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Tesi di Laurea

PROGNOSTIC AND PREDICTIVE ROLE OF LIPIDOMIC PROFILE AND T CELL CHARACTERIZATION IN ADVANCED BREAST CANCER PATIENTS TREATED WITH CDK4/6 INHIBITORS.

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Index

LIST OF ABBREVIATIONS

ACC	Acetyl-Coa Carboxylase
AI	Aromatase Inhibitor
AMPK	AMP-Activated Protein Kinase
ATGL	Adipose Triglyceride Lipase
BC	Breast Cancer
BMI	Body Mass Index
CDK4/6	Cyclin-Dependent Kinase 4/6
CDK4/6i	Cyclin-Dependent Kinase 4/6 Inhibitors
Cer	Ceramides
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DAG	Diacylglycerol
DC	Dendritic Cell
DCIS	Ductal Carcinoma In Situ
eBC	Early Breast Cancer
ER	Estrogen Receptors
ET	Endocrine Therapy
FA	Fatty Acid
FACS	Fluorescence-Activated Cell Sorting
FAO	Fatty Acid Oxidation
FAS	Fatty Acid Synthesis
FASN	Fatty Acid Synthase
FISH	Fluorescence In Situ Hybridization
FOXP3	Forkhead Box P3
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hormone Receptors
HRT	Hormonal Replacement Therapy
HSL	Hormone-Sensitive Lipase
IDC	Invasive Ductal Carcinoma
IDO	Indoleamine 2,3-Dioxygenase

IFN	Interferon
IGF-1R	Insulin-Like Growth Factor 1
IHC	Immunohistochemistry
IL	Interleukin
ILC	Invasive Lobular Carcinoma
KLRG-1	Killer Cell Lectin-Like Receptor G1
LAG-3	Lymphocyte-Activation Gene 3
LPC	Lysophosphatidylcholines
LS-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
MAGL	Monoacylglycerol Lipase
mBC	Metastatic Breast Cancer
MDSCs	Myeloid-Derived Suppressor Cells
MHC	Major Histocompatibility Complex
MUFA	Monounsaturated Fatty Acid
NA	Not Available
NAE	N-Acylethanolamines
NFAT	Nuclear Factor Of Activated T
NST	Non-Special Type
OEA	Oleoylethanolamine
OFS	Ovarian Function Suppression
OS	Overall Survival
PBMCs	Peripheral Blood Mononuclear Cells
PC	Phosphatidylcholine
PD	Progression Disease
PD-1	Programmed Cell Death Protein 1
PD-L1/2	Programmed Death-Ligand 1/2
PE	Phosphatidylethanolamine
PFS	Progression Free Survival
PG	Prostaglandin
PgR	Progesterone Receptors
PIP	Phosphatidylinositol Phosphate
PL	Phospholipids

PUFA	Polyunsaturated Fatty Acids
Rb	Retinoblastoma
RECIST	Response Evaluation Criteria In Solid Tumors
ROS	Reactive Species Of Oxygen
SAGA	Senescence After Growth Arrest
SASP	Senescence-Associated Secretory Phenotype
SCD	Stearoyl-Coa Desaturase
SD	Stable Disease
SL	Sphingolipids
SM	Sphingomyelin
SREBP	Sterol Regulatory Element-Binding Protein
T reg	Regulatory T Cell
TAG	Triacylglycerol
TAM	Tumor-Associated Macrophage
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor B
TIGIT	T Cell Immunoreceptor With Ig And ITIM Domains
TILs	Tumor Infiltrating Lymphocytes
Tim-3	T-Cell Immunoglobulin And Mucin-Domain Containing-3
TME	Tumoral Microenvironment
TNBC	Triple Negative Breast Cancer
TNF	Tumor Necrosis Factor
VD	Visceral Disease

INTRODUCTION

BREAST CANCER

Epidemiology

Breast cancer (BC) is the most frequently diagnosed cancer and represents the second leading cause of cancer-related deaths among women worldwide. It's estimated that one in eight to ten women will get breast cancer during their lifetime. (1)

Incidence and mortality vary in different regions of the world, with a higher rate and a lower lethality in the West compared to the East. (1,2) Incidence of breast cancer has increased in recent years worldwide. This trend reflects a significant increase compared to previous decades, driven by numerous factors including changes in lifestyle, aging populations, and improved diagnostic capabilities. High-Income Countries (HICs) have the highest incidence of breast cancer, but advancements in screening and treatment have led to higher survival rates. (3) This change is attributable to the combination of greater population awareness, an increase in early detection, timely diagnosis, and effective treatment strategies. Conversely, in Low- and Middle-Income Countries (LMICs), while incidence rates are also rising, the mortality rates remain disproportionately high due to limited access to quality healthcare services. (3)

In Europe, breast cancer covers about 30% of all female tumours, representing the neoplastic condition with the highest prevalence among women. (4) Specifically, in Italy, 834,200 women are estimated to live after a breast cancer diagnosis. In 2023, there were an estimated 55,900 new cases of breast cancer among women. Concerning mortality, in 2022 there were approximately 15,500 deaths attributed to the disease, however data indicates a 6% decrease in breast cancer mortality from 2007 to 2019. (5)

Thanks to the introduction of mammographic screening campaigns and increased awareness among women, the majority of malignant breast tumours are now diagnosed at an early stage. (5) At this stage, surgical treatment can often be more conservative,

and the adopted therapy more effective, leading to remarkably high 5-year survival rates, currently around 88% in Italy. (5) However, the lifetime risk of recurrence in these treated patients, considered disease-free, is around 20-30%. Indeed, in Italy, approximately 37,000 women live with a diagnosis of metastatic breast cancer. (5) Only 6-7% of these cases are metastatic at diagnosis, whereas in most scenarios, advanced breast cancer represents a recurrence of the disease after treatment for an initial form of breast cancer. (6)

Metastatic breast cancer is a condition that is not curable by definition. Despite that, overall survival for patients with metastatic disease has significantly increased in recent years due to diagnostic and therapeutic advances, availability of new anticancer drugs, improved supportive therapies and better integration of systemic and local therapies. (7)

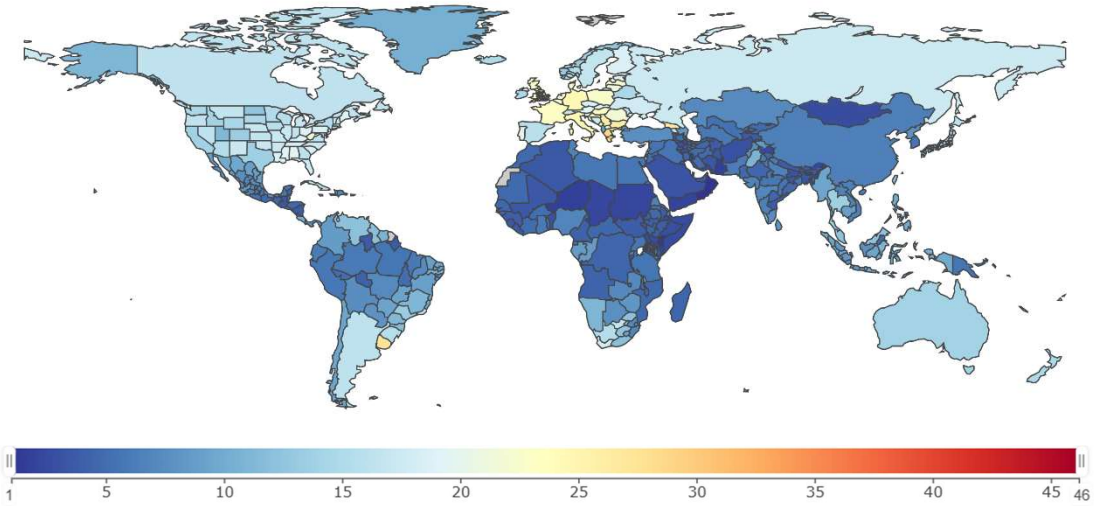


Figure 1 - Breast cancer, deaths per 100.000 (2021)

Risk factors

Breast cancer is a multifactorial disease and, as such, presents multiple associated risk factors. The number of risk factors of breast cancer is significant and includes both modifiable factors and non-modifiable factors. (8)

Concerning non-modifiable factors, female sex, older age, family history, genetic mutations, ethnicity, reproductive factors, previous history of breast cancer or other breast disease and previous radiation therapy are the most crucial factors playing a role

in breast cancer development. (8) The risk of developing breast cancer increases with age. The probability of developing breast cancer is 2.3% up to the age of 49 (1 in 43 women), 5.4% between 50 and 69 years old (1 in 18 women), and 4.5% in the 70-84 age group (1 in 22 women). This correlation appears to be linked to the continuous endocrine proliferative stimulation of the mammary epithelium, combined with progressive DNA damage and the accumulation of epigenetic alterations that disturb the balance between oncogene expression and tumour suppressor genes. Reproductive factors, related to an increased estrogen exposure, also play a role as risk factors: long duration of the fertile period, nulliparity, a first full-term pregnancy after the age of 30, not breastfeeding, and hormonal factors including the use of estrogen-progestin both for contraceptive purposes and as hormone replacement therapy during menopause may increase the risk of breast cancer. Genetic factors are equally important, both in terms of family history and heredity. Although most breast cancers are sporadic, 5%-7% are hereditary, a quarter of which are due to mutations in two genes: BRCA-1 and BRCA-2. Other genes that may play a role are ATM, PALB2, p53, PTEN, STK11/LKB. (5,6)

Many modifiable factors also play a role in increasing breast cancer development risk. Among them we can find hormonal replacement therapy (HRT), insufficient vitamin supplementation, drugs, but biggest role is played by various lifestyle factors, consequently linking breast cancer risk also with socioeconomic status. These include dietary factors such as high consumption of animal fats and processed food, low consumption of vegetable fibres, high alcohol consumption, smoking, low physical activity. Metabolic factors like obesity and metabolic syndrome also contribute to increase the risk of develop breast cancer.

Classifications

Histological classification

Most breast cancers are carcinomas, arising from the epithelial component of the breast, which consists of the cells that line the lobules and terminal ducts. Within the large group of carcinomas, there are many diverse types of breast cancer identified based on their pathological features and invasiveness.

Ductal carcinoma in situ (DCIS) is a non-invasive or pre-invasive breast cancer, which develops inside of pre-existing normal ducts, but has high potential to become invasive cancer, so early and adequate treatment is important in preventing the patient from developing an invasive cancer. (9,10)

Infiltrating breast cancer have cancer cells that invade and spread outside of the normal breast lobules and ducts, growing into the surrounding breast stromal tissue. They have the potential to spread to other sites of the body, such as the lymph nodes or distant organs. Based on the tissue and cell types involved, invasive breast cancers are further divided into Invasive Ductal Carcinoma (IDC), now defined as Non-Special Type (NST), and Invasive Lobular Carcinoma (ILC). IDC is the most common type of breast cancer representing about 80% of all breast cancers. It is characterized by a large diversity in terms of cancer cell morphology and the presence of tubular or glandular structures. ILC is the second most common type of breast cancers and accounts for approximately 10-15% of all breast cancers. It is characterized by a small diversity of cancer cells, very frequent expression of steroid receptors and extremely rare overexpression of the Her-2 receptor. ILC and IDC show similarities and differences, both regarding clinical, molecular and genetic features, thus presenting different prognoses and treatment options. Rare types of breast cancers include inflammatory breast cancer, Paget disease of the breast, angiosarcoma of the breast, phyllodes tumour etc. (9,10)

Metastatic breast cancers are late-stage tumours which have spread to other organs in the body. Metastases from breast cancers can be found in lymph nodes and/or in distant sites such as the lung, liver, bone, and brain. Patients may initially be diagnosed with metastatic disease (de novo), or they may develop metastases after receiving initial treatment (recurrent). Approximately 30% of the women diagnosed with early-stage breast cancer will develop a metastatic form of the disease. Indeed, even after primary tumour treatment, microscopic tumour cells or micro-metastases may remain in the body, which allows the cancer to return and disseminate. Recurrence risk is not clearly understood and not fully predictable as it depends on different features, largely depending on the unique molecular biology of the tumour and the stage at the time of the original diagnosis. (9,10)

Immunohistochemical classification

Routinely determined elements of the pathomorphological examination are insufficient to predict the clinical course of breast cancer, which makes it difficult to make appropriate therapeutic decisions. The diverse clinical course of cancers with similar morphological characteristics is due to their molecular heterogeneity. (10)

Breast cancer encompasses a heterogeneous and phenotypically diverse group of diseases. Several biological subtypes exist and show distinct behaviours and therapeutic targets, differing significantly in prognosis as well as in responses to therapy. The current biological classification of invasive breast tumours is based on the immunohistochemical (IHC) assessment of estrogen and progesterone receptors (ER and PgR) expression, Ki67 proliferation marker, and HER2/neu receptor. The combined evaluation of such IHC biomarkers allows to identify four subtypes of breast tumours, with different clinical behaviours, prognosis, and response to treatments. (10)

Luminal A-like tumours account for about 40% of all breast cancers and show positive ER and/or PR, no HER2 and low levels of Ki-67 expression. They are low-grade, slow growing, and tend to have the best prognosis.

Luminal B-like account for less than 20% of all breast cancers, is ER and/or PR positive, HER2 negative and presents elevated levels of Ki-67. They grow faster than luminal A and their prognosis is worse.

HER2-enriched makes up 15-20% of all breast cancers, can be either ER/PgR negative or positive, and it is characterized by a high HER2 expression. They grow faster than luminal cancers and have a generally worse prognosis however, they can be successfully treated with targeted therapies aimed at the HER2 protein.

Triple-negative breast cancer (TNBC) accounts for approximately 20% of all breast cancers, and is characterized as ER-negative, PgR-negative and HER2-negative. This subtype is more common in women with BRCA1 gene mutations as well as among women younger than 40 years of age and African American and usually behaves more aggressively than other types. (10)

Table 1 - IHC classification

BC subtype	HR	HER2	Ki67	Frequency
Luminal A-like	+	-	↓	40%
Luminal B-like	+	-	↑	~ 20%
HER2-enriched	±	+	↑	15-20%
TNBC	-	-	↑	~ 20%

Anatomopathological classification

TNM is the most used classification for breast cancer staging. This histopathological examination assesses the size of the primary tumour (T), the condition of the axillary lymph nodes (N) and the presence of distant metastasis (M). Its 8th edition, published in 2018, includes further prognostic factors such as tumour grade, ER/PgR expression, HER2 expression/amplification and multigenic tests. Correct assessment of all elements of the TNM classification makes it possible to determine the stage of cancer, which is the most important prognostic factor. (10,11)

Table 2: TNM classification of breast cancer, 8th edition (AJCC).

