OBESITY AND BREAST CANCER:

AN INTEGRATIVE APPROACH

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Resumo

A ligação entre obesidade e cancro tem sido extensivamente abordada e investigada, revelando uma relação complexa e multifacetada. A obesidade é atualmente uma condição preocupante de saúde pública com proporções alarmantes em todo o mundo. É caracterizada pela acumulação excessiva de tecido adiposo e representa um fator de risco em inúmeras condições patológicas incluindo diabetes tipo 2, doenças cardiovasculares e cancro, nomeadamente no cancro de mama. O cancro de mama, por outro lado, é atualmente, o cancro com maiores taxas de incidência e mortalidade nas mulheres em todo o mundo. O cancro de mama caracteriza-se pela heterogeneidade na sua apresentação clínica e molecular. A sua classificação clínica pode basear-se na localização, tipo e extensão da doença enquanto a classificação molecular baseia-se na expressão de marcadores como recetores hormonais, de estrogénio e progesterona, e do gene HER2. A etiologia do cancro de mama é multifatorial, envolvendo fatores genéticos – não modificáveis e ambientais – modificáveis. Incluída nos fatores ambientais encontra-se a obesidade.

Muitas têm sido as hipóteses apresentadas do impacto da obesidade no cancro de mama e como esta pode, não só ser um fator de risco, mas também um fator de mau prognóstico na progressão da doença. As alterações promovidas pela obesidade podem resultar num estado inflamatório persistente de baixo grau, num perfil modificado de adipocinas, na remodelação da matriz celular, em alterações da microbiota, em flutuações nos níveis hormonais devido à expressão da aromatase e na desregulação do metabolismo. Dentro das alterações metabólicas observadas na obesidade, este trabalho concentra-se no metabolismo dos carbohidratos no seguimento dos resultados obtidos num estudo prévio onde foi descrito o "efeito de Warburg inverso". Em linhagens celulares tumorais de mama expostas a um ambiente que simula a obesidade, observa-se que as células recorrem ao processo de gliconeogénese, em contraste ao processo esperado da glicólise. Os resultados de uma análise metabolómica permitiram inferir que moléculas, como os aminoácidos, funcionavam como fonte de carbono para a produção de glicose. Este mecanismo estava associado a um aumento da agressividade tumoral. O presente plano de trabalhos deriva desta observação sendo um dos seus principais objetivos compreender as alterações promovidas pela obesidade no metabolismo central dos carbohidratos do cancro de mama.

Nesse sentido, abordamos não só os mecanismos celulares associados ao metabolismo dos carbohidratos em ensaios *in vitro* e em ensaios *in situ*, como também o contexto epidemiológico dos desfechos de pacientes com cancro de mama considerando a sua categoria de índice de massa corporal (IMC). Em ensaios *in vitro* procedeu-se a ensaios de cultura celular de 4 linhas representativas dos subtipos moleculares de cancro de mama (luminal A, luminal B, HER2 positivo e triplo negativo) sujeitos a secretoma da cultura celular da diferenciação de adipócitos humanos. Após tratamento, realizou-se uma análise dos metabolitos da glicólise/gliconeogénese assim como a quantificação da expressão das enzimas limitantes destas vias. Adicionalmente determinou-se a presença dos principais aminoácidos e lípidos. Em ensaios *in situ*, biópsias de tecido tumoral de

pacientes com peso normal e com obesidade foram obtidos através de um protocolo estabelecido com o Instituto Português de Oncologia (IPO-Porto). Foi aplicada a técnica de imunohistoquímica para as enzimas limitantes da glicólise e da gliconeogénese. Os resultados foram posteriormente cruzados com os parâmetros clinicopatológicos dos pacientes. Em contexto epidemiológico foram identificadas 2246 pacientes com cancro de mama e estratificados de acordo com o seu IMC. Procedeu-se posteriormente a análise estatística de variados parâmetros clinicopatológicos e demográficos assim como dos procedimentos cirúrgicos.

Dos resultados epidemiológicos obtidos destaca-se uma idade mais avançada em pacientes com sobrepeso e com obesidade. Nas mesmas categorias de IMC, a história familiar de cancro de mama é menos comum. A obesidade está associada a uma maior probabilidade de ter cancro de mama positivo para o recetor de progesterona. Mulheres com obesidade são mais suscetíveis a tumores pouco diferenciados, a tumores primários maiores, embora paradoxalmente, com menos casos de metástase à distância. Como esperado, pacientes com excesso de peso e obesidade geralmente apresentam taxas de sobrevida global mais baixas. Em termos de tipo de cirurgia, mulheres com excesso de peso têm maior probabilidade de serem submetidas a biópsia do nódulo sentinela, enquanto mulheres com excesso de peso e obesidade são mais propensas a serem submetidas a cirurgia conservadora e menos propensas a serem submetidas a mastectomia total. No caso de cancro de mama bilateral, os resultados revelam que todas as mulheres obesas apresentaram tumores pouco diferenciados. Mulheres com excesso de peso demonstraram melhor sobrevida global, embora sem significância estatística.

Os resultados dos ensaios celulares revelaram que todas as linhas celulares representativas dos subtipos moleculares basearam o seu perfil metabólico no efeito Warburg, com consumo de glicose e produção de lactato, mesmo em ambiente mimetizador da obesidade. Todos os subtipos moleculares apresentam redução no consumo de glutamina, valina e isoleucina, sendo a linha celular triplo negativo a mais afetada. O perfil metabólico da alanina muda de produção para consumo nas linhas celulares Luminal A e triplo negativo quando expostas a condições obesogénicas. Foi também observado um aumento no consumo de lípidos em todos os subtipos. Todas as enzimas limitantes da taxa glicolítica e gliconeogénica estavam elevadas em HER2+. A fosfofrutoquinase foi a única enzima com expressão aumentada nas linhas celulares Luminal A e triplo negativo. A análise revelou um padrão semelhante na expressão das enzimas glicolíticas e gliconeogénica de casos obesos nos grupos com HER2 positivo (Luminal B e HER2+) e o grupo com HER2 negativo (Luminal A e triplo negativo).

