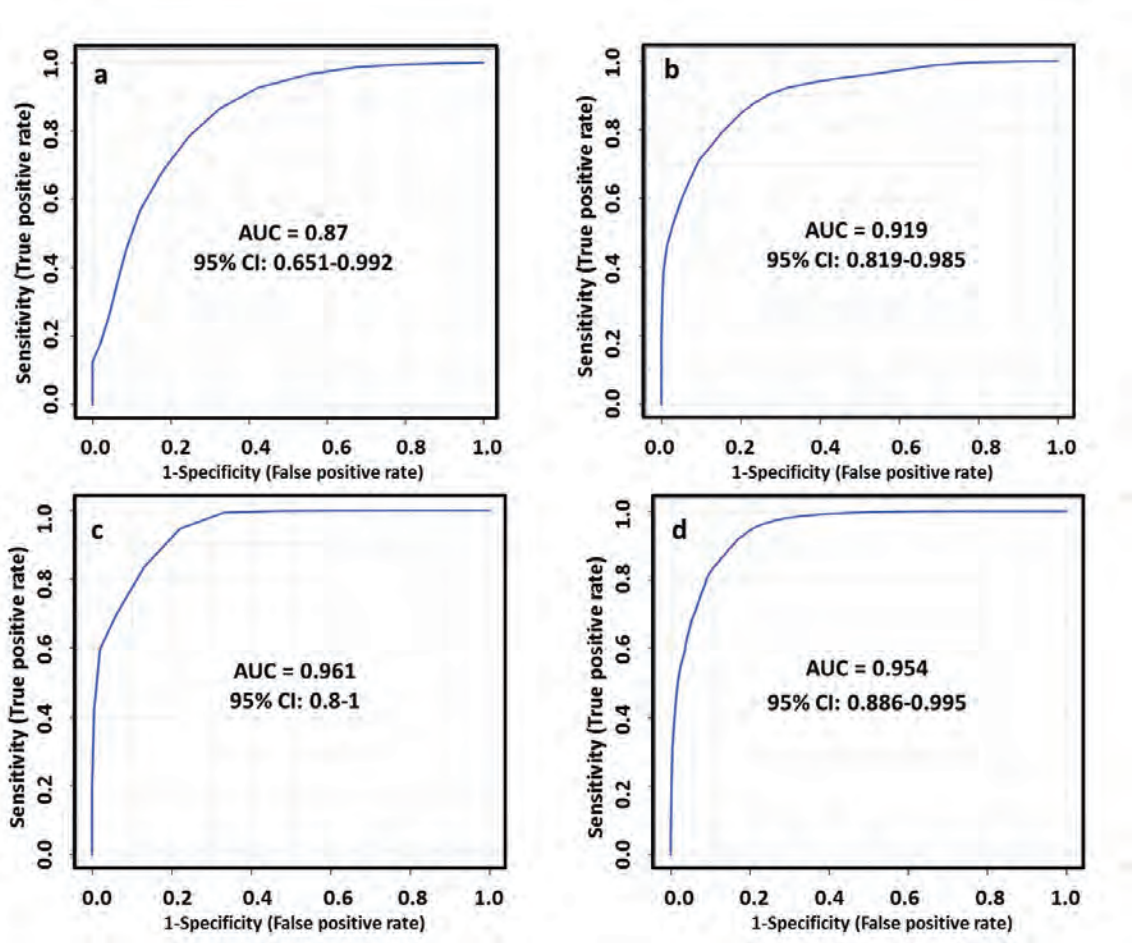


FIGURE 5. ROC curves for combined biomarkers model in LA_BC (a), HER2_BC (b), TN_BC (c) and LB_BC (d) by ESI+ and ESI−; 100 cross-validations were performed, and the results were averaged to generate the plot.

TABLE 1. Differential identified metabolites of molecular subtypes in BC.

BC molecular subtype	Tentative ID	m/z	RT	mass error (ppm)	*p* (FDR)	FC* (BC/HC)	Adduct	Molecular Formula
				ESI +				
LB	LysoPE(18:2)	478.2916	11.34	-2.5	1.670E-08	0.6008	[M+H]	C23H44NO7P
	LysoPE(18:1(11Z/9Z))	480.3108	12.15	4.8	5.365E-03	0.6303	[M+H]	C23H46NO7P
	LysoPE(18:1(11Z/9Z))	480.3073	12.47	-2.5	9.058E-10	0.4713	[M+H]	C23H46NO7P
	LysoPC(20:3)	546.3539	12.16	-2.7	2.214E-02	0.7303	[M+H]	C28H52NO7P
	Biliverdin	583.2566	8.95	2.6	7.390E-09	1.5681	[M+H]	C33H34N4O6
LA	L-Tryptophan[1]	188.0707	3.73	0.5	2.503E-02	0.6362	[M+H-NH3]	C11H12N2O2
	LysoPC(14:0)	468.3084	9.66	-0.2	3.745E-02	0.5849	[M+H]	C22H46NO7P
HER2	LysoPE(18:1(11Z)/9Z)	480.3109	12.31	5	6.192E-03	0.6407	[M+H]	C23H46NO7P
	LysoPC(0:0/16:0)	496.3411	11.71	2.6	6.396E-06	0.6701	[M+H]	C24H50NO7P
	Biliverdin	583.2525	8.65	-4.5	2.0621E-06	1.6265579	[M+H]	C33H34N4O6
TN	L-Tryptophan[1]	188.0702	3.4	2.1	4.153E-02	0.625911	[M+H-NH3]	C11H12N2O2

	LysoPC(16:0/0:0)	518.3224	10.07	1.3	0.03043	0.5289669	[M+Na]	C24H50NO7P
				ESI –				
LB	LysoPE(16:0)	452.2796	5.71	2.9	5.427E-14	0.5342	[M-H-H2O]	C21H44NO7P
	LysoPE(18:2)	476.2804	5.59	4.4	1.304E-08	0.5498	[M-H]	C23H44NO7P
LA	L-Tryptophan[2]	203.0824	1.27	-1	1.637E-02	0.6543	[M-H]	C11H12N2O2
	Glycoursodeoxycholic acid[3]	448.3066	3.24	-0.4	2.861E-02	0.5646	[M-H]	C18H34O4
	LysoPE(18:2)	476.2766	5	-3.6	3.489E-02	0.6711	[M-H]	C23H44NO7P
HER2	L-Tryptophan[2]	203.0836	1	4.9	7.536E-05	0.6744	[M-H]	C11H12N2O2
	LysoPE(18:2)	514.2381	5.5	7.8	3.403E-04	0.6408	[M+K-2H]	C23H44NO7P
TN	LysoPE(18:1(11Z)/9Z)	957.5976	5.86	2.6	0.027908	0.4407772	[2M-H]	C23H46NO7P

Features statistically significant (FDR < 0.05 and FC > 1.3) with a tentative identification based on their accurate mass (m/z), MS/MS pattern or comparison with commercial standards 1,2,3, were selected to create the proposed multivariate model. * Fold change (FC) expressed as the ratio of two averages (BC/HC); BC varies depending on the molecular subtype. BC: breast cancer; HC: healthy control; LA: luminal A; LB: luminal B; HER2: overexpressing human epidermal growth factor 2; TN: triple negative.

TABLE 2. AUC scores of selected biomarkers (BM) for the proposed models and confusion matrices of the BC subtypes.

BC molecular subtype	BM	AUC	95% CI	Confusion Matrix	
				BC	HC
LA	5	0.87	0.651-0.992	14/20	16/21
HER2	5	0.919	0.819-0.985	26/31	28/34
TN	3	0.961	0.8-1	13/15	14/15
LB	7	0.954	0.886-0.995	50/56	54/62

TABLE 3. Altered pathways associated with BC molecular subtypes by ESI+ and ESI−.

Altered pathways	BC molecular subtype	*p* value
Porphyrin and chlorophyll metabolism	LB and HER2	0.038347
Glycerophospholipid metabolism	LA, LB, TN and HER2	0.045927

TABLE 4. Demographic and clinical characteristics of breast cancer patients and healthy control subjects.

	LB	**HC**	**LA**	**HC**	**TN**	**HC**	**HER2**	**HC**
Subjects	61	64	21	21	15	15	34	34
Age	49	50	50	49	49	51	51	49
(Range)	(27-75)	(42-56)	(32-81)	(34-60)	(29-71)	(26-63)	(33-70)	(28-62)
BMI (Kg·m-2)	25.63 (16.9-40.5)	25.35 (19.8-30.0)	24.90 (20.0-37.2)	25.00 (18.0-28.3)	27.60 (21.60-41.23)	26.5 (21.3-30.0)	26.10 (21.0-33.3)	25.30 (20.80-29.80)
HER2	Negative	-	Negative	-	Negative	-	Positive	-
PR	Neg/Pos	-	Neg/Pos	-	Negative	-	Neg/Pos	-
ER	Positive	-	Positive	-	Negative	-	Neg/Pos	-
Ki67	>20%	-	<20%	-	-	-	-	-
TNM-stage IA	0	-	1	-	0	-	1	-
TNM-stage IIA	26	-	10	-	9	-	9	-
TNM-stage IIIA	12	-	0	-	0	-	3	-
TNM-stage IIB	19	-	9	-	3	-	19	-
TNM-stage IIIB	2	-	1	-	2	-	1	-
TNM-stage IC	2	-	0	-	1	-	1	-

HC and BC patients were matched in terms of age and BMI. BC: breast cancer; LB: luminal B; HC: healthy control; LA: luminal A; TN: triple negative; HER2: human epidermal growth factor receptor 2 positive; BMI: body mass index; PR: progesterone receptor; ER: estrogen receptor; TNM: tumor nodes metastasis.

T A B L E S1. Extracted peaks from RPLC ESI+ and ESI- HRMS, significant candidates and statistical quality model description.

BC molecular Subtype	Total	Monoisotopics	RSD>30%	OS filtering	PCA evaluation	Altered metabolites	R^2	Q^2
				ESI +				
LB	1497	542	168	255	119	80	0.76	0.6
LA	1322	419	99	152	168	62	0.75	0.49
TN	2129	529	261	93	175	7	0.95	0.38
HER2	2384	646	108	344	194	115	0.84	0.55
				ESI -				
LB	1584	410	8	154	248	197	0.99	0.98
LA	821	144	17	77	50	32	0.98	0.96
TN	1254	309	5	52	252	10	0.96	0.59
HER2	1467	442	5	80	357	164	0.7	0.45

Marker View software provided a data matrix containing the extracted peaks. This software allowed us to apply different filter steps to finally detect the features responsible for the discrimination between the groups. The non-parametric Wilcoxon rank-sum test (p, FDR< 0.05) was used to validate the significance of the difference in intensities between variables. R^2 and Q^2 parameters were calculated to evaluate the statistical quality model description.

TABLE S2. Features identified by accurate mass (m/z) and retention time (RT).

BC molecular Subtype	m/z	RT	*p* (FDR)	FC (BC/HC)*
		ESI+		
LB	188.0694	3.54	0.000181	0.661659764
	424.3436	9.88	0.047649	0.720931714
	494.3241	10.94	0.016454	0.696788467
	500.2729	11.35	1.01E-09	0.567782517
	502.2877	12.43	2.93E-09	0.526618524
	508.3396	11.77	0.047049	0.765045405
	516.3074	10.96	0.036772	0.702130928
	518.3239	10.68	0.00633	0.649721236
	520.3401	11.53	5.43E-07	0.632905958
	522.3486	11.44	4.61E-05	0.62972251
	522.3588	12.72	0.022614	0.719381574
	539.3099	11.51	2.70E-08	0.548812571
	541.3302	12.7	7.17E-06	0.605368254
	544.3354	12.61	0.044136	0.707257547
	544.3392	12.74	0.027313	0.730365454
	548.3685	13.28	0.049492	0.747556702
	558.2988	11.52	0.001147	0.702232228
	566.3205	11.46	0.031742	0.721545192
	762.981	12.2	0.001961	0.719055992

	798.9749	11.47	3.73E-09	0.420999989
	801.9816	11.53	3.36E-09	0.447644268
	991.6758	12.19	0.000488	0.681636976
	1002.6569	12.22	8.65E-05	0.651087195
	1010.6433	12.21	3.75E-05	0.660973934
	1014.157	12.17	0.00117	0.688398189
	1017.6837	12.31	2.45E-07	0.603190274
	1041.69	12.19	1.71E-06	0.610901465
	1043.6957	12.72	9.83E-07	0.493623718
	1061.6563	11.51	3.73E-09	0.426939569
	1062.6515	11.52	3.10E-09	0.41806545
	1063.6633	11.5	2.53E-09	0.45355252
	1065.689	12.7	1.56E-07	0.477682583
	1085.6556	11.51	3.31E-07	0.533432221
	1261.3227	12.19	0.000185	0.607528629
	1508.9915	12.22	0.000198	0.613783086

	482.3212	10.63	0.008477	0.612964935
	518.6578	10.86	0.034272	0.635888274
LA	521.3444	10.88	0.039792	0.638710336
	522.3439	10.61	0.011563	0.59654187
	542.3196	10.61	0.037448	0.604686277

	758.4347	10.61	0.02503	0.605662817
	798.9807	10.86	0.016881	0.457333326
	801.982	10.86	0.032804	0.541499037
	810.978	10.87	0.008477	0.510034942
	991.6718	11.4	0.005927	0.607791282
	997.6209	10.81	0.014809	0.451747844
	1002.659	11.4	0.014809	0.658313957
	1010.643	11.41	0.014809	0.646775446
	1062.65	10.85	0.007173	0.472831286
	1063.661	10.87	0.003475	0.412957109
	1085.646	10.88	0.011563	0.573309571
	329.2457	15.87	0.001163	1.329118996
	450.3221	9.66	0.015067	0.573750651
	476.2761	10.45	0.014926	0.68770148
	480.3111	12.01	0.043154	0.67273135
HER2	496.3396	12.06	0.019999	0.712534412
	502.2883	12.31	0.033225	0.683612
	515.3145	12.05	0.019433	0.727539976
	515.2627	4.15	0.002221	0.374716794
	521.3429	11.31	0.017469	0.689239322
	522.3458	11.11	0.02931	0.701052267

524.3716	13.97	0.039263	0.706184276
542.3228	11.31	0.011049	0.639305036
542.3228	11.31	0.011049	0.639305036
585.2708	7.3	7.34E-05	1.527313773
599.4391	12.14	0.000144	0.658826728
610.3079	11.11	0.007454	0.68190286
623.4416	11.38	0.048891	0.661109942
754.992	12.09	3.65E-07	0.615305186
762.9782	12.09	3.66E-06	0.617498696
765.9881	12.05	0.00019	0.695053185
771.9705	12.05	0.002302	0.650196234
798.9802	11.37	0.011482	0.591346447
801.9857	11.41	0.014926	0.642295525
805.0046	12.57	0.003006	0.615981099
810.9778	11.39	0.003427	0.621959193
932.5261	11.72	2.56E-05	0.62900932
955.5781	11.23	0.00458	0.626984118
956.5262	11.12	0.007454	0.558480779
991.6731	11.72	4.66E-08	0.350103215
991.6721	12.06	8.50E-08	0.512209654
1002.661	12.05	4.66E-08	0.508449895
1010.637	12.06	1.21E-07	0.519220446
1013.6497	11.71	8.50E-08	0.312418306
1013.6568	12.08	5.74E-05	0.689294135

	1014.1575	12.06	2.25E-07	0.583683555
	1017.6787	12.24	2.53E-05	0.564801879
	1039.6682	11.37	0.003546	0.522726991
	1039.6638	12.19	8.01E-05	0.63472339
	1041.7014	12.02	0.000903	0.605781811
	1043.6986	12.59	0.012336	0.629198894
	1061.6562	11.37	0.011482	0.642010974
	1063.6685	11.39	0.002633	0.574875198
	1065.667	11.37	0.005994	0.601601277
	1065.6847	12.59	0.010275	0.627315539
	1073.6651	11.38	0.00458	0.589103205
	1085.6489	11.39	0.044253	0.694602855
	1258.3102	12.08	6.52E-07	0.447635622
	1258.81	12.07	6.52E-07	0.427070417
	1261.3215	12.05	4.66E-08	0.442607045
	1261.8226	12.05	2.25E-07	0.458332604
	1508.9825	12.08	2.25E-07	0.445220732
TN	409.1587	9.31	0.03043	1.807452654
	425.1359	9.33	0.03043	1.820459243

		ESI -			
LB	199.1707	3.72	1.45E-11	1.438373242
	225.1863	3.93	6.11E-11	1.443604784
	238.0805	2.32	1.05E-05	1.582350023
	241.1963	5.6	6.49E-16	1.310097924
	303.2349	4.73	1.53E-14	1.596269769
	305.2499	4.87	2.43E-10	1.388192357
	331.1923	4.53	3.17E-16	2.292562288
	355.1701	6.64	5.46E-20	3.17342607
	367.1612	3.32	1.35E-06	1.649502118
	369.1765	3.11	9.42E-06	1.40701092
	369.1744	3.54	5.75E-08	1.824429281
	383.1538	2.9	1.96E-07	1.616166787
	385.1716	2.64	6.24E-08	1.696586057
	385.1812	6.64	5.46E-20	3.108817515
	391.289	3.52	0.000788	0.317178793
	395.1914	3.71	2.88E-11	2.197940531
	397.2086	3.51	1.70E-10	2.168436878
	397.206	3.87	0.000182	2.005603465
	399.2234	3.6	9.02E-07	2.615232098
	407.2795	3.27	0.000429	0.203073672

409.2392	7.56	6.15E-07	0.661343969
413.2028	3.21	1.45E-11	1.720133144
421.1571	6.64	5.46E-20	2.96387748
427.2173	3.54	8.52E-05	1.426932244
435.2545	7.57	3.40E-07	0.674320154
435.2536	7.8	0.000462	0.688626077
436.2862	5.89	3.82E-09	1.395241773
437.2716	8.46	1.22E-15	0.439588975
437.2714	8.69	5.55E-07	0.563739994
448.1777	6.64	5.46E-20	2.920654647
449.1532	3.73	5.46E-20	12.42037049
459.2552	7.19	8.99E-06	0.637042437
460.2592	7.2	1.31E-05	0.630757381
461.2718	7.86	9.46E-05	0.675847406
462.3005	6.13	1.03E-12	1.637399978
465.2536	3.2	5.58E-06	1.514013551
465.3061	6.45	1.72E-12	1.522051505
473.3672	4.04	0.040611	0.603563783
478.2981	5.92	6.27E-14	0.409165151
479.3729	4.12	2.98E-14	1.45323045
480.3056	6.43	0.000224	0.605032068
494.323	7.56	1.49E-06	0.663194812
495.3005	3.44	0.002154	2.071991354
498.2624	5.53	6.95E-07	0.586808354

500.2796	5.92	5.31E-12	0.446895021
508.2844	5.68	5.46E-20	2.256078377
511.2949	3.22	0.000237	1.313327037
518.3237	7.18	2.88E-06	0.626038938
537.3326	7.57	0.002128	0.699994088
540.2727	4.05	1.63E-14	0.466732668
540.2757	4.59	5.46E-20	0.047582144
540.3368	7.48	4.63E-06	0.630137922
540.3315	7.56	6.82E-07	0.66062606
540.2727	9.88	5.46E-20	0.186376564
540.8199	1.66	1.05E-09	1.361998162
554.3434	7.56	2.11E-05	0.672001144
556.3287	7.55	2.85E-08	0.660066798
557.4643	4.55	6.33E-10	0.341584753
558.3401	7.48	3.67E-05	0.655130023
560.2705	5.53	6.09E-06	0.641304003
561.3341	7.19	0.000197	0.650665458
563.3499	7.81	0.046753	0.718724427
564.337	7.17	6.09E-06	0.631363624
566.3444	7.81	0.001027	0.695284278
567.5368	5.24	2.19E-12	1.353710489
568.3603	8.45	1.92E-15	0.434058749
568.3675	8.69	1.68E-07	0.516084703

570.3444	7.97	0.003009	0.653275679
573.4619	4.08	0.007093	0.515972634
573.4588	4.24	0.002642	0.487966171
578.3528	7.17	4.74E-06	0.628832325
580.3332	7.17	4.82E-06	0.624295471
580.371	7.82	0.000207	0.672308285
582.3374	7.17	7.42E-05	0.651869084
582.3459	7.8	0.000392	0.686786579
584.3583	8.45	3.06E-15	0.441814117
584.3656	8.67	2.97E-07	0.549971899
585.361	8.65	7.02E-07	0.552641203
590.3195	7.5	0.000207	0.659589983
590.3223	7.56	8.39E-05	0.698952048
591.4696	3.94	0.027099	0.479219449
593.4829	4.13	0.017147	0.585140289
602.7984	1.64	2.22E-15	1.337511598
606.3402	8.49	8.01E-15	0.469353112
608.3642	8.09	7.02E-07	0.640526956
614.3166	7.13	5.10E-05	0.656174146
616.3321	7.82	0.002674	0.705227251
619.2935	4.72	1.92E-07	0.570427603
659.4169	8.49	0.000423	0.668615024
668.3394	8.45	9.26E-15	0.46417904
668.34	8.68	0.003998	0.666116978

671.2656	9.88	9.38E-06	0.586439383
678.3005	9.88	1.20E-09	0.51610498
681.3031	9.88	1.93E-10	0.506723816
695.3179	9.87	8.10E-10	0.508550763
697.298	9.88	6.47E-11	0.495852767
698.4262	11.22	5.46E-20	2.459756411
712.4004	10.93	1.70E-10	0.398465484
714.4221	10.11	5.46E-20	5.578054658
714.417	10.91	6.81E-19	1.748179883
714.4107	11.25	1.31E-13	0.487365074
730.415	11.12	5.46E-20	0.110698181
730.4133	9.35	5.46E-20	3.088994098
730.4138	9.36	0.043704	0.725181709
730.4107	9.9	1.48E-19	2.159996657
734.4027	11.22	5.46E-20	2.3543829
746.4014	10.9	5.46E-20	0.032685023
746.4076	9.88	1.50E-14	0.398782137
750.3965	10.09	5.46E-20	4.526014424
750.3987	11.29	4.63E-12	0.472602827
757.4203	11.28	6.97E-08	0.5582306
761.4241	11.23	5.46E-20	2.020445815
764.3618	10.95	1.46E-17	0.087260903
766.394	11.11	5.46E-20	0.130556068