Tumour	**T0/Tis**	**T1**	**T2**	**T3**	**T4**
	T0: No primary tumour Tis: confined to ducts or lobules	≤ 2 cm	> 2 cm ≤ 5 cm	> 5cm	Any size, extension to the chest wall/skin
Nodes	**N0**	**N1**	**N2**	**N3**	
	No lymph nodes metastasis	1-3 axillary lymph nodes	4-9 axillary lymph nodes	Infra- or supraclavicular lymph nodes or ≥ 10 axillary lymph nodes	
Metastasis	**M0**		**M1**		
	No distant metastasis		Evidence of distant metastasis		

Prognostic and predictive factors

Breast cancer presents numerous prognostic factors such as axillary lymph nodes status, tumour size, patient age, histologic grade and subtype, proliferation rate (Ki67), hormone receptors (HR) status, human epidermal growth factor receptor 2 (HER2) expression/amplification, gene expression profiles and tumor infiltrating lymphocytes (TILs). Ki67, HR, HER2 expression, gene expression profiles and TILs have also shown a predictive role for different type of systemic therapies such as chemotherapy, endocrine therapy, target therapy and immunotherapy.

Therapy principles

Breast cancer therapy is a multifaceted approach aimed either at eradicating cancer or chronify the disease, respectively for early and advanced breast cancers. The choice of therapy depends on several factors, including the type and stage of cancer, HR and HER2 status, patient global health, and preferences. Therapeutic choices comprehend surgery, radiotherapy and systemic treatments, including chemotherapy, endocrine therapy, target therapy and immunotherapy.

METASTATIC BREAST CANCER

Metastatic breast cancer (mBC) represents the condition where the disease has grown beyond mammary gland and regional lymph-nodes and has spread to other organs. In 6-7% of cases, breast cancer presents as metastatic already at diagnosis, however, most of mBC patients have experienced disease recurrence after treatment for an initial form of breast carcinoma. (5) Although survival rates of mBC have dramatically improved over the past decades, this disease still represents an incurable condition with a median overall survival (OS) of 3 years and a 5-year survival of only 25%. (12,13) Recurrent mBC patients are much more difficult to treat since they have usually already received very potent adjuvant therapies. De novo mBC show a better 5-year survival rate compared to recurrent disease (44% vs 21%). Despite that, in recent years the landscape of mBC has undergone profound advancements leading to a progressive prolongation of progression-free survival (PFS) and, in some cases, also of overall survival (OS) particularly in de novo mBC. These results indicate that the increasing

concept of metastatic breast cancer as a chronic disease controlled by sequential therapies over a prolonged period is realistic, at least for certain subgroups. (1,6)

Generally, systemic therapy is the first therapeutic choice in metastatic breast cancer and locoregional therapy (e.g., surgery, radiotherapy) can be added in specific situations (such as primary, symptomatic bone, or brain metastases). (14) Nowadays, the treatment of advanced breast cancer aims to chronify the disease rather than aiming for a cure, maintaining quality of life and palliation of symptoms. Such treatment involves the use of long-term systemic therapies, with drug selection based on the evaluation of biological parameters and tumour characteristics, as well as the psycho-physical condition of the woman and the previous treatments received. (5) Because of potential heterogeneity between primary tumour and metastasis and even between metastases, gain of a therapeutic target is important for choice of therapy. For this reason, if clinically feasible, biopsy of the first metastatic site is recommended to verify breast cancer histology and determine again the tumour biology. (14)

HR+/HER2- metastatic breast cancer and CDK4/6i

HR+/HER2- mBC accounts for approximately two thirds of all mBC and show the better outcomes between other subtypes. (13,15) Endocrine therapy has been the preferred option for HR-positive disease for years, even in the presence of visceral disease, unless there is visceral crisis or concern/proof of endocrine resistance. (13) In recent years the addition of cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) to hormonal therapy in HR positive and HER2 negative mBC has played a relevant role in increasing therapeutic success, becoming the standard-of-care. (7) Approved drugs of this class (Palbociclib, Ribociclib and Abemaciclib), when combined with hormonal therapy, have shown to double progression free survival (PFS) both in the first line and subsequent lines compared to exclusive hormonal therapy, while maintaining an acceptable toxicity profile. Treatment with Palbociclib associated with Fulvestrant resulted also in longer OS than treatment with Placebo associated to Fulvestrant. (16–22)

Cyclin-dependent kinase 4 and 6 (CDK4/6) are two serine/threonine kinases modulated by D-type cyclins (D1, D2, and D3). (23) They play key roles in cell

proliferation mainly by controlling the G1 (pre-DNA synthesis) to S (DNA synthesis) cell cycle checkpoint. (24) Dysregulation of D-cyclin-dependent kinase 4/6-retinoblastoma (Rb) pathway has been frequently observed in cancer, including breast cancer. Particularly, CDK4/6 have been identified as key drivers of proliferation in HR-positive BC, justifying the use of CDK4/6i in these patients. (24–26) Upon activation by mitogenic signalling pathways, cyclins D associate with CDK4 or CDK6. Cyclin D-CDK4/6 complexes are able to phosphorylate Rb proteins (p110, p107 and p130). The hyperphosphorylation of Rb limit promotes dissociation of the transcriptionally repressive Rb-E2F complex, reducing the affinity for E2F. This leads to release of E2F transcription factors, that are free to reach the nucleus and activate genes required for entry into S phase and DNA replication, promoting cell cycle progression. (23,24,27,28)

Increased cyclin D-CDK4/6 activity can occur through several mechanisms. Overexpression of D-type cyclins, mutation or amplification of CDK4/6, loss of cyclin D-CDK4/6 negative regulators and oncogenic signalling pathways that promote cyclin D-CDK4/6 activity have shown to play a role in enhancing this pathway in breast cancer. (23,24)

CCND1 (encoding cyclin-D1 protein) amplification is seen in up to 35% of breast cancer cases, while his overexpression has been seen to occur in approximately 50% of breast cancers. (29,30) CCND1 is a transcriptional target of the ER, one of the most important mechanism responsible for overexpression of cyclin D in breast cancer is estrogen receptor activation that bind directly to the CCND1 promoter, enhancing the cyclin D1 expression and modulating the mitosis process. In addition, also the RAS-RAF-MEK-ERK pathway and the HER2-PI3K-AKT axis play a significant role in regulating cyclin D1 expression. This explains how amplification and overexpression of CCND1 oncogene is more frequent within luminal A (29%), luminal B (58%), and HER2 enriched (38%) subtypes. Similarly, CDK4 gene amplification, frequently demonstrated in breast cancer, has been observed more specifically in 14% of luminal A, in 25% of luminal B, and in 24% of HER2 enriched tumours. (23)

Endocrine therapy, hallmark of HR+ breast cancer, inhibits activation of this pathway, while CDK4/6 inhibitors trigger cell cycle arrest in Rb protein-competent cells. (25)

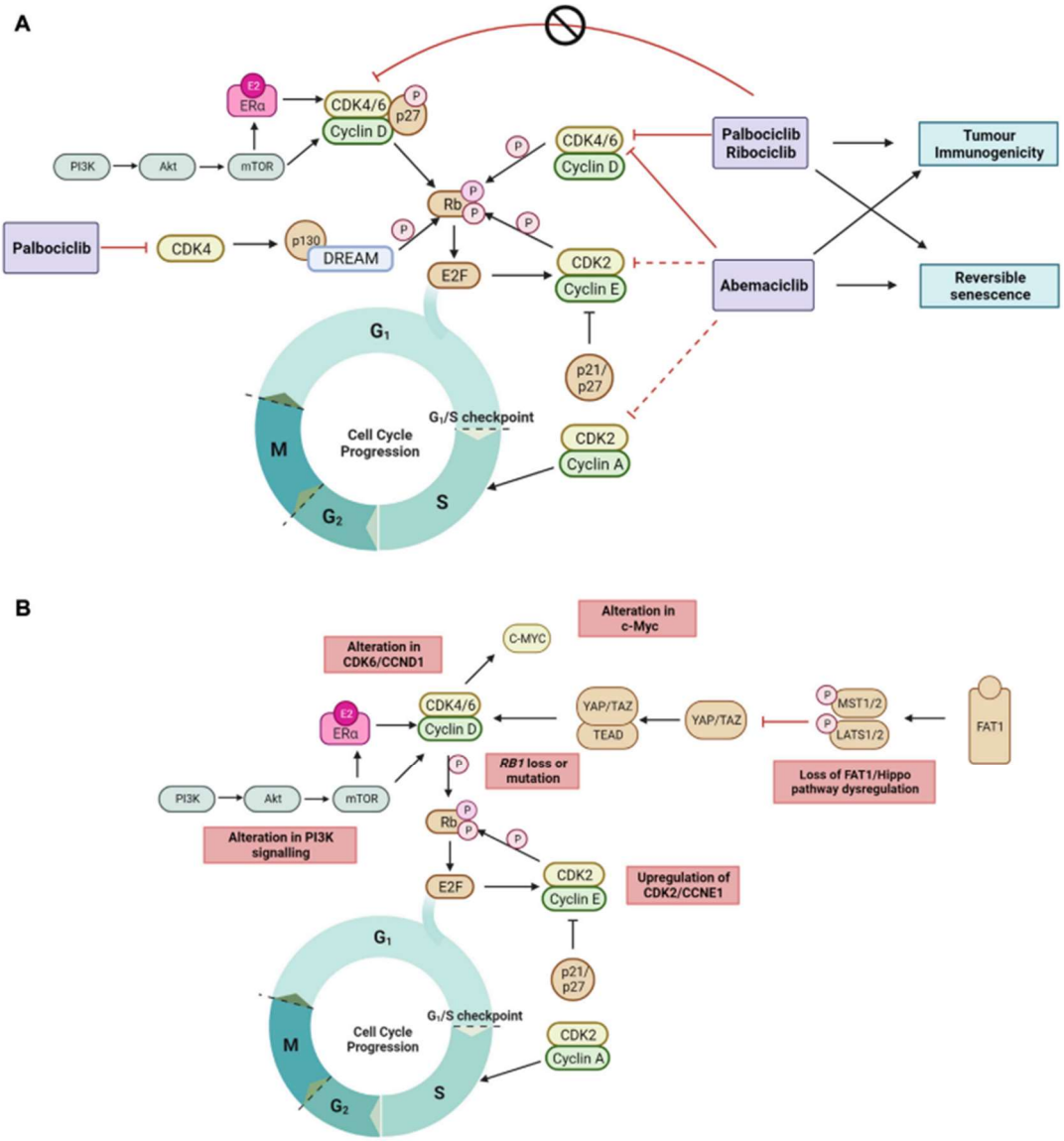


Figure 2 - signalling pathway of CDK 4/6-Cycle D complex and mechanism of action of CDK 4/6i. (31)

Recent evidence suggests that CDK4/6i alter tumour cell biology in other ways apart from blocking cell cycle progression, interacting with non-canonical CDK4/6 substrates, given that RB is the canonical CDK4/6 substrate. It has been demonstrated that CDKs 4 and 6 directly phosphorylate dozens if not hundreds of other proteins, and that CDK4/6 inhibition can thus impact tumour cell biology in an Rb-independent manner. Some notable examples include the FOXM1 transcription factor, key metabolic enzymes including 6-phosphofructokinase and pyruvate kinase M2, the signal transduction protein IRS2, the deubiquitinase DUB3, and the ubiquitin ligase adaptor SPOP. The consequences of inhibiting the phosphorylation of these substrates

are far-reaching and have been shown that they include modulation of tumour cell senescence, cellular metabolism, invasion and metastasis, and immunogenicity. (32,33)

Prolonged exposure of cancer cells to CDK4/6i has been shown to induce not only quiescence, but also a senescence-like phenotype in some cells. This transition from quiescence into senescence has been termed geroconversion and can also be described as senescence after growth arrest (SAGA). Unlike quiescent cells, that are temporary exit from cell cycle, senescent cells will not return to the cell cycle and are generally refractory to other proliferation-inducing signals. (33) CDK4/6i induces a phenotype that reproduces many of the morphological hallmarks of cellular senescence, a similar condition, but not an exact phenocopy compared to classical senescence induced by DNA damage. Induced changes include cellular enlargement, increased beta-galactosidase activity, and development of senescence-associated heterochromatin foci. (32) In addition, CDK4/6 inhibition upregulates a transcriptional program for cytokines and chemokines secretion known as senescence-associated secretory phenotype (SASP). SAPS has seen to influence the immune response: on one hand, it can induce the recruitment of immune cells that will mediate tumour clearance or promote paracrine senescence, on the other hand, it can create an immunosuppressive and protumourigenic environment. (33)

It has long been recognized that cell division is coordinated with metabolic state. A high number of non-Rb targets for CDK4/6 have been identified in the metabolic machinery. Among CDK4/6i effects on metabolism we can find different alterations of glycolytic and oxidative metabolism. These changesets leads to a stress response with an increase in reactive species of oxygen (ROS). CDK4/6 inhibition also depletes the antioxidants NADPH and glutathione. Increasing ROS and depleting antioxidants are known factors implicated in cancer cell autophagy and apoptosis. (32,33)

Moreover, CDK4/6i have shown important effects on non-tumoral cells within the tumoral microenvironment (TME), that can impair or enhance tumour responses to therapy. (33,34) For instance, CDK4/6 pathway regulates both proliferation and senescence in fibroblasts and a recent study demonstrated that CDK4/6 inhibitor-treated fibroblasts develop a senescent phenotype characterized by secretion of a large

number of pro-inflammatory cytokines. Hence, cancer-associated senescent fibroblasts may have the potential to enhance tumour growth by both directly stimulating tumour cells and also suppressing anti-tumour responses. (32)

Conversely, CDK4/6 inhibitors may have effects on specific lymphocyte populations that can potentiate anti-tumour immunity. The immunomodulatory activity of CDK4/6i is due to their interaction both with tumour cells and immune cells and includes increased tumour antigen presentation, promotion of immunogenic cell death, activation of effector T cells, depletion of immunosuppressive Treg, induction of T-cell memory formation, increase in B and NK cells and reduction of Myeloid-Derived Suppressor Cells (MDSCs) infiltration. (35,36)

After CDK4/6i treatment number of CD3+ cells recruited into the tumour mass increases, as well as cancer cells' processing and presentation of tumour neoantigens on Major Histocompatibility Complex (MHC) Class I molecules. (32–34) It is possible that CDK4/6 inhibitor-induced enhancement of tumour cell antigen presentation is one component of the senescent phenotype. (32) CDK6 inhibition enhances the nuclear translocation and activity of Nuclear Factor of Activated T cell (NFAT) family members, resulting in increased IL2 levels and increased expression of interferon-stimulated genes including those encoding for the antigen presentation machinery. In addition, CDK4/6 inhibitors reduced the activity of DNA methyltransferase, an E2F target protein which promotes cytotoxic T cell-mediated tumour inhibition. (32,37)

Moreover, CDK4 is necessary for the development of CD4+ FOXP3+ Treg cells. CDK4/6i suppress the proliferation of immunosuppressive regulatory T cells more than other T cell subsets, resulting in a decreased number of these cells in TME. (32,33) These changesets enhances the immunogenicity of the tumour cells, potentially shifting the immune balance in favour of an anti-tumour immune response. (32)