In situ observou-se uma associação estatística entre o IMC e o estadio patológico, grau histológico e subtipo molecular. Casos luminais exibem frequentemente estadios patológicos e graus histológicos mais baixos. Dentro do subtipo Luminal A, observou-se uma regulação negativa dos genes de proliferação, indicando um prognóstico mais favorável. Quando estratificados por subtipo molecular, houve diferença significativa na expressão das enzimas piruvato carboxilase, fosfoenolpiruvato carboxiquinase e da

fosfofrutoquinase, significância que se mantém após estratificação por subtipo molecular e IMC. Estas mesmas enzimas, correlacionaram-se significativamente com características clinicopatológicas dos pacientes (recetores hormonais e HER2 e grau histológico). Mulheres obesas com subtipo molecular triplo negativo tendem a expressar níveis elevados de todas as enzimas glicolíticas. A infiltração do tecido adiposo e de células inflamatórias não revelou nenhuma associação com a expressão enzimática. Em relação às condições da síndrome metabólica (diabetes, hipertensão e dislipidemia), foi observada uma correlação positiva entre a fosfoenolpiruvato carboxiquinase e a diabetes e entre a fosfofrutoquinase com a hipertensão e com a dislipidemia.

Os resultados obtidos destacam a importância da pesquisa contínua de abordar o metabolismo da glicose para melhor entender o impacto da obesidade no cancro de mama. A gliconeogénese, especificamente, parece desempenhar um papel crucial na remodelação da paisagem metabólica dentro do tumor, influenciando o seu comportamento.

Abstract

The link between obesity and cancer has been extensively studied and investigated, revealing a complex and multifaceted relationship. Obesity is currently a concerning public health condition with alarming proportions worldwide. It is characterized by the excessive accumulation of adipose tissue and represents a risk factor for numerous pathological conditions, including type 2 diabetes, cardiovascular diseases, and cancer, particularly breast cancer. Breast cancer, on the other hand, is currently the cancer with the highest incidence and mortality rates in women worldwide. Breast cancer is characterized by its heterogeneity in its clinical and molecular exhibition. Its clinical classification can be based on the location, type, and extent of the disease, while the molecular classification is based on the expression of biomarkers such as hormone receptors, estrogen, and progesterone, and the HER2 gene. The etiology of breast cancer is multifactorial, involving non-modifiable factors such as genetics and modifiable environmental factors, including obesity.

Various conjunctures have been proposed regarding the impact of obesity on breast cancer, suggesting that obesity is not only a risk factor but also a poor prognostic factor in disease progression. Alterations promoted by obesity can lead to a persistent low-grade inflammatory state, a modified profile of adipokines, a remodulation of the cellular matrix, alterations in the microbiota, fluctuations in hormone levels due to aromatase expression, and metabolic dysregulation. Within the observed metabolic changes in obesity, this work focuses on carbohydrate metabolism following the results obtained in a previous study describing the "Warburg effect inversion". In breast cancer tumor cell lines exposed to an obesity-mimicking environment, it is observed that the cells resort to the process of gluconeogenesis, contrary to the expected glycolysis process. Metabolomic analysis results suggest that molecules, such as amino acids, function as a carbon source for glucose production. This mechanism is associated with increased tumor aggressiveness. The present work plan arises from this observation, with one of its main objectives being to understand the changes promoted by obesity in the central carbohydrate metabolism of breast cancer.

In this context, we addressed not only the cellular mechanisms associated with the carbohydrate metabolism in *in vitro* and *in situ* assays but also the epidemiological context of the outcomes of breast cancer patients considering their body mass index (BMI) category. In *in vitro* assays, four molecular representative cell lines (luminal A, luminal B, HER2-positive, and triple-negative) were subjected to secretome from the differentiation of human adipocytes. After treatment, an analysis of glycolysis/ gluconeogenesis metabolites was performed, as well as quantification of the expression of the limiting enzymes of these pathways. Additionally, the presence of major amino acids and lipids was determined. In *in situ* assays, tissue biopsies from breast cancer patients with normal weight and obesity were obtained through a protocol established with the Portuguese Oncology Institute (IPO-Porto). Immunohistochemistry was applied for the limiting enzymes of glycolysis and gluconeogenesis. Results were subsequently cross-referenced with the clinicopathological parameters of the patients. In the

epidemiological context, 2246 breast cancer patients were and stratified according to their BMI. Subsequently, a statistical analysis of various clinicopathological and demographic parameters was conducted, as well as surgical procedures.

Epidemiological results highlight an older age in overweight and obese patients. In the same BMI categories, a family history of breast cancer is less common. Obesity is associated with a higher probability of positive progesterone receptor. Women with obesity are more susceptible to poorly differentiated tumors, larger primary tumors, although paradoxically, with fewer cases of distant metastasis. As expected, overweight and obese patients generally present lower overall survival rates. Regarding surgical interventions, overweight women are more likely to undergo sentinel node biopsy, while overweight and obese women are more likely to undergo conservative surgery and less likely to undergo total mastectomy. In the case of bilateral breast cancer, the results reveal that all obese women presented poorly differentiated tumors. Overweight women demonstrated better overall survival, although without statistical significance.

Cellular based assays revealed that all molecular representative cell lines based their metabolic profile on the Warburg effect, with glucose consumption and lactate production, even in an obesity-mimicking environment. All molecular subtypes show a reduction in the consumption of glutamine, valine, and isoleucine, with the triple-negative cell line being the most affected. The metabolic profile of alanine changes from production to consumption in Luminal A and triple-negative cell lines when exposed to obesogenic conditions. An increase in lipid consumption was also observed in all subtypes. All rate limiting enzymes of glycolytic and gluconeogenic pathways were increased in HER2+. Phosphofructokinase was the only enzyme with increased expression in Luminal A and triple-negative cell lines. The analysis revealed a similar pattern in the expression of glycolytic and gluconeogenic enzymes in obese cases in groups with HER2-positive (Luminal B and HER2+) and the HER2-negative group (Luminal A and triple-negative).