766.382	9.39	3.22E-16	0.397493252
768.3951	9.36	8.21E-16	0.386022448
775.4007	10.9	7.91E-11	0.413058921
777.4156	10.09	5.46E-20	3.577938579
777.4163	11.32	1.00E-13	0.457894786
782.3805	10.86	5.46E-20	0.035440369
791.3928	10.98	8.25E-18	0.090092625
793.4098	11.04	5.46E-20	0.129887089
904.5777	7.55	1.14E-12	0.546884212
918.5875	7.55	2.06E-12	0.531768963
952.5745	7.18	3.17E-09	0.481593909
956.6125	7.84	3.19E-08	0.552639615
976.5738	7.24	1.24E-05	0.598945011
989.6758	7.56	2.01E-13	0.54914282
1035.6755	7.56	6.92E-10	0.551998883
1037.6753	7.19	9.40E-09	0.493304993
1041.6998	7.83	1.70E-08	0.538034313
1051.6721	7.46	1.22E-15	0.353525035
1051.6559	7.56	5.69E-13	0.552133485
1073.6529	7.53	0.00057	0.695610816
1099.6742	7.17	6.75E-09	0.488465793
1103.698	7.82	1.37E-08	0.532730131
1121.657	7.17	8.38E-07	0.586531839
1123.6707	7.2	2.36E-05	0.60350591

	1135.6532	7.55	0.002642	0.70277207
	1183.6658	7.17	4.74E-06	0.592968652
LA	253.2182	4.03	0.01134	1.543582497
	255.2339	4.26	3.72E-06	1.395733146
	281.2487	4.33	0.030446	1.88602715
	286.214	4.27	7.17E-06	1.500205102
	313.2395	4.36	0.001924	1.97821063
	351.2194	4.29	4.71E-06	1.380483283
	367.1581	3.13	0.038789	0.629369591
	377.2347	4.37	0.031318	1.869997431
	389.2745	4.37	0.030446	1.831135114
	391.2821	3.49	0.025021	0.233415595
	407.2761	3.25	0.046014	0.134123139
	413.1983	5.99	6.19E-11	3.122262519
	447.1301	3.56	3.60E-09	0.30481595
	465.3038	5.12	6.19E-11	5.101262081
	500.2743	4.98	0.005684	0.693522754
	523.2927	4.07	0.034844	1.410911548
	524.2733	4.98	0.018667	0.732511821
	540.272	4.02	6.19E-11	0.02640082
	551.3252	4.36	0.031318	1.727496137

	580.3218	11.84	0.018667	0.577213127
	602.3004	11.82	0.005586	0.582257098
	613.2726	4.07	0.020789	1.44768918
	641.3003	4.38	0.02779	1.791668744
	643.317	4.6	8.42E-09	2.052066351
	653.2979	4.49	5.57E-10	0.157001633
	664.301	11.84	0.002697	0.560615407
	225.1866	3.89	0.002148	1.409120314
	253.2174	4.36	0.002084	1.366445531
	277.2179	4.3	0.041601	1.560883434
	279.2338	4.53	0.004836	1.555074692
	281.2475	4.83	0.009389	1.473024803
	297.2451	4.83	0.004429	1.54607755
	303.2338	4.61	0.003754	1.428186991
HER2	309.2819	5.26	0.006765	1.307058039
	377.236	4.83	0.006123	1.467860128
	409.2356	7.36	0.001598	0.747175491
	416.8413	1.89	9.28E-08	0.638046131
	448.3069	3.57	0.047398	0.747279385
	450.8298	1.94	2.17E-05	0.708987921
	465.8403	1.97	2.38E-08	1.534379444

480.3101	7.49	0.031827	0.759247047
486.2645	3.57	0.002587	0.603816223
494.3294	7.36	0.000761	0.747991165
508.3434	8.41	0.020992	0.765569101
508.3426	8.61	0.017295	0.763137463
522.804	2.09	4.77E-07	0.628761733
538.7805	2.11	9.27E-09	0.420443774
540.3342	7.34	0.005021	0.756415884
550.7944	2	1.98E-06	0.671107518
563.5014	4.83	0.014832	2.427858684
568.3643	8.42	0.021481	0.752554061
572.7695	2	5.94E-08	0.549397622
583.7535	2.11	8.14E-08	0.514337475
584.3601	8.41	0.014095	0.739897009
584.3567	8.61	0.024108	0.763099038
593.8019	1.89	2.60E-08	1.356101965
611.4896	4.82	0.021481	1.772913291
611.7626	1.94	4.71E-06	0.663956408
628.7725	1.84	5.31E-05	0.708331848
643.777	2.04	3.65E-08	1.305538691
644.7408	2	1.36E-08	0.461977904
660.7137	2	1.36E-08	0.440777209
676.694	2.06	1.36E-08	0.428084929

678.7327	1.94	3.65E-08	0.586403516
694.7091	2	9.28E-08	0.553390049
714.7602	1.94	0.000928	0.72510449
736.7468	2.01	0.002905	0.730976843
742.747	1.93	0.025722	0.759168768
750.7213	2.09	6.29E-08	0.605366658
751.7348	1.84	0.002795	0.703461577
768.7278	1.93	0.005619	0.745626815
771.7401	1.89	1.05E-08	1.634477131
778.6954	1.96	1.65E-07	0.588741679
784.6968	2.08	3.05E-07	0.644847895
790.701	1.94	6.26E-05	0.699017898
796.6584	1.92	0.041601	0.765151292
817.6818	1.94	9.28E-08	0.523775149
856.6782	1.93	1.72E-06	0.655711746
862.6614	1.93	5.83E-07	0.625372605
872.642	2.11	1.74E-07	0.610930585
882.699	2	0.046318	0.766298791
904.5728	7.34	3.34E-05	0.631142893
904.5692	7.53	0.008125	0.74504555
918.5862	7.53	0.006592	0.740383453
952.5661	7.16	0.022024	0.716582334
952.6562	2	0.001047	0.670422214

	953.5715	5.48	0.030844	0.720157173
	960.6318	8.61	0.006765	0.658813078
	978.6174	2.04	6.29E-08	0.603800853
	989.6597	7.53	8.29E-05	0.735065347
	1035.661	7.53	0.001757	0.7445087
	1037.67	7.16	0.014458	0.700021507
	1051.663	7.34	5.56E-05	0.62213411
	1051.658	7.54	0.000394	0.749866322
	1061.651	7.17	0.038037	0.738628266
	1099.674	7.18	0.012703	0.702771133
	1107.727	8.63	0.000539	0.620406573
	1546.993	7.53	0.002005	0.669029124
TN	824.7259	1.29	0.001651	2.006859929

Potential biomarkers were selected according to the non-parametric Wilcoxon rank-sum test (*p*, FDR< 0.05) and fold change > 1.3. Identification of theses features was based on their accurate mass (m/z) and retention time since their tentative ID could not be clarify by comparison of MS/MS spectra or commercial standards. *Fold change (FC) expressed as the ratio of two averages (BC/HC); BC varies depending on the molecular subtype. LA: luminal A; LB: luminal B; HER2: overexpressing human epidermal growth factor 2; TN: triple negative.

TABLE S3. Altered non-significant pathways associated with BC molecular subtypes by ESI+ and ESI-.

Altered pathways	BC molecular subtype	*p* value
Tryptophan metabolism	LA, HER2 and TN	> 0.05
Aminoacyl-tRNA biosynthesis	LA. HER2 and TN	> 0.05

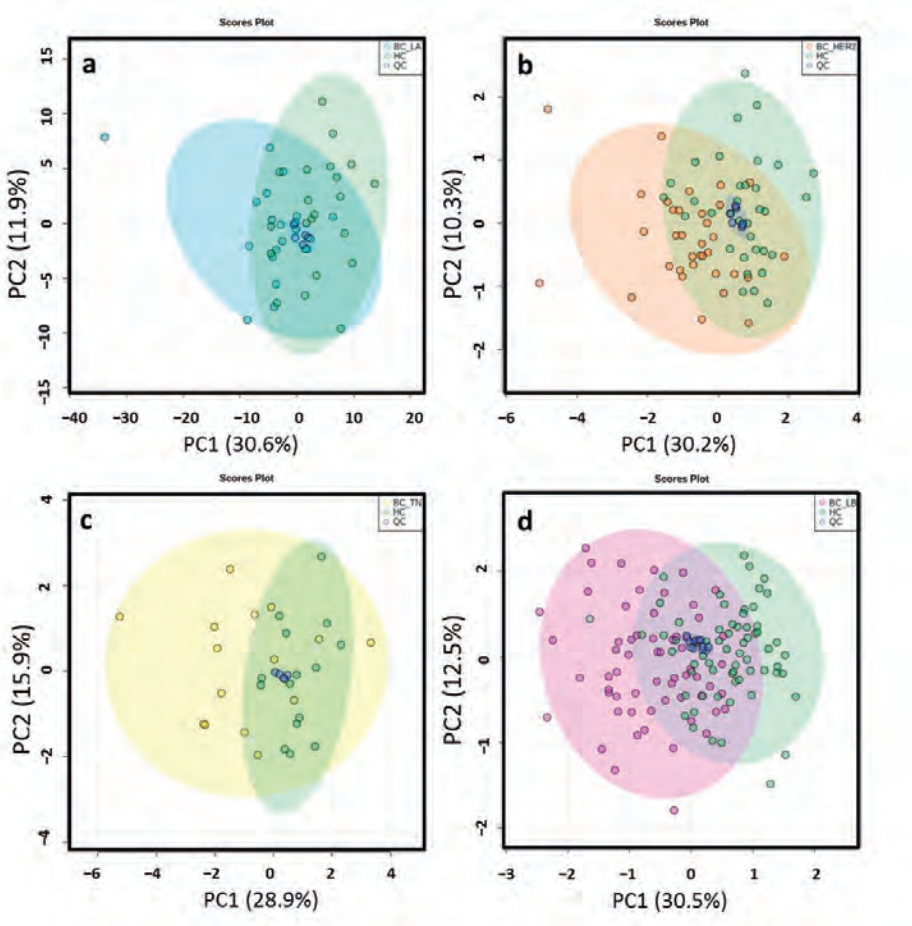


FIGURE S1. 2D score plots of the unsupervised PCA of HC group (green) and LA_BC (light blue) (**a**), HER2_BC (orange) (**b**), TN_BC (yellow) (**c**) and LB_BC (pink) (**d**) patients by ESI + showed that the separation observed between the groups was due to biological reasons according to the close clustering of the QC samples (dark blue).

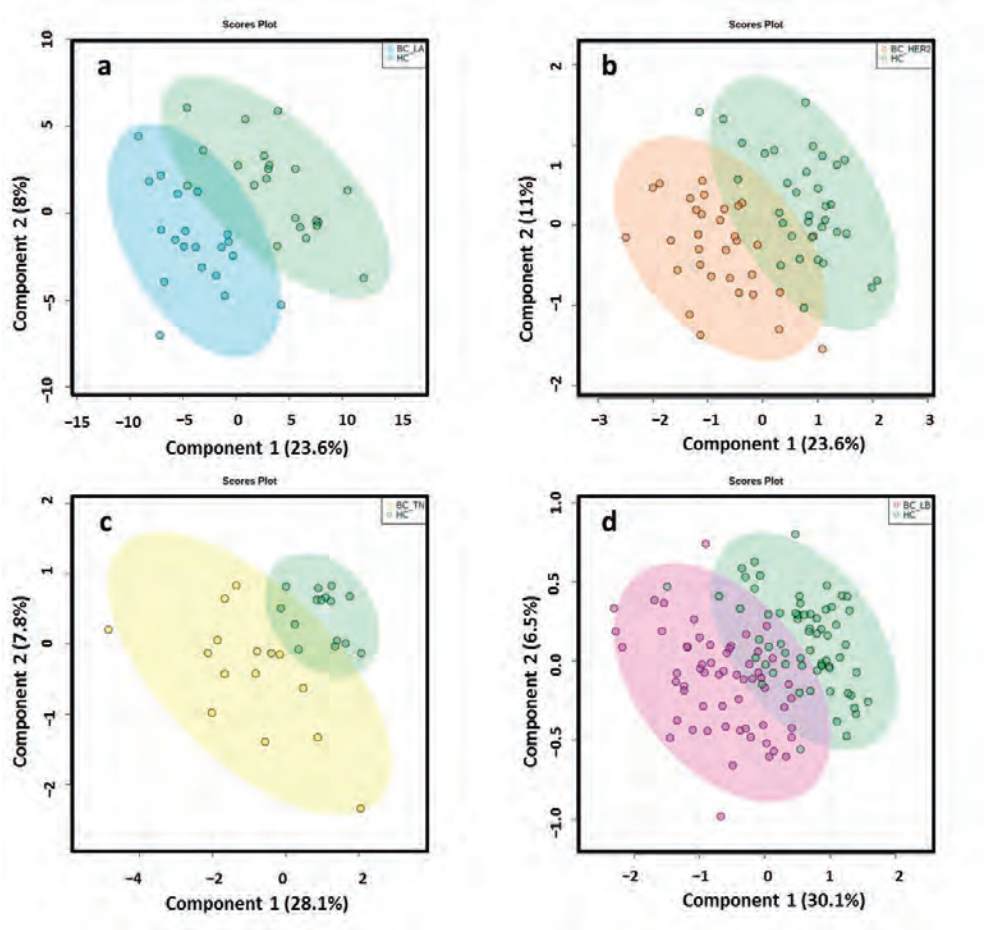


FIGURE S2. 2D score plots of the supervised PLS-DA of HC group (green) and LA_BC (light blue) (**a**), HER2_BC (orange) (**b**), TN_BC (yellow) (**c**) and LB_BC (pink) (**d**) patients by ESI+ determined a notably separation between BC molecular subtypes and matched controls.

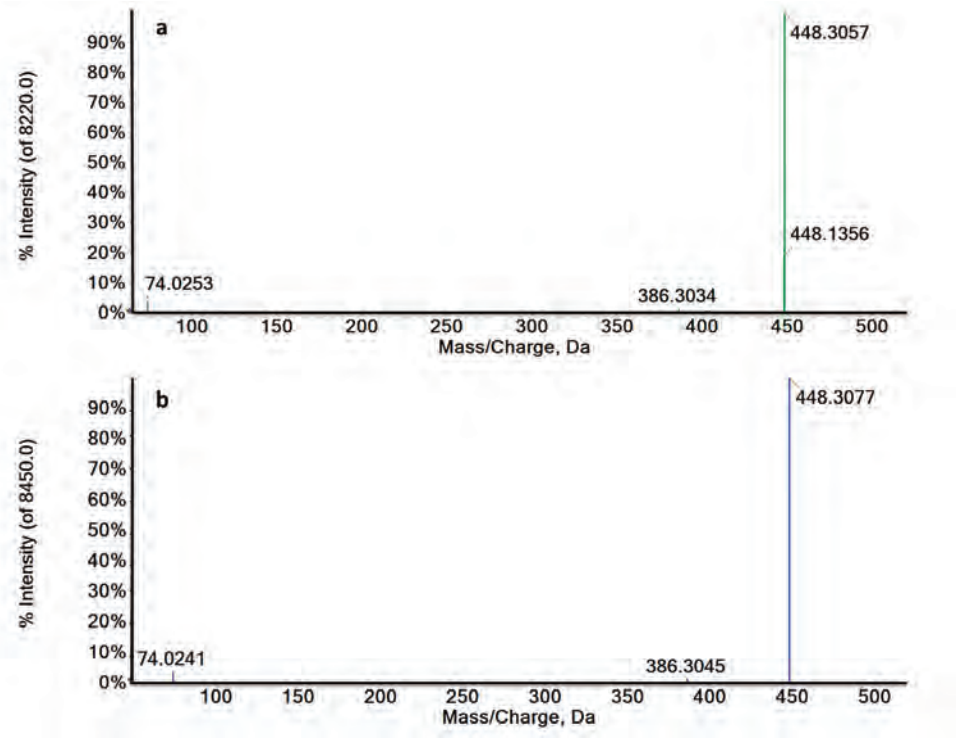


FIGURE S3. Characteristic MS/MS spectra of m/z 448.3066 in a biological sample (green) (**a**) and the glycoursodeoxycholic acid (GUDCA) standard (blue) (**b**) at 3.24 min. MS/MS spectra revealed the characteristic fragmentation pattern of GUDCA in ESI-.

Chapter 2

Chapter 2: Original Research Article.

Predicting dynamic response to neoadjuvant chemotherapy in breast cancer: a novel metabolomics approach.

Caridad Díaz, **Carmen González-Olmedo**, Leticia Díaz-Beltrán, José Camacho, Patricia Mena García, Ariadna Martín-Blázquez, Mónica Fernández-Navarro, Ana Laura Ortega-Granados, Fernando Gálvez-Montosa, Juan Antonio Marchal, Francisca Vicente, José Pérez del Palacio and Pedro Sánchez-Rovira

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Abstract

Neoadjuvant chemotherapy (NACT) outcomes vary according to breast cancer (BC) subtype. Since pathologic complete response is one of the most important target endpoints of NACT, further investigation of NACT out- comes in BC is crucial. Thus, identifying sensitive and specific predictors of treatment response for each phenotype would enable early detection of chemoresistance and residual disease, decreasing exposures to ineffective therapies and enhancing overall survival rates. We used liquid chromatography high-resolution mass spectrometry (LC-HRMS)-based untargeted metabolomics to detect molecular changes in plasma of three different BC subtypes following the same NACT regimen, with the aim of searching for potential predictors of response. The metabolomics data set was analysed by combining univariate and multivariate statistical strategies. By using ASCA, we were able to deter- mine the prognostic value of potential biomarker candidates of response to NACT in the triple-negative (TN) subtype. Higher concentrations of docosahexaenoic acid and secondary bile acids were found at basal and presurgery samples, respectively, in the responders group. In addition, the glycohyocholic and glycodeoxycholic acids were able to classify TN patients according to response to treatment and overall survival with an area under the curve model > 0.77. In relation to luminal B (LB) and HER2+ subjects, it should be noted that significant differences were related to time and individual factors.

Specifically, tryptophan was identified to be decreased over time in HER2+ patients, whereas LysoPE (22:6) appeared to be increased, but could not be associated with response to NACT. Therefore, the combination of untargeted-based metabolomics along with longitudinal statistical approaches may represent a very useful tool for the improvement of treatment and in administering a more personalized BC follow-up in the clinical practice.

Keywords: ASCA; breast cancer; LC-HRMS; neoadjuvant chemotherapy; personalized medicine; treatment response

Abbreviations

AcN, acetonitrile; ASCA, ANOVA–simultaneous component analysis; AUC, area under the curve; BC, breast cancer; BMI, body mass index; CCs, cancer cells; DHA, docosahexaenoic acid; ER, estrogen receptors; FC, fold change; FDR, false discovery rate; FISH, fluorescent in situ hybridization; GDCA, glycodeoxycholic acid; GHCA, glycohyocholic acid; HER2, human epidermal growth factor 2; IDA, information dependent acquisition; Kyn, kynurenine; LB, luminal B; LC-HRMS, liquid chromatography high-resolution mass spectrometry; MP, Miller and Payne; MVA, multivariate analysis; NACT, neoadjuvant chemotherapy; NR, nonresponders; PCA, principal component analysis; pCR, pathological complete response; PL, phospholipids; PR, progesterone receptors; QC, quality control; R, responders; ROC, receiver-operating characteristic; RP, reverse phase; RSD, relative standard deviation; SVM-linear, linear kernel support vector machine; t1, time 1; t2, time 2; t3, time 3; TN, triple negative; TNM, tumour nodes metastasis; TOF, time of flight; Trp, tryptophan; UVA, univariate analysis.

1. Introduction

Breast cancer (BC) incidence continues rising, being the leading cause of cancer death in women in the last Global Cancer Statistics 2020 [1]. Resistance to chemotherapeutic drugs is still the main obstacle for any cancer treatment. Some cancer cells (CCs) have innate chemotherapy resistance while others acquire it during exposure. Thus, pathological nonresponse to the chemo agents facilitates tumour cell survival and uncontrolled proliferation or metastasis after treatment administration [2–4]. Nowadays, undergoing surgery after a successive combination of drugs is considered the gold standard for assessing tumour response [5,6]. However, not all BC patients benefit from the neoadjuvant chemotherapy (NACT) setting and, therefore, it is critical to differentiate between the subjects that will respond positively and those who will not, in order to choose alternative and more effective therapies. Regarding NACT efficacy, recent studies tackle the relationship between BC phenotypes and treatment outcomes [7–9], revealing pathological complete response (pCR) as a surrogate biomarker of response and survival [10,11]. Nevertheless, this procedure is invasive and time-consuming. Thus, faster, less invasive and more sensitive tools are required in order to detect useful molecu- lar and/or clinical predictors of pCR [12,13]. On this point, metabolomics has quickly risen up as a novel approach in the cancer biomarker field for overcoming the current limitations of standard diagnostic and prognostic techniques [14,15]. This expanding research area, combined with high-throughput screening technologies, may help to unravel the subjacent molecular factors conferring true chemosensitivity to tumour recurrence, yet unknown. Indeed, it appears as the -omics science that better reflects the complex interactions from the genome expression to the phenotypic variations. Common metabolites directly or indirectly involved in the biology of cancer may serve as disease evaluators in group of patients. Several studies have already been conducted to explore the possibility of using panels of metabolites as biomarkers for early diagnosis and tumour characterization [16–22]. The abnormally accumulated metabolites derived from disrupted cancer metabolic pathways are newly described as oncometabolites, for example, D2-hydroxyglutarate has an important function in prognosis and diagnosis of breast cancer and leukemia patients [23–25]. Thus, although

detection of metabolic markers with an important role in oncological processes is appearing, research focused on finding discriminant biomarkers of NACT response in BC, and therefore, clinical outcome prognosis, is still sparse [12,26–28]. Notably, the development of metabolic fingerprinting to find a molecular pattern that might predict chemoresistance depending on the molecular BC subtype would support the evidence for its use in the clinical practice. Large-scale data sets resulting from the untargeted metabolomics approach, in combination with other factors, such as time, are becoming increasingly intricate to analyse, and the use of traditional biostatistical methods cannot be applied straightforwardly to extract clear and definite results. Hence, the incorporation of advanced methods such as ANOVA–simultaneous component analysis (ASCA) has become crucial for understanding the complexity and heterogeneity of biological information. ASCA is a direct generalization of the analysis of variance for univariate data applied to the multivariate case [29,30]. In consequence, longitudinal intervention studies over time, combined with untargeted metabolomics, may arise as an essential type of experimental approach in BC clinical research for discovering highly accurate markers or proven targets for tailored therapeutic treatments, detected in plasma of individuals with this disease [12,30,31]. However, to date, the definition of best practices for the analysis and interpretation of longitudinal metabolomics data is still a matter of research [32]. With this aim in view, here we explore whether untargeted metabolomics is able to determine molecular profiles of prediction to NACT response in a follow-up of 92 BC patients with different phenotypes, integrating univariate analysis and ASCA. Grounded in a liquid chromatography–high-resolution mass spectrometry (LC-HRMS) platform-based metabolomics analysis, plasma samples were studied at three differ- ent time points. Therefore, we propose and test the notion that metabolic fingerprinting in a longitudinal study may characterize potential clinical biomarkers and provide new insights into the response to a partic- ular treatment according to different BC phenotypes.