Finally, CDK4/6 inhibitors have been shown to inhibit the proliferation of bone marrow hematopoietic stem and progenitor cells. And endothelial cell proliferation and angiogenesis are CDK4/6 dependent phenomena, but the effects of CDK4/6 inhibition on tumour angiogenesis are not known. (32)

CDK4/6i resistance and predictive factors

Despite the clinical benefit that CDK4/6i offer to HR+/HER2- advanced breast cancer patients, the development of resistance remains a critical challenge in these patients. (37) Pharmacological resistance to this class of drugs is not fully understood yet. Several mechanisms have been described as possible drivers of resistance to CDK4/6i, however, many cases of resistance find no explanation in the molecular mechanisms studied so far. Among the described mechanism we can find loss of function of Rb, PTEN loss, FAT1 loss, overactivation of CDK2, aberrant cyclin E signalling, increased activity of CDK4/6, alteration of the MET-FAK/CDK2 or c-myc/miR-29B-3p/CDK6 axis, activation of tyrosine kinase receptor signalling (such as RAS, FGFR2, HER2). (38) Moreover, early data suggest that insulin-like growth factor 1 receptor (IGF-1R) amplification may be associated with resistance to CDK4/6 inhibition. (38) Recently, also deregulated immune related pathways were found to be associated with CDK4/6i resistance such as: deregulation of INF signalling, high PD-L1 expression, MDSCs recruitment. (35,37,39–43)

LIPIDS IN BREAST CANCER

Lipids biology in cancer cells

Lipids are involved in numerous cellular processes, many of which are linked to the oncogenic process. Triglycerides are mainly used for energy storage as they serve as an independent source for fatty acids (FAs) oxidation, which plays a critical role in promoting cell proliferation and tumour growth. Phosphoglycerates, sterols, and sphingolipids contribute to the structural components of cellular membranes, modulating their fluidity and forming lipid rafts involved in specific signal transduction pathways. Nevertheless, lipids also act as important metabolic signalling messengers and steroid hormones such as estrogen, progesterone and testosterone. (44)

Cancer cells high metabolism requires, in addition to an increase in glucose, glutamine and amino acid, the reprogramming of lipid metabolism. (45) Uptake, anabolism, and catabolism of lipids are increased in these cells to meet their multiple needs. Moreover, evidence is emerging that altered lipid metabolism, interfering with numerous

signalling pathways, plays a leading role in promoting tumour cell survival, growth, proliferation, differentiation, immune escape, and migration, thereby supporting cancer development in a changing and hostile environment. (44,46,47)

It has been known from decades that cancer cells largely rely on glucose for energy production and can use glucose more efficiently compared to normal cells, even in the presence of normal levels of oxygen, as described in Warburg effect. (44) However, it is now established that glucose is preferentially used by cancer cells as a carbon source for anabolic processes. While lipids play a fundamental role as energy sources, in order to face cancer cells' high energy request. (47) FA metabolism plays a pivotal role in cancers. The body obtains FAs through endogenous synthesis, primarily occurring in the liver, adipocytes, and lactating breast tissues, and from dietary intake providing exogenous FAs as free molecules or complexed with proteins in lipoproteins. While non-tumour cells rely more on exogenous sources, with limited endogenous FAs synthesis, in tumour cells one key aspect of lipid metabolism changesets is the upregulation of FAs synthesis. This is driven by the increased activity of key enzymes such as FASN (fatty acid synthase), ACC (acetyl-CoA carboxylase). Also, cancer cells show enhanced lipolysis and higher levels of FAs uptake through the upregulation of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL). (44) Fatty acid oxidation is also increased to assists cancer cells to not only sustain their rapid proliferative rate but also offer a vital energy source during conditions of metabolic stress and increased ATP demand such as hypoxia, cell invasion, resistance to anoikis. (47–49)

In cancer cells we also assist to cholesterol and cholesterol ester synthesis upregulation. (44) These lipids are vital for cancer cells to feed membrane production, essential process for cell proliferation and migration. In addition, they play a role in balancing membrane lipid content in order to protect cells from lipotoxicity and endoplasmic reticulum stress, preventing lipid peroxidation and ferroptosis. (50) In fact, saturated FAs are toxic to cells at prominent levels. SCD1 (steaoryl-CoA desaturase 1), overexpressed along with FASN in lipogenic tumours, has a key role in protecting cells from lipotoxicity, desaturating a substantial fraction of saturated FAs. (51) Moreover, membrane lipid composition changes, especially regarding content of

cholesterol and phospholipids containing saturated FAs, are known to dramatically alter membrane function and membrane fluidity. Membrane fluidity alterations have shown to stimulate metastasis formation and to alter the uptake of certain chemotherapeutics such as doxorubicin, thus influencing drug resistance. Chemo resistant cancer cells exhibit decreased fluidity in their lipid bilayers, hindering the uptake of chemotherapeutic agents through passive diffusion or endocytosis. (44,47,52)

Furthermore, membrane lipids are not uniformly distributed but, depending on their biophysical properties, tend to cluster into specific microdomains: lipid rafts. Lipid rafts are overall enriched in sphingolipids and cholesterol, and, depending on their specific lipid composition, they create optimal biophysical conditions for certain **signalling** proteins to be recruited and to cluster, acting as platforms for growth factor or cell death receptor signalling. For instance, cholesterol-rich lipid rafts allow the accumulation of tyrosine kinase receptors such as HER2 and IGF-1, thus enhancing oncogenic signalling. Lipids can also regulate signalling through post-translational modifications of proteins, as it happens with prenylation or palmitoylation of important oncogenes like EGFR and RAS, essential to their localization and function.

Another key role of lipids in cancer cells is balance of oxidative stress that characterize these cells due to oncogenic transformation, altered metabolism, deregulated redox homeostasis and hypoxia. Increased ROS levels have been shown to contribute to tumorigenesis, however an excess of ROS can induce cell death. Involved mechanisms comprehend degradation of lipid hydroperoxides by GPX4 (glutathione peroxidase 4), balancing membrane relative degree of saturation (through FAS activation, membrane monodesaturation mediated by SCD, MUFAs up taking and incorporating into membrane PLs, PUFA incorporation into TAGs), maintaining antioxidant levels (through FAO derived NADPH as well as FSP1-driven ubiquinone recycling). (47)

Moreover, lipids-inducted signalling alteration occurs also in the TME. In hypoxic, acidic, and nutrition-deficient TMEs, not only cancer cells, but also cancer associated cells, tend to rely on lipids for storage of energy, cellular building blocks for membrane formation, and sources of signalling molecules. Abnormally regulated lipids in the TME can dramatically influence tumorigenesis, subsequent cancer development, and

metastasis formation. (46)

Moreover, lipids function as precursor for important extra- and intra-cellular signals, such as oxylipins, LPa, ceramide and sphingosine-1-phosphate, DAG, PIPs, able to functionally influence cells in the TME. For instance, altered lipid metabolism reduces levels of the pro-apoptotic signals and increases the levels proliferative factors within the TME. Lipids are also able to influence the TME in an immunosuppressive direction, thus enhancing cancer cells immune escaping. It has been shown that in endothelial cells, acetyl-CoA produced through FAO is essential for de novo nucleotide synthesis. In this way, FAO drives pathological angiogenesis in vivo. (53) Finally, cancer cells have shown to generate lipid-enclosed microvesicles able to convey signals in distant sites and playing a role in metastases establishment. (47)

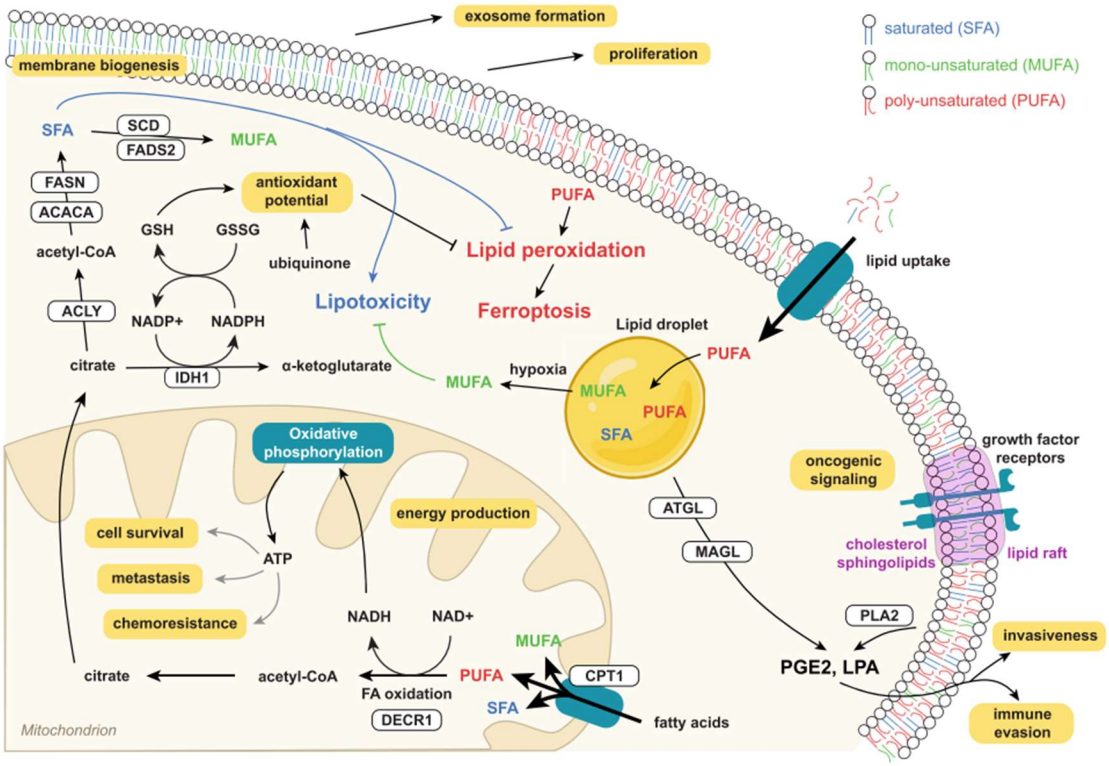


Figure 3 - Main mechanisms by which lipids contribute to cancer biology. (47)

Lipid metabolism in breast cancer

It is nowadays recognised that lipids play an essential role in the physiopathology of

many cancers, included breast cancer. This has been hypothesized for decades, due to the well-documented association between overweight and breast cancer reported since 1976. In these reports, obese BC patients exhibited significantly larger and more invasive tumours, along with lower survival rates compared to the control group. (54) Elevated BMI, obesity and metabolic syndrome are nowadays recognized as risk factors, as well as negative prognostic factors, for breast cancer. (6)

Excess of adipose tissue, especially in postmenopausal women where it represents the main source of circulating estrogens synthesis, represents a risk factor for breast cancer leading to excessive hormonal stimulation of the mammary gland. (6) Metabolic syndrome is a clinical condition characterized by the presence of at least three of the following factors: abdominal obesity, altered glycaemic metabolism (diabetes or prediabetes), elevated lipid levels (cholesterol and/or triglycerides), and high blood pressure. (6) Insulin resistance, correlated to being overweight and leading to increased blood insulin levels, is also linked to certain cancers, including breast cancer. Indeed, insulin acts on the membrane receptor of IGF-1, activating intracellular signalling pathways fundamental for neoplastic growth and described among drug resistance mechanisms. (9) Dyslipidaemia, defined as imbalanced blood lipid levels and strongly linked to obesity, may also contribute to breast cancer development as well as poorer outcome and drug resistance. (44) Indeed, dietary cholesterol has been associated with an increased risk to develop BC for many years. (55)

While much attention has always been given to metabolic disorders such as obesity and metabolic syndrome, recently many studies are exploring changes in lipid metabolism within tumour cells to understand their association with tumorigenesis, prognosis, and treatment efficacy.

Breast cancer is strongly associated with the biological function of FA metabolism owing to the abundant presence of adipocytes in breast tissue. (48) However, breast cancer consists of a group of heterogeneous subtypes in which different pathways dysregulation are involved. Many important pathways have been described as responsible of lipid metabolism alteration. Particularly, regarding HR+ breast cancers, it has been reported that the estrogen (ER) and progesterone (PR) receptor pathways has a key role in lipid metabolism alterations. For instance, de novo FAS and FA

transportation rate are also modulated based on hormone status and show increased levels in HR+ tumours compared to HR- ones. HR status is also related to lipid membrane modulation. Moreover, it has been shown that FASN, in turn, regulates the ER signalling pathway in mammary carcinoma cells. (56)

Lipids have been seen to play a role also in many drug resistance mechanisms, representing possible candidates as predictive biomarkers. For instance, numerous studies demonstrate that changes in the lipid composition of cellular membranes, as well as changes in FA metabolism, are linked to chemotherapy resistance. (52,56–60) Concerning endocrine therapy, resistant BC cell line models, compared to isogenic sensitive lines, show SREBP1-driven upregulation of genes involved in lipid, notably cholesterol, biosynthesis and targeting of SREBP has demonstrated to be effective in reducing the growth of these resistant cell lines. (61,62)

Lipidomic as biomarker in breast cancer

The potential for lipids as biomarkers has come to the forefront thanks to great advances in technologies enabling the quantitative analysis of complex lipids, including mass spectrometry-based lipidomics. Lipidomics is a new omics approach which studies the lipid profile of a cell, tissue, or organism. In recent years, several publications are providing initial data on potential roles of studying lipid profiles in breast cancer, such as: differentiating breast carcinoma from healthy tissues and differentiating subtypes of breast carcinoma (63–68), distinguishing breast cancer patients from healthy controls (65,69–74), highlighting tumours with increased metastatic potential and worse prognosis (75–77), providing insights for the development of therapeutic targets (78–88), predicting response to radiotherapy (89) and chemotherapy (90,91). However, nowadays few of these markers are currently being used in the clinic and the role of lipidomic studies in other settings, such as CDK4/6i treatments in HR+ metastatic breast cancer, have still to be explored.