In situ, a statistical association between BMI and pathological stage, histological grade, and molecular subtype was observed. Luminal cases often exhibit lower pathological stages and histological grades. Within the Luminal A subtype, negative regulation of proliferation genes was observed, indicating a more favorable prognosis. When stratified by molecular subtype, there was a significant difference in the expression of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and phosphofructokinase enzymes, significance that remains after stratification by molecular subtype and BMI. These same enzymes correlated significantly with clinicopathological characteristics of patients (hormone receptors/HER2 and histological grade). Obese women with triple-negative molecular subtype tend to express higher levels of all glycolytic enzymes. Infiltration of adipose tissue and inflammatory cells revealed no association with enzyme expression. Regarding the conditions of metabolic syndrome (diabetes, hypertension, and dyslipidemia), a positive correlation was observed between phosphoenolpyruvate carboxykinase and diabetes and between phosphofructokinase with hypertension and dyslipidemia.

The results highlight the importance of continuous research to address glucose metabolism to better understand the impact of obesity on breast cancer. Gluconeogenesis, specifically, appears to play a crucial role in remodeling the metabolic landscape within the tumor, influencing its behavior.

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GA3P – glyceraldehyde 3-phosphate; 3PG – 3-phosphoglycerate; LDH – lactate dehydrogenase; PHGDH – phosphoglycerate dehydrogenase; AcCoA – acetyl coenzyme A; CPT1/2 – carnitine palmitoyltransferase 1 and 2; TCA – tricarboxylic acid cycle; GLS – glutaminase; FASN – fatty acid synthase; UDP-GlcNAc – uridine diphosphate N-acetylglucosamine.

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Chapter III - Epidemiological approach to obesity and breast cancer

A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer

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A narrative review in why breast cancer is diagnosed later in obese women.

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Bilateral breast cancer and the influence of body mass index in clinicopathological features and overall survival

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Does body mass index influence surgical options and overall survival in breast cancer patients?

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Appendix C - Integrative approach on enzymatic isoforms

Glycolytic and Gluconeogenic Enzymes Isoforms: Implications in Breast Cancer Metabolism

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A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer

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Bilateral breast cancer and the influence of body mass index in clinicopathological features and overall survival

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Does body mass index influence surgical options and overall survival in breast cancer patients?

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Chapter IV - Cell-based approach to obesity and breast cancer

The Influence of Adipocyte Secretome on Selected Metabolic Fingerprints of Breast Cancer Cell Lines Representing the Four Major Breast Cancer Subtypes

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Chapter V - Tissue based approach to obesity and breast cancer

Breast Cancer Molecular Subtypes Differentially Express Gluconeogenic Rate-Limiting Enzymes—Obesity as a Crucial Player

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Appendix B - Clinical approach on Metabolically Healthy Obese

Distinguishing Metabolically (Un)Healthy Obese Women. Metabolic stratification of health parameters

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Appendix C - Integrative approach on enzymatic isoforms

Glycolytic and Gluconeogenic Enzymes Isoforms: Implications in Breast Cancer Metabolism

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List of abbreviations

2PC – 2-phosphoglycerate

3PC – 3-Phosphoglycerate

ABC – Avidin biotin complex

AD – Axillary dissection

AKT – AKT serine/threonine protein kinase 1

AKT – Protein kinase B

ALDO – Fructose-bisphosphate aldolase

AMF – Autocrine motility factor

AMFR – Autocrine motility factor receptor

AMPK – AMP-activated protein kinase

AS – Adipocyte secretome

ASCO/CAP – American Society of Clinical Oncology/College of American Pathologists