2. Materials and methods

2.1. *Participants and ethics*

A total of 92 BC female patients were enrolled in our study at the Medical Oncology Unit of the University

Hospital of Jaén (Spain), in order to detect metabolomics changes associated with the efficiency of NACT. BC was divided into different subtypes by immunohistochemical and gene expression testing of the human epidermal growth factor 2 (HER2), hormone receptors of estrogen (ER) and progesterone (PR) and Ki-67. Specifically, luminal B (LB) patients were diagnosed with HER2 negative (HER2?) and ER+ with a positive Ki-67 finding defined as >15%. Patients who neither expressed hormone receptors (PR-, ER-) nor overexpressed HER2 were considered as triple-negative (TN) patients; and, finally, patients overexpressing human epidermal growth factor 2 were diagnosed as HER2-positive (HER2+) patients. Concretely, the evaluation of HER2 was done following the ASCO/CAP 2018 guidelines, by immunohistochemistry (IHC) staining and by fluorescent in situ hybridization (FISH): scores 0 and 1+ were considered negative, 3+ was considered HER2+, while a dual-probe FISH was carried out for 2+ scores of the same specimen, or additional IHC or FISH for a new specimen [33]. Cancer stage was classified according to the 2010 Tumour Nodes Metastasis (TNM) system [34]. The main characteristics of these subjects are summarized in Table 1. Evaluation of potential confounding variables was performed using the Shapiro–Wilk normality test and, subsequently, Levene's test for the equality of variances between responders (R) and nonresponders (NR), depending on age and body mass index (BMI) for each BC phenotype. U-Mann and Whitney Wilcoxon test was performed for the data that presented a nonparametric distribution. The association analysis of the menopausal status with treatment response was checked with the Pearson chi-square test. In addition, this statistical test allowed to evaluate whether the overall survival was related to the outcome to NACT in the TN phenotype. To know the intensity of the association, the Cramer's V test was used. Venous blood samples were collected under fasting conditions at three different time points: before the first therapy cure with anthracyclines (basal); once they received taxol (presurgery); and after they went into surgery (postsurgery). The blood collection campaign was conducted over a timeframe period of eight years. Every patient provided a signed informed consent for participation prior to basal sample extraction. This study was approved by the institutional review board of the Clinical Research Ethics Committee of

Jaén. All clinical investigations were conducted under Helsinki Declaration guidelines and International Conference on Harmonization-Good Clinical Practices (ICH-GCP).

2.2. *Neoadjuvant chemotherapy*

All patients received NACT consisting on bi-weekly dose-dense cycles of anthracyclines (epirubicin 90 mg·m-2 and cyclophosphamide 600 mg·m-2) followed by 12 weekly cycles of taxanes (paclitaxel 80 mg·m-2). Cycletimeadministrationcouldbemodified according to the Common Toxicity Criteria (CTC v5.0). Anti-HER2 therapy (trastuzumab and pertuzumab) was added in HER2-positive BC patients [35].

2.3. *Response evaluation*

Samples obtained during surgery underwent a histopathological analysis in order to determine the postsurgery Miller and Payne (MP) grade [36]. Patho- logical complete response was assessed from the five- step scale based on reduction in malignant cellularity after treatment. Following these criteria, MP5 is considered as pCR with no malignant cells; MP4 is a very good response with <10% of malignant cells remaining, near the pCR; in MP3 the significant loss of tumour cells is too variable between 30 and 90%; MP2 shows a reduction of tumour cells < 30%, and MP1 has no reduction in malignant cells. Herein, we defined a response group (MP grades 4–5) and a nonresponse group (MP grades 1–3) according to the prognostic potential of the MP grading system [37–40].

2.4. *Sample collection and preparation*

Blood samples were extracted using standard venipuncture processes and collected in EDTA tubes. Plasma was obtained by centrifugation at 1400xg for 10 min at 4 °C. All samples were kept at -80 °C until the analysis was made.

2.5. *Metabolite extraction*

An aliquot of 75 µL of plasma was mixed with 600 µL of cold acetonitrile (AcN) containing the analytical standard (roxithromycin). Then, it was shacked for 2 min at 2500 r.p.m. All the samples were centrifugated at 21 982 g for 10 min at 4 °C. Collected supernatants were transferred into new vials for evaporation and reconstituted in 210 µL of water/acetonitrile (50/ 50) with 0.1% formic acid.

2.6. *Liquid chromatography coupled to high- resolution mass spectrometry analysis*

The analytical separation was achieved using liquid chromatography (LC) with an Agilent series 1290 (Agilent Technologies, Santa Clara, CA, USA) in

reverse phase mode (RP) using Atlantis T3 C18 column (2.1 mm 9 150 mm, 3 µm) from Waters (Water Corporation, Milford, MA, USA). The mobile phase A consisted of water/acetonitrile (90/10) and 0.1% formic acid. The mobile phase B consisted of acetonitrile/water (90/ 10) and 0.1% formic acid. The chromatographic run was 20 min. The gradient elution consisted of 0.0– 0.5 min 1% eluent B; 0.5–11.0 min 99% eluent B, 11.0– 15.5 min 99% eluent B and 15.5–15.6 min 1% eluent B. and 15.6–20.0 min 1% eluent B. Mass detection was performed using Triple TOF 5600 quadrupole time-of-flight mass spectrometer (SCIEX, Concord, ON, Canada). The mass spectrometer was operated using electrospray ionization in positive mode and an information-dependent acquisition (IDA) method, and the eight most intense signals were fragmented. The exact mass calibration was automatically performed every six injections. Three different LC-HRMS analyses were made in positive ionization mode, in order to detect molecular differences within the subtypes of BC (LB, TN, and HER2+) depending on their response to neoadjuvant chemotherapy after surgery. A total of 144 samples were analysed for LB phenotype, 69 samples for HER2, and 63 for TN, and blanks and quality control (QC) samples were also used in each metabolomics analysis.

2.7. *Data set creation*

Peak View software (version 1.1.2; AB SCIEX) was used to evaluate the retention time and mass-to-charge (m/z) variability of three peaks over at different time points and m/z values. This allowed us to determine the ranges for the alignment. Peak detection, alignment, and data filtering were achieved using Marker view software (version 1.2.1; SCIEX). Collection parameters were set as follows: retention time window 0.10 min, noise threshold 70 cps, and mass tolerance 5.0 ppm. Additionally, only monoisotopic peaks were considered in order to decrease mass redundancy and improve true molecular features selection. Blank samples were used to remove contaminants and signals provided by solvents.

2.8. *Analytical method validation and normalization*

Principal component analysis (PCA) was used to assess the quality of the analytical system performance. QC samples clustering representation in this multivariate analysis (MVA) were useful to validate the analytical system's stability. The relative standard deviation (RSD) was calculated for all the features in the QC samples after the data set

creation. Variables with variability higher than 30% were discarded (Table S1). Data normalization by a QC reference sample (probabilistic quotient normalization), logarithmic transformation, and autoscaling were performed in order to obtain a Gaussian-type distribution.

2.9. *Univariate statistical analysis*

Two different statistical approaches were used in this work in order to determine the broadest range of metabolites that might differ between the groups of study when comparing them at a specific point or over time. Univariate statistical analysis was performed using the Student's t-test, which enabled assessing differences between R and NR patients of the TN, LB, and HER2+ molecular subtypes. Univariate statistical analysis (UVA) was applied at three different time points independently: before the first therapy cure with anthracyclines (basal, time 1), once the patients received treatment with taxol (presurgery, time 2), and after the breast-conserving surgery (postsurgery, time 3). A P-value < 0.05 was determined as the cutoff threshold with a Benjamini–Hochberg False Discovery Rate post hoc correction (FDR < 0.1). This analysis was carried out using Metaboanalyst 4.0 [41]. Eventually, discriminant metabolites selection was also based on their fold change (FC > 1.3).

2.10. *Multivariate statistical analysis (ASCA)*

The metabolomics data set shows a multilevel structure with multiple types of variation: the metabolic dynamism within the individual, the statistical differences between the subjects, and their combination. To deal with such complex information, we used ASCA, which factorizes the original data set into subsets describing the variation between response and nonresponse, the variation in time and their interaction [29]. To deal with unbalanced data, we used the ASCA+ version [42]. We tested for significance using exact and approximated permutation tests for the main factors (response, time, and patient) and interactions, respectively [43]. Significant factorized data were visualized using PCA. From statistically significant factors, we derived a list of relevant metabolites, ordered by the sum of squares of the difference between R and NR. All computations were done with the MEDA Toolbox for Matlab [44].

2.11. *Identification of differential metabolites*

Peak View software was used to establish a molecular formula according

to the experimental exact mass, fragmentation spectrum, and isotope pattern. The identification of molecular components was achieved through comparative searches of available mass spectra using several databases such as Metlin, the Human Metabolome DataBase, Lipid Maps, NIST 2012, and mass bank mainly. Additional MS/MS analysis was carried out when necessary. Also, we used the information at the experimental conditions, ionization behaviour, and/or retention time in order to assign a tentative identification. In those cases in which it was not possible to assign it, scientific literature was con- sulted. Finally, mass error of all the candidates was equal or lower than 5 ppm.

2.12. *Biomarker evaluation*

The area under the receiver-operating characteristic curves was used to test the clinical relevance of candidate metabolites with corrected P-value < 0.05. Assessment of the classifier performance was carried out with linear kernel support vector machine (SVM-linear) and random forest models, using the Biomarker Analysis provided by Metaboanalyst.

3. Results

3.1. *Patient's characteristics*

Regarding the patients eligible for analysis, a number of 55 BC subjects out of 92 were classified as R to NACT, in contrast to 37 NR subjects. Considering the BC phenotype, 16 out of 23 human epidermal growth factor 2 positive (HER2+) patients responded (69.56%) and 7 out of 23 showed a nonresponse according to the MP grading system (30.44%). In the case of luminal B (LB) molecular subtype, 26 out of 48 responded (54.16%), while 22 out of 48 did not show treatment response (45.84%). Last, the TN phenotype showed 13 out of 21 patients with response to NACT (61.9%) and 8 out of 21 patients with nonresponse (38.1%). Assessment of the confounding vari- ables, body mass index, age, and menopausal status showed no significant differences in relation to response when the corresponding t-test was applied for each BC phenotype (Table S2). In the case of the survival analysis, a moderate association with outcome to NACT was obtained for the TN phenotype (Table S3).

3.2. *Metabolomic profiling from univariate analysis*

Significant identified metabolites, selected according to P-value corrected by FDR < 0.1 and FC > 1.3, are shown in

Table 2. Other altered metabolites with P-value < 0.05 (FDR > 0.1) and FC < 1.3 are identified in Table S4, and those not able to be identified are listed in Table S5. However, a lot of spectral information, and the availability of analytical standards, is still needed. In this study, tentative identities were classified at level 2 as reported by the Schymansky classification [45], validated by their MS/MS spectra (Fig. S1) after several searches in diverse databases (Metlin, Human Metabolome Database, Lipid Maps, NIST 2012 mass spectral library, or mass bank). Specifically, in the TN molecular subtype, a total of four signals were selected as significant at time 1 (t1) and time 2 (t2) but none at time 3 (t3). Candidate metabolites identified as cis-4,7,10,13,16,19- Docosahexaenoic acid and LysoPE (18:1) were found at t1. At t2, 2 significant metabolites were tentatively identified as 2 bile acids (glycodeoxycholic and glycohyocholic acid). The analysis for the LB phenotype showed three significant signals at t1 but none at t2 or t3. Candidate metabolites at t1 were tentatively identified as LysoPE (18:2), LysoPC (16:0), and tridecanoyl carnitine (Table 2). Signals shown in Table S4, corresponding to 23 different m/z in TN, 2 in LB, and 1 in HER2+, would be expected to have significant values in larger and balanced cohorts. At the three time points, some m/z were detected as the same tentative identification with different adducts. There were 12 signals that could not be identified for the TN molecular subtype, 2 m/z for the LB, and no altered signals were detected at basal or at postsurgery levels, when comparing the response in HER2+ patients, as shown in Table S5.

3.3. *Metabolic profile from multivariate analysis*

ANOVA–simultaneous component analysis (ASCA) provided the statistically significant factors (Table 3) from which we drew up a list of associated relevant metabolites (Table 4). In our multivariate analysis, time and patient factors were statistically significant for the HER2+ and LB molecular subtypes (Figs S2 and S3), while response and patient factors were statistically significant for the TN (Fig. S4). To interpret the time factor, we used ASCA score and loading plots, that is, the PCA plots of the data factorized by ASCA. This is shown in Fig. 1 (A and B, respectively). Score plots in Fig. 1 illustrate samples of HER2+ (A1) and LB (A2) subjects corresponding to different time points (t1 in red, t2 in blue, and t3 in green), which can be interpreted in combination with the loading plots in Fig. 1B1, B2), where only most relevant signals are labeled (see also Table 4 and

Table S6). Score plots include data ellipses at 0.05 significance level, although we did not use confidence levels, due to unbalanced data [46]. For instance, the metabolite 526.2915 [LysoPE (22:6/0:0)] at the upper right corner of Fig. 1 (B1) is correlated with the green scores in Fig. 1 (A1), which represent HER2+ postsurgery measurements. Also, metabolite 188.07 (tryptophan) is right in the opposite direction. These signals can be identified as the ones that change the most after surgery (Fig. S5): 526.2915 and 188.07 present a generalized higher and lower value, respectively, after surgery. The same can be inferred in Fig. 1 (A2 and B2) but for 247.1443 (tryptophan betaine) and m/z 452.3214, with lower and higher values, respectively, after surgery in LB patients (Fig. S6). Lastly, ASCA of TN showed significance in response factor. Following the same approach using one PCA score/loading plots, we selected metabolites 448.3047 (glycohyocholic acid), 450.32 (glycodeoxycholic acid), and 572.3699 [LysoPC (22:4)] as the most differential between R and NR (Fig. 2). Metabolites 448.3047 and 450.32 in R tend to be generally higher than in NR, observation that agrees with significant results after FDR correction in Table 2. Otherwise, m/z 572.3699 tends to be mostly higher in NR than in R.

3.4. *Candidate biomarker evaluation*

Significant metabolites were checked for their diagnostic potential with a multivariate receiver-operating characteristic (ROC) analysis. The area under curve (AUC) obtained for the 448.3047 (glycohyocholic acid) and 450.32 (glycodeoxycholic acid) in combination (0.946, 95% CI: 0.875–1) indicates how well these candidate biomarkers distinguish between our groups of study (Fig. S7a). Based on this model, only 3 out of 13 TN R were wrongly classified as NR, whereas all TN NR were correctly classified (Fig. S7b). Finally, the prognostic power of these bile acids in combination was tested with an AUC performance of 0.777 (95% CI: 0.541–1). The model indicates a good classification of patient subgroups with survival expectancy of more than 2 years (Fig. S8). However, an independent cohort would be required to validate the prognostic power of these promising candidates.

4. Discussion

Neoadjuvant chemotherapy constitutes a standard treatment for the management of BC with several benefits, although there are yet unresolved questions that concern a

high percentage of women that suffer from this heterogeneous disease. Some challenges faced in the clinical practice that affect the efficiency of this systemic treatment are the lack of early predictors of response, as well as the establishment of the pCR prognostic value. Stratification of BC patients according to underlying molecular factors that confer NACT resistance would be a great step toward personalized medicine. In this work, the untargeted LC-HRMS-based meta- bolomics approach used enables the detection of different small molecules that may be involved in the behaviour of three BC phenotypes against NACT. For this purpose, two statistical analyses—univariate and multivariate—were carried out. As an outcome of UVA, alteration of the metabolome in LB and HER2+ subjects only appeared at basal or presurgery levels, while the TN molecular subtype showed the highest variability in response to treatment at all time points. The use of ASCA is of great relevance for better under- standing the greater metabolome impact over time and to properly select the biomarkers that might be potential predictors of the chemotherapy response associated with the phenotype. This prominent multivariate method allows analyzing complex metabolomics data sets with simultaneously measured covariates considering the experimental design [26,29,31,32,47].

Thus, our longitudinal study analysed the influence that factors such as the individual itself, response to treatment, time, and their interaction, may have on the dynamic metabolome of 92 BC patients. Clearly, the significance of the patient factor, obtained in the ASCA results of all the molecular subtypes studied, reflects the need for a tailored follow-up in BC [14,16,48–50]. On the contrary, the significance of the time factor (with nonsignificant response) should be interpreted as a homogeneous change in the metabolome of the HER2+ and LB patients after treatment, regardless they are classified as R or NR. Regarding the outcomes obtained in the LB and HER2+ analyses, we highlight the reprogramming of cellular metabolism as a hallmark of BC. Herein, in the UVA of LB molecular subtype, lysophospholipids were increased at basal levels of responder patients while carnitines appeared as decreased. So that, we suggest that phospholipids (PL) and carnitines may be considered as useful targets for cancer therapy and as BC biomarkers, as described in previous observations [51–55]. In addition, alteration of amino acids was also found. The lower tryptophan betaine

levels detected postsurgery in LB patients could give insights into its potential role in the phenotype behaviour [56,57]. Like- wise, research on larger cohorts would help to validate whether the increased concentration of LysoPE (22:6) at t3 in HER2+ could be a decisive biomarker of residual disease. It should be especially noted the dysregulation of the tryptophan (Trp) metabolism in the HER2+ molecular subtype. Specifically postsurgery, a significant decrease in Trp plasmatic concentrations was observed, as previously reported in serum and plasma of BC patients [58–60]. In this regard, Trp catabolism dysregulation is known to indirectly contribute to cancer progression by the kynurenine (Kyn) pathway [61–63], although no associations with response or sen- sitivity to chemotherapy were observed in previous studies, which coincides with our observations [22,64]. In this line, further investigating the metabolome alteration related to treatment response is still needed to better understand the behaviour of the LB and HER2+ molecular subtypes in response to NACT. Unlike the molecular subtypes LB and HER2+, this approach notably differentiates TN patients that respond to NACT and those who do not. From the ASCA results, given that treatment response is statistically significant, but time is not, we could conclude that there is a difference between R and NR sustained across the three time points in the TN phenotype. In particular, this metabolic difference may relate to treatment effectiveness and, if validated in future analyses, to treatment selection. It should be pointed out that, while response over time was not found to be significant, the effect size of this interaction in TN doubles the one obtained in HER2+ and triples the one in LB. Hence, it may be of great interest to further investigate the interaction between time and response in order to determine the prognostic applicability of the candidate biomarkers proposed for treatment efficiency prediction in BC phenotypes. In our findings, both statistical strategies supplement the results in the TN analyses in relation to response. At basal plasma levels, the docosahexaenoic acid (DHA) concentrations are significantly higher in TN R than in NR. The dysregulation of DHA is of great importance since it has been shown to be involved in cell signaling, leading to the reduction in cancer cell viability and proliferation both in vivo and in vitro [65–67]. Indeed, DHA supplementation in combination with NACT is being explored in the interventional study NCT03831178

(ClinicalTrial.gov). This observation clearly supports that the measurement of this fatty acid may be considered as a biomarker for an early detection of chemoresistance at the diagnosis of the disease. Furthermore, two bile acids (BAs), glycodeoxycholic acid (GDCA) and glycohyocholic acid (GHCA), were significant in TN presurgery R. The predictive biomarker model with these candidates was evaluated with a multivariate ROC analysis, which showed excellent performance since all the TN NR were correctly classified at t2. Their prognostic power was also assessed, obtaining a good classification between patients with survival expectancy of more than 2 years. Additionally, the role of bile acids in carcinogenesis is increasingly being studied. Thus, paradoxical functions of these bioactive molecules have been observed depending on the tissue affected and BA receptor activation (FXRa, TGR5) in cancer [68,69]. However, not many studies have been able to shed light on how their dysregulation may affect BC development and behaviour [70–72]. Nonetheless, we observed that plasma levels of conjugated secondary bile acids, GDCA and GHCA, are higher in TN R when compared to NR at pre- and postsurgery time points. Nevertheless, GDCA and GHCA were not found at basal levels, which may be the reason why interaction between response and time factors is not significant in our ASCA outcome. Being secondary BAs directly related to the intestinal microbiota, the study of its potential role in the behaviour of BC should be investigated to a greater extent [73]. In this regard, different clinical trials gather more information about the effect of chemotherapy on gut bacteria and the affection that gut microbiota might have on the NACT-induced immunosurveillance in TN patients (NCT02370277 and NCT03586297, ClinicalTrial. gov). Notwithstanding these promising results, further analysis would be needed in order to better understand the effects of medical interventions on the microbiome, as well as the relevance of independent bile acids as constituents of the BC tumour microenvironment. Thus, a good noninvasive prognostic strategy for the aggressive TN phenotype is suggested in this study by detection of BAs in plasma using LC-HRMS. On the contrary, variations in the composition of plasma phospholipids compared with the treatment response appeared at different time points in our analyses. Specifically, the increased concentration of phos- phatidylethanolamines [LysoPE (18:1) and (18:2)] at t1 in TN NR

is supported by the increased demand for PE in BC cells under metabolic stress [74,75]. Other- wise, the phosphatidylcholine LysoPC (22:4) was deter- mined from the ASCA results as a significantly increased metabolite for nonresponder TN patients. In line with our findings, it could be inferred that evolving knowledge of these candidate metabolites' behaviour in the BC process would improve the stratification of the BC patients for better therapy decision- making.