IMMUNE SYSTEM IN BREAST CANCER

Immune system in cancer

Cancer immune surveillance refers to the process by which the immune system

monitors, recognizes, and eliminates newly formed tumour cells. This process involves three critical phases: elimination, equilibrium, and escape, through which tumour development progresses. One of the recognized hallmarks of cancer is its ability to evade immune surveillance. Cancer cells not only develop mechanisms to suppress the immune response but can also manipulate the immune system to support tumour growth. However, the mechanisms behind this process are not yet fully understood. (92)

Several types of immune cells such as lymphocytes, macrophages, and neutrophils, infiltrate the TME in response to the tumour presence. However, tumour infiltrate composition plays conflicting and seemingly counterintuitive roles in creating a tumour-antagonizing or tumour-promoting environment. In the elimination phase, immune cells can control and eliminate cancer cells, but later in the escape phase they often contribute to cancer development. Indeed, inflammatory cells and cytokines found in tumours tent to contribute to promote immunosuppression, rather than effective antitumour responses. The paradoxical role of immune cells is due to functional plasticity of myeloid and lymphoid cells, whose phenotype changes based on the stimuli they receive within the TME. (92)

The most well characterized mechanisms by which breast cancer cells evade the immune response include the expression of immune inhibitory costimulatory receptors (such as PD-1, CTLA-4, LAG-3), the secretion of tumour-derived immunosuppressive factors (TGF-β, IL-10, IDO) and the infiltration of suppressive immune cells (Tregs, MDSCs, TAMs) into the TME. This creates an immunosuppressive environment that can, in turn, influence tumour-associated cells, both immune and stromal cells, affecting their capability to fight cancer cells and giving them the power to enhance tumour cells' survival and proliferation. (92)

T cells dysfunctional states

It is now well recognized that an efficacy immune state is a key factor for successful antitumour immunity. (93) However, it is also demonstrated that the immunosuppressive TME can induce dysfunctional states in T cells. High numbers of tumour-specific functionally hyporesponsive T cells in the TME have been reported,

resulting in failure of cancer cells elimination and counteraction of tumour progression. Senescence and exhaustion of T cells are two dominant dysfunctional states, which represent a challenging issue for antitumour immunity. (94)

Senescent and exhausted T cells comprise different subpopulations and their actual definitions remain confusing since both states share several phenotypic and functional characteristics, such as defective proliferative activity, impaired cytotoxic activity, and increased cell cycle arrest. However, each state has unique molecular and developmental signatures, such as surface molecules, cytokines, and transcriptional profiles. (94)

T-cell exhaustion was initially described in chronic viral infections with increased expression of a panel of inhibitory receptors (IR), including PD-1, CTLA-4, Tim-3, LAG-3, BTLA, TIGIT, CD244 (2B4), and CD160. (94) Exhausted T cells are incapable of proliferating and show impaired cytotoxicity and effector cytokine production, including IL-2, TNF, and IFN-γ. (95).

Cellular senescence was first described as a biological process in cells with a finite lifetime and low rate of proliferation after extensive serial passages in vitro. Senescence also occurs in human T cells with aging, as well as in chronic viral infections and diverse types of cancers. (94) Cellular senescence processes are triggered by telomere shortening (replicative senescence) and/or damage signals (premature senescence), including oxidative stress, cell culture stress, DNA-damaging chemotherapeutic agents, and mitogenic oncogenes. Senescent T cells present a high expression of SA-β-Gal, dramatically downregulated expression of the costimulatory molecules CD27 and CD28, and high expression of additional senescence-associated markers, including Tim-3, CD57, CD45RA and KLRG-1. (94,95) Similar to exhausted T cells, senescent T cells do not proliferate after TCR stimulation due to the loss of several key molecules involved in the TCR signalling machinery. Additionally, they exhibit upregulation of cell cycle regulatory genes (such as p16, p21, and p53), displaying cell cycle arrest. (94) Furthermore, far from being dysfunctional, senescent T cells retain robust capacity to produce effector cytokines as they acquire a unique senescence-associated secretory phenotype (SASP), producing high amounts of proinflammatory cytokines, such as IL-2, IL-6, IL-8, TNF, and IFN-γ. In contrast,

senescent T cells also secret the suppressive cytokines IL-10 and TGF-β and show reduced expression of the effector molecules perforin and granzyme B. (95)

Increased senescent and exhausted T cells have been found in both the circulation and tumour sites in a variety of cancers, including breast cancer. Exhaustion in TILs, emerging evidence indicates that cancer cells themselves can directly induce T-cell exhaustion during cross talk. (94) Concerning senescence, recent studies have demonstrated that both tumour-derived Treg cells and multiple types of tumour cells can directly trigger T-cell senescence. Additionally, senescent T cells can directly suppress other immune cells in the TME thanks to their unique SASP secretion. (96,97) The growing body of evidence strongly suggests that that T-cell senescence is an alternative novel mechanism utilized by malignant tumours for immune evasion. (94)

It has been shown that untreated breast tumours exhibit an accumulation of CD8+ and CD4+ senescent T cells in peripheral blood. These cells were also found to infiltrate tumours as well as tumour-draining lymph nodes, both invaded and non-invaded. (95)

Immune infiltrate as a biomarker

Lymphocytic infiltrate has proven to be a strong prognostic and predictive indicator in several types of cancers, included breast cancer. However, the composition of tumour infiltrate can exhibit a wide range of phenotypes, and it has been demonstrated that specific TIL subtypes possess distinct prognostic and predictive value.

For instance, high levels of PD1+ or FOXP3+ TILs as well as increased levels of exhausted T cells are positively associated with a poor prognosis (94,95), while higher levels of CD8+ TILs predict a good prognosis. (98) High levels of CD8+ TILs alone, CD83+ DCs, CD20+ B cells, and CXCL13- producing CD4+ follicular helper T cells (Tfh) have all be correlated with pathological complete response (pCR) in BC patients. (92)

Furthermore, initial data demonstrates that T cell senescence/exhaustion profiles in cancer patient are altered by antitumoral therapies, and these modifications could predict response to therapy. For instance, neoadjuvant chemotherapy appears

correlated to reduced expression of exhaustion markers in T cells and increased levels of CD25- CD127- CD4+ T and CD8+ T cells, demonstrating its ability to stimulate the immune system against cancer. This induced immune activation of seems to be correlated with clinical response. (95,99,100) Concerning CDK4/6i, one preclinical study shows that CD8+ TILs from CDK4/6i-treated mice express lower levels of exhaustion markers compared to not treated mice. (101)

LIPID METABOLISM AND IMMUNE STATUS

A link exists between lipid metabolism and T cell immune status: evidence shows the importance of lipid metabolism in the functioning of the immune system. (47) It's demonstrated that lipids, such as cholesterol and FAs, play a crucial has a role in controlling T cell differentiation, survival, and effector functions. (102) Dysregulated lipid metabolism in cancer cells can disrupt immune cell activation, infiltration, and effector functions, thereby facilitating immune evasion. (45,103)

TILs require nutrients found within the TME to support proliferation and differentiation. Increasing evidence shows that malignant tumour cells and neighbouring cells can establish nutrient-deprived conditions, through competitive uptake of glucose, amino acids, glutamine, fatty acids and other metabolites or growth factors, thus functionally impairing immune cells in the TME. (94,104) In addition to nutrient consumption, metabolites produced by cancer cells, such as lactate, glutamine, PGE2, arginine, tryptophan, FAs, cholesterol, oxysterols also have a deleterious effect on immune function. (104) Hence, the functionality of T cells will not only be affected by their intrinsic metabolic alterations, but also by the unfavourable metabolic milieu within the TME. (93) Metabolic reprogramming represents one of the critical factors controlling the development of both exhausted and senescent T cells in the TME. (94,104)

T cells rely on lipid metabolism to function properly. Particularly, effector T cells need to expand their energy sources to meet their increased energy and ATP demands. (47) However, a lipid enriched TME is not sufficient to fulfil the energy needs of effector T cells. Moreover, certain T cells, especially CD8+, lack the ability to synthesize all the necessary enzymes for complete catabolism of different types of FAs. As a result,

lipotoxicity increases, leading to T cells exhaustion. (105) Additionally, cholesterol and FAs are vital sources for cell membranes necessary for immune cell proliferation, they play a key role in immune cells differentiation, and they can directly affect the function of immune cells, for example inhibiting TCR signalling and thus impairing T cell proliferation and cytokine production. (47,104)

Furthermore, it has been shown that high levels of cholesterol in the TME promote the expression of exhaustion markers, such as suppressive immune checkpoints of T cells, including PD-1, LAG-3, and TIM-3, protecting tumour cells from immune surveillance. (106) PD-1, PD-L1, CTLA4, IDO1, CD96, TIGIT, LAG-3, and PVR have also shown higher expression levels in cancer cells with high FA metabolism-related genes expression. (48) PI3K/Akt/mTOR signalling, often activated in breast cancers cells and involved in lipids metabolism control, has been seen to promote the expression of PD-L1. (107,108) PD-1/PD-L1 expression is also induced by AMPK pathway, hyperactivated during scarcity of energy sources, that negatively regulates gluconeogenesis, lipid, and protein synthesis and involved in T cells senescence induction. (107) In turn, inhibitor receptors play a role in lipid metabolism. PD-1 engagement promotes FAO and lipolysis (104), while it suppresses glycolysis and oxidative phosphorylation. CTLA-4 signalling inhibits glycolysis as well. (94)

Accumulated free FAs in cancer cells, cancer and tumour-associated cells preferential anaerobic metabolism, and consequent high levels of lactate, low pH and hypoxia can increase ROS production. (104) (94) The increased oxidative stress leads to damage to cellular components, including DNA, proteins, and lipids and can enhance the secretion of immunosuppressive cytokines in the TME such as IL-10 and TGF-β. (48,109,110)

Effector immune cell grow rapidly and require lipids formed by fatty acid synthesis to build cell membranes during proliferation, while memory immune cells grow slowly and biosynthesis needs relatively little, so during memory formation, T cells revert to oxidative phosphorylation and become increasingly dependent on mitochondrial FAO, which plays an essential role in the generation and maintenance of memory T cell. (93,104).

Moreover, FAO can regulate the balance between effector and regulatory T cells. In regulatory T cells, FAO is mainly used to provide energy, while in effector T cells it is inhibited. (104) FOXP3, the essential regulator of Treg development and function, promotes FA uptake and FAO and enhances Treg resistance to lipotoxic environments to allow for expansion. (111)

The aberrant accumulation of lipid metabolites in tumour-infiltrating myeloid cells, including MDSCs, DCs and TAMs, has been shown to skew these cells towards immunosuppressive and anti-inflammatory phenotypes via metabolic reprogramming. FAs accumulation in monocytes, as well as cancer cells inducted cholesterol efflux from macrophages, leads to macrophages reprogramming to the M2 phenotype, known to have an anti-inflammatory and pro-tumoral phenotype. (44,47,104) Those cells mainly rely on FA oxidation as its source of energy. (112) Lipid accumulation in DCs and macrophages impairs their pivotal role of MHC expression and antigen presentation to T cells. (113) Indeed, it has been observed a relationship between increased lipid levels in tumour-infiltrated DCs and the immunosuppression of antitumour T cells. (44) Cancer-associated adipocytes and fibroblast markers (respectively IL-6 and FAP) increased levels have also been detected in breast cancer and are thought to exacerbate these cancer–stroma interactions, releasing pro-inflammatory cytokines and FAs and transferring FAs to breast cancer cells, thus leading to increased cell proliferation and migration. (48,56)

THESIS OBJECTIVES

As described in the introduction, HR+/HER2- mBC patients have demonstrated significant benefits from CDK4/6i-based therapy, showing a substantial increase in PFS and OS compared to the previous standard of care. However, patients are destined to undergo disease progression due to drug resistance. Various mechanisms have been reported to play a role in CDK4/6i resistance, however, none of them are used as tools in clinical practice. This underlines the importance of identifying new and more effective biomarkers to allow the early detection of patients who are unlikely to have a prolonged benefit from this treatment, identify mechanism of resistance to CDK4/6i, and find rescue treatment strategies for these patients.

Based on these premises, an observational, prospective, and translational study was initiated at the A.O.U. Maggiore della Carità in Novara. This study aims to identify new prognostic biomarkers in patients with HR+/HER2- advanced breast cancer undergoing treatment with CDK4/6 inhibitors. The data presented in this thesis are from a preliminary analysis of this study.

Evidence demonstrates that lipid metabolism alteration and dysfunctional T cell states, such as T cell senescence and exhaustion, can have a key role in tumorigenesis, progression and response to treatments in breast cancer patients, thus representing valid candidates as prognostic and predictive markers for these patients. Seen this, the study's objective is to evaluate the possible prognostic and/or predictive role of lipidomic profile and immunophenotypic T cell characterization in the peripheral blood of HR+/HER2- mBC patients.

MATERIALS AND METHODS

STUDY DESIGN AND SETTING

The study from which the presented data are taken is an observational, prospective, and translational study. It has been, and continues to be, conducted as a single centre study at the A.O.U Maggiore della Carità in Novara. The biological samples required for the research have been analysed at the UPO-CAAD (Center for Autoimmune and Allergic Diseases) in Novara.

ELIGIBILITY CRITERIA

Inclusion Criteria

Only patients able to meet the following criteria were included in the study:

- Capability and willingness to read, understand and sign the informed consent form prior to the initiation of any study-related procedure.
- Age ≥ 18 years.
- Pathologically documented diagnosis of HR+/HER2- metastatic breast cancer.
- Indication for treatment with endocrine therapy (ET) and CDK4/6 inhibitors (Abemaciclib, Ribociclib, Palbociclib), according to standard of care.

Exclusion Criteria

The only exclusion criterion for this study concerns subjects with psychological, familial or social conditions that could contraindicate compliance and adherence to the proposed therapies.

RECRUITMENT AND DATA COLLECTION

During an initial or periodic follow-up visit, patients who met the inclusion and exclusion criteria were identified and, after a preliminary evaluation, were invited to

participate in the study. Upon receiving the appropriate information, consenting patients provided written consent to join the initiative. Subsequently, clinical evaluations and blood sample collections were conducted before the initiation of CDK4/6i-based therapy. The blood samples were delivered and processed at the UPO-CAAD Research Laboratory. No additional non-routine visits or procedures were conducted.

Clinical evaluation

The baseline clinical evaluation included personal data, anthropometric data, physiological anamnesis, pharmacological anamnesis, and medical history, including oncological history and any other past or concurrent illnesses. During follow-up visits, any treatment changes such as treatment cessation or modification were evaluated. At the time of reassessments, the type of treatment response was assessed and defined as complete response, partial response, stable disease, or progressive disease based on standardized criteria such as Response Evaluation Criteria in Solid Tumors (RECIST).

Peripheral blood analysis

At the time of collection, approximately 30 ml of blood were drawn and collected into EDTA-containing tubes. The material was maintained at room temperature and delivered to the immuno-oncology laboratory at the University Center for Translational Research on Autoimmune and Allergic Diseases (UPO-CAAD) in Novara. From sample collection to processing, no more than 24 hours elapsed.

The samples underwent the following analyses:

- Density gradient centrifugation using Ficoll/Hypaque to collect serum and peripheral blood mononuclear cells (PBMCs) from blood samples.
    - PBMCs were used for flow cytometric analysis to characterize the senescent and exhausted phenotype of CD3+ T lymphocytes.
    - Serum was used for lipidomic profiling using LS-MS/MS technology to assess the lipidomic profile of the patients.

T cell characterization

Quantitative and qualitative evaluation of senescent and exhausted CD3+ lymphocytes of peripheral blood was conducted for each blood sample. After obtaining peripheral venous blood, PBMCs were isolated using a density gradient centrifugation method. The isolated cells were then stained and incubated with a viability dye and specific fluorochrome-conjugated antibodies targeting surface markers associated with T cell senescence and exhaustion. Following staining, the cells were washed and analysed for T-cell phenotyping using Fluorescence-Activated Cell Sorting (FACS) with the latest version of FlowJo software.