ATF4 – Activating Transcription Factor 4

ATP – Adenosine-5-triphosphate

BAD – BCL2 Associated Agonist of Cell Death

BC – Breast cancer

BCAA – Branched-chain amino acid

BMP2 - Bone morphogenetic protein 2

C – Control

CAA – Cancer associated adipocytes

CAF – Cancer associated fibroblasts

CCNB1 – G2/mitotic-specific cyclin B1

CDK1 – Cyclin dependent kinase 1

CHUSJ – São João hospital and university centre

CI – Confidence interval

circFOXP1 – FOXP1 (Forkhead box P1) circular RNA

CQ – Central quadrant

CS – Conservative surgery

CXCL12 – C-X-C motif chemokine ligand 12

CXCR4 – C-X-C chemokine receptor type 4

DAB – 3,3-diaminobenzidine

DFS – Disease-free survival

DOC – Deciphering obesity and breast cancer

DSH17B – Hydroxysteroid 17-beta dehydrogenase

EGF - Epidermal growth factor

ENO – Enolase

ER – Estrogen receptor

ERBB – erb-b2 receptor tyrosine kinase

F1-6BP – Fructose-1,6-biphosphate

F6P – Fructose-6-phosphate

FBPase – Fructose-1,6-bisphosphatase

FBS – Fetal bovine serum

FISH – Fluorescence *in situ* hybridization

G1P – Glucose-1-phosphatase

G6P – Glucose-6-phosphate

G6Pase – Glucose-6-phosphatase

G6PC – Glucose-6-phosphatase catalytic subunit

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GDP – Guanosine diphosphate

GEPIA – Gene Expression Profiling Interactive Analysis Dataset

GK – Glucokinase

GPI - Glucose-6-phosphate isomerase

HbA1c – Glycated hemoglobin

HDL – High density lipoproteins

HER 2 – Human epidermal growth factor receptor 2

HGF – Hepatocyte growth factor

HIF1α – Hypoxia-inducible factor 1-alpha

HK – Hexokinase

HR – Hazard ratio

IDC – Invasive Ductal Carcinoma

IFN – Interferon

IGF1 – Insulin-like growth factor 1

IIQ – Inferior inner quadrant

IL – Interleukin

ILC – Invasive Lobular Carcinoma

IOQ – Inferior outer quadrant

KEAP – Kelch like ECH associated protein

Kg – Kilograms

LDL – Low density lipoproteins

LIF – Leukaemia inhibitory factor

LumA – Luminal A

LumB – Luminal B

m^2 – Squared meters

mBMI – Molecular subtype and BMI

MHO – Metabolically Healthy Obese

MR – Mastectomy and reconstruction

MS – Molecular subtype

MTB – *Mycobacterium tuberculosis*

mTOR – mammalian target of rapamycin

MUO – Metabolically Unhealthy Obese

MYBL2 – MYB Proto-oncogene like 2

MYCBP – MYC proto-oncogene binding protein

N – Normal

NCS – Newborn calf serum

NFE2L2 – Nuclear factor erythroid 2-related factor 2

NMR – Nuclear magnetic resonance

NRF – Nuclear respiratory factor

NST – No special type

NUP – Nucleoporin

O – Obese

OR – Odd ratio

OS – Overall Survival

PC – Pyruvate carboxylase

PEP – Phosphoenolpyruvate

PEPCK – Phosphoenolpyruvate Carboxykinase

PFK – Phosphofructokinase

PGAM – Phosphoglycerate mutase

PGK – Phosphoglycerate kinase

PI3K - Phosphoinositide 3-kinase

PK – Pyruvate kinase

PPAR – Peroxisome proliferator-activated receptor

PR – Progesterone receptor

RM – Radical mastectomy

SD – Standard deviation

SIQ – Superior inner quadrant

SLC25A1 – Solute Carrier Family 25 Member 1

SNB – Sentinel node biopsy

SOQ – Superior outer quadrant

STAT3 – Signal transducer and activator of transcription 3

TCA – Tricarboxylic acid

TCR – T-cell receptor

TG – Triglycerides

TGF – Transforming growth factor

TIGAR – TP53 induced glycolysis regulatory phosphatase

TM – Total mastectomy

TME – Tumour microenvironment

TMN – Tumour, node, metastasis system

TN – Triple negative

Treg – Regulatory T Cells

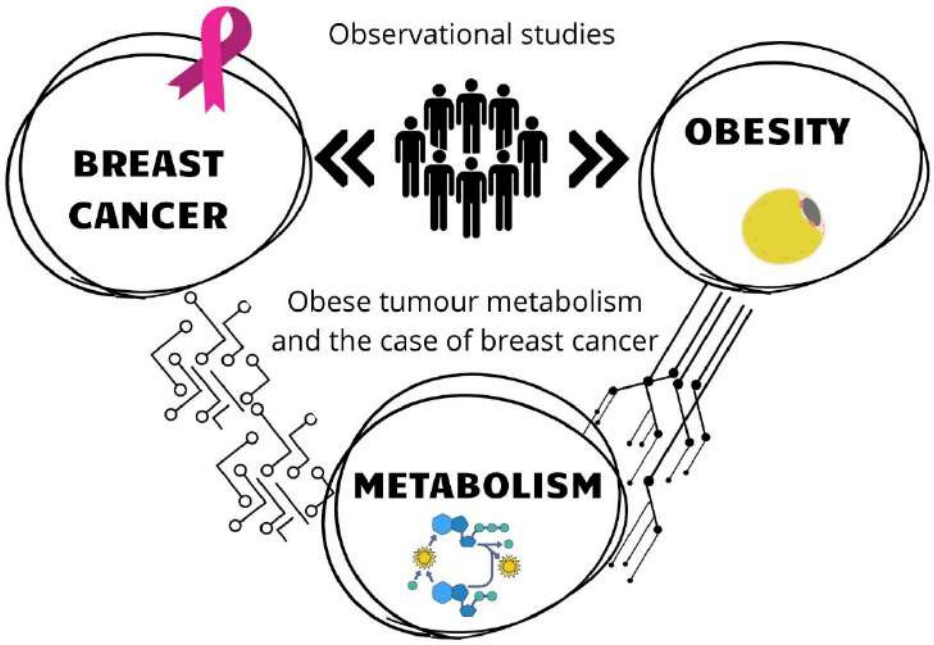
UDP – Uridine diphosphate

VDAC – Voltage-dependent anion channel

WHO – World Health Organization

CHAPTER I

Introduction



LIST OF CONTENTS:

- The breast cancer "syndrome"
- Obesity: an ongoing epidemic
- The observational link between breast cancer and obesity
- The obese tumour metabolism and the case of breast cancer

CHAPTER I

1. Introduction

Breast cancer and obesity are two metabolically associated pathologies with high and increasing prevalence rates[1]. Strong evidence suggests that obesity-related factors regulate and modulate tumorigenesis and tumour progression. A poor diet and a sedentary life are the common causes to promote the increased fat deposition which results in the development of several metabolic pathologies such as diabetes, cardiovascular diseases, and cancer[2]. It is established that obesity has a significant impact in breast cancer metabolism due to the increase levels of local oestrogen and aromatase activity[3]. Additionally, obesity related factors can promote alterations in the central biochemistry metabolism. A preliminary study carried out by our group on the metabolism of breast tumour cells revealed that obesity-mimicking conditions promoted growth and aggressiveness with an alteration of the basal energy metabolism (from glycolysis to gluconeogenesis)[4]. This chapter is dedicated to the state of the art of both pathologies and the metabolic crosstalk between them. An extensive literature review performed in scientific databases and manuscripts published in peer-review indexed journals was included.

1.1. The breast cancer "syndrome"

Breast cancer (BC) complexity is vast and incorporates so many different classifications that one could almost accept them as a syndrome (a set of conditions that occur in the presence of a certain disease). Breast cancer can be classified according to molecular subtype, histological type, differentiation grade, disease extension, pathological and clinical stage, which represents different prognosis with different treatment strategies.

The combination of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression determines the molecular subtype. The existence of four molecular subtypes: Luminal A (ER/PR+, HER2-), Luminal B (ER+, PR-/+, HER2-), HER2-enriched (ER-/PR-/HER2+) and Triple Negative (ER-/PR-/HER2-). This classification is of extreme importance since it determines the treatment strategy specifically endocrine therapy for Luminal A and B and trastuzumab (Herceptin) to HER2-enriched[5]. But new emerging subtypes and potential receptors are being the focus of translational and clinical research. In 2007, a new subtype was described as claudin-low BC[6] characterized by the lack of tight junction proteins (Claudin-3 and E-cadherin) and it is still a poorly characterized subtype with a wide broad of heterogeneity[7]. Microarray panels identify other potential subtypes, such as interferon-enriched[8], apocrine subtype[9] and six more subgroups of triple-negative BC[10]. The Lehman's classification categorizes triple negative BC in six subtypes: Basal-like 1 and 2, mesenchymal-like, mesenchymal/stem-like, immunomodulatory, and luminal androgen receptor[10], each one with a gene profiling signature and specific therapeutic targets. For instance, androgen

receptor (AR) can be a potential target in quadruple negative BC, i.e., triple negative with AR negative[11].