5. Conclusion

In conclusion, our work presents dynamic metabolic changes at the individual level in all the phenotypic analyses carried out during disease and treatment. The complete set of small molecules within a biological sample can be influenced by pathological processes, treatment, as well as the microbiome, thus affecting its consequent relationship with the metabolome. The high level of individual variability makes it difficult to find a single metabolic signature to classify our groups of patients. Nevertheless, the results obtained in TN sub- type between R and NR may point toward new approaches in the fight against cancer. A larger sample size and number of balanced cohorts would help to corroborate and validate the findings reported in this work. Lastly, the combination of untargeted metabolomics and ASCA appears to be a highly valuable tool for deciphering the behaviour of BC treated with NACT and, thus, open up the possibility of an early modification of this therapy according to the future response to treatment, improving prognosis for these patients.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

CD, CG-O, and LD-B contributed to conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, and visualization. JC contributed to methodology, software, formal analysis, data curation, writing—original draft preparation, and writing—review and editing. PMG con- tributed to methodology. AM-B contributed to methodology. MF-N contributed to investigation and resources. ALO-G contributed to resources. FG-M contributed to resources. JAM contributed to writing —review and editing and visualization. FV contributed to resources, writing—review and editing, and supervision. JPdP contributed to writing—review and editing and visualization. PS-R contributed to conceptualization, writing—review and editing, visualization, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data accessibility

The data analysed and generated in our work are avail- able upon request from the corresponding author. The data are not publicly available due to patient confidentiality, participant privacy, and ethical restrictions.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Experimental MS/MS spectrum obtained in our analysis for the secondary bile acids a) gly-codeoxycholic acid and b) glycohyocholic acid.

Fig. S2. Reference distribution for HER2+ significance testing with resampling in ANOVA–simultaneous com- ponent analysis: time factor (left, P-value = 0.002) and patient factor (right, P-value = 0.013).

Fig. S3. Reference distribution for LB significance testing with resampling in ANOVA–simultaneous component analysis: time factor (left, P-value = 0.001) and patient factor (right, P-value = 0.001).

Fig. S4. Reference distribution for TN significance testing with resampling in ANOVA–simultaneous com- ponent analysis: time factor (left, P-value = 0.031) and patient factor (right, P-value = 0.002).

Fig. S5. Differential expression of 526.2915 [LysoPE (22:6) and 188.07 (tryptophan)] according to the pathological response group (R,

responders; NR, non- responders) in HER2+ at time 1 (t1, basal), time 2 (t2, presurgery) and time 3 (t3, postsurgery) detected using ANOVA–simultaneous component analysis.

Fig. S6. Differential expression of 247.1443 (tryptophan betaine) and 452.3214 (not identified) according to the pathological response group (R, responders; NR, non- responders) in LB at time 1 (t1, basal level), time 2 (t2, presurgery), and time 3 (t3, postsurgery) detected using ANOVA–simultaneous component analysis.

Fig. S7. ROC curve plot for the model obtained from combination of the significant candidates identified in TN breast cancer molecular subtype [448.3047 (glycohy- ocholic acid) and 450.32 (glycodeoxycholic acid)]: (a) ROC curve plot was created from the averaged results of 100 cross-validations; (b) as an outcome the model provides with the distinction of all nonresponders TN patients and 3 out of 13 responders misclassified.

Fig. S8. ROC curve plot for the prognostic model obtained from combination of the significant candi- dates identified in TN breast cancer molecular subtype [448.3047 (glycohyocholic acid) and 450.32 (gly- codeoxycholic acid)]: (a) ROC curve plot was created from the averaged results of 100 cross-validations; (b) as an outcome the model provides with the distinction of 2 out of 7 patients from the nonsurvival group and 5 out of 14 survivors misclassified.

Table S1. Selected variables from the untargeted meta- bolomics analysis for each breast cancer molecular subtype.
Table S2. Values of significance for normality and homoscedasticity tests of the continuous variables: age and BMI; and for association tests of the categorical variable: menopausal status.
Table S3. Association tests of the survival and treat- ment response data in the TN phenotype.

Table S4. Tentative identification of the differential metabolites between response groups in UVA.

Table S5. Differential signals between response groups without a tentative identification according to the breast cancer molecular subtype detected in UVA.

Table S6. Differential signals without a tentative identification detected in ASCA according to time and patient factors.

T A B L E 1. Pathological and clinical characteristics of the subjects of study. N, nodes; P.R, pathologic response; post, postmenopause; pre, premenopause; T, tumour.

BC molecular subtypes	LB		TN		HER2+	
Subjects	48		21		23	
P.R	R	NR	R	NR	R	NR
MP grading system	25	23	13	8	16	7
MP1	-	1	-	2	-	1
MP2	-	3	-	2	-	1
MP3	-	19	-	4	-	5
MP4	14	-	5	-	5	-
MP5	11	-	8	-	11	-
Age (range)	49 (33-62)	52 (34-76)	53 (31-76)	48 (33-58)	48 (35-63)	58 (34-70)
BMI (Kg·m-2)	26 (19.3-38.7)	27 (20.1-36.5)	30 (22.1-41.7)	32 (22.1-38.9)	28 (19.6-40.6)	26 (19.0-32.5)
Menopausal Status						
pre	16	12	7	6	10	2
post	9	11	6	2	6	5
HER2+ Status	Negative		Negative		Positive	
PR Status	Neg/Pos		Negative		Neg/Pos	
ER Status	Positive		Negative		Neg/Pos	
Ki-67	> 15%		-		-	
Stage						
T1	5	2	0	0	0	0
T2	18	16	14	5	14	5
T3-4	2	5	2	2	2	2
N+	10	10	8	3	8	3
N-	14	13	6	4	6	4

TABLE 2. Tentative identification of the significant metabolites detected in the comparison between response groups in UVA. Δppm, mass error; FC, fold change > 1 indicates that the average normalized peak area ratio in R patients is larger than that in NR patients; RT, retention time; t1, before starting the therapy cure, basal level; t2, once the patients received taxol, presurgery; UVA, univariate analysis (Student's t-test).

Time point	BC molecular subtype	*m/z*	RT (min)	Molecular formula	Tentative identification	Δppm	Adduct	*P*–value (FDR)	FC
t1	TN	329.246	14.39	C22H32O2	cis-4,7,10,13,16,19-Docosahexaenoic acid	0.3	[M+H]	0.059	2.198
		502.287	11.59	C23H46NO7P	LysoPE(18:1/0:0)	3.2	[M+Na]	0.059	-1.351
t1	LB	358.295	8.11	C20H39NO4	Tridecanoyl carnitine	1.2	[M+H]	0.032	-1.742
		478.293	10.79	C23H44NO7P	LysoPE(18:2/0:0)	0.4	[M+H]	0.084	1.352
		518.323	10.17	C24H50NO7P	LysoPC(16:0/0:0)	0.2	[M+Na]	0.032	1.694
t2	TN	448.305	8.45	C26H43NO6	Glycohyocholic acid	-1.5	[M+H-H2O]	0.004	3.404
		450.320	9.19	C26H43NO5	Glycodeoxycholic acid	0.7	[M+H]	0.004	3.967

TABLE 3. Significant factors detected in ASCA.

BC molecular subtype	Factor	*P*–value
TN	Patient	0.0020
	Response	0.0310
HER2+	Patient	0.001
	Time	0.001
LB	Patient	0.013
	Time	0.002

TABLE 4. Tentative identification of the metabolites significatively detected in ASCA. Δppm, mass error; RT, retention time.

BC molecular subtype	*m/z*	RT (min)	Molecular formula	Tentative identification	Δppm	Adduct
TN	448.3047*	8.45	C26H43NO6	Glycohyocholic acid	-1.5	[M+H-H2O]
	450.3200*	9.19	C26H43NO5	Glycodeoxycholic acid	0.7	[M+H]
HER2+	572.3699	11.87	C30H54NO7P	LysoPC(22:4/0:0)	0.6	[M+H]
	188.0700	3.57	C11H12N2O2	Tryptophan	0.5	[M+H-NH3]
	454.2922	11.19	C21H44NO7P	LysoPE(16:0/0:0)	-0.9	[M+H]
	566.3175	10.54	C28H50NO7P	LysoPC(20:4/0:0)	-1.3	[M+Na]
	583.2567	8.39	C33H34N4O6	Biliverdin	-0.9	[M+H]
	526.2915	10.62	C27H44NO7P	LysoPE(22:6/0:0)	-1.7	[M+H]
	568.3416	10.68	C30H50NO7P	LysoPC(22:6/0:0)	-2.2	[M+H]
	590.322	10.69	C30H50NO7P		-2.7	[M+Na]
LB	247.1443	3.86	C14H18N2O2	Tryptophan-betaine	0.8	[M+H]
	342.2631	7.38	C19H35NO4	Dodecenoylcarnitine	-0.5	[M+H]
	363.2163	6.96	C21H30O5	Cortisol	0	[M+H]
	454.2935	11.36	C21H44NO7P	LysoPE(16:0/0:0)	0.2	[M+H]
	502.2921	10.5	C25H44NO7P	LysoPE(20:4/0:0)	-2	[M+H]

[a] m/z found also as significant in univariate analysis.

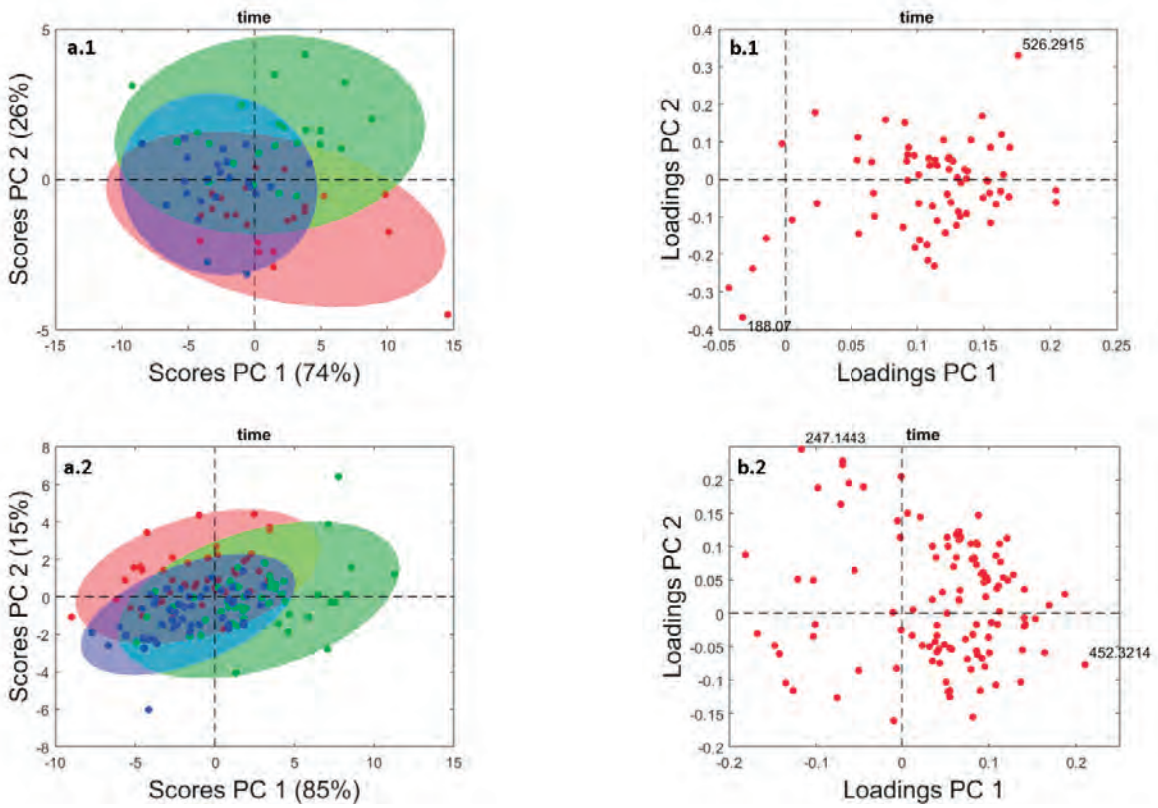
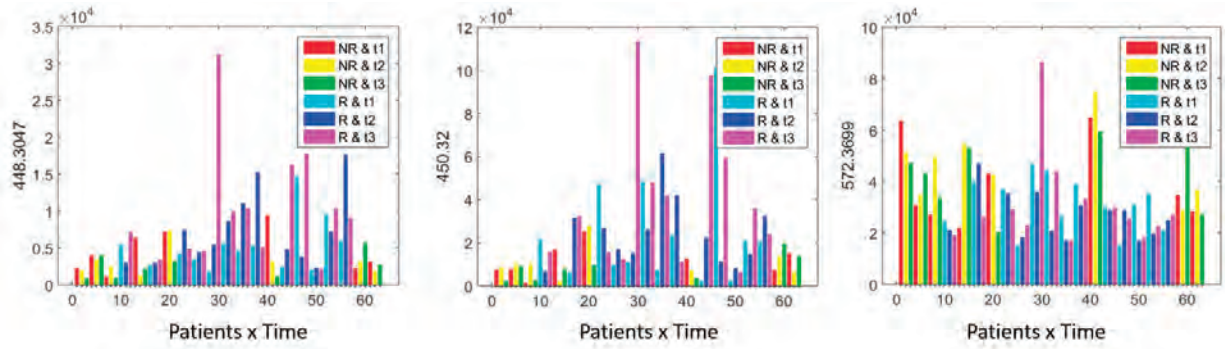


FIGURE 1. HER2+ and Luminal B phenotype longitudinal study using ANOVA–simultaneous component analysis (ASCA). The score plots represent the variation of the patient samples over time (basal, presurgery and postsurgery) in relation to the concentration of metabolites present in each of them, and the loading plots show the metabolites that are contributing to the significant differences over time in patients with luminal B and HER2+ phenotypes. (A1) 2D score plot of HER2+ patient samples over time. (A2) 2D score plot of Luminal B patient samples over time. (B1) The molecular ion at m/z 526.2915 [LysoPE (22:6/0:0)] and 188.07 (tryptophan) represent the metabolites most differential over time for the HER2+ phenotype. (B2) The molecular ion at m/z 247.1443 (tryptophan betaine) and 452.3214 represent the metabolites most differential over time for the luminal B phenotype. The red, blue, and green dots correspond to the basal, presurgery, and postsurgery time, respectively.



F I G U R E 2. Differential metabolites according to the pathological response to neoadjuvant chemotherapy in triple-negative breast cancer using phenotype ANOVA–simultaneous components analysis (ASCA). The molecular ions at m/z 448.3047 (glycohyocholic acid) and 450.32 (glycodeoxycholic acid) were found elevated in responders. The molecular ion at m/z 572.3699 [LysoPC (22:4)] appeared decreased in responders. R, responders; NR, nonresponders; t1, basal time; t2, presurgery; t3, postsurgery time.

SUPPLEMENTARY TABLE 1. Selected variables from the untargeted metabolomics analysis for each breast cancer molecular subtype.

BC molecular subtype	Total	Monoisotopics	Contaminant's filtering	Discarded (RSD>30%)
LB	2670	946	124	117
TN	2378	968	113	112
HER2+	2537	775	71	70

BC: breast cancer; LB: luminal B; TN: triple negative; HER2+: human epidermal growth factor receptor 2 positive; RSD: relative standard deviation

SUPPLEMENTARY TABLE 2. Values of significance for normality and homoscedasticity tests of the continuous variables: age and BMI; and for association tests of the categorical variable: menopausal status.

	BC Molecular Subtype	Shapiro-Wilk	Levene's Test	U Mann Whitney	Pearson Chi Square	Cramer's V
Age	TN	0.362	0.401	NA	NA	NA
	LB	0.495	0.111	NA	NA	NA
	HER2+	0.424	0.124	NA	NA	NA
BMI	TN	0.159	0.989	NA	NA	NA
	LB	0.016	NA	0.09	NA	NA
	HER2+	0.109	0.21	NA	NA	NA
Menopausal Status	TN	NA	NA	NA	0.112	NA
	LB	NA	NA	NA	0.281	NA
	HER2+	NA	NA	NA	0.134	NA

BC: breast cancer; TN: triple negative; LB: luminal B; HER2+: human epidermal growth factor receptor 2 positive; BMI: body mass index; NA: not applicable

SUPPLEMENTARY TABLE 3. Association tests of the survival and treatment response data in the TN phenotype.

	BC Molecular Subtype	TN	
Subjects		21	
P.R		**R**	**NR**
		13	8
Overall Survival	> 24 months	11	3
	exitus	2	5
(median)	follow up time, months	53	39
Association tests	**Pearson Chi Square**	0.026	
	Cramer's V	0.485	

BC: breast cancer**;** TN: triple negative; R: responders; NR: non-responders; P.R: pathologic response

SUPPLEMENTARY TABLE 4. Tentative identification of the differential metabolites between response groups in UVA.

Time point	BC molecular subtype	m/z	RT (min)	Molecular formula	Tentative identification	Δppm	Adduct	*p* – value*	FC
t1	LB	450.321	7.76	C26H43NO5	Glycodeoxycholic acid	-0.2	[M+H]	0.045	1.625
		478.293	10.79	C23H44NO7P	LysoPE(18:2/0:0)	0.4	[M+H]	0.002	1.352
t1	TN	480.306	11.76	C23H46NO7P	LysoPE(18:1/0:0)	0.3	[M+H]	0.009	1.37
		1039.67	10.83	C26H50NO7P	LysoPC(18:2/0:0)	2	[2M+H]	0.022	1.529
		478.294	10.64	C23H44NO7P	LysoPE(18:2/0:0)	0.4	[M+H]	0.021	-1.340
		500.274	10.62	C23H44NO7P		-0.7	[M+Na]	0.012	-1.365
		508.341	11.01	C25H50NO7P	LysoPC(17:1/0:0)	-0.5	[M+H]	0.0099	1.383

	414.301	8.94	C26H43NO5			-0.2	[M+H-2H2O]	0.021	1.658
t2	432.311	8.96	C26H43NO5	Glycodeoxycholic acid		0.4	[M+H-H2O]	0.019	1.706
	450.323	8.94	C26H43NO5			-0.4	[M+H]	0.024	1.671
	472.303	8.94	C26H43NO5			-0.9	[M+Na]	0.012	1.744
	466.317	7.62	C26H43NO6	Glycocholic acid		-2.4	[M+H]	0.006	2.183
TN	496.338	11.27	C24H50NO7P	LysoPC(O-14:0/2:0)		0.1	[M+H]	0.015	-1.339
	991.672	11.29	C48H100N2O14P2			0.1	[2M+H]	0.045	-1.980
	542.323	10.03	C28H48NO7P	LysoPC(20:5/0:0)		0	[M+H]	0.010	1.343
	564.305	10.02	C28H48NO7P			0.2	[M+Na]	0.009	1.323
	566.321	10.74	C28H50NO7P	LysoPC(20:4/0:0)		0.2	[M+Na]	0.029	-1.437

t2	**HER2+**	426.357	9.89	C25H47NO4	Oleoylcarnitine	-3.3	[M+H]	0.045	-1.365
		414.301	8.94	C26H43NO5	Glycodeoxycholic acid	-0.2	[M+H-2H2O]	0.006	2.25
		432.310	8.96	C26H43NO5		0.4	[M+H-H2O]	0.005	2.44
		450.32	9.19	C26H43NO5		0.7	[M+H]	0.004	2.22
		450.323	8.94	C26H43NO5		-0.4	[M+H]	0.008	2.5
		472.303	8.94	C26H43NO5		-0.9	[M+Na]	0.005	2.3
t3	**TN**	448.305	8.45	C26H43NO6	Glycohyocholic acid	-1.5	[M+H-H2O]	0.001	2.31
		466.317	7.62	C26H43NO6	Glycocholic acid	-2.4	[M+H]	0.034	1.71
		524.374	12.77	C26H54NO7P	LysoPC(O-16:0/2:0)	-1.3	[M+H]	0.028	-1.471
		572.370	11.87	C30H54NO7P	LysoPC(22:4/0:0)	0.6	[M+H]	0.008	-1.754
		991.672	11.29	C48H100N2O14P2	LysoPC(O-14:0/2:0)	0.1	[2M+H]	0.018	-1.667

UVA: univariate analysis (Student's *t*-test); BC: breast cancer; TN: triple negative; LB: luminal B; HER2+: human epidermal growth factor receptor 2 positive; m/z: mass-to-charge ratio; RT: retention time; *p*–value*: *p*-value which FDR > 0.1; FC: fold change > 1 indicates that the average normalized peak area ratio in responder patients is larger than that in non-responder patients; t1: before starting the therapy cure at basal level; t2: once the patients received taxol, pre-surgery; t3: after going to the breast conserving surgery, post-surgery

SUPPLEMENTARY TABLE 5. Differential signals between response groups without a tentative identification according to the breast cancer molecular subtype detected in UVA.