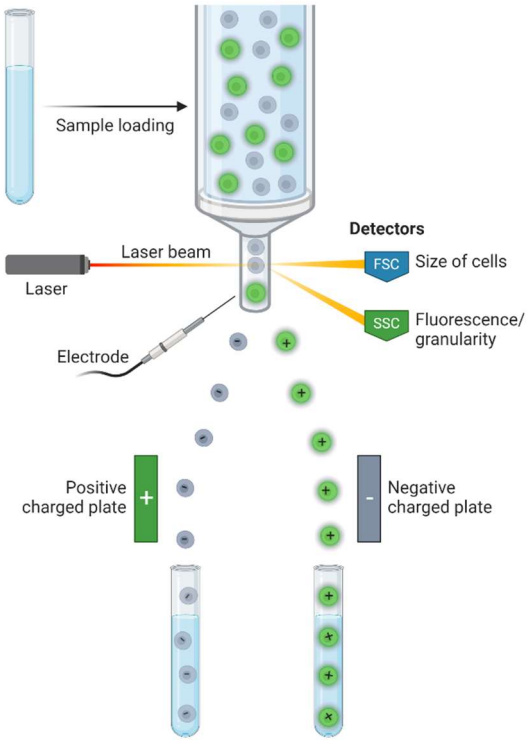


Figure 4 - FACS (Fluorescence-Activated Cell Sorting)

The expression of the following surface markers was evaluated by FACS analysis:

- CD4 and CD8 are transmembrane glycoproteins that serves as a co-receptor for the T cell receptor (TCR) and respectively define the T helper and the T cytotoxic phenotype.
- CD28 is a co-stimulatory molecule found on T cells, it provides essential signals for T cell activation and survival when it binds to its ligands.

(CD80/CD86) on APCs. It is usually unexpressed by senescent T cells, while exhausted T cells express it on their surface.

- PD1 (Programmed cell death protein 1) is an inhibitory receptor able to reduce T cell activity after binding its ligands (PD-L1 and PD-L2) maintaining immune tolerance and prevent autoimmunity. It is a key regulator of T cell exhaustion.
- TIGIT (T cell immunoreceptor with Ig and ITIM domains) is an inhibitory receptor expressed on T cells, it binds to its ligands (CD155 and CD112) to inhibit T cell activation and proliferation, contributing to anti-tumor immunity suppression. It is expressed in exhausted T cells.
- LAG3 (Lymphocyte-activation gene 3) is an inhibitory receptor that binds to MHC class II molecules and negatively regulates T cell proliferation and activation. It contributes to T cell exhaustion.
- CD57 is a marker found on a subset of NK cells and T cells, particularly those with a history of repeated activation. It is associated with terminal differentiation and cellular senescence in T cells, indicating a less proliferative but cytotoxic phenotype.
- KLRG1 (Killer cell lectin-like receptor G1) is an inhibitory receptor that binds to E-cadherin on target cells and delivers inhibitory signals that reduce cell proliferation and cytotoxicity. KLRG1 expression is associated with T cell exhaustion and senescence.

Overall, exhausted T lymphocytes have been identified as CD4+/CD8+ CD28+PD1+TIGIT+LAG3+ cells, while senescent T lymphocytes as CD4+/CD8+ CD28-CD57+KLRG1+ cells.

Lipidomic profiling

Lipidomic analysis was performed for baseline blood samples using an untargeted LS-MS/MS (liquid chromatography–tandem mass spectrometry) based approach. This is a powerful analytical technique used to comprehensively characterize the lipid composition of biological samples, allowing for the identification and quantification of hundreds of different circulating lipids in both absolute and relative terms. This type of analysis offers valuable insights to overcome the current limitations of standard

prognostic and predictive technologies, defining a new field for personalized medicine application.

After obtaining peripheral venous blood, serum was isolated using a density gradient centrifugation method. Subsequently, lipids were extracted from the serum samples and injected into a liquid chromatography system capable of separating lipids based on their physicochemical properties such as polarity, hydrophobicity, and size. After this, tandem mass spectrometry was performed to further characterize individual lipid species. Following chromatography, tandem mass spectrometry (MS/MS) was performed to further characterize individual lipid species. This technique selects specific lipid ions of interest (precursor ions) and fragments them into smaller ions (product ions) using collision-induced dissociation (CID) or other fragmentation methods. Analysis of the fragmentation patterns allows for the identification and quantification of lipid species based on their head group and fatty acid composition. The acquired MS/MS data were then processed and analysed using specialized software tools.

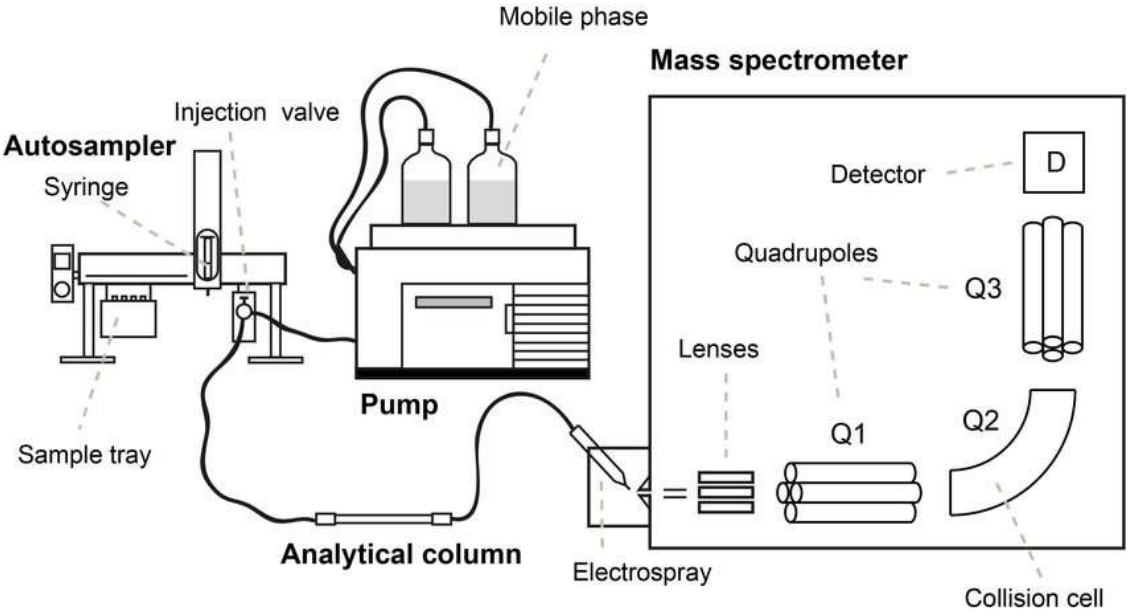


Figure 5 - LS-MS/MS (liquid chromatography–tandem mass spectrometry)

DATA MANAGEMENT AND STATISTICAL ANALYSIS

The data were collected and stored in an electronic database in a pseudonymized manner. Each patient was assigned a unique code, which was used to correlate clinical and laboratory data in compliance with privacy regulations. The database contained information obtained from clinical evaluations, medical records, and laboratory analyses, including:

- Personal data: patient code, gender, date of birth;
- Anthropometric data: weight, height, BMI;
- Physiological anamnesis: smoking, alcohol use, dietary habits, menopausal status;
- Pharmacological anamnesis;
- Medical history
    - Oncological history: date of first breast cancer diagnosis, disease stage at diagnosis, date of metastatic disease diagnosis, sites of metastatic disease, histological type, tumour grading, ER/PgR/Ki67/HER2 expression levels, BRCA mutation, prescribed oncological treatment (drugs, start date, therapy line), eventual prior oncological treatments, endocrine resistance;
    - Other past or concurrent relevant illnesses;
- Total cholesterol, HDL, LDL, triglycerides, insulin, glucose, HOMA-IR index evaluated at T0 and T1;
- Date of eventual progressive disease or death;
- Eventual time and cause of treatment cessation or modification;
- Last contact date.

Endocrine sensibility was defined as either de novo mBC recurrent disease without previous adjuvant ET for early BC, or recurrent disease occurring at least 12 months after adjuvant ET end date. Primary endocrine resistance was defined as either disease recurrence during the first 2 years of adjuvant ET for eBC or disease progression

within the first 6 months of first line ET for mBC. Secondary endocrine resistance was defined as disease recurrence at least 2 years after the start of adjuvant ET and within 12 months from end date of adjuvant ET, or disease progression after 6 months of first line ET in case of mBC.

Laboratory data at baseline included:

- Biochemical profile, including total cholesterol, HDL, LDL, triglycerides, insulin, glucose;
- Senescent and exhausted T cells levels, both expressed as a percentage;
- Lipidomic profile.

The prognostic role of serum lipids and circulating T cells populations was evaluated by assessing their association with progression-free survival (PFS), overall survival (OS), and overall response rate (ORR). Rresults at an alpha level of 0.05 were then selected to be fitted in multivariable models accounting for age at diagnosis (younger than 65 years versus older), and presence of visceral disease (yes versus not). Kaplan-Meier survival curves, log-rank test, and Cox's proportional hazard regression were performed for survival analysis. Logistic regression was performed to assess the association with ORR.

To better identify biomarkers associated with early progression, clinical benefit rate (CBR) was also evaluated. Clinical benefit rate was defined as the percentage of patients who achieved a complete response, a partial response, or a stable disease at six months after the starting of treatment. In addition, a potential correlation between the lipidomic profile and metastatic sites, distinguishing between bone/soft tissue metastases and visceral metastases, will be examined. The distribution of circulating biomarkers according to CBR and metastatic sites was assessed by means of Mann Whitney U test, and Kruskal-Wallis test as appropriate.

Expression levels of markers on T cells has been evaluated and compared between patients of two groups

- PD/death: patients who experienced PD or death during CDK4/6i treatment.

- response/SD: patients who did not experience PD or death during CDK4/6i treatment.

Firstly, CD3+ T lymphocytes were considered altogether, then CD4+ T helper and CD8+ T cytotoxic lymphocytes levels were evaluated separately. The frequency T-cell subtypes, categorized based on treatment response (response/SD vs PD/death), was assessed using Mann Whitney U and Wilcoxon matched-pairs rank test.

Spearman's correlation test between serum lipids and circulating T cells was performed to investigate the link between patients lipidic profile and host's antitumor immunity. Normally distributed data were presented as mean and Standard Deviation (SD). For descriptive purposes, baseline characteristics were defined as categorical variables and reported as values and percentages. Data analyses were performed using Graph Pad prism v.8 and FlowJo 10.9.0. and R software, v1.4 All reported P values were two-tailed and $P \leq 0.05$ was considered statistically significant.

ETHICAL CONSIDERATIONS

This study was conducted in accordance with the protocol and with the following consensus ethical principles derived from international guidelines including the Declaration of Helsinki and Council of International Organizations of Medical Sciences (CIOMS) international ethical guidelines, applicable ICH Good Clinical Practice (GCP) guidelines and applicable laws and regulations.

RESULTS

PATIENTS CHARACTERISTICS

A total of 63 patients have been enrolled in the study from 2021 to today.

All the enrolled patients were female (n = 63; 100%), with a mean and median age of 66 and 69 years, respectively (range 38-87 years). Most patients were postmenopausal (n=52, 83%), while only few were premenopausal (n=11, 17%).

Nearly half of the enrolled patients were healthy weight, with a BMI between 18,5 and 25 (n=32, 51%). Overweight, defined as BMI comprised between 25 and 30, and obesity, defined as BMI greater than 30, represented 25% and 19% of the total, respectively (n=16; n=12). Only 3 patients were underweight, with a BMI lower than 18,5 (5%).

Some patients presented with comorbidities and concomitant chronic treatments. Among these, we evaluated those that could affect variables considered in the study, such as lipid metabolism and immune system functionality. Seventeen percent had a previous diagnosis of cancer (n=11), ten percent had a diagnosis of diabetes and took antidiabetic medications chronically (n=6), eight percent were affected by dyslipidaemia and were treated with statins (n=5).

Table 3 - Patient general characteristics

Characteristic	**Number of patients**	**% of patients**
Enrolled patients	63	100%
Sex		
Female	63	100%
Male	0	0%
Age		
< 65 years	26	41%
≥ 65 years	37	59%

Menopausal status		
Post-menopausal	11	17%
Pre-menopausal	52	83%
BMI		
Underweight (< 18,5)	3	5%
Healthy weight (18,5-25)	32	51%
Overweight (25-30)	16	25%
Obesity (> 30)	12	19%
Comorbidities		
Diabetes mellitus	6	10%
Dyslipidaemia	5	8%
Another cancer	11	17%
Concomitant chronic treatments		
Anditiabetics	6	10%
Statins	5	8%

All patients presented with ER+/HER2- advanced breast cancer. Among them, 57% had a recurrent mBC (n=36), 37% de novo mBC (n=23), and 6% non-operable locally advanced breast cancer (n=4). Most of the metastatic patients presented with a single metastatic site (n=25, 40%), while 22% (n=14), 29% (n=18) and 10% (n=6) of the patients showed 2, 3 or 4 metastatic sites, respectively. However, patients with more than one metastatic site represented 60% of the total (n= 38). Patients with or without visceral disease were 32 (51%) and 31 (49%), respectively. The most common metastatic site was bone (n=39, 62%), followed by lymph nodes (n=32, 51%), soft tissues (n=13, 21%), lung (n=13, 21%), liver (n=12, 19%) and brain (n=3, 5%).

Table 4 - Metastatic breast cancer characteristics

Characteristic	Number of patients	% of patients
Enrolled patients	63	100%
Presentation		
Recurrent	36	57%
De novo	23	37%
LA	4	6%
Number of metastatic sites		
1	25	40%
2	14	22%
3	18	29%
4	6	10%
Visceral vs non visceral disease		
Visceral disease	32	51%
Non visceral disease	31	49%
Metastatic sites		
Bone	39	62%
Lymph nodes	32	51%
Soft tissue	13	21%
Lung	13	21%
Liver	12	19%
Brain	3	5%

Regarding tumour characteristics, the most prevalent histopathological type was invasive ductal carcinoma (n=44, 70%), followed by invasive lobular carcinoma (n=14, 22%). ER expression ranged from 5% to 100%, with an average and median value of 87% and 95%, respectively. Indeed, most of the patients showed a high ER expression, defined as expression level greater than 50% (n=58, 92%). Only few patients demonstrated an intermediate (n=4, 6%) or low (n=1, 2%) ER expression, defined as between 10% and 50% or lower than 10%, respectively. Regarding HER2 status, the number of patients with HER2-negative disease (n=30, 48%), defined as 0 at immunohistochemistry (IHC), was similar to those with HER2-low expression, defined as IHC 1+ or 2+ with a negative FISH (n=32, 51%). For only one patient this information was missing. Tumour characteristics assessment was performed on metastatic disease biopsy for most of patients (n=48, 76%).