According to the World Health Organization (WHO) there are 23 main histological subtypes of breast cancer[12], the more incident ones are the invasive ductal carcinoma (IDC) accountable for 70-80% of all invasive tumours and termed as "no specific type" due to the lack of sufficient morphological characterization to be classified otherwise except the ductal localization. Lobular carcinoma with invasive phenotype designated invasive lobular carcinoma (ILC), diagnosed in 5-15%[13]. Histological classification also includes mixed invasive ductal and lobular carcinoma (IDLC), diagnosed in approximately 5% of the cases[14]. The histological type not only indicates whether the tumour is circumscribed to (or spread from) the mammary ducts or lobes but also describes cell and secretion characteristics as well as distinctive cellular morphological architecture[15]. For instance, medullary carcinoma is characterized by the presence of lymphocytic infiltrate and large and pleomorphic tumour cells[16]. Other "special subtypes" include invasive micropapillary carcinoma, mucinous carcinoma, tubular adenocarcinoma, metaplasic carcinoma, invasive intraductal papillary adenocarcinoma, cribriform carcinoma, glycogen-rich carcinoma, adenocarcinoma with neuroendocrine differentiation, intracystic carcinoma, apocrine adenocarcinoma, clear cell adenocarcinoma, liposarcoma, malignant myoepithelioma, adenocarcinoma with pavement metaplasia, inflammatory carcinoma, papillary carcinoma, among others[12].

Differentiation grade is associated to the process in which cells suffer "metamorphosis", when it switches from a normal original cell/tissue to a malignant one. Differentiation grade is applied to all histological types, and it is usually measured according to the Nottingham grading system (NGS)[17]. NGS takes in consideration: gland/tubular formation, mitotic activity, and nuclear features, each is classified from 1 to 3. Differentiation grade is then stratified in 3 grades: (1) Grade 1 (total score: 3-5) is well differentiated with a low proliferation rate and is phenotypically similar to normal breast cells, (2) Grade 2 (total score: 6-7) is moderately differentiated and is intermediate between grade 1 and 3 for proliferation and phenotype and (3) Grade 3 (total score: 8-9) which is poorly differentiated with an increased growth rate and phenotypically distinctive from normal breast cells[18]. Nowadays, many authors and some international institutes are already using the Multifactorial Nottingham prognostic index to therapeutical recommendations, this index include the NGS along with tumour size and lymph node infiltration[19].

Tumour size, nodal involvement along with metastasis (TMN system) are the variables included in the most commonly used index for the classification of the pathological stage of BC: the American Joint Committee on cancer staging system. The 7th edition had an entirely anatomic method based on clinical and pathological evaluations, and presented relevant limitations associated with biological factors such as receptors, tumour grade and proliferation activity by Ki67, important decision maker modulators in the treatment strategy and response to therapy. The 8th edition is the most updated version and integrates the TMN system alongside with molecular biomarkers[20]. Additionally,

multigene panels, genomic profiles, signature scores can be used to clinical support, such may include the following tools: EndoPredict, IHC4, Mamaprint®, Oncotype DX ®, PAM50 (ProSigna®)[21]. A summary of BC classifications is presented in Figure 1.

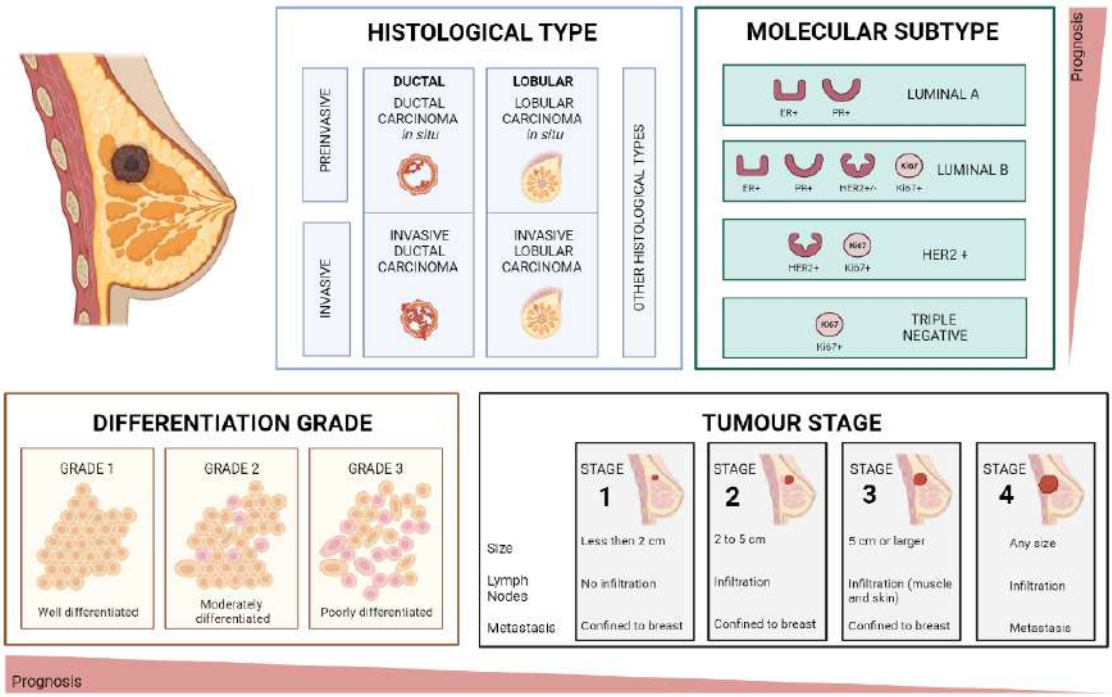


Figure 1 – Summary of breast cancer characteristics and associated prognosis. (*Created in Biorender.com*).