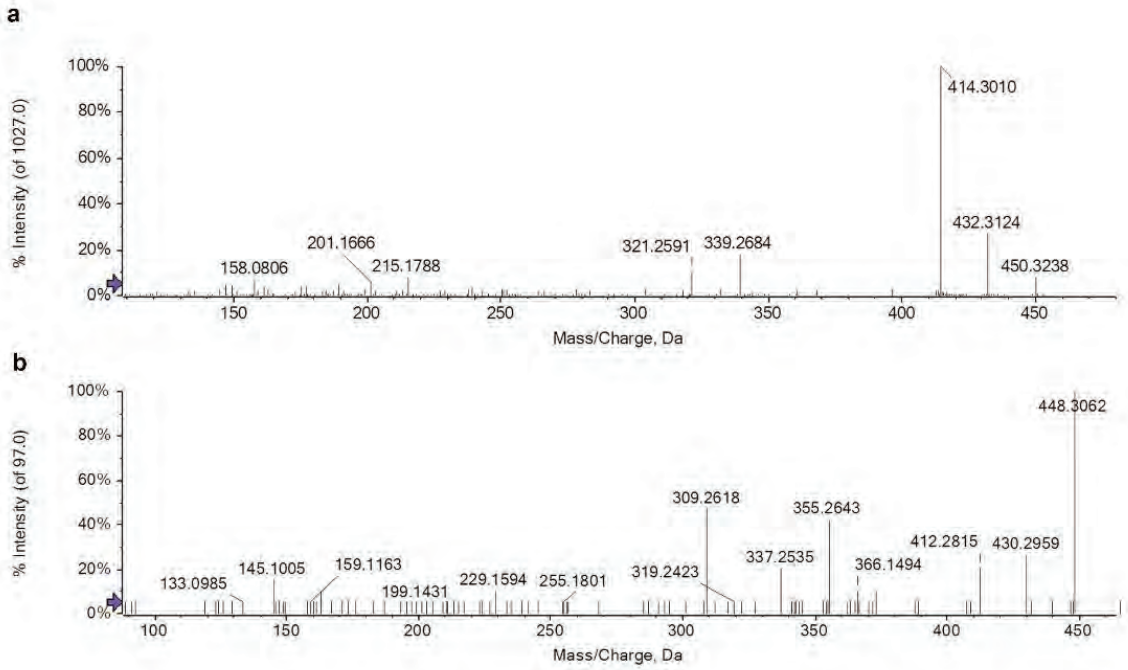
Time point	BC molecular subtype	m/z	RT (min)	*p*–value*	FC
t1	**LB**	546.7947	10.83	0.028	1.515
		1041.681	11.43	0.018	1.350
t2	**TN**	754.9921	11.29	0.021	-1.567
		755.9839	11.27	0.044	-1.519
		762.9844	11.27	0.045	-1.516
		765.9889	11.27	0.011	-1.413
		1010.65	11.29	0.044	-1.980
		1013.65	11.32	0.019	-1.926
		1258.309	11.29	0.032	-1.442
t3	**TN**	754.9921	11.29	0.023	-1.515
		755.9839	11.27	0.049	-1.515
		1002.6574	11.29	0.018	-1.786
		1013.6496	11.32	0.023	-1.449
		1258.3088	11.29	0.018	-2.128

UVA: univariate analysis (Student's *t*-test); BC: breast cancer; TN: triple negative; LB: luminal B; m/z: mass-to-charge ratio; RT: retention time; *p* – value*: *p*-value which FDR > 0.1; FC: fold change > 1 indicates that the average normalized peak area ratio in responder patients is larger than that in non-responder patients; t1: basal level; t2: pre-surgery; t3: post-surgery.

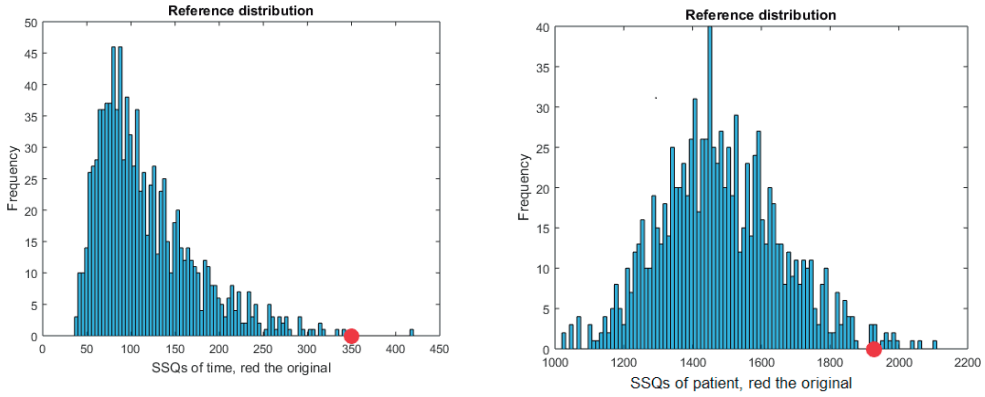
SUPPLEMENTARY TABLE 6. Differential signals without a tentative identification detected in ASCA according to time and patient factors.

BC molecular subtype	m/z	RT (min)
HER2+	576.3276	10.75
LB	452.3214	8.99
	515.2623	3.86
	409.1604	9.3

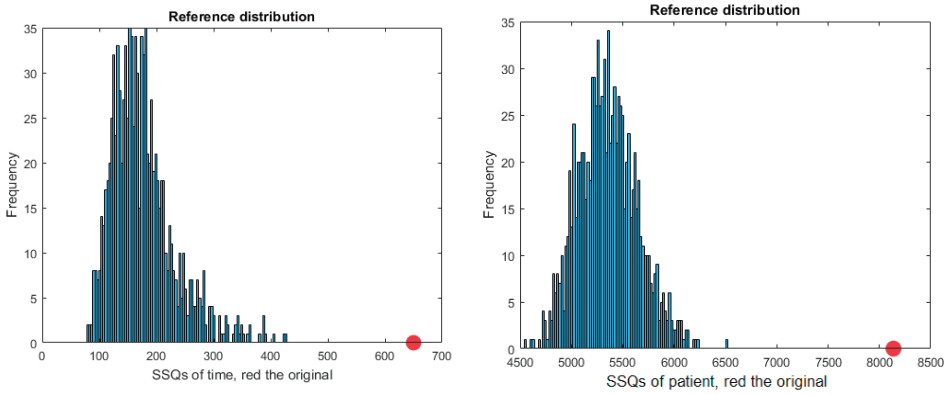
BC: breast cancer; HER2+: human epidermal growth factor receptor 2 positive; LB: luminal B; m/z: mass-to-charge ratio; RT: retention time



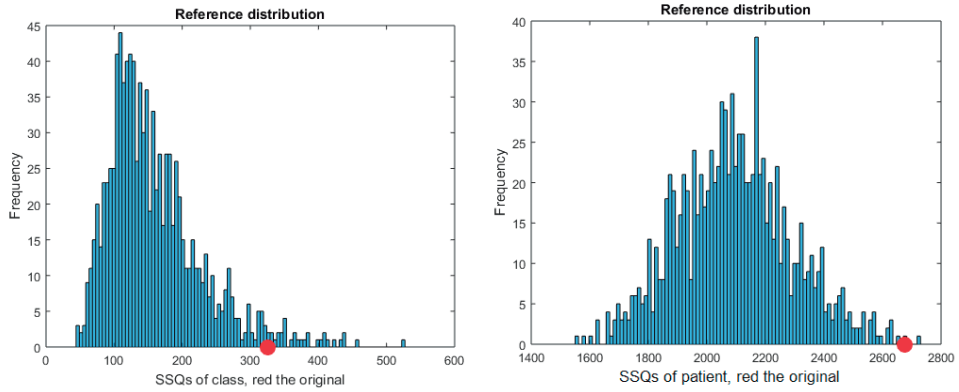
SUPPLEMENTARY FIGURE 1 Experimental MS/MS spectrum obtained in our analysis for the secondary bile acids a) glycodeoxycholic acid and b) glycohyocholic acid.



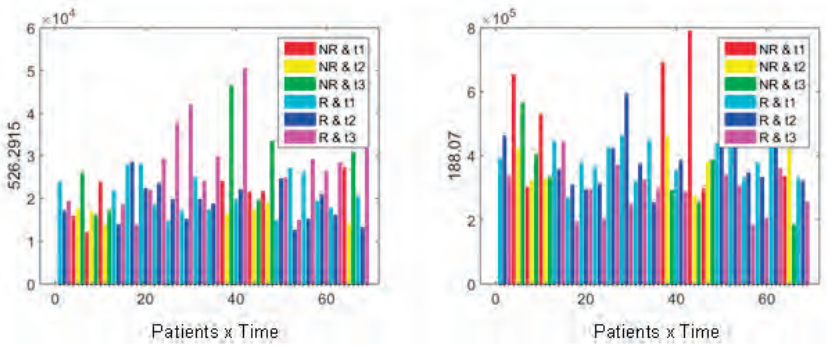
SUPPLEMENTARY FIGURE 2. Reference distribution for HER2+ significance testing with resampling in ANOVA-simultaneous component analysis: time factor (left, p – value = 0.002) and patient factor (right, p - value = 0.013).



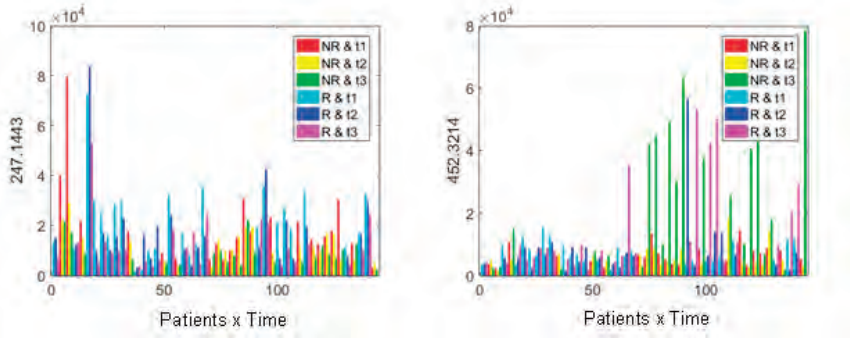
SUPPLEMENTARY FIGURE 3. Reference distribution for LB significance testing with resampling in ANOVA-simultaneous component analysis: time factor (left, *p*-value = 0.001) and patient factor (right, *p*-value = 0.001).



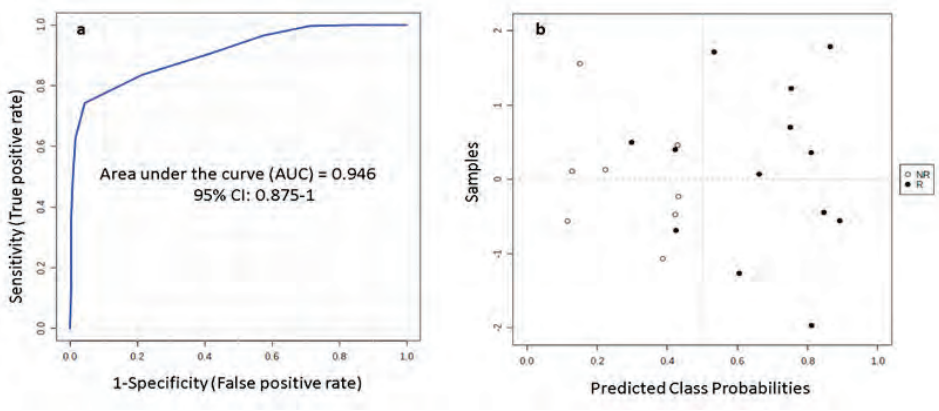
SUPPLEMENTARY FIGURE 4. Reference distribution for TN significance testing with resampling in ANOVA-simultaneous component analysis: time factor (left, *p*-value = 0.031) and patient factor (right, *p*-value = 0.002).



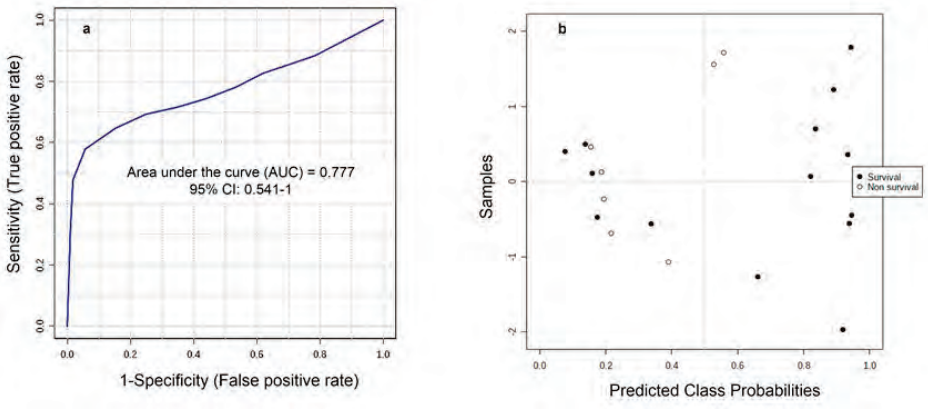
SUPPLEMENTARY FIGURE 5. Differential expression of 526.2915 (LysoPE (22:6) and 188.07 (tryptophan) according to the pathological response group (R, responders; NR, non-responders) in HER2+ at time 1 (t1, basal), time 2 (t2, pre-surgery) and time 3 (t3, post-surgery) detected using ANOVA-simultaneous component analysis.



SUPPLEMENTARY FIGURE 6. Differential expression of 247.1443 (tryptophan-betaine) and 452.3214 (not identified) according to the pathological response group (R, responders; NR, non-responders) in LB at time 1 (t1, basal level), time 2 (t2, pre-surgery) and time 3 (t3, post-surgery) detected using ANOVA-simultaneous component analysis.



SUPPLEMENTARY FIGURE 7. ROC curve plot for the model obtained from combination of the significant candidates identified in TN breast cancer molecular subtype (448.3047 (glycohyocholic acid) and 450.32 (glycodeoxycholic acid)): (a) ROC curve plot was created from the averaged results of 100 cross-validations; (b) as an outcome the model provides with the distinction of all non-responders TN patients and 3 out of 13 responders misclassified.



SUPPLEMENTARY FIGURE 8. ROC curve plot for the prognostic model obtained from combination of the significant candidates identified in TN breast cancer molecular subtype (448.3047 (glycohyocholic acid) and 450.32 (glycodeoxycholic acid)): (a) ROC curve plot was created from the averaged results of 100 cross-validations; (b) as an outcome the model provides with the distinction of 2 out of 7 patients from the non-survival group and 5 out of 14 survivors misclassified.

Chapter 3

Chapter 3: Letter to the Journal.

Metabolomics signature as a survival predictor in patients with resectable colorectal liver metastasis.

Carmen González-Olmedo, Francisco José García-Verdejo, Antonio Reguera-Teba[5], Carmen Rosa-Garrido, José Antonio López-López, Leticia Díaz-Beltrán, Patricia Mena García, Natalia Luque-Caro, Fernando Gálvez-Montosa, Ana Laura Ortega-Granados, María Ruiz-Sanjuan, Antonio Cózar-Ibáñez, Juan Sainz, Juan Antonio Marchal, José Camacho, José Pérez del Palacio, Rosario Fernández-Godino, Caridad Díaz, Pedro Sánchez-Rovira

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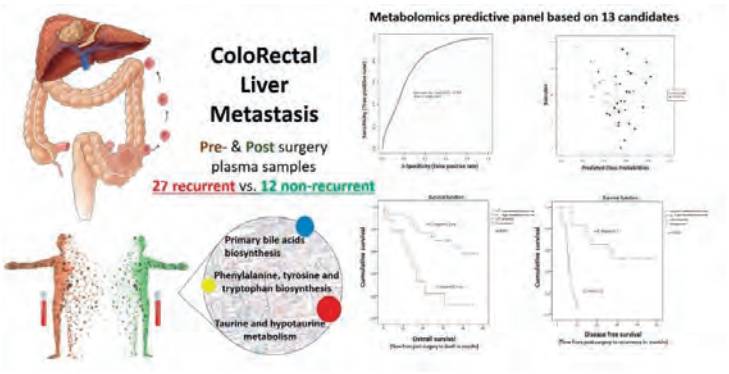
Graphical Abstract



Headlights

1. There is an urgent need to define reliable biomarkers of prognosis that allow an optimal therapeutic decision-making in metastatic colorectal cancer, which causes the death of almost 1 M patients every year.
2. Liquid chromatography-mass spectrometry provides a non-invasive, cost-effective, and rapid tool to detect onco-metabolites in plasma that can be used as prognostic biomarkers of liver metastasis in colorectal cancer patients.

3. We have identified 13 candidates in plasma samples of colorectal cancer patients with liver metastasis that can predict recurrence with an area under the ROC curve (AUC) of 0.793 and a metabolomics risk score for survival expectancy with a $P \leq .001$.
4. The detection of prognostic metabolites in plasma after resection of liver metastasis confirms the hypothesis that metabolomics can be used to detect minimal residual disease and predict survival expectancy.

Keywords: colorectal cancer, liver metastasis, metabolomics risk score, prognostic biomarkers.

Dear Editor,

Colorectal cancer (CRC) is the third most common cancer diagnosed worldwide, with over 1.9 million new cases per year (0.9 million deaths) in 2020.[1] In Spain, CRC was the most frequent cancer in 2022, with colorectal liver metastasis (CRLM) representing the leading cause of death.[2] The treatment of choice for metastatic patients with potential survival benefit is surgery, but more than 50% relapse.[3] Therefore, there is an urgent need to anticipate disease progression and prolong survival by defining predictive and prognostic biomarkers in CRLM patients after hepatic resection. Metabolomics has been previously used to detect CRC biomarkers,[4] but this is the first report that identifies specific metabolome alterations related to survival expectancy in a metastatic setting.[5]

In this pilot study (Figure 1), we analysed paired plasma samples of 39 patients with CRLM from the University Hospital of Jaén (Supporting Information Sections -SIS 1.1, 2.1 and Table S1) according to pre- and post-hepatic resection using untargeted metabolomics (Figure S1). Our research aims to determine metabolomics differences after surgery, when metastatic disease is still present versus successfully eliminated. This will shed light on the specific metabolic changes associated with relapse and survival, enabling the creation of a risk metabolomics score. The risk score could help to define which patients need close monitoring or more intensive treatment, even before the manifestation of recurrence symptoms.

Once we filtered metabolomics data matrices, the clustering of quality control samples (QCs) in unsupervised principal component analysis (PCA) confirmed the analytical stability of our methodology (SIS 1.2 and Figure S1). The ability to discriminate between presence or absence of metastasis in CRLM patients was determined by supervised partial least square-discriminant analysis (PLS-DA) (Figure 2A, 2B). Dysregulated metabolites between paired samples with a *P* value lower than 0.05 by student *t*-test with Benjamini-Hochberg false discovery rate (FDR) correction were selected. Metabolites with a fold change (FC) > 1.3 were identified between pre- and post-surgery samples (SIS 1.4-1.6 and 2.2). Statistical analyses showed that 346 metabolites were differentially expressed pre- and post-surgery, which could be used to discriminate the metastatic status. Besides the paired

analysis, we hypothesized that in patients where the disease is still present after resection of metastasis, the detection of onco-metabolites could predict CRC prognosis. For this analysis, we considered only post-surgery samples and metabolites with a FC > 1.3 and variable important of projection (VIP) > 1 between the groups of recurrence. Interestingly, we found that PLS-DA could discriminate between recurrent and non-recurrent patients (Figure 1C, 1D). The discrimination was based on 57 differentially expressed metabolites between these groups. Among the 57 metabolites, 20 candidates were identified (Table 1 and SIS 1.5) and the rest remained unidentified (Table S6). The differences involved molecular changes in the metabolism of taurine and hypotaurine, the biosynthesis of primary bile acids, and the biosynthesis of phenylalanine, tyrosine, and tryptophan (Figure 2E and SIS 1.7 and 2.2).

The results demonstrate that the early detection of onco-metabolites could help in predicting the risk of disease recurrence after surgery and guide treatment decisions for optimal clinical management in a metastatic scenario. We built a metabolomics model with the most predictive markers identified according to the value of the multivariate area under the curve (AUC-ROC). A precise model including 13 compounds showed the highest prediction ability (AUC = 0.793, 95% CI: 0.585-0.974, P = 0.023; SIS 1.7 and Figure S2).

Finally, to assess the prognostic value of these candidate metabolites, we used a univariate Cox-regression analysis and Kaplan-Meier curves (SIS 1.8, 2.3, and Table S7). The stratification of patients based on a potential metabolomics risk score (mRS) revealed that patients with an mRS of more than 6 candidate metabolites (high risk to relapse) had a 13-fold increased risk of recurrence (crude hazard ratio - cHR = 13.307 [3.826-46.281], $P < 0.001$), while patients with an mRS of more than 7 candidates (high risk to die) had a 4-fold increased risk of death (cHR = 4.241 [1.674-10.742], $P = 0.002$). Accordingly, Kaplan-Meier curves showed significant differences in the survival expectancy of patients per metabolomics risk group and event of study ($P \leq 0.001$) (Figure 3). Detailed results and methodologies are described in SIS I–II.

Previous studies have demonstrated that cancer cell metabolism is impaired, and metabolic rewiring of CRC cells can alter the expression of critical energy

metabolites, which leads to proliferation and spreading to other organs.[6] These findings line up with the decreased levels of circulating glycerophospholipids (lysoPCs and lysoPEs), taurine, and hypoxanthine found in recurrent patients with CRLM (Table 1). A possible explanation could be (1) a rapid clearance of lysoPC from the circulation for the synthesis of phosphatidylcholine (the most abundant phospholipid of mammalian cell types in the liver); (2) a high demand of energy for cell membrane-remodeling during cancer proliferation, previously reported as a shift of lysoPC concentrations between cancer tissue and blood[7], (3) a higher demand for hypoxanthine by an up-regulated purine metabolism typically associated with cellular differentiation and aggressiveness.[8] Also, inversely correlated levels of taurine and taurine-conjugated bile acids (BAs) may obey to a higher metabolism rate of taurine required for CRC disease progression. According to this hypothesis, it has been reported that increased secondary BAs metabolites may promote tumourigenic signaling pathways in the intestine.[9] Furthermore, changes in the BAs metabolism are associated with the intestinal microbiota composition, heavily influenced by the diet, which has a role in CRC tumourigenesis.[10]

In conclusion, our study shows that easily detectable onco-metabolites in plasma samples might be used to predict disease recurrence and have a prognostic value for CRLM patients undergoing surgery. In addition, the identification of a model based on 13 metabolites enables a precise risk stratification of disease progression and, consequently, a personalized follow-up in the clinical setting. Our study is limited by a relatively low sample size and collection timepoints, therefore, validations in larger cohorts are required to corroborate the prognostic value of the metabolomics signature.

Author Contributions

Conceptualisation: Caridad Díaz, Antonio Reguera-Teba and Pedro Sánchez-Rovira. *Data curation*: Carmen González-Olmedo, Leticia Díaz-Beltrán, Francisco José García-Verdejo, José Antonio López-López, Natalia Luque-Caro, Fernando Gálvez-Montosa and Ana Laura Ortega-Granados. *Investigation and methodology*: Patricia Mena García, Caridad Díaz and Carmen González-Olmedo. *Formal analysis*: Caridad Díaz, Carmen González-Olmedo, José Camacho and Carmen Rosa-Garrido. *Funding acquisition*: Pedro Sánchez-

Rovira. *Project administration*: Leticia Díaz-Beltrán, María Ruiz-Sanjuan and Carmen González-Olmedo. *Resources*: AntonioCózar-Ibáñez, Antonio Reguera-Teba, Rosario Fernández-Godino and Pedro Sánchez-Rovira. *Supervision*: Francisco José García-Verdejo, Antonio Reguera-Teba and Pedro Sánchez-Rovira. *Visualisation*: Caridad Díaz, Francisco José García-Verdejo, José Antonio López-López, Pedro Sánchez-Rovira, Rosario Fernández Godino and Carmen González-Olmedo. *Writing – original draft*: Carmen González-Olmedo, Caridad Díaz, Francisco José García-Verdejo, Rosario Fernández-Godino and Pedro Sánchez-Rovira. *Writing – review and editing*: José Antonio López-López, Leticia Díaz-Beltrán, Caridad Díaz, Juan Sainz, Juan Antonio Marchal, José Camacho, José Pérez del Palacio, Rosario Fernández-Godino and Pedro Sánchez-Rovira.