Table 5 - Biologic and molecular features

Characteristic	**Number of patients**	**% of patients**
Enrolled patients	63	100%
Histology		
CDI	44	70%
CLI	14	22%
Other subtypes	5	8%
ER expression		
Low (<10%)	1	2%
Intermediate (10-50%)	4	6%
High (>50%)	58	92%
HER2 status		
Negative (IHC 0)	30	48%
Low (IHC 1+ or 2+/FISH-)	32	51%
NA	1	2%

All enrolled patients received a CDK4/6i-based treatment, comprising a CDK4/6i among Abemaciclib (n=23, 37%), Ribociclib (n=15, 24%), and Palbociclib (n=25, 40%). Endocrine therapy was chosen based on endocrine sensibility and menopausal status of the patient, according to clinical practice guidelines. Most of the patients were post-menopausal, while 11 (17%) were premenopausal and were therefore treated with ovarian function suppression (OFS) in addition to ET. Most patients were endocrine sensible (n=39, 62%), while 6 (10%) and 18 showed primary and secondary (29%) endocrine resistance, respectively. Most patients received aromatase inhibitors (AIs), (n=43, 68%), fewer patients received Fulvestrant (n=20, 32%).

CDK4/6i-based treatment represented first line therapy for the majority of patients (n=50, 79%), only few patients had already received other treatments for mBC (13, 21%). Regarding previous oncological treatments, 29 (46%) patients had been treated with previous chemotherapy (n=29, 46%), either in the (neo)adjuvant or metastatic setting. At the time of data cutoff, most patients were still on treatment with CDK4/6i (n=37, 59%). Disease progression, death, and drug toxicity were reasons for treatment discontinuation in 20 (32%), 3 (5%), and 3 (5%) patients, respectively. Overall, 11 deaths were observed during the study observation period (17%).

Table 6 - CDK4/6i treatment

Characteristic	**Number of patients**	**Percentage of patients**
Enrolled patients	63	100%
CDK4/6i		
Palbociclib	25	40%
Abemaciclib	23	37%
Ribociclib	15	24%
Associate ET		
AI	43	68%
Fulvestrant	20	32%
OFS		
Yes	11	17%

No	52	83%
Hormone resistance		
No	39	62%
Primary	6	10%
Secondary	18	29%
Treatment line		
First	50	79%
Subsequent	13	21%
Previous CT		
Yes	29	46%
No	34	54%
Current status		
CDK4/6i ongoing	37	59%
CDK4/6i stopped	26	41%
- PD	20	32%
- Death	3	5%
- Toxicity	3	5%
Death	11	17%

LIPIDOMIC ANALYSIS

Untargeted LS-MS/MS-based lipidomic analysis was performed from blood samples of 30 enrolled patients. Four samples were excluded for low output quality, and the concentration of 1139 circulating lipids was obtained for the remaining 26 samples. Of them, 14 were from patient who experienced PD or died during study observation. Overall, 9 lipids showed a statistically significant association with an increased risk of PFS event: phosphatidylcholine (PC.O.36.4), lyso-phosphatidylcholine (LPC.O.26.1), phosphatidylethanolamines (PE.P.38.1.PE.P.20.0_18.1, PE.P.40.5.PE.P.18.0_22.5),

sphingomyelins (SM.44.4.3O, SM.44.2.2O.SM.18.1.2O.26.1), N-acylethanolamine (NAE.18.1), lysophosphatidylinositol (LPI.18.1) and diacylglycerol (DG.30.5).

Table 7 - Cox regression univariate analysis of circulating lipids

Variable	N	**N event**	**HR**	**Lower HR**	**Upper HR**	**p-value**
PC.O.36.4	26	14	1.10	1.03	1.18	0.006
SM.44.4.3O	26	14	1.25	1.04	1.50	0.013
LPC.O.26.1	26	14	1.25	1.04	1.50	0.017
NAE.18.1	26	14	1.27	1.04	1.56	0.021
PE.P.38.1.PE.P.20.0_18.1	26	14	3.16	1.18	8.48	0.022
LPI.18.1	26	14	1364.31	1.53	1215303.27	0.037
PE.P.40.5.PE.P.18.0_22.5	26	14	2.78	1.01	7.67	0.049

Cox regression multivariate analysis was performed and six out of nine associations remained significant after adjustments for age at diagnosis and presence of visceral disease (VD). Results showed that high serum concentrations of sphingomyelin (SM 44:4;3O HR 1.28 [1.05 – 1.55]), phosphatidylethanolamine (PE P-38:1 HR 3.16 [1.18 - 8.48]), N-acylethanolamine (NAE 18:1 HR 1.27 [1.04 - 1.56]) and phosphatidylcholines (PC O-36:4 HR 1.10 [1.03 - 1.18], LPC O-26:1 HR 1.25 [1.04 - 1.50]) were associated with poorer PFS.

Table 8 - Cox regression multivariate analysis of circulating lipids

Variable	N	**N event**	**HR**	**Lower HR**	**Upper HR**	**p-value**
PC.O.36.4	**26**	**14**	**1.14**	**1.03**	**1.25**	**0.009**
Age ≥65 vs <65	26	14	0.51	0.13	2.02	0.337
VD vs non VD	26	14	0.73	0.20	2.73	0.640

SM.44.4.3O	**26**	**14**	**1.29**	**1.04**	**1.61**	**0.020**
Age ≥65 vs <65	26	14	1.46	0.45	4.67	0.527
VD vs non VD	26	14	0.94	0.26	3.47	0.929
LPC.O.26.1	**26**	**14**	**1.25**	**1.04**	**1.51**	**0.017**
Age ≥65 vs <65	26	14	1.66	0.52	5.34	0.394
VD vs non VD	26	14	1.07	0.30	3.81	0.913
NAE.18.1	**26**	**14**	**1.36**	**1.07**	**1.73**	**0.011**
Age ≥65 vs <65	26	14	2.33	0.66	8.20	0.187
VD vs non VD	26	14	0.86	0.23	3.20	0.826
PE.P.38.1.PE.P.20.0_18.1	**26**	**14**	**3.23**	**1.18**	**8.84**	**0.023**
Age ≥65 vs <65	26	14	1.49	0.48	4.59	0.492
VD vs non VD	26	14	1.06	0.32	3.53	0.920
SM.44.2.2O.SM.18.1.2O.26.1	**26**	**14**	**1.52**	**1.02**	**2.27**	**0.039**
Age ≥65 vs <65	26	14	1.88	0.57	6.19	0.302
VD vs non VD	26	14	1.05	0.31	3.58	0.941

To assess whether significant lipids were able to identify prognostic subgroups, we stratified patients according to different cutoff of lipids concentrations (i.e. median, tertiles, quartiles) and derived Kaplan-Meier survival curves. Notably, five out of six lipids were able to identify groups of patients with statistically significant and clinically relevant differences in PFS.

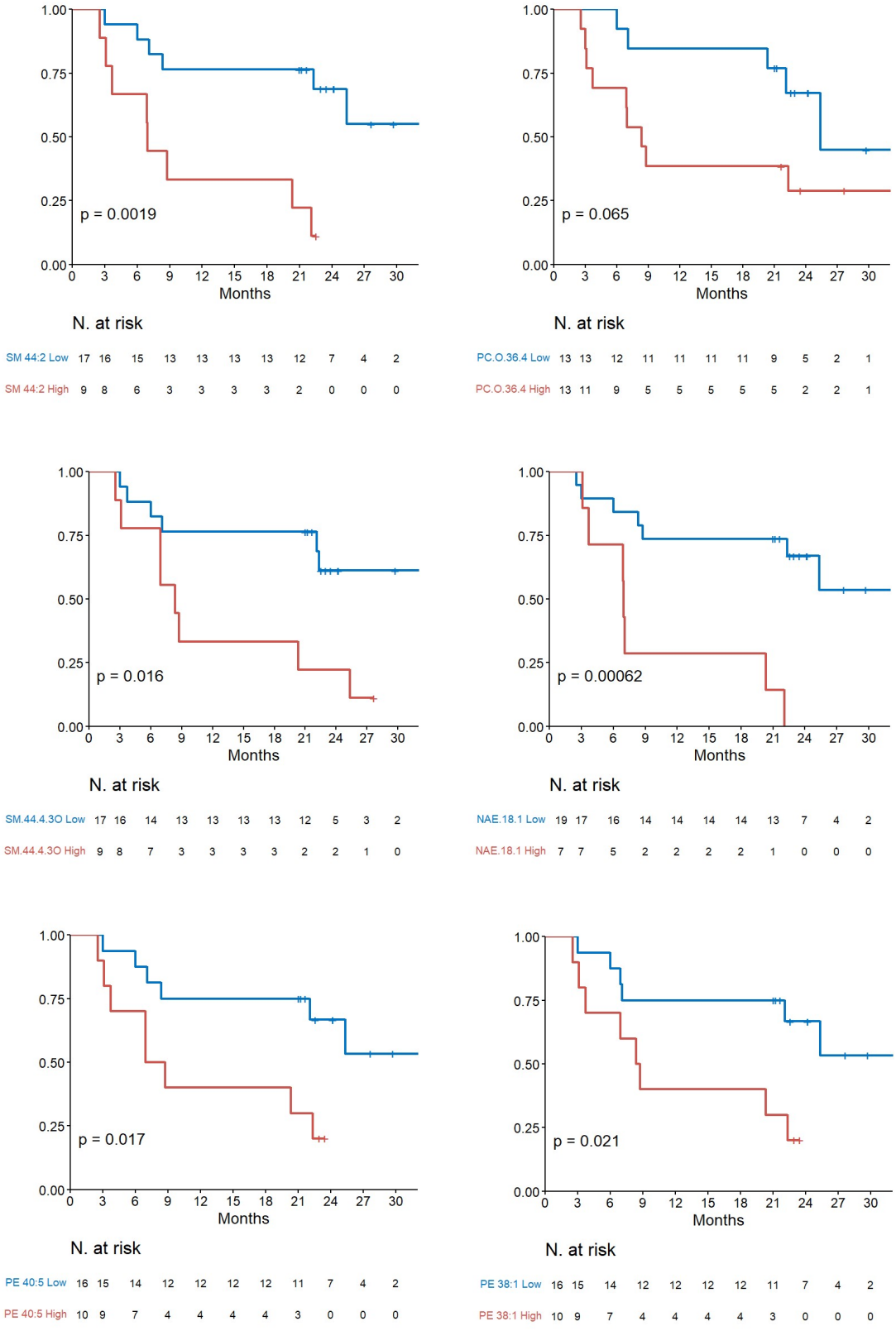


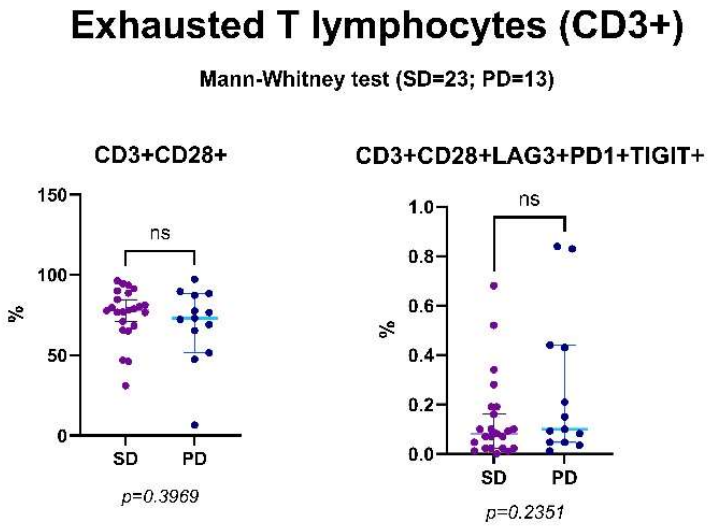
Figure 6, 7, 8, 9 – Kaplan-Meier curves of circulating lipids statistically significantly associated with PFS.

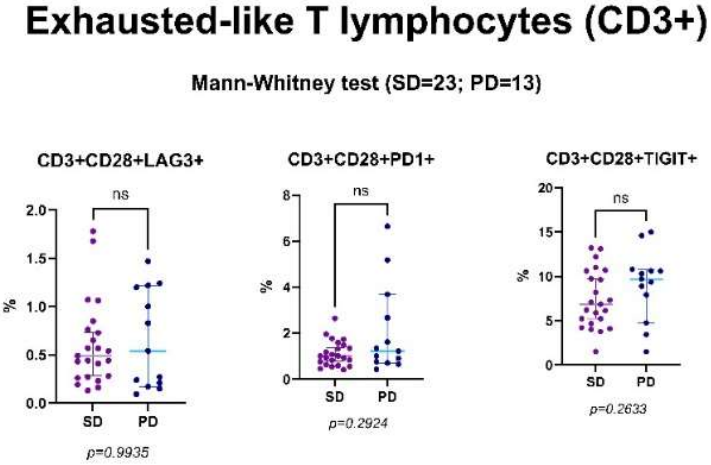
T CELL CHARACTERIZATION

Blood samples from 44 patients were collected for T cell characterization. After exclusion of 4 samples due to low lymphocytes viability, downstream analysis were performed for the remaining 36 samples. Among these patients, 13 developed PD or died due to breast cancer during follow-up.

T cells were isolated from peripheral blood and immune phenotyping was conducted to identify cells who showed senescence or exhaustion features.

No statistically significant differences between the two groups were observed when comparing levels of senescence or exhaustion profiles, nor when comparing levels of individual senescent or exhaustion markers both in CD3+ T cells or CD3+CD8+ cytotoxic lymphocytes.