The different metrics and characteristics used to classify breast cancer has the ultimately intention to decipher the best treatment to improve quality and time of life. Currently, treatment options include surgery, radio- and chemotherapy and endocrine pharmaceuticals. They can be addressed to the primary tumour (local) or to the nodal infiltration or distant metastasis (systemic), but surgery is still the cornerstone in BC treatment and provides control of the primary tumour and of potential nodal infiltrations[22]. Breast cancer surgery evolved significantly in the last decades. In the 70s, radical mastectomy was the only surgical procedure, characterized by its invasive and traumatizing intervention. Nowadays, BC surgery has a more comprehensive approach with several conservative and targeted methods, which comprises an aesthetic basis[23]. Conservative surgery (CS) or partial mastectomy, consists in the removal of the malignant tissue and surrounding margins, results exhibit a satisfactory outcome regarding long-term survival and aesthetical result[24]. CS is considered a safe procedure, associated with low rates of local and distant recurrence, low positive margins rate and low surgical complications[25]. Total mastectomy (TM) implies the excision of all breast tissue, skin, nipple, and areola. Same for radical mastectomy (RM), which additional remotion of chest wall muscles and lymph node dissection. Another surgical alternative is mastectomy with immediate reconstruction (MR), associated with considerable improvements on the aesthetic outcomes of breast cancer patients[26]. Surgical approaches also include Sentinel node biopsy (SNB) and axillary dissection (AD), this last procedure is effective to maintain

local control but is associated with several co-morbidities[27] and previous studies uncovered no oncological advantages in axillary dissection[28].

Radiation therapy (RT) is usually used as an adjuvant treatment to surgery to control metastasis and prevent residual tumour cell proliferation, although it is associated with acute and chronic complications. For several decades, adjuvant RT in BC was associated with cardiac mortality due to radiation exposure of cardiac tissues, especially in left sided BC[29]. With technique modernization, RT evolution focus on more precise and harmless techniques like three-dimensional conformal RT (3DCRT), forward planned intensity-modulated radiation therapy (FP-IMRT), inverse planned IMRT (IP-IMRT), volumetric modulated arc therapy (VMAT) – a type of IP-IMRT[29]. Each technique is applied according to the extent of the disease and of individual features. The most innovative RT intervention is brachytherapy, which has already revealed favourable results. It consists in the injection of a radioactive substance into or next the malignant breast tissue. It is a promising technique but not fully optimized[30].

Chemotherapy uses chemotherapeutical agents that take advantage of tumour cell characteristics to reduce proliferation (cytostatic) and/or to promote apoptosis (cytotoxic). It is normally used in combination with other treatment options to enhance the treatment efficacy. The spectrum of drugs applied in BC has been increasing towards optimized results, not only increasing efficacy but diminishing side-effects and drug resistance[31]. Types of chemotherapeutics can include direct damage to cell cycle by *alkylating agents*, nucleobases analogues that by incorporation stop DNA/RNA replication (*antimetabolites*), antibiotics which target DNA replication enzymes (*anthracyclines*) and others such as mitotic inhibitors, hormone, and immunologic chemotherapeutical drugs, etc[32]. In disease management, systemic therapy can be given before surgery (neoadjuvant therapy) or after surgery (adjuvant therapy). Treatment endorsement depends of several patient/cancer characteristics such as patient age and postmenopausal status or molecular subtype and metastatic development[33], usually recommended according specific/regional guidelines (USA, EU, and UK national guidelines)[31]. Treatment options are presented in Figure 2.

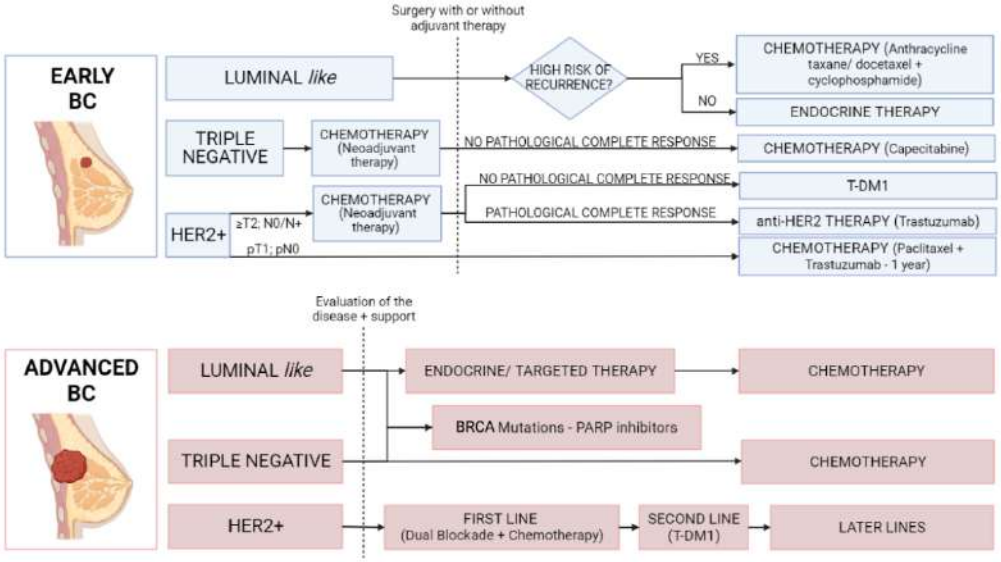


Figure 2 – Algorithm for early and advanced breast cancer treatments based in the study from (Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, Cardoso F. Breast cancer. Nat Rev Dis Primers. 2019 Sep 23;5(1):66)[33]. *Legend*: BC: breast cancer, pT: pathological Tumour size; T: Tumour size; N: Nodal infiltration; PARP: poly ADP ribose polymerase; T_DM1: ado-trastuzumab emtansine. (*Created in biorender.com*)

For an improved outcome of BC treatments, most countries worldwide believe on an early diagnosis and have implemented screening programs to decrease not only BC associated mortality but also adverse effects from BC treatments and associated economic burden (Figure 3). Portuguese BC screening program consists in a mammography exam, every two years, in women between 50 and 69 years old. In 2020, the Portuguese screening program covered 62% of women in a 78% of geographic territory which exposed 0.6% primary positive results (equivalent to 1053 women)[34]. Screening programs can vary in screening age and exam interval along with different exam methods. For example, Japanese screening program decreased the screening age due to the observed incidence rate in women between 45 and 49 years-old[35]. The American Cancer Society recommends that women between 40 and 54 should be screened every year and, after 55 years old, screened every 2 years[36]. Studies on screening follow-up are scattered and limited. A systemic review on controlled trials, performed by Fitzpatrick-Lewis, has an update of the United States Preventive Services Task Force (USPSTF) 2009 with the main objective of determining the mammography screening efficacy in mortality rates. Main conclusions unravel that mammography screening reduced breast cancer mortality in women between 40 and 69 years old, but no significant alteration was observed in women with more than 70 years old[37].