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Conflict of Interest Statement

The authors declare they have no conflicts of interest.

Data Availability Statement

All data generated or analysed during this study are included in this published article and its supplemental and supporting materials. Metabolomics data are not publicly available yet, since they contain information that could compromise the publication of future methodological work, but are available from the corresponding author on reasonable request.

Ethics Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and the International

Conference on Harmonization-Good Clinical Practices, and approved by the Institutional Review Board of the Clinical Research Ethics Committee of Jaén (protocol code: 6.2.05.2017 and date of approval: 25th May, 2017).

Informed Consent Statement

Informed consent was obtained from all study patients.

Correspondence

Caridad Díaz, Avenida del Conocimiento, 34 Parque Tecnológico de Ciencias de la Salud, 18016 Granada, Spain, caridad.diaz@fundacionmedina.es

Caridad Díaz and Pedro Sánchez-Rovira are co-senior authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section at the end of this article (QR).

TABLE 1. Tentative identification of the candidate biomarkers of CRLM recurrence post-surgery, detected by two LC-HRMS strategies.

LC-HRMS	*m/z*	R.T (min)	FC (RtoNR)	VIP	Molecular Formula	Putative ID	Adduct	Mass error (ppm)
HILIC	124.0067*	3.8	0.751	1.988	C2H7NO3S	Taurine	M-H	3.3
ESI -	135.0295*	2.85	0.652	1.442	C5H4N4O	Hypoxanthine	M-H	-4.7
	193.0358*	3.87	0.549	1.447	C6H10O7	Galacturonic acid	M-H	1.7
	307.1507	5.21	1.434	1.515	C12H24N2O7	Fructose-Lysine	M-H	3.0
	336.0895	3.91	0.484	1.059	C6H12O7	D-Gluconic-related acid	M-H	0.0
	353.1582	1.27	4.047	1.323	C18H26O7	Propofol glucuronide	M-H	-2.7
	369.1727	1.65	1.428	1.593	C19H30O5S	Androsterone sulfate	M-H	-2.3
	452.2789*	1.82	0.743	2.145	C21H44NO7P	LysoPE(16:0)	M-H	-3.2
	464.299	1.34	2.175	1.837	C26H43NO6	Glicocholic acid	M-H	0.9
	498.2887*	2.54	1.296	1.296	C26H45NO6S	Taurochenodeoxycholic acid	M-H	2.6
	514.2818*	2.97	1.837	1.837	C26H45NO7S	Taurocholic acid	M-H	-2.9
	583.2544*	1.18	1.360	1.360	C33H36N4O6	Bilirubin	M-H	-1.7
RPLC	454.2918*	11.2	0.735	1.571	C21H44NO7P	LysoPE(16:0)	M+H	-2.2
ESI +	478.2925*	10.6	0.736	1.511	C23H44NO7P	LysoPE(18:2)	M+H	-0.7
	480.3439*	12.2	0.781	1.682	C24H50NO6P	LysoPC(P-16:0)	M+H	-2.0
	482.3211*	13.2	0.773	1.452	C23H48NO7P	LysoPE(18:0)	M+H	-4.4
	512.3348*	10.0	0.773	1.632	C24H50NO8P	Unknown LysoPC	M+H	1.0
	526.2912*	10.6	0.768	1.407	C27H44NO7P	LysoPE(22:6)	M+H	1.3
	548.2693	10.6	0.757	1.486			M+Na	-1.3
	564.3056	10.0	0.725	1.054	C28H48NO7P	LysoPC(20:5)	M+Na	-0.8

m/z: mass/charge ratio; R.T: retention time; FC: fold change > 1.3 indicates that the average normalized peak area ratio in post-surgery samples of recurrent patients with colorectal cancer liver metastasis (R) is larger than that in non-recurrent patients (NR); VIP: variable of importance in projection; ID: identification; *: candidates selected for the diagnostic model; LysoPE: lysophosphatidylethanolamines; LysoPC: lysophosphatidylcholines.

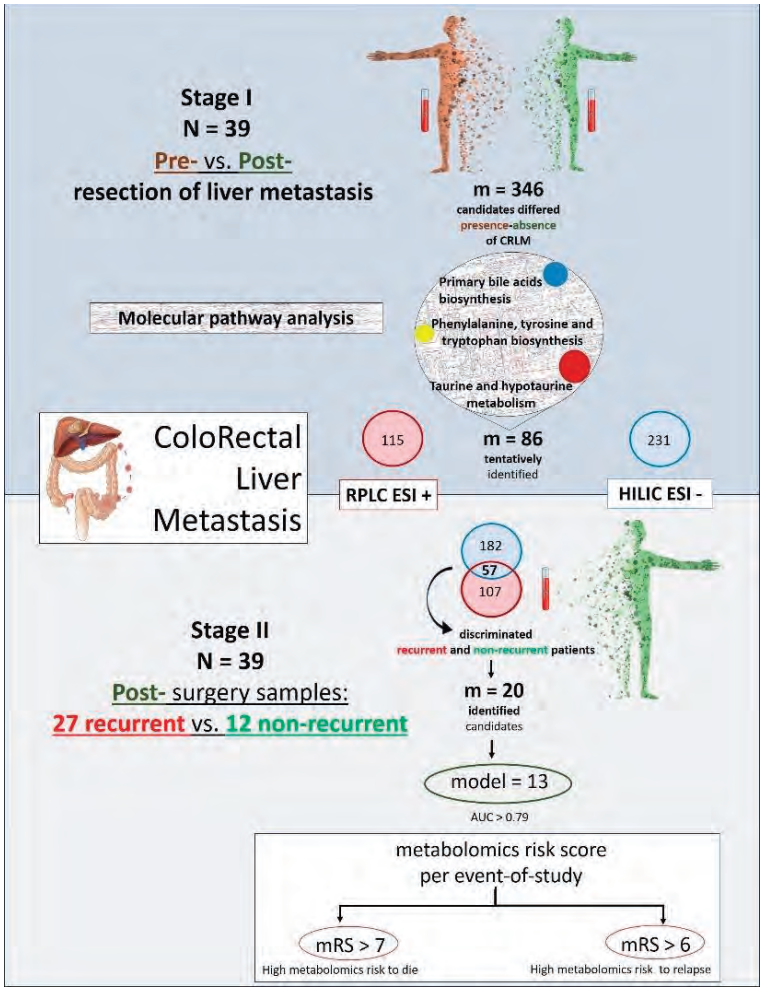
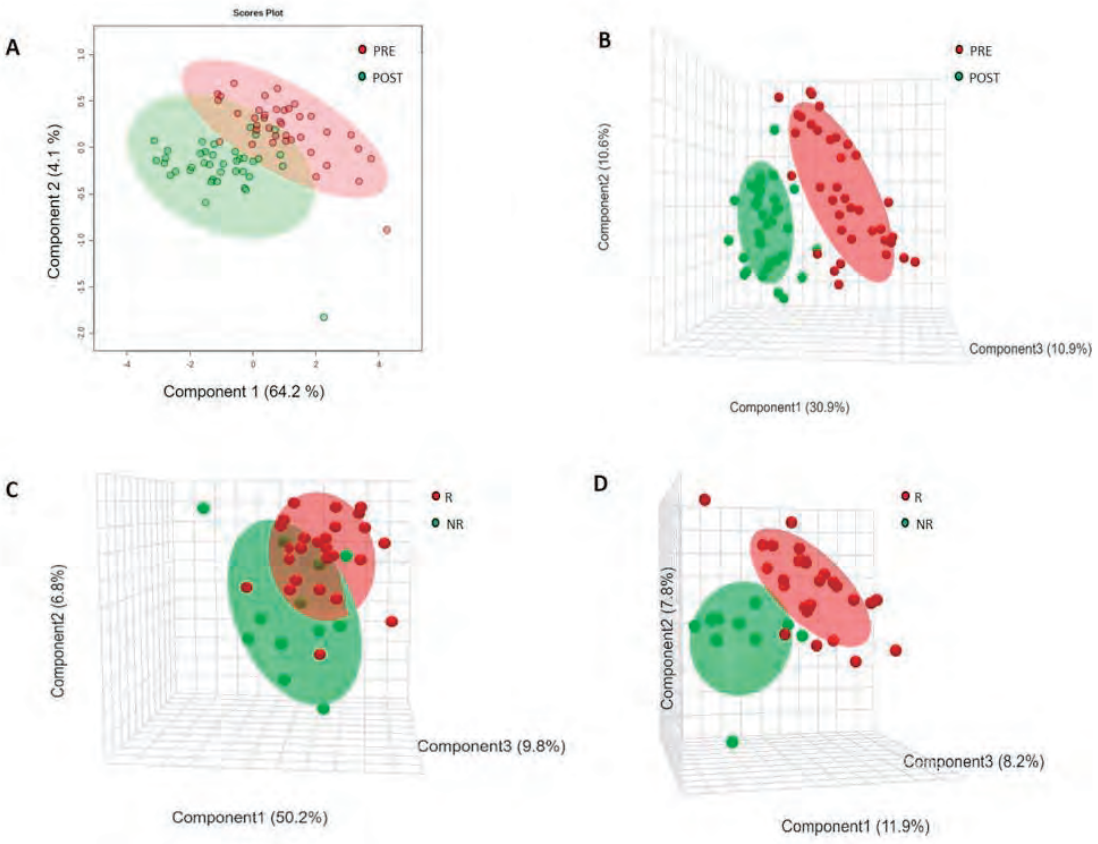


FIGURE 1. Study workflow. In Stage I, out of 346 metabolites found altered between paired samples (pre- and post- resection of liver metastasis), 115 were detected by RPLC + mode, 231 by HILIC – mode, and 86 were tentatively identified. In Stage II, the metabolomics alterations found in plasma that differed if metastases were present, were further analysed for their potential association with recurrence. Specifically, 107 metabolites found by RPLC + mode that discriminated paired plasma samples of patients with CRLM, were not associated with disease recurrence. Similarly, 182 metabolites found by HILIC - mode that differentiated the metastatic status pre- and post-surgery were not associated with relapse after surgery. Finally, 57 out of the 346 metabolites were found to discriminate between recurrent and non-recurrent patients.

Importantly, 20 out of the 57 candidates were identified and 13 showed the highest prediction ability so were used for the survival analysis. Based on the expression of these 13 candidate metabolites, we could define two metabolomics risk groups according to the event of study. CRLM: colorectal liver metastasis; N: sample size; m: metabolites; RPLC ESI +: reverse phase liquid chromatography and positive electrospray ionization mode; HILIC ESI -: hydrophilic interaction liquid chromatography and negative ionization mode; AUC: area under the curve; mRS: metabolomics risk score.



E

Altered pathways	Groups of study	*P*
Taurine and hypotaurine metabolism	Pre- to post-surgery	0.003
	R to NR	0.0008
Primary bile acid biosynthesis	Pre- to post-surgery	0.010
	R to NR	0.002
Phenylalanine, tyrosine and tryptophan biosynthesis	Pre- to post-surgery	0.041

R: recurrent patients with CRLM; NR: non-recurrent patients; *P*: *P* – value.

FIGURE 2. Supervised PLS-DA score plots shows the discrimination between pre- and post-surgery plasma samples (red and green dots respectively), using two components in RPLC ESI + (A) and three components for HILIC ESI – (B) methods. PLS-DA score plots illustrate the differentiation in post-surgery samples of recurrent (red dots, R) and non-recurrent (green dots, NR) patients with CRLM, by using three components in RPLC ESI + (C) and HILIC ESI – (D) analyses. Molecular pathways significantly altered between the experimental groups (E).

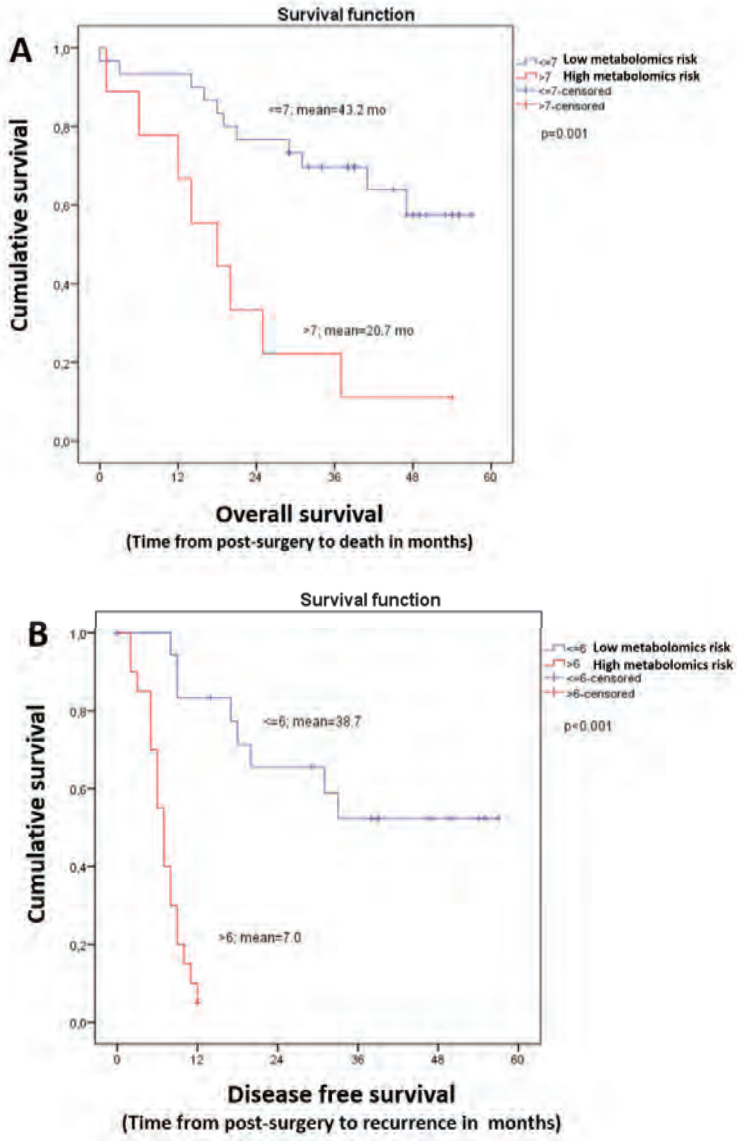


FIGURE 3. Survival expectancy in months from surgery of metastasis to death (A) and recurrence (B), according to the metabolomics score per group of risk represented by Kaplan-Meier curves. The mean overall survival time (A) of the high metabolomics risk group (mRS > 7 candidate metabolites) was 20.7 months compared to 43.2 months observed in patients with a low metabolomics risk to die (mRS ≤ 7). On the other hand, for the disease-free survival (B), the mean survival time of the high metabolomics risk group (mRS > 6 candidates) was 7.0 months versus 38.7 months for the patients with low metabolomics risk to relapse (mRS ≤ 6). mo: months; mRS: metabolomics risk score.

DISCUSSION

6. DISCUSSION

6.1 Contribution to current knowledge

6.1.1 *Metabolomics and cancer detection*

Currently, early cancer detection might be delayed, occurring when disease symptoms are already visible, being the mortality risk increased a 6 to 13% when the treatment is postponed by one month (Hanna et al. 2020). Moreover, cancer detection is accompanied of reasonably invasive procedures since population based-screenings such as mammograms, human papillomavirus or Pap tests and colonoscopies can cause potential harms including physical, psychological, emotional, inaccurate results with unnecessary follow-up of the patients and subsequent financial effects (Shieh et al. 2016). In this sense, the advent of -omics sciences provide with high-throughput technologies leading to the discovery of new potential biomarkers. These innovative chemical entities may pave the way for a better understanding of the underlying molecular behaviour of the disease as well as for the development of more precise therapeutic agents (Hebar, Valent, and Selzer 2013).

Lately, research in the context of this thesis proposal has shed light into the use of untargeted metabolomic approaches to detect and identify candidate biomarkers of cancer, both diagnostic and prognostic, with potential value for the clinical practice (Clish 2015; Kowalczyk et al. 2020; Vignoli et al. 2021). Whereas genomics, transcriptomics or proteomics provide indirect information about cellular function, metabolomics offers unique advantages in capturing a more direct and dynamic insight into the functional biological system. Thus, the emergence of metabolomics has provided with the identification of molecular alterations closely linked to the cellular phenotype and physiological status (Stine et al. 2022). Herein, in order to achieve the main objective of this doctoral thesis, two *Original Research Articles* and a *Letter to the Journal* proved that non-targeted metabolomics-based approaches might characterize the metabolic idiosyncrasy of cancer behaviour according to three different biological scenarios: *Chapter 1)* metabolomic differentiation of BC subtypes, *Chapter 2)* BC subtype-specific metabolomic dynamism in response to NACT, and *Chapter 3)* CRC metabolomics-based prediction of recurrence and survival after resection of liver metastasis. Nevertheless, the early diagnosis of cancer by detection of precancerous changes at the earliest time point is still facing some fundamental challenges (Crosby et al. 2022): the understanding

of the biology and behaviour of the early disease; determining the risk of developing cancer; the discovery and validation of biomarkers for an early detection of the disease; the technological capacity and the translation of biological insights into clinical trials.

In cancer metabolomics, preliminary results have demonstrated alterations in normal metabolic processes occurring in cancerous cells that do not occur in healthy cells (Griffin and Shockcor 2004; Stine et al. 2022). The metabolism adaptation of malignant cells towards glycolysis in the hypoxic microenvironment of tumour tissues generate the key metabolic intermediates that provide important building blocks for DNA and the synthesis of FAs or redox regulation (Weinberg et al. 2010). However, whereas metabolic aberrations are the cause of cancer or the consequence of its development is still a matter of debate (Garber 2006). In this sense, the term "oncometabolite" was born to refer to that molecules that are increased in tumours relative to normal cells (Khatami, Kazem Aghamir, and Tavangar 2019). For instance, the 2-hydroxybutyrate, succinate, and fumarate are molecules that can be produced due to the mutation of nuclear encoded mitochondrial enzymes. Regarding this, increasing research is focused to prove the contribution of other oncometabolites in cancer development and progression (Ježek 2020; Pan, Li, and Simon 2021).

6.1.2 *Metabolomics and breast cancer*

On the basis of the results presented in the metabolomic differentiation of BC subtypes, we proved that untargeted LC-HRMS-based metabolomics clearly differentiates the BC molecular subtypes from HC and that main significantly altered features were metabolites from the porphyrin metabolism, glycerophospholipid metabolism, tryptophan metabolism and aminoacyl-tRNA biosynthesis. Likewise, the potential use of metabolomic BC screening was evidenced by a LC-MS fingerprinting that showed a 100% accuracy identifying between BC plasma samples and HC (Jové et al. 2017). Additionally, the HER2 and luminal BC molecular subtypes significantly differed by using the glutamate/glutamine ratio and aerobic glycolysis as biomarkers (Budczies et al. 2013; Alakwaa, Chaudhary, and Garmire 2018). Therefore, further investigation into the metabolic-associated pathways in BC would enhance comprehension of the underlying molecular mechanisms triggering the initiation of the disease as well as the BC molecular stratification between the different subtypes. In this sense, metabolic reprogramming in cancer is linked to important oncogenes mediating energetic pathways (C. V. Dang, Le, and Gao 2009; Matoba et al. 2006; Bensaad et al.

2006). So, measuring metabolomics changes might provide with an easy valuable reflect of the dynamic response to genetic alterations.

Additionally, our recent evaluation of untargeted metabolomics warrants its use not only for an early detection of BC and molecular stratification according to the phenotype, but also as a promising strategy for differentiating BC stages and aggressiveness (González Olmedo et al. 2024). In this regard, previous metabolomic analyses highlighted the significant alteration of the metabolome in relation to the therapy response in BC patients under a NA regimen. In accordance with these findings, alteration of phenotype-specific molecular pathways might allow with the prediction of response in a more personalized way (Zapater-Moros et al. 2023). Moreover, a wider comprehension of the underlying molecular behaviour of therapy resistance or the presence of MRD would provide with therapeutical potential targets for a better precision oncology. Taking advantage of the study provided by BC subtype-specific metabolomic dynamism in response to NACT, we could perform a temporal metabolomic approach in combination with ASCA. Herein, the implication of several factors on the alteration of the metabolome could be deciphered according to the diversity of response to the NACT in BC molecular subtypes. Thus, the factors time, response and their interaction were analysed together with the significance at the individual level. Interestingly, the patient factor was significant between all the BC phenotypes, which points to the importance of improving personalized management of BC molecular heterogeneity.

6.1.3 *Metabolomics and colorectal cancer*

Drawing on the results of CRC metabolomics-based prediction of recurrence and survival after resection of liver metastasis, a metabolomic signature was revealed to be capable of distinguishing recurrence with prognostic value for survival in mCRC (González-Olmedo et al. 2024). Similarly to BC, various metabolomic strategies have been successfully applied in CRC to characterize molecular pathways linked to the disease, thereby offering potential targets for tailored therapies and better clinical management. In this regard, previous research has demonstrated the utility of untargeted and targeted metabolomics in CRC detection, identifying tentative diagnostic biomarkers, and showing potential as a minimally invasive tool for monitoring CRC progression (Tan et al. 2013; Long et al. 2017; Yachida et al. 2019; Martín-Blázquez et al. 2019; Martini et al. 2020).

For instance, Jonas J. *et al*. conducted a targeted metabolomic analysis to assess the risk of early disease recurrence following CRLM surgery, using pre-surgery plasma samples (Jonas et al. 2021). In line with our findings, they suggested that circulating metabolites could be useful in detecting early CRC recurrence. However, their model was focused on circulating glycerophospholipids, while our approach detected significant metabolites involved in the bile acids metabolism and biosynthesis, and the metabolism of amino acids, highlighting the methodological differences and outcomes between strategies (González-Olmedo et al. 2024).