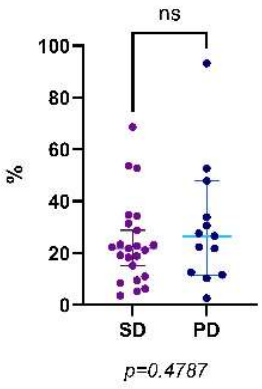




Senescent T lymphocytes (CD3+)

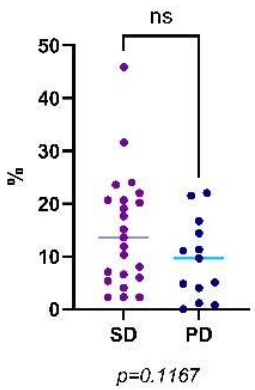
Mann-Whitney test (SD=23; PD=13)

CD3+CD28-



p=0.4787

CD3+CD28-CD57+KLRG1+

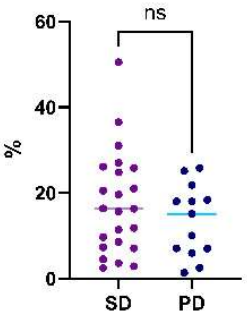


p=0.1167

Senescent-like T lymphocytes (CD3+)

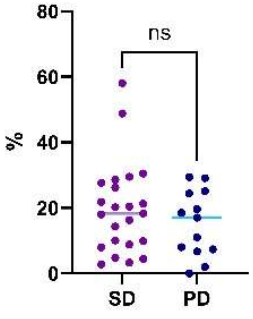
Mann-Whitney test (SD=23; PD=13)

CD3+CD28-CD57+



p=0.3570

CD3+CD28-KLRG1+

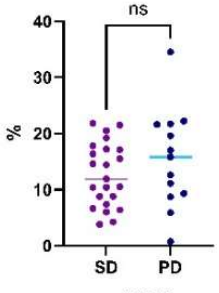


p=0.4345

Exhausted T cytotoxic lymphocytes (CD8+)

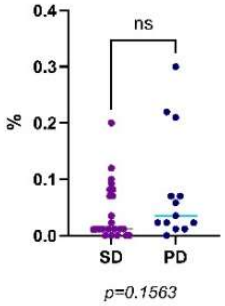
Mann-Whitney test (SD=23; PD=13)

CD8+CD28+



p=0.3240

CD8+CD28+LAG3+PD1+TIGIT+



p=0.1563

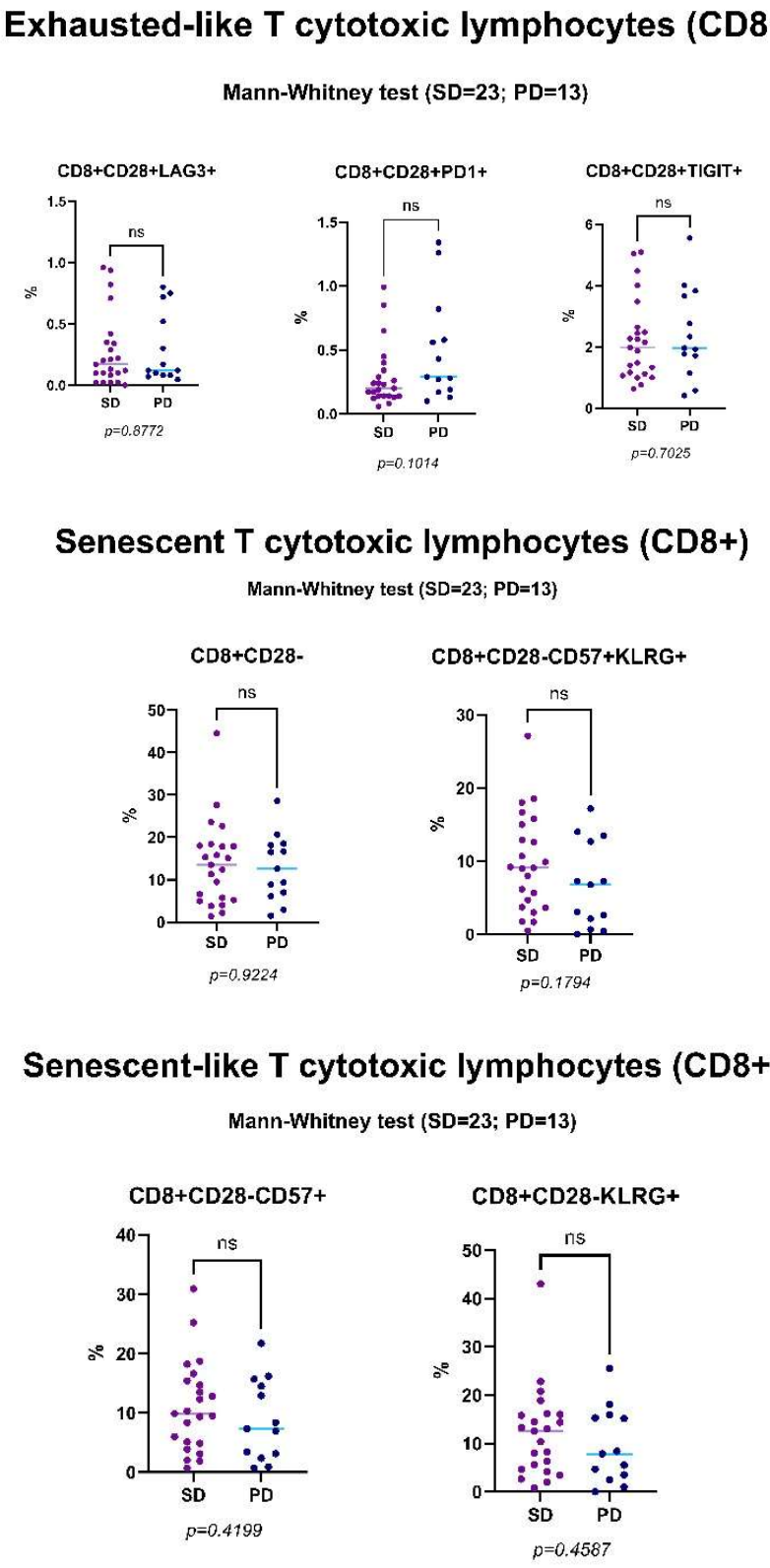
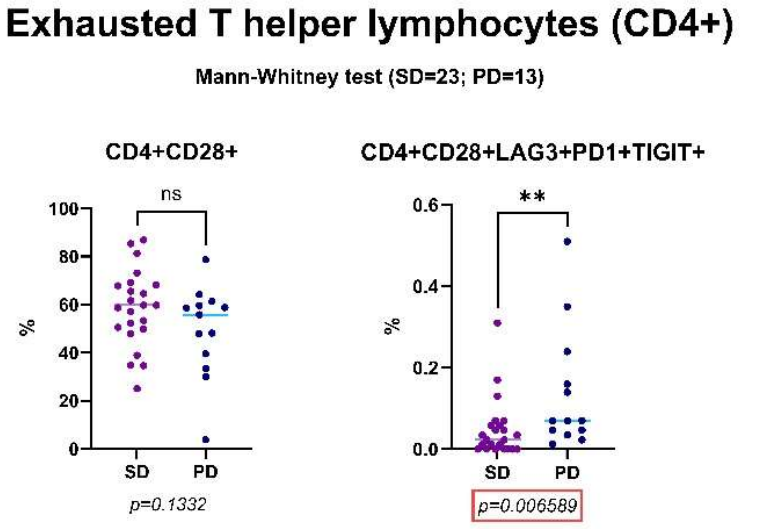
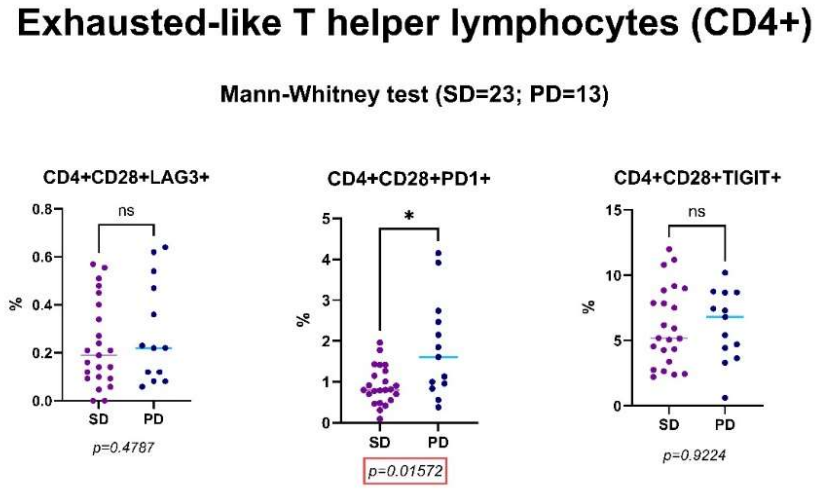


Figure 10, 11, 12, 13, 14, 15, 16, 17 – Mann-Whitney test, CD3+ T lymphocytes and CD8+ T cytotoxic lymphocytes characterization

No statistically significant differences in levels of senescent CD4+ T lymphocytes and in expression levels of senescent markers on T helper were found comparing the two groups. However, higher level of exhausted T helper lymphocytes, defined as CD4+CD28+PD1+LAG3+TIGIT+ T-cells, have been observed in the PD/death group compared to response/SD group (0.05 ± 0.07 vs 0.14 ± 0.15, p= 0.0066). Moreover, considering exhaustion markers separately, T helper lymphocytes expressing PD1+ have showed higher levels in the PD/death group compared to response/SD group (0.91 ± 0.47 vs 1.83 ± 1.22, p= 0.0117). Other exhaustion markers (LAG3, TIGIT) did not show different expression in the two groups, when evaluated separately.





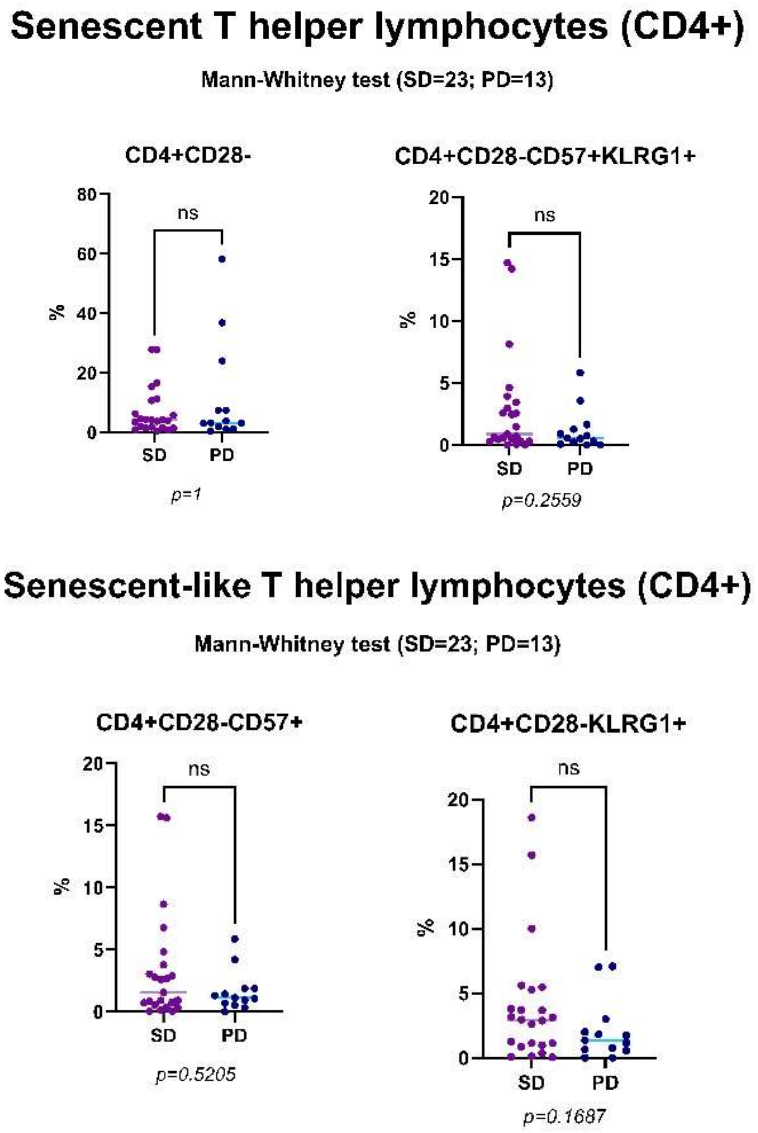


Figure 18, 19, 20, 21 – Mann-Whitney test, CD4+ T helper lymphocytes characterization

At the survival analysis high frequency of CD3+ T lymphocytes expressing TIGIT (HR 1.25 [1.01 - 1.53]) and of PD1+ T helper lymphocytes (HR 1.99 [1.18 - 3.34]) were associated with statistically significant increased risk of PD or death. Other two populations showed a similar trend but were not statistically significant at an alpha level of 0.05 : exhausted T helper lymphocytes (CD4+CD28+LAG3+PD-1+TIGIT+) and PD-1 expressing T cells (CD3+CD28+PD-1+).

Table 9 - Cox univariate analysis of T cell populations

Population	N	N event	HR	Lower HR	Upper HR	p-value
CD4+CD28+PD-1+	33	10	1.99	1.18	3.34	0.010
CD3+CD28+TIGIT+	33	10	1.25	1.01	1.53	0.037
CD4+CD28+LAG3+PD-1+TIGIT+	33	10	43.68	0.87	2194.01	0.059
CD3+CD28+PD-1+	33	10	1.36	0.99	1.88	0.059

Cox regression multivariate analysis was performed and, after age and visceral metastases adjustments, significant associations remained as such for CD4+CD28+PD-1+ and CD3+CD28+TIGIT+ T lymphocytes.

Table 10 - Cox multivariate analysis of T cell populations

Population	N	N event	HR	Lower HR	Upper HR	p-value
CD4+CD28+PD-1+	33	10	2.06	1.16	3.66	0.013
VD vs non VD	33	10	0.90	0.20	4.09	0.889
Age >=65 vs <65	33	10	0.74	0.20	2.77	0.657
CD3+CD28+TIGIT+	33	10	1.27	1.02	1.58	0.013
VD vs non VD	33	10	1.34	0.32	5.51	0.688
Age >=65 vs <65	33	10	0.59	0.15	2.28	0.439

Next patients were stratified according to arbitrary cutoffs (i.e. median, tertiles, quartiles) in the frequencies in lymphocytes that showed significant association in previous analysis: exhausted T helper lymphocytes, exhausted-like T helper lymphocytes expressing PD1 and T cells expressing the exhaustion marker TIGIT. All of them showed a statistically significant correlation with poorer PFS when present in high circulating levels.