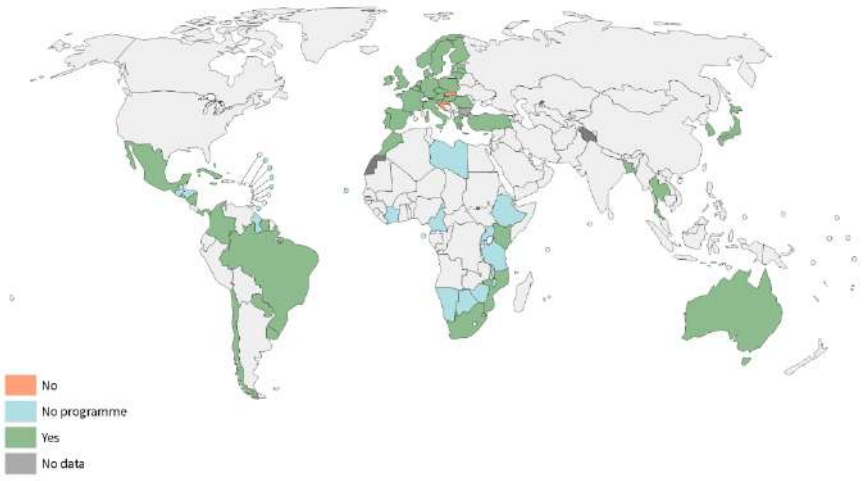


Figure 3 – National breast screening policies for all ages in all continents, data from *CanScreen5* (*Source: World Health Organization 2022*)[38].

Nonetheless, the motto remains that prevention is better than the treatment ("better safe than sorrow"). Risk factors can be of upmost importance in tumorigenesis and cancer development. It is well described that breast cancer incidence is associated with several

risk factors like genetics and environment/lifestyle[39]. Inherited breast cancers are prevalent in syndromes such as Ataxia telangiectasia and Li Fraumeni and in BRCA1 and BRCA2 genes mutation carriers, two suppressor genes involved in DNA repair. Such genetic alterations are accountable for approximately 10% of breast cancers[33]. Regarding environment/lifestyle, breast cancer epidemiology distinguished two main segments, reproductive and modifiable factors. Reproductive factors include late age for first pregnancy, early menarche, and late-onset menopause[33]. Modifiable risk factors include physical inactivity, alcohol consumption and obesity, and they are responsible for approximately 20% of breast cancers[33]. The present study will specifically focus on the impact of obesity on BC. Obesity not only influences tumour initiation but also its proliferation, aggressiveness, dissemination, bilaterality, diagnosis, response to therapy and other not fully understood relevant outcomes.

1.2. Obesity: an ongoing epidemic

In 1998, the WHO classified obesity as a "global epidemic"[40]. Nowadays, although the term is still used, it is not totally accurate to classify obesity as an epidemic since epidemics usually starts with an outbreak and resolves itself, either by death of the infected individuals or by their cure. On the contrary, obesity has been progressively increasing in the last decades. From 1975 to 2016 the worldwide prevalence of obesity has nearly tripled[41] and prevalence estimation for the next years is a continuing growth, with alarming proportions (Figure 4). For instance, the incidence rate of obesity in Saudi Arabian women is projected to be 49,1% and in Egyptian women of 49,4% in 2025, being women the most affected group.

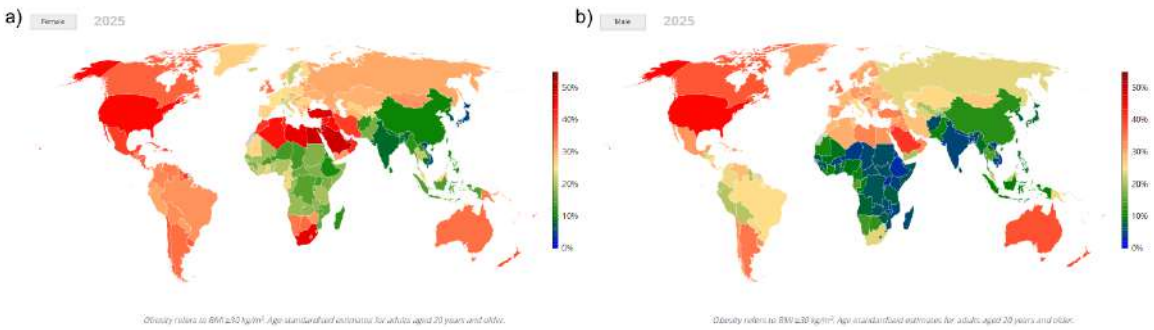


Figure 4 - Projected obesity prevalence in 2025 according to NCD Risk Factor Collaboration (NCD RisC) in adult females (a) and males (b). (*Figure created in the NCD RisC website and generated from data published in* [41]).

Overall, obesity can be defined by the excessive accumulation of adipose tissue or by the abnormal distribution of body fat due to both environmental and genetic factors[42]. The estimated genetic contribution for obesity can vary between 40% and 70%[42] although environmental risk factors are the most problematic and difficult to address, consequence of the XXI century lifestyle. A poor and imbalanced nutritional diet, the

reduced physical activity, overall stress, and circadian desynchronization associated with sleep deprivation[43] are the most prevalent.