Additionally, similar to the metabolic reprogramming observed in BC, common mutations in oncogenes or tumour suppressors in CRC, such as mutations in RAS and BRAF, significantly influence cellular metabolism. Thus, genetically impacted metabolic pathways may be reflected in circulating metabolites, making metabolomics a valuable tool for identifying potential therapeutic targets. In this sense, genetic profiling of CRC patients has greatly improved clinical management by guiding the selection of optimal therapies based on these genetic alterations (Heide et al. 2022), and novel therapeutic strategies could be developed and validated by integrating genomics with metabolomics and using minimally invasive methods such as liquid biopsy (Ullah et al. 2022).

However, despite these advances, the role of individual genes may not fully explain the complexity of our biological system (Krop et al. 2018), neither of CRC biology, particularly given that most of CRC cases are sporadic whereas genetic predisposition is relatively low (De La Chapelle 2004; Valle et al. 2019). In this scenario, prevention of CRC through liquid biopsy-based biomarkers is essential to improve survival rates by enabling intervention before tumour detection or metastasis spread.

6.2 Main findings

To address the main objective of this study, three clinical scenarios were analysed throughout the whole dissertation. As a result, our metabolomic findings unveiled that some distinctive metabolic features might be correlated across cancers located in different anatomical sites, while others could be tumour-specific. In particular, employing untargeted metabolomics methodologies, we discerned unique molecular

landscapes of BC in comparison to HC, BC responders to non-responders and last, recurrent to non-recurrent mCRC patients.

6.2.1 *Association between purine metabolism and cancer*

Purines (adenine and guanine) and pyrimidines (thymine, uracil, and cytosine) are the main nitrogenous bases of nucleotides, which are the basic building blocks for the biosynthesis of DNA and RNA. Under normal physiological conditions, nucleotide metabolism is balanced between the biosynthetic and degradation pathways. In cancerous cells, however, large quantities of nucleotides are required to support the rapid and uncontrolled cell proliferation characteristic of tumours (Traut 1994). This demand implies an upregulation of the metabolic genes and encoded enzymes, which are primarily responsible for maintaining the intracellular nucleotide concentrations (Mullen and Singh 2023). To meet the increased demand for nucleic acid precursors, cancer cells rely predominantly on the *de novo* biosynthesis pathway and, to a lesser extent, the nucleotide salvage pathway. Both routes are metabolite-depending on the 5-phosphoribosyl-1-pyrophosphate , but involve different regulated genes and enzymes, which are discussed in detail elsewhere (Lane and Fan 2015).

As part of the results from this dissertation, we observed an alteration in purine metabolism within the mCRC cohort. Specifically, in our CRC metabolomics-based prediction of recurrence and survival after resection of liver metastasis, we found that circulating hypoxanthine levels were lower in recurrent mCRC patients compared to non-recurrent patients. Hypoxanthine plays a dual role in purine metabolism: 1) it is a byproduct of ATP breakdown and an intermediate in the catabolism of inosine, adenosine and guanine-monophosphate; and 2) it is a biosynthetic precursor produced by the hypoxanthine-guanine phosphoribosyltransferase reaction in the salvage pathway. Under normal conditions, most purine nitrogenous bases are recycled through the salvage pathway, with 90% of hypoxanthine being reused to form inosine monophosphate. However, under hypoxic conditions, hypoxanthine can be further metabolized into xanthine and uric acid by xanthine oxidase , resulting in increased production of ROS during the final steps of purine catabolism (Kuwabara et al. 2003; Saugstad 1988).

In this context, the enzyme involved in the catabolism of hypoxanthine is the xanthine oxidoreductase (XOR) which is activated under hypoxic conditions to degrade DNA and RNA. Although XOR regulation in cancer is not well understood, previous

studies have shown that: 1) XOR is downregulated in CRC mouse models and cell lines as well as in several tumours such as BC, gastric cancer or CRC (Battelli et al. 2016); 2) XOR is upregulated during the differentiation and progression of colon cancer cells (H. Li et al. 2021; Linder et al. 2009). This dysregulation of XOR could explain that decreased hypoxanthine levels observed in our study may be related to the progression of the disease. Nevertheless, further research is required to validate whether circulating hypoxanthine levels may truly reflect aberrant XOR activity during CRC development.

6.2.2 *Association between tryptophan (Trp) metabolism and cancer*

Imbalances in Trp metabolism have been extensively studied in relation to several pathophysiological states. In humans, Trp is exclusively obtained from diet and a 95% of it is catabolized in the Kyn pathway (Platten et al. 2019; Takikawa 2005). The Kyn pathway-related enzymes and metabolites play major roles in immune regulation, neuronal function and intestinal homeostasis (Platten et al. 2019; Cervenka, Agudelo, and Ruas 2017; Turner 2017; Zelante et al. 2013). Regarding Trp catabolism in cancer, it is increasingly recognized that this essential amino acid may have an indispensable function in immune activation and tolerance, which is largely regulated by expression of IDO1. While deficiency of the IDO1 in mouse models lead to autoimmune disease (Mellor et al. 2003), aberrant expression of this enzyme in several tumours results in immune suppressive effects (Meireson, Devos, and Brochez 2020; Lijie Zhai, Stefani Spranger, David C. Binder, Galina Gritsina, Kristen L. Lauing, Francis J. Giles 2015; Soliman et al. 2013). Moreover, activation of IDO1, 2 or the tryptophan 2,3-dioxygenase regulates the Trp breakdown in several cells and tissues, inducing upregulation of its catabolites. In this sense, downstream metabolites such as Kyn are responsible for T regulatory cell infiltration and differentiation through the AhR as well as inhibition of T lymphocytes or cytotoxic cells, which permit cancer cell growth (X. han Liu and Zhai 2021; Onesti et al. 2019; Platten, Wick, and Van Den Eynde 2012).

Specifically, in our study we observed decreased levels of circulating Trp in the LA, TN and HER2 molecular subtypes when compared to HCs. Interestingly, in the LB and HER2 phenotypes we could also detect a significant alteration of Trp and Trp-betaine in a temporal manner from the diagnosis till the time after surgery. In accordance

with these results, reduced expression of plasmatic Trp has been previously associated with different types of cancer such as BC, lung cancer or digestive system tumours (Yu, Lu, and Du 2024; Eniu et al. 2019; Smith et al. 2012). One main reason for the uptake of Trp during cancer growth is its role in the *de novo* synthesis of nicotinamide adenine dinucleotide. This cofactor supports essential metabolic pathways in cancerous cells (Navas and Carnero 2020), mitochondrial energy production (Castro-Portuguez and Sutphin 2021) and it has been observed that it may also confer resistance to treatments *via* activation of the DNA repair proteins, which help the cancer cell to survive under genotoxic stress caused by therapy (Vareki et al. 2014). Nevertheless, although decreased levels of Trp were found in BC patients in comparison to HCs, we did not find any dysregulation of plasmatic Trp in relation to the NACT factor. This observation may be due to the low sample size per molecular subtype as previously described elsewhere (Onesti et al. 2019).

6.2.3 *Association between porphyrin metabolism and cancer*

Porphyrin metabolism is the metabolic pathway responsible for the production of heme substrates, intermediates and end products. In this context, HO-1 is the key enzyme responsible for heme catabolism producing ferrous iron, carbon monoxide and BV. BV is then converted to bilirubin by the biliverdin reductase (BVR). Then, BR is associated to albumin (indirect bilirubin) in order to become soluble and circulate to the liver to be mainly cleared by: 1) reoxidation to BV, or 2) conjugation with glucuronic acid (direct BR) by the uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1). Finally, direct BR is excreted into the bile conduct where it is reduced to urobilinogen which may be: 1) further metabolized into stercobilin, or 2) reabsorbed into the blood circulation to form urobilin in the liver (B. Wu, Wu, and Tang 2019).

Heme-derived compounds are crucial for normal cell physiology, but their over-down expression may result in harmful effects. Indeed, recently, *porphyrin overdrive* in cancerous cells has been determined as a potential therapeutic target since it appeared to be cancer cell-essential and specific, and absent in normal cells (Adapa et al. 2024). Herein, our findings show an aberrant porphyrin metabolism in both the BC cohort, and the mCRC cohort. Interestingly in the metabolomic differentiation of BC subtypes, we found that in the BC plasma samples of the LB and HER2 molecular subtypes, the circulating levels of BV were elevated in comparison to HC. Moreover, in the BC subtype-specific metabolomic dynamics in response to NACT, the expression of BV

showed a temporal change from high plasma levels at diagnosis to lower levels post-surgery specifically in the HER2 phenotype. Otherwise, in the CRC metabolomics-based prediction of recurrence and survival after resection of liver metastasis, we observed high levels of circulating BR post-surgery, as well as in plasma of recurrent mCRC patients in comparison with non-recurrent. This observation may be due to the increase of the porphyrin metabolism in cancer cells or during the progression of the disease.

In this sense, while bile pigments have traditionally been considered as non-toxic molecules that protect cells by reducing ROS (Weaver et al. 2018), their role in cancer pathophysiology seems more complex. For instance, BR can paradoxically (hyper) activate the ERK1/2 signalling pathway, helping colon cancer cells evade death (Öllinger et al. 2007), despite its described ability to evoke apoptosis *in vitro* (Keshavan et al. 2004). More recent evidence highlights the pro-carcinogenic effect of BV and BR, since high expression of BVR has also been linked to the epithelial-mesenchymal transition in BC, a process that promotes tumour progression (Zhang et al. 2018, 2016). Besides, BV and BR have been identified as endogenous activators of the aryl hydrocarbon receptor, a transcription factor that regulates tumour growth, survival and invasion (Therachiyil et al. 2022; Safe, Lee, and Jin 2013; Phelan et al. 1998). Last, whereas total circulating BR and direct BR may correlate with poor prognosis in CRC (Jia et al. 2021), elevated levels of indirect BR appear to have a protective effect (X. Zheng et al. 2021; Sticova and Jirsa 2013), possibly due to due to genetic variations such as a polymorphism in the UGT1A1 gene (Jirásková et al. 2012). This observation suggests that the specific form of BR plays a critical role in cancer outcomes.

Accordingly, our findings lead to the inference that increment of BV and BR in plasma might be potential diagnostic and prognostic biomarkers of BC and CRC respectively. Thereby, determining salutary or damaging thresholds for these compounds will allow with a more precise stratification of the molecular risk to develop each type of cancer or for monitoring the disease. Hence, it is of utmost importance to further investigate and clarify the signalling-related pathways or tissue-specific gene expression implied in the progression of the disease, either by an up-regulation of the HO-1, a dysregulation of the BVR or from variations in the UGT1A1 gene.

6.2.4 *Association between bile acids' metabolism and cancer*

Bile acids (BAs) are amphipathic compounds which molecular structure in mammals is characteristic due to a 24 carbon atoms chain that conforms the steroid nucleus (three

six-member rings) and a five-carbon ring with a carboxyl at the C-24 position. BAs are classified as primary or secondary according to their synthesis. Primary BAs, cholic acid and chenodeoxycholic, are synthesized from cholesterol in the liver by the classical or the alternative pathways; then, they are conjugated with glycine or taurine and stored in gallbladder. When they are released into the intestine, different enzymes and intestinal microbiota deconjugate and modify their molecular structure into the secondary BAs: deoxycholic acid, litocholic acid and ursodeoxycholic acid. Most of the BAs (95%) are transported to liver while the remaining (5%) is excreted in stool. Last, reabsorbed secondary BAs may also be tauro- and glyco-conjugated and stored in gallbladder or recycled to the enterohepatic circulation to mainly participate in digestive processes as detergent compounds (Monte et al. 2009).

In the last decades, diverse functions of BAs have been deciphered in pathophysiological conditions. Within their roles, BAs act as potent antimicrobial agents as well as signalling molecules for cell metabolism and immunity regulation. Through activation of a membrane G-protein receptor and the nuclear farnesoid X receptor (FXR), BAs lead to tissue-specific effects that may seem paradoxical (Baptissart et al. 2013). In BC, FXR expression was correlated with proliferative markers of the disease in luminal subtypes (Journe et al. 2009), while in advanced BC, higher expression of FXR was associated with a better prognosis (Y. H. Lee and Song 2013). In liver and CRC has been observed that BAs metabolism *via* the FXR enhance an anti-inflammatory and tumour-suppressing environment (X. Huang, Fan, and Huang 2022; Di Ciaula et al. 2017; X. F. Huang, Zhao, and Huang 2015). Moreover, loss of FXR expression has been linked to liver cancer, and its expression in CRC is inversely related to disease progression (Fu et al. 2019; Jiang et al. 2013).

Despite the clear dysregulation of BAs metabolism in several cancers, more research is needed to clarify its potential role as a therapeutic target or prognostic marker. This dissertation highlights changes in circulating BAs levels in BC and CRC, demonstrating tissue-specific associations. For instance, low levels of conjugated-secondary BAs were found in plasma of BC patients, while high levels of conjugated-primary BAs were detected in recurrent mCRC subjects and after the resection of liver metastasis. Herein, a possible diet-microbial link to the origin of BAs dysregulation appeal for attention.

First, the pool of secondary BAs is regulated by gut microbes, which are tightly influenced by diet. Research in BC has shown that intestinal dysbiosis, particularly of

beta-glucuronidase-producing bacteria, may affect disease progression through estrogen-dependent mechanisms, while other microbes produce protective bioactive metabolites (Ruo et al. 2021). In this regard, a plant-based diet can decrease beta-glucuronidase activity, promoting estrogen excretion and lowering exposure. Within our results, we found reduced levels of secondary circulating BAs in BC patients, specifically glycoursodeoxycholic acid in estrogen-receptor positive cases, and glycohyocholic and glycodeoxycholic acids in non-responder TN patients. It is noteworthy that further investigation into diet, dysbiosis and the imbalance of these conjugated-secondary BAs may offer new therapeutic or prognostic tools for improving BC management.

Second, our results also demonstrated reduced taurine but increased taurine-conjugated BAs (taurocholic and taurochenodeoxycholic acids) in the blood of recurrent mCRC patients right after the resection of liver metastasis. In this context, CRC risk has been previously linked to diets rich in animal products which increase taurine and taurine-conjugated BAs, promoting the production of genotoxic secondary metabolites (Ridlon, Wolf, and Gaskins 2016). Specifically, taurine-derived compounds support the growth of microbes that produce pro-oncogenic molecules like hydrogen sulfide and deoxycholic acid (Wolf et al. 2020), which are linked to cancer development. Last, growing interest in gut microbiota and its correlation with diet in BC and CRC development is reflected in ongoing clinical trials for characterization of microbiota modulators as detailed elsewhere (L. Y. Zhao et al. 2023; Álvarez-Mercado et al. 2023).

6.2.5 *Association between lipids' dysregulation and cancer*

Metabolic reprogramming is a firmly established cancer hallmark (Hanahan and Weinberg 2000; Pavlova and Thompson 2016; Salita et al. 2022) consequence of the Warburg effect, which implies an exacerbated synthesis of essential biomolecules for cell renewal in such a harsh tumour microenvironment. Specifically, lipids' dysregulation is a constant in the BC and CRC metabolomic studies (Ward, Anderson, and Sartorius 2021; Costantini et al. 2023). However, lipidomics profiling according to each cancer type is underdeveloped in comparison to genomics or proteomics.

FAs, triglycerids and PL are multifunctional biomolecules that may act as structural components for cell membranes, signalling molecules or energy suppliers. The principal component of cell membranes are PL (PCs and PEs), glycolipids and sterols (Hishikawa et al. 2014). Otherwise, FAs also mediate the storage of energy *via* triglycerids and

provide the precursors for the biosynthesis of PLs. Furthermore, previous research has demonstrated that all these lipidic compounds may play major roles in molecular signalling resulting in the aberrant phenotype of cancer cells (Yuan TL; Cantley LC 2008; Hannun and Obeid 2008; Y. H. Huang and Sauer 2010).

Deregulation of lipids' metabolism is directly related to key metabolic enzymes in maintaining cellular lipid homeostasis by control of the *de novo* biosynthesis of FAs and fatty acid oxidation (FAO): adenosine triphosphate citrate lyase convers citrate into acetyl-CoA and oxaloacetate for cholesterol and FA synthesis; acetyl-CoA carboxylase carboxylates acetyl-CoA to malonyl-CoA which act as substrate for lipogenesis and as inhibitor of carnitine palmitoyltransferase 1 to regulate the FAO; fatty acid synthase produces the 16-carbon palmitate – PC (16:0) - by adding seven malonyl-CoA molecules to one acetyl-CoA. Therefore, a genomics alteration may reflect the aberrant FA, choline and ethanolamine phospholipids' metabolism, since evaluation of these metabolic-related enzymes has unveiled their involvement in tumorigenesis and cancer progression (Menendez and Lupu 2007; Zaidi, Swinnen, and Smans 2012). So, a better understanding of the altered associated mechanisms of the above-mentioned enzymes would broaden the underlying molecular factors that may lead with cancer origin or progression.

Lysophospholipids

From the experimental results of this dissertation, the list of identified lipidic compounds belonged to several different classes. Overall, we found a decreased expression of lysophospholipids (lysoPC and lysoPE) in plasma samples of BC molecular subtypes compared to HC: lysoPC (20:3), lysoPEs (18:1 and 18:2) in LB molecular subtype; lysoPC (14:0) in the LA; lysoPE (18:1) and lysoPC (16:0) in HER2 phenotype; and lysoPC (16:0) in the TN. Interestingly, we observed a similar behaviour for the lysoPE (18:2) and lysoPC (16:0) in non-responders LB patients whereas the expression of lysoPE (18:1) was decreased in plasma of responders LB patients. In this context, by using ASCA for metabolomic data analysis, we could add some more information about the lysophospholipids' alteration through the clinical BC process under a NACT regimen. Thus, in LB patients the lysoPEs (16:0 and 20:4) varied their expression towards the diagnosis, post-treatment and post-surgery while in HER2 patients we observed alteration of circulating lysoPC (20:4 and 22:6), and lysoPE (16:0

and 22:6). Last but not least, we found that plasma levels of lysoPC (22:4) differed between TN patients according to their response to NACT.

In line with our results, higher plasma levels of lysophospholipids are related to lower cancer risk throughout the literature. Indeed, decreased circulating lysoPC (16:0, 18:0, 18:1 and 18:2) have been previously reported in CRC (Z. Zhao et al. 2007) while higher expression of lysoPC (18:0) is associated with low risk of BC, prostate cancer and CRC (Kühn et al. 2016). In this sense, the common lipidic remodelling may be driven by an upregulation of the *de novo* FA synthesis in cancers such as CRC, lung or liver, whereas an increased expression of fatty acid synthase has been associated to a worse prognosis in BC or prostate cancer (Thupari, Pinn, and Kuhajda 2001; Brusselmans et al. 2005; Menendez and Lupu 2007). Moreover, cancer cells are able to obtain extracellular FAs under hypoxic conditions in order to maintain their lipids demands. In this instance, lysophospholipids may be utilized by cancer cells in several ways: (1) to conform cell membranes, (2) for energy storage or (3) as signalling molecules (P. Liu et al. 2020; Z. Chen et al. 2023; Jin et al. 2023). So, the uptake of exogenous lysophospholipids provide with the fuel for cells' survival and progression (Koundouros and Poulogiannis 2020).

Similarly, we found that levels of lysoPEs (16:0, 18:0, 18:2 and 22:6) and lysoPCs (P-16:0, 20:5, and the unknown LysoPC) were also decreased in plasma samples of recurrent mCRC patients after the resection of liver metastasis. In accordance, the largest recent study on pre-diagnostic lipid levels and CRC risk demonstrated an inverse relationship between these energy compounds and CRC tumorigenesis (Harewood et al. 2024). However, it is noteworthy that under our study conditions, the levels of these glycerophospholipids were higher in plasma samples of mCRC previous the resection of the liver metastasis in comparison with those samples obtained after the surgery. Interestingly, in the context of early-stage cholangiocharcinoma, higher levels of lipids in pre-operative blood samples were associated with the existence of CSCs, which benefits disease recurrence (Padthaisong et al. 2021). Also, in BCSCs the FAO is elevated to facilitate cell renewal and chemoresistance. Otherwise, inverse concentrations of circulating lysoPC (18:1) have been previously observed in non-advanced CRC (Z. Zhao et al. 2007) and mCRC (Martín-Blázquez et al. 2019). Herein, our findings suggest that circulating levels of glycerophospholipids in the metastatic setting may vary in comparison with that found in localized disease, which denotes the importance for

further investigation of this molecular pathway in order to define useful biomarkers of the cancer progression or potential new targets for a better precision medicine.

Acylcarnitines

Besides the glycerophospholipids above described, the aberrant plasma expression of two acylcarnitines (tridecanoyl carnitine and dodecanoyl carnitine) were found to be altered in the BC temporal metabolomics-based analysis. In this case, increased levels of the long-chain tridecanoylcarnitine were found in the non-responders LB patients at the time of diagnosis while the medium-chain dodecenoylcarnitine could not be associate to the LB response at the time of diagnosis, but it related to a temporal change within this BC phenotype. Consistent with our results, the carnitine system was also altered in the LB patients when analyzing this BC cohort of patients with a different metabolomics strategy (Zapater-Moros et al. 2023). Accordingly, His M. *et al*. have demonstrated direct associations between plasma acylcarnitine C2 and risk of breast cancer (His et al. 2019). In this regard, regulation of the carnitine system is pivotal in supplying energetic and biosynthetic demands in malignant cells (Melone et al. 2018). The shuttle of carnitine/acylcarnitine from the cytosol to the mitochondria provides with the acyl groups for the FAO, one of the main metabolic strategies promoted in some type of tumours due to its highest rate of ATP production in comparison to the oxidation of other nutrients (Caro et al. 2012; X. Wu et al. 2014). Indeed, it should be noted that recent observations in BC tissue pointed to the upregulation of key metabolic enzymes for the FAO (Sun et al. 2020). However, the role of acylcarnitines' circulating levels in the aetiology of cancer is not well-described, since they may be influenced by different factors such as sex, age, BMI, the intake of certain foods, metabolism of branched-chain amino acids and fasting status at blood collection (Wedekind et al. 2022). In this context, we could suggest that plasma concentrations of acylcarnitines may be a reflection of disease progression in LB patients which requires higher rates of the FAO pathway and lipolysis, in a similar way that those higher concentrations of medium and long-chain acylcarnitines found during fasting conditions (Dambrova et al. 2022; Liepinsh et al. 2014; Makrecka et al. 2014).