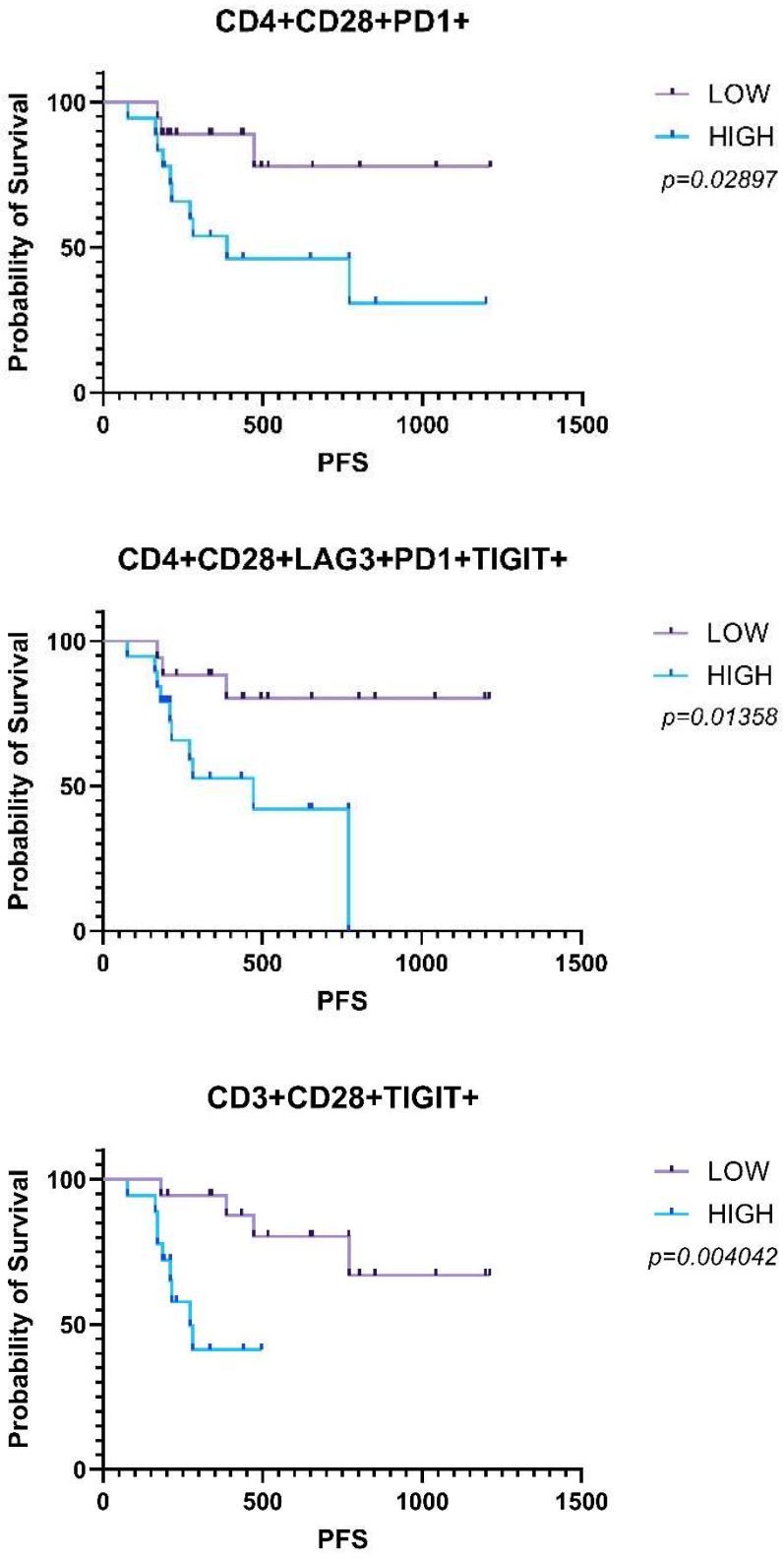


Figure 22, 23, 24 – Kaplan-Meier curves of T lymphocyte populations significantly associated with PFS.

LIPIDOMIC PROFILE AND IMMUNOPHENOTYPE CORRELATION

Preliminary analysis of a potential correlation between results obtained from lipidomic analysis and T cell characterization was conducted. Spearman's correlation test was performed to assess a possible association between lipids and T cell populations that were statistically significant in previous analyses. A moderate correlation was found for PD1+ T helper lymphocytes with NAE 18:1 (R=0.56, p=0.05) and PE P-38:1 (R=0.66, p=0.017).

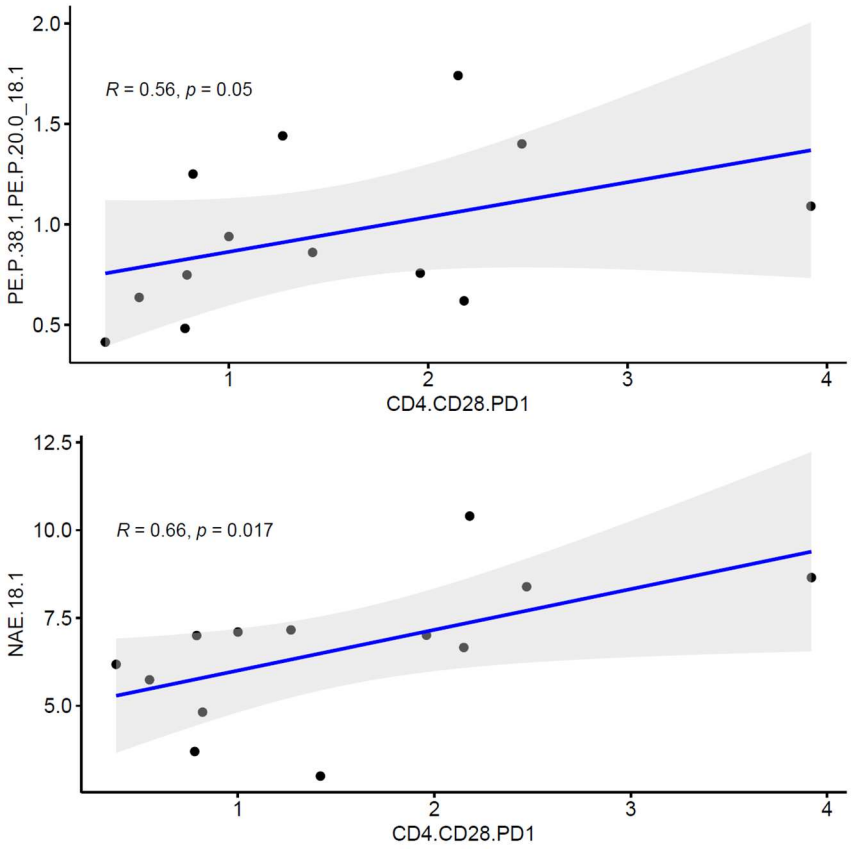


Figure 25 - Spearman's correlation test, PD1+ T helper lymphocytes association with NAE 18:1 and PE P-38:1

DISCUSSION

PROGNOSTIC ROLE OF LIPIDOMIC PROFILE

Lipids have demonstrated to play a key role, not only in tumorigenesis and tumour growth, but also in increasing aggressiveness and metastatic potential of cancers, as well as influencing different drug resistance mechanisms in a wide variety of cancers, including breast cancer. Therefore, lipid profile represents a possible candidate as a prognostic and predictive biomarker for breast cancers patients.

Phospholipids (PL) are an essential and ubiquitous class of lipids, serving as key components of cell membrane lipid bilayers and participating in various metabolic and signaling pathways. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most dominant phospholipids in the majority of biological membranes. (114) Increased phospholipid metabolism has been reported in breast cancer tissue. Particularly, higher levels of PC and PE or their precursor molecules (PCho and phosphoethanolamine) have been detected in tissue samples from breast cancer patients compared with healthy tissue. The same association has been observed in breast cancer cell lines. (115–118) Moreover, significant difference in serum phospholipids content was found, especially PC and PE, between breast cancer patients and cancer-free individuals. These lipids are apparently specific products of tumor cells released into the blood stream. (119) Lysophosphatidylcholines (LPC) has been detected in a relevant abundance in blood microvescicles from BC patients compared to controls. Moreover, high concentrations of specific LPC and PC species have been associated with shorter overall survival. (120) LPC/PC ratio was found to be higher in breast cancer tissues compared to healthy tissue. (121) According to literature we found increased levels of specific circulating phospholipids to be correlated to a poorer PFS in a cohort of patients treated with CDK4/6i-based treatment. In particular, greater levels of PC.O.36.4, PE.P.38.1.PE.P.20.0_18.1 and LPC.O.26.1 have been associated with an increased risk of PD.

Dysregulation of sphingolipids (SL) homeostasis has been seen to play a critical role in the development and progression of cancer. (122) Sphingomyelin (SM) is one of

the most abundant SL in biological membranes, where it plays important structural roles and regulates numerous signalling pathways. Sphingomyelins have been detected in higher levels in blood microvescicles from BC compared to controls. (120) Moreover, total levels of SM, as well as all some specific SM species were found in significantly higher levels in breast cancer tissues than in normal breast tissue. (122) Ceramides are precursor of SM, they are known to promote Fas-mediated apoptosis through interaction with key molecules in the Fas-FasL pathway, such as Bak, Bax, and Bcl-2. Higer levels of sphingomyelin synthases, the enzyme responsible to SM synthesis by converting ceramides (Cer) into SM, have been associated with higher BC aggressivity, promoting cancer cell proliferation by suppressing a Cer-associated apoptotic pathway, and cancer cell migration and invasiveness by enhancing EMT through the activation of TGF-β/Smad signalling pathway. Higher levels of sphingomyelinases, the enzyme catalyzing the hydrolysis of SM regenerating Cer, as well as increased Cer levels, have been correlated to less BC aggressivity. Sphingomyelinases have also been seen to play an essential role for the efficacy of chemotherapy and radiotherapy, and many studies have also linked these enzymes with drug resistance. (122) Indeed, SL metabolism in dox-resistant MCF-7 cells has been seen to be oriented toward the downregulation of Cer and the concomitant increase in SM. (90) In line with what has been described in literature we found higher levels of circulating SM.44.4.3O to be associated with a higher risk of PD, therefore correlating with a poorer PFS.

Fatty acids have been observed to be associated with a poorer prognosis in breast cancer, as well as to play a role in many drug resistance mechanisms. (91) Within the class of fatty acyl lipids, the N-acylethanolamines (NAEs) have garnered attention as a family of bioactive fatty acid amides with roles in diverse biological processes, probably including cancer-involved processes. Altered NAE levels have been reported in various forms of cancer. Increased expression of NAE receptors has been detected in breast cancer, and an association between NAE receptors and cancer outcome has also been described. Finally, they have also been associated with resistance to anticancer drugs. (123) We found higher levels of NAE 18:1, also known as N-Oleoylethanolamine (OEA), to be associated with worse PFS. OEA is the amide of oleic acid and ethanolamine and acts mostly activating PPAR-α nuclear receptors,

moreover, it also works as an agonist for GPR119 receptor, and TRPV1 channel, as well as a ceramidase inhibitor.

PROGNOSTIC ROLE OF T CELLS EXHAUSTION

Exhausted T lymphocytes are T cells incapable of proliferating showing an impaired functionality. Their cytotoxicity and effector cytokine production are deficient. Therefore, we can deduce their ability to control tumor cells to be impaired as well. In addition, exhaustion profiles have been seen to be altered by antitumoral therapies. Inhibition of CDK4 and CDK6 has been demonstrated to increase PD-L1 protein levels by impeding cyclin D-CDK4-mediated phosphorylation of speckle-type POZ protein (SPOP) and thereby promoting SPOP degradation and PD-L1 expression. (124)Deregulated immune related pathways, including high PD-L1 expression, were found to be associated with CDK4/6i resistance. PD-L1 acts as the ligand of PD-1, a key regulator of T cell exhaustion. Increased exhausted T cells have been found in both the circulation and tumour sites in a variety of tumours, including breast cancer. Moreover, patients with elevated levels of exhausted T cells have been found to be associated with a poorer prognosis. Our results, according to literature, show a correlation between circulating exhausted and exhausted-like T lymphocytes levels with a worse response to CDK4/6i-based treatment and a poorer prognosis. In particular, higher levels of circulating exhausted T helper lymphocytes were found in blood sample from patients who experienced PD compared to patient whose disease remained stable or responded to therapy. A similar association was found for T helper lymphocytes expressing the exhaustion marker PD-1. Moreover, greater levels of these two populations as well as higher levels of circulating T cells expressing the exhaustion marker TIGIT, were associated with a poorer PFS. Therefore, we found that patients with elevated levels of exhausted/exhausted-like T cells have a higher risk to experience PD during CDK4/6i-based treatment compared to those showing lower levels. Moreover, seen the described role of PD-1/PD-L1 pathway in CDK4/6i resistance, we can hypothesise that increased levels of circulating exhausted and exhausted-like T helper lymphocytes, both expressing PD-1, can be involved in CDK4/6i resistance. Our results also shed a light on the potential benefits of

combination treatment with CDK4/6 inhibitors and PD-1/PD-L1 immune checkpoint blockade in breast cancer patients, currently in study in different clinical trials.

LIPIDS AND IMMUNOPHENOTYPE CORRELATION

It has been described that lipids can influence the anti-tumoral immune response, enhancing an immunosuppressive environment. Among other mechanisms, lipids can facilitate immune escape by promoting the expression of exhaustion markers. In turn, inhibitory receptors expressed on the surfaces of exhausted lymphocytes play a key role in lipid metabolism of tumour cells. We found that exhausted-like T helper lymphocytes expressing PD1 are moderately associated with NAE 18:1 and PE P-38:1 blood levels. Our preliminary results, in accordance with the literature, show a connection between lipid metabolism and T cell dysfunctional states.

LIMITS OF THE STUDY

Our study presents few noteworthy limitations, which will be addressed in the final analysis of the study.

Firstly, when evaluating lipidomic profiles, it is important to consider that plasma lipid content is strongly influenced by various potential confounding factors, such as diet, concurrent metabolic pathologies like obesity, dyslipidaemia, metabolic syndrome, and concomitant chronic treatments such as statins. It is well known that a greater BMI, as well as higher levels of circulating cholesterol and triglycerides predict a worse prognosis in breast cancer patients. In contrast, statin use has been associated with a better prognosis in breast cancer patients in terms of decreased risk of breast cancer recurrence. (125,126) Therefore, adjusting our results for BMI and statin could be useful to better confirm observed associations.

In addition, our study considered both endocrine-sensitive and endocrine-resistant diseases together. However, it is recognized that patients with endocrine-resistant tumours are more likely to experience early PD and show worse OS. Moreover, it has been observed that lipid content, particularly total phospholipids, as well as PC and PE, differ between hormone-sensitive and highly hormone-resistant breast cancer cell

lines. (114) Therefore, stratification based on hormone sensitivity could help avoid confounding factors.

Finally, evaluating biomarkers using serum samples is surely advantageous since it does not require invasive procedures, resulting in an easy, non-painful cheap procedure, and allows to assess a whole body scenario. However, tissue samples have the advantage of directly analysing the biological characteristics of tumor tissues compared to serum samples that can introduce various confounding factors. Furthermore, tumour-released exosomes provide a more specific source of cancer-associated lipids than whole serum while not requiring invasive procedures. Therefore, as a future prospective assessing lipidomic and T cells profiles on tissue samples and circulating microvescicles could serve to have a more tumour specific visual.

CONCLUSIONS

Despite notable advances obtained in breast cancer care, treating advanced disease remains a medical challenge. In this setting more effective prognostic and predictive biomarkers are needed to better predict disease behaviour and response to therapy and allow better treatment individualization.

Results obtained from our research support the hypothesis that plasma lipidomic profiling and circulating T lymphocytes characterization could represent valid methods to assess prognosis and treatment benefit in advanced HR+/HER2- breast cancer patients treated with CDK4/6i. Lipidic and T cell profiling may help to recognize patients with a poorer outcome and allow the early detection of patients who are unlikely to have a prolonged benefit from this treatment.

These results warrant further validation in future studies to allow the implementation of serum lipidic profile and circulating T cells as novel biomarkers to guide treatment choices in advanced HR+/HER2- breast cancer.

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