Docosahexaenoic acid (DHA)

Lastly, the detection of significantly altered DHA levels in the most aggressive BC phenotype highlights the potential influence of this omega-3 long-chain polyunsaturated FA on the disease. Specifically, we found higher baseline DHA levels

in plasma of TN responders compared to non-responders (Díaz et al. 2022). In this regard, DHA's anti-cancer effects in the BC-TN molecular subtype have been previously described, primarily by inducing cancer cell death *via* apoptosis and, to a lesser extent, pyroptosis. Studies committed to investigate DHA's role in inhibiting BC growth have shown a tight association with cancer cell death through caspase signalling activation: gasdermin D cleavage *via* caspase-1 activation in pyroptosis (Pizato et al. 2018), or caspase-3 and poly(ADP-ribose) polymerase activation *via* caspase-12 cleavage during endoplasmic reticulum stress in apoptosis and autophagy (T. tian Wang et al. 2021).

Additionally, a recent LC-MS metabolomics-based analysis demonstrated DHA's potential as part of a biomarker model that distinguished BC patients from HC, with increased DHA plasma levels correlating with protective or anti-cancer effects (Park et al. 2019). Moreover, multiple *in vivo* and *in vitro* observations support the evidence about DHA's role in reducing BC cell viability and proliferation, as well as enhancing chemotherapy efficacy by integrating into tumour membrane phospholipids (Mason et al. 2015; Molfino et al. 2017; Newell, Brun, and Field 2019).

Although the molecular mechanisms underlying the anti-cancer effects of omega-3 polyunsaturated FAs are not yet fully understood, ongoing clinical trials (i.e. NCT03383835, NCT03831178, NCT01849250 in ClinicalTrial.gov) are investigating DHA supplementation alongside the chemotherapy setting. This fact underscores the potential therapeutic value of DHA in improving BC outcomes (Newell et al. 2019).

6.3 Methodological considerations

Discovery-based research has produced big amount of data from several -omics high-throughput technologies that claimed to discriminate for cancer diagnosis and prognosis. During this dissertation, some strengths and weaknesses were found when using untargeted metabolomics-based approaches for biomarkers' discovery. In spite that hundreds of molecular features might differentially discriminate the experimental groups under study, many of them could not be tentatively identified and, for those with a putative identification, caution is needed for their biological interpretation.

6.3.1 *Study design and sampling*

Sample size determination during design of discovery-based studies using untargeted metabolomics copes with some determinant issues due to the nature of

global phenotyping. This challenging task requires *a priori* assumptions, or hypothesis, to calculate the minimum expected difference and estimated variability. However, global metabolomics is "hypothesis generating" and potential metabolic targets are unknown before the analysis is completed. Moreover, metabolome complexity is higher than other -omics ensembles and high-resolution platforms do not provide with detection of the whole set of known and unknown variables present in a sample. Thus, we must be aware about bias from theoretical and measured metabolome differences since using metabolomics different signals may correspond to the same molecular compound. Last, high-throughput platforms provide with big amounts of data, much greater than the number of samples tested, which have to face multiple hypothesis-testing contexts, estimation of effect size for power analysis while increasing the risk for false discoveries (Billoir, Navratil, and Blaise 2014).

It should be highlighted that in reality, there not exist standardization in the statistical methodology for sample size calculations in global metabolomics-based analyses. Furthermore, the number of samples analysed will also depend on the costs and available funds for patients' recruitment, clinical follow-up, samples and data collection, analysis and interpretation. In this sense, multicentric studies provide with higher probability to increase the patients' ratio, mitigating some of these drawbacks. An outstanding tool to address the heterogeneity of metabolomics data and the limitations inherent in sample collection is the advent of AI for biomedical research. By applying machine learning algorithms and AI-driven analytical tools, it is now possible to handle complex and large-scale datasets more effectively. AI enhances pattern recognition, allowing for the identification of hidden relationships between variables and improving the accuracy of biomarker discovery. Moreover, AI can help optimize sample size and minimize bias by predicting variability more accurately and adapting analysis in real-time as new data is integrated. This makes AI a powerful tool to overcome traditional bottlenecks in metabolomics, ultimately driving forward precision medicine initiatives (Camacho, Díaz, and Sánchez-Rovira 2022; W. Chong et al. 2022).

On the basis of this dissertation, three main types of cancer cohorts were analysed with the aim to answer three different questions regarding the utility of untargeted metabolomics for detection and identification of diagnostic or prognostic biomarkers of cancer for clinical use. In every case, we did expect to find the *maximum* number of metabolomic features as possible to differentiate between the groups under study. In

this regard, we are aware that our studies are limited by a relatively low sample size. Nevertheless, obtaining a homogeneous cohort can be very difficult to deal with, particularly when collecting biological samples at different time points. In the case of the TN-BC phenotype is even more challenging since it is the most aggressive of the BC molecular subtypes and, unfortunately, for some patients, it was not possible to obtain the three samples to meet their eligibility for the study. Similarly, the metastatic and surgical setting hinder the collection of biological samples since the eligible population for undergoing interventions of hepatic metastasis sin CRC represent approximately 10% of liver metastatic CRC patients, which would be equivalent to 12 or 15 patients annually in our hospital. However, we warrant future recruitments of higher number of patients which enable obtaining more rigorous results and to further validate the potential of the metabolomic signatures found in larger and external cohorts.

6.3.2 *Confounding and bias*

Metabolome is significantly influenced by intrinsic and extrinsic factors from the genome, proteome and their interaction, to the environment, diet, hydration or medication. Moreover, during a metabolomic analysis, technical confounders may also be a source of bias. So that, confounding factors must be considered in order to reach a clear and causal conclusion from the analysis (J. Zheng et al. 2022). In this regard, major efforts are made in order to avoid the bias of analysing raw data without considering these variables.

Through this dissertation, tested confounding factors that may affect the set of small molecules product from our metabolism were fundamentally age, sex and BMI. Except for the mCRC cohort, any of these variables were found to differentially determine the outcomes of our studies. In the CRC metabolomics-based prediction of recurrence and survival after resection of liver metastasis, though, we could observe that the chance of recurrence appeared to be influenced by age. In this regard, it is well-established that age is associated with prognosis in CRC (Lund et al. 2018; Álvaro et al. 2019; Mima et al. 2020; Kunst et al. 2020). Thus, our results confirm that age should be considered when performing large observational studies due to:

1. Comorbidities and general health status (Van Eeghen et al. 2015). Sometimes, advanced age implies greater comorbidity that may constrain treatment options as well as increase the risk of complications, translating into a worse prognosis and a higher probability of relapse.

2. Immune response (Roxburgh et al. 2013; Thoma, Neurath, and Waldner 2021). Immunosenescence or the age-related poor capacity for the immune system might hinder the ability to fight cancer, affecting disease progression.
3. Treatment tolerance (Zare-Bandamiri et al. 2017; Lund et al. 2018). Age may also influence tolerance to chemotherapy both before and after surgery which may also promote relapse.
4. Biological characteristics of the tumour (Álvaro et al. 2019; Lan et al. 2021). The molecular characteristics of the tumour may also vary according to age and these differences might contribute to an increased rate of recurrence or come along with a decreased overall survival rate.

6.3.3 *Untargeted LC-HRMS metabolomics*

To date, the application of LC-HRMS-based metabolomics in the study of cancer has provided with the identification of a myriad of phenotypic variations which are necessary for the survival of cancer cells and that can be used to differentiate them from healthy cells. However, by using the metabolic phenotyping strategy you may not measure every metabolite in the organism of sample under study and the measurements are not always biologically informative due to the following issues:

1. First, the metabolic picture will vary significantly based on the source of the biological material studied, being crucial to select the most adequate type of fluid to analyse (Breiding 2014). Under our study conditions, plasma samples were used in every case and analysed by using untargeted LC-HRMS-based metabolomics. Regarding the use of plasma samples, this type of biological source is advantageous in a clinical setting due to its ease of collection. It is minimally invasive and can be obtained through the same track used for treatment administration, thereby reducing additional exposure or unnecessary pain for patients. Moreover, the sample procedure is simpler and faster than that needed when obtaining serum.
2. Second, due to the complexity and the multifactorial character of cancer, deciphering the molecular aetiology or the triggers of cells' progression should be developed by the integration of several approaches and not just limiting to one methodology. In our case, two main approaches of LC-HRMS, by using RPLC and HILIC combining different ionization modes, were performed in order to cover the maximum range of physicochemical molecular features within our samples of choice. Moreover, analysis of samples collected at several time points should be a

requisite in order to unravels the dynamism of the disease behaviour. In this sense, untargeted longitudinal metabolomics is much more advantageous to provide the cascade effects and biochemical amplifications that may occur during cells' metabolism.

3. Last but no less, the primary bottleneck in data interpretation for untargeted metabolomics-based strategies is the identification of metabolomic features. Although many novel spectral libraries have emerged over the years, facilitating faster and more accurate basic interpretation of metabolomics experiments, further spectrum annotation is still needed to enhance the biological knowledge provided by untargeted metabolomics (Bittremieux, Wang, and Dorrestein 2023). Additionally, the use of AI holds great promise in the data interpretation process as it will significantly enhance the elucidation of new structures at a much faster pace than in previous years.

In contrast to the metabolomics approach used in this dissertation, targeted metabolomics can detect and identify of hundreds to thousands of metabolites simultaneously, along with their quantification. This capability facilitates the comparison of metabolomics data with data from different cohorts, research centers and -omics technologies. Moreover, as far as targeted metabolomics identifies known metabolites based on their comparison with native and isotopically-labelled standards, false positives detection decreases. One of the major utilities of targeted metabolomics is to establish baseline thresholds for metabolite levels, from which the altered state of an individual or signalling pathway could be defined. However, the main disadvantage of this methodology is the limited scope for metabolites' detection, which may lead to the omission of potentially important but unknown features (Ribbenstedt Id, Ziarrusta Id, and Benskin 2018).

Thereby, study designs restricted to the use of just one of these main metabolomics approaches may decrease the discovery of novel and potential biomarkers or pathways for improving tailored therapeutic interventions in the oncological practice. Hence, using different approaches by a systematic performance of combined (un)targeted metabolomics in cancer research may solve the weaknesses of each of these strategies when carried out separately.

6.3.4 *Biomarkers discovery*

Regarding the phases of translational research reviewed in the *Introduction* of this thesis proposal, the findings above-mentioned and exposed would be understood as a T1 phase that needs further validation to get proper biological insights before its application into the clinical practice as a T2 phase (Fort et al. 2017; Mandal, Ponnambath, and Parija 2017). In this sense, accomplishment of three main items would enable us to establish our tentatively identified compounds as reliable diagnostic and/or prognostic biomarkers of BC or CRC:

1. Prospective recruitment. In the search of reliable biomarkers, the best source of samples is prospectively obtained or even collected in the past and properly banked to be analysed in the future.
2. Independent reproducibility. In order to avoid bias and threat from chance when finding useful biomarkers, a rigorous validation in a larger and external cohort of patients must be performed following exactly the same methodology that is object of validation.
3. REporting recommendations for tumour MARKer prognostic studies (REMARK). Study design is the most critical point when performing biomarkers' research; hence, following REMARK guidelines allows the analytical validity and clinical utility while promotes structured reporting of analyses (Hayes, Sauerbrei, and McShane 2023).

A lot of research and published articles have provided with an unprecedent number of biological markers for cancer in the last decades. Unfortunately, most of them have emerged as clinically not useful due to the inconsistence between the research studies or contradiction of the promising results. Hereafter, inadequate conclusions are consequence of the lack of standardized and rigorous methodologies with poor study designs and small sample sizes (McShane et al. 2005). To address this main issue, the National Cancer Institute-European Organisation for Research and Treatment of Cancer recommended the development of common guidelines for reporting tumour marker studies (First International Meeting on Cancer Diagnostics; From Discovery to Clinical Practice: Diagnostic Innovation, Implementation, and Evaluation; Nyborg, Denmark, July 2000). However, the last and critical step for translation of potential biomarkers to the clinical practice involves their validation in TR-driven clinical trials. This combination, though, is full of obstacles and still needs further accurate improvements

from their analytical validation, clinical validation in prospective studies, regulatory approval, to their implementation and incorporation into the clinical guidelines and practice.

6.4 Further research in pursuit of precision oncology

In the medical practice, the only way to pursuit a real precision oncology is by supporting and improving high quality TR-driven clinical trials as they play a critical role at the healthcare system (Shahzad et al. 2011; Gu et al. 2021). A pragmatic and close example is the Medical Oncology-TR Unit at the University Hospital of Jaén, where this doctoral thesis was performed in collaboration with the Fundación MEDINA. At our Medical Oncology-TR Unit, three main labors are carried out by a multidisciplinary team: 1) medical oncology practice by oncologists and specialized nurses; 2) clinical trials performance by professionals specialized as coordinators or data-entry; 3) translational research on clinical samples by biomedical specialists. Each of these labors are essential and (indirectly) co-dependent since: 1) the medical oncology practice is based on evidence-based research and accurately proved by 2) previous clinical trials which, at the same time, aimed to validate 3) scientific observations obtained at a more basic level. Thereby, the results presented through this whole dissertation might be understood as the future of precision oncology to a more or less extent.

6.4.1 *Implications in precision oncology*

It is noteworthy that targeting the impaired cancer metabolism has opened up new avenues for a better precision therapy in cancer (Anand et al. 2023). Nonetheless, despite the progress since the use of targeted treatments in precision medicine with higher overall survival and free disease survival rates, there are still subjects without response, limited clinical applications tailoring tumour-specific metabolic targets while mitigating systemic toxicity, neither the disease is eradicated in every case. This phenomenon brings to light that current research and understanding of the disease miss some pieces in the cancer puzzle. As it can be inferred from our findings, BC and CRC may share alterations at some molecular levels while other molecular aberrations appear to be more tissue-specific. In this sense, although we did not conduct a direct comparative metabolomic analysis between the aforementioned cancer types, main molecular similarities and distinctions were elucidated from the different clinical contexts:

1. Hypoxanthine was found differently expressed between pre- and post-surgery in mCRC samples.
2. Perturbations in the tryptophan metabolism were predominantly observed in BC molecular subtypes.
3. The porphyrin metabolism was altered in both BC and mCRC with a similar metabolic pattern.
4. Differential expression of secondary BAs was observed in the BC cohort; specifically, we found reduced levels of glycodeoxycholic and glycohyocholic acids in non-responders TN patients at pre- and post-surgery time points. In this context, primary BA biosynthesis along with taurine and hypotaurine metabolism exhibited notable variations primarily in the mCRC cohort.
5. While glycerophospholipid metabolism manifested significantly altered both in BC and mCRC cohorts, the fatty acid DHA and two acylcarnitines were only identified as differently expressed in plasma of BC patients in response to NACT.

According to the aberrant molecular compounds determined in our work, previous and recent research on BC and CRC could also identify the alteration of the related metabolic pathways in several clinical contexts. Hence, further investigation on these common features would allow with determining novel molecular targets for precision oncology regarding the metabolic hallmark of cancerous cells. In this sense, an interesting argument raises from our observations: the detection of circulating subproduct metabolites from the different identified pathways in plasma samples of cancer patients points to their relevance in the development or progression of the disease. Nevertheless, as extensively exposed during this dissertation, the information given by a unique biomolecule type should not be considered as an exclusively determinant of disease behaviour since many other molecules could interfere in their expression. Thus, metabolomic expression patterns in a clinical context should be integrated with the proteomics and genomics expression of enzymes and associated-genes and *viceversa* in order to obtain the most comprehensive picture of the disease molecular basis. Grounded on this argument, an integrative strategy for further deciphering the role of the molecular pathways identified as altered through this dissertation would consider a targeted analysis of: 1) candidate metabolites, 2) enzymes involved in the catabolism of this metabolites, 3) associated and codifying genes of the selected enzymes.

In this regard, the above-mentioned approach represents a promising line of research to enhance precision oncology and it promises to deepen our understanding of cancer metabolism, accelerate biomarker discovery, and facilitate the development of personalized therapy regimens tailored to the molecular characteristics of individual tumours. Thereby, this approach holds significant potential to improve patient outcomes and advance the field towards more effective and precise cancer care.

6.4.2 *Future research*

Grounded on the just presented results, different research lines may come up as worthwhile to be further developed in the future:

1. Metabolomics and oncobiosis. The alteration of intestinal and tumour microbiota might affect the expression of microbiome-specific metabolites easily detectable in faecal and plasma samples during cancer progression.
2. Cancer metabolomics and proteo-genomics. Aberrant circulating metabolites are the result from altered molecular pathways in which several enzymes are involved. DNA and RNA sequencing of the oncometabolic enzymes and their codifying genes might lead to the confirmation that measuring levels of oncometabolites in plasma are a functional reflection of related enzymes activity or gene expression in a minimally invasive way.
3. Cancer metabolomics and big data. Integration of all the above-mentioned data in a temporal manner would not be possible without a proper bioinformatic analysis. Since high-throughput methodologies are bid data generators *per excellence*, combining multi-omics approaches by machine learning or AI algorithms is required. This strategy will facilitate the comprehensive dynamic study of the disease behaviour, in order to provide with the most accurate molecular risk score that will reveal the presence of tumoural cells, by an easy and cost-effective monitoring of MRD in cancer patients.

In addition, a new hypothesis emerges from the presented observations: molecular pan-tumoral aberrations may serve as the basis for a novel cancer nosology, shifting the focus away from prioritizing the tissue of origin. Hence, a more rational approach would involve universal genomic testing of all tumours, followed by (un)targeted proteo-metabolomics analyses to detect unexpected druggable alterations or biomarkers. This strategy aims to enable a more accurate, personalized, and molecular-based management of the disease.

CONCLUSIONS

7. CONCLUSIONS

1. The circulating metabolome of BC molecular subtypes shows significant differences when compared to HC. This indicates that each phenotype of BC may have unique metabolic signatures to be used for both BC subtyping and diagnosis.

2. Untargeted LC-HRMS-based metabolomics enables the identification of molecular pathways involved in the metabolic dysregulation of BC phenotypes. The most relevant biological routes were the metabolism of glycerophospholipids and porphyrin related compounds.

3. Temporal untargeted metabolomics combined with ASCA data analysis allows a better understanding of metabolome dynamics during BC pathology process, serving as a valuable tool to decipher disease behaviour under a NACT regimen.

4. Potential predictive biomarkers of response in the TN phenotype were identified by the temporal untargeted metabolomics using ASCA data analysis. We found higher levels of DHA in baseline plasma of responders TN and higher conjugated-secondary BAs (glycodeoxycholic and glycohyocholic acids) at pre- and post-surgery time points. Assessing these compounds may allow for response monitoring, adjusting therapy to ensure that patients receive the most appropriate and effective treatment, tailored to their unique molecular profile.

5. Using untargeted metabolomics, we identified significant differences in the plasma metabolomic profiles of CRC patients before and after liver metastasis resection. The main molecular pathways implicated in these changes were related to the metabolism of taurine and hypotaurine, the biosynthesis of primary BAs, and the biosynthesis of phenylalanine, tyrosine and tryptophan.

6. Identifying a predictive and prognostic metabolomic signature in CRLM patients based on 13 metabolites enables precise risk stratification of disease progression. This model may help tailor post-surgical treatments and personalize follow-up strategies, ultimately improving patient outcomes and optimizing clinical resources.

7. Eventually, untargeted metabolomics offers a promising tool for biomarker discovery in cancer, laying the groundwork for the integration of metabolomic profiling into future molecular models that may become new resources to guide personalized treatment in precision oncology.

CONCLUSIONES

8. CONCLUSIONES

1. El metaboloma circulante de los subtipos moleculares del CM muestra diferencias significativas en comparación con el de sujetos sanos. Esto indica que pueden existir firmas metabólicas exclusivas de cada fenotipo, que pueden utilizarse tanto para la subtipificación como para el diagnóstico.

2. La metabolómica no dirigida mediante cromatografía líquida acoplada a espectrometría de masas posibilita la detección e identificación de rutas moleculares involucradas en la desregulación metabólica de los subtipos del CM. Las rutas biológicas más relevantes implicadas están relacionadas con el metabolismo de los glicerofosfolípidos y los compuestos relacionados con la porfirina.

3. La metabolómica no dirigida combinada con el análisis de datos longitudinales mediante ASCA, permite una mejor comprensión de la dinámica del metaboloma durante el proceso patológico del CM. Siendo, además, una herramienta valiosa para descifrar el comportamiento de la enfermedad bajo un régimen de quimioterapia neoadyuvante.

4. Identificamos potenciales biomarcadores predictivos de respuesta mediante metabolómica temporal no dirigida y el análisis de datos ASCA. Encontramos niveles elevados de DHA en el plasma basal de pacientes TN respondedoras y niveles altos de ácidos biliares secundarios conjugados (ácidos glicodeoxicólico y glicohiocólico) en los tiempos pre- y postquirúrgicos. La evaluación de estos compuestos podría permitir la monitorización de la respuesta, ajustando la terapia para que las pacientes reciban la más adecuada y efectiva, adaptada a su perfil molecular único.

5. Mediante metabolómica no dirigida, identificamos diferencias significativas en los perfiles metabolómicos del plasma de pacientes con CCR antes y después de la resección de metástasis hepáticas. Las principales vías moleculares implicadas en estos cambios estaban relacionadas con el metabolismo de la taurina y la hipotaurina, la biosíntesis de ácidos biliares primarios y la biosíntesis de fenilalanina, tirosina y triptófano.

6. La identificación de una firma metabolómica predictiva y pronóstica en pacientes con CCR y metástasis hepáticas, basada en 13 metabolitos, permite una estratificación precisa del riesgo de progresión de la enfermedad. Este modelo puede ayudar a adaptar los tratamientos postquirúrgicos y personalizar las estrategias de seguimiento, mejorando así los resultados de los pacientes y optimizando los recursos clínicos.

7. Finalmente, se establece la metabolómica no dirigida como una herramienta prometedora para el descubrimiento de biomarcadores en cáncer, sentando las bases para la integración del perfilado metabolómico en futuros modelos moleculares que se conviertan en nuevos recursos para guiar el tratamiento personalizado en la oncología médica de precisión.

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