Body Mass Index (BMI, kg/m^2) or Quetelet index[44], is the most common classification used, however, debatable in terms of accuracy. Nowadays, obesity phenotypes are being discussed with no global consensus for the lack of proper biomarkers. A better characterization, with a consensual set of proteins, genes or cells as biomarkers would largely improve disease management. Mayoral et al, 2017, reviewed four proposed subtypes of obese categories presented in Figure 5. This study included a literature review and results were cross-checked with potential biomarkers[45]. Several authors support the concept that an obese individual doesn't necessarily have to be an unhealthy one. A person may have high BMI, yet without metabolic impairments associated. On the other hand, normal-weight individuals can exhibit obesity-like features like insulin resistance, hypertriglyceridemia, hyperinsulinemia, hypertension and be classified as unhealthy. The loss of muscle mass – sarcopenia, can also be observed in certain obese individuals.

Obese groups	Definition	Other terminology for this group	Notes
MHO	Absence of metabolic disorders, including type 2 diabetes mellitus, dyslipidaemia, and hypertension	Metabolically normal obese, metabolically benign obese, metabolically healthy overweight/obese	Definitions vary in different studies, manly based on inflammatory markers and cut-off values
MAO	Defined by 2 main factors, BMI and metabolic status, which is classified as having three or more points from the NCEP-ATP III, to define MetS	MUO	Several definitions of MetS have been published since 1999, the first was proposed by the WHO
MONW	Individuals are characterized by a BMI <25 kg/m^2, hyperinsulinaemia and (or) insulin resistance, increase abdominal and visceral adiposity, atherogenic lipid profile, unfavourable adipokine profile, as well as hypertriglyceridaemia and hypertension, and higher levels of oxidative stress	Metabolically obese healthy	Some definitions consider other variables such as BMI, FFM, VAT, HOMA, ATP III
Sarcopenic obese	BMI <25 kg/m^2, low muscle mass and weak muscle strength lack physical exercise	Sarcopenic overweight	

MHO, metabolically healthy obese; MAO, metabolically abnormal obese; MONW, metabolically obese, normal weight; MetS, metabolic syndrome; BMI, body mass index; NCEP-ATP III, National Cholesterol Education Program Adult Treatment Panel III; FFM, fat-free mass; VAT, visceral adipose tissue; HOMA, homeostasis model assessment; ATP III, adult treatment program III; MUO, metabolically unhealthy obese

Figure 5 – Description of obesity subtypes (*source: Mayoral, Laura Perez-Campos, et al. "Obesity subtypes, related biomarkers & heterogeneity." The Indian journal of medical research 151.1 (2020): 11.*)[45].

Control of body fat is one of the greatest health challenges. Although there are several treatment options available like surgery and anti-obesity pharmaceuticals, lifestyle interventions are still the foundation of disease management. Moreover, the most difficult achievement in obesity is maintaining the weight loss at a long term. Figure 6, obtained from a study by Müller and colleagues[43], demonstrated the weight loss efficacy of different treatments. The intervention with a more pronounced weight loss was bariatric surgery. A meta-analysis performed by Buchwald and colleagues on morbid obese patients (mean BMI of 46.9 kg/m^2) submitted to bariatric surgery also observed a complete remission of diabetes in 76.8% of the population, 70% of the patients improved the state of hyperlipidemia, hypertension was also resolved in 61.7% of the cases and

85.7% of the patients that underwent bariatric surgery had their obstructive sleep apnea resolved[46]. But advantages of bariatric surgery were also described in cancer, a follow-up study of 701 patients with obesity and diabetes for 21.3 years, observed a decrease of cancer incidence of patients who underwent bariatric surgery when compared to usual obesity disease management (9.1 per 1,000 person-years VS 14.1 per 1,000 person-years). Women, particularly, were significant associated with decreased cancer incidence[47]. Bariatric surgery seems to ameliorate obesity-associated comorbidities and improve overall survival[48]. Nonetheless, bariatric surgery is also associated with surgical complications such as bowel obstruction, nutrient deficiencies, nausea, infection, stenosis, pancreatic exocrine insufficiency, bone loss and higher fracture risk[49–51]. Currently, bariatric surgery seems to be the most promising treatment option, although novel pharmacological approaches are being developed with interesting results.

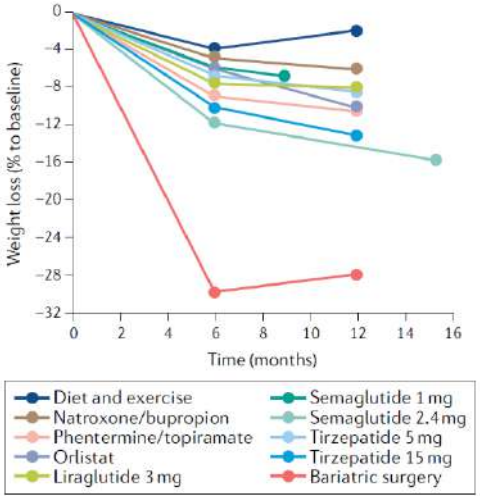


Figure 6 – Body weight loss obtained from different obesity treatments in % to baseline (*Source: Müller, Timo D., et al. "Anti-obesity drug discovery: Advances and challenges." Nature Reviews Drug Discovery 21.3 (2022): 201-223.*)[43].

A poor weight management leads to a systemic dysmetabolism correlated with several life-threatening pathologies including type 2 diabetes, hypertension, hyperlipidaemia, hypertriglyceridemia, infertility, insulin resistance, hyperinsulinemia, cardiovascular disease, and obesity-related cancers (Meningioma, thyroid, oesophagus, breast, liver, stomach, gallbladder, pancreas, kidney, bowel, ovarian, womb, myeloma)[52]. It was previously described that in overweight individuals, a 5-unit increase in BMI enhances the overall mortality in 29%, diabetes related mortality in 210%, and vascular disease-associated mortality in 41%[53]. In cancer, 4.6% of overall cancer deaths were attributed to obesity[54] and a 5 kg/m^2 increase in BMI corresponds to a 2% increased risk in breast cancer for women[55].

But obesity consequences are not limited to physical pathologies. It also comprises financial issues and mental health problems as illustrated in Figure 7, an overview in causes, consequences, and treatments of obesity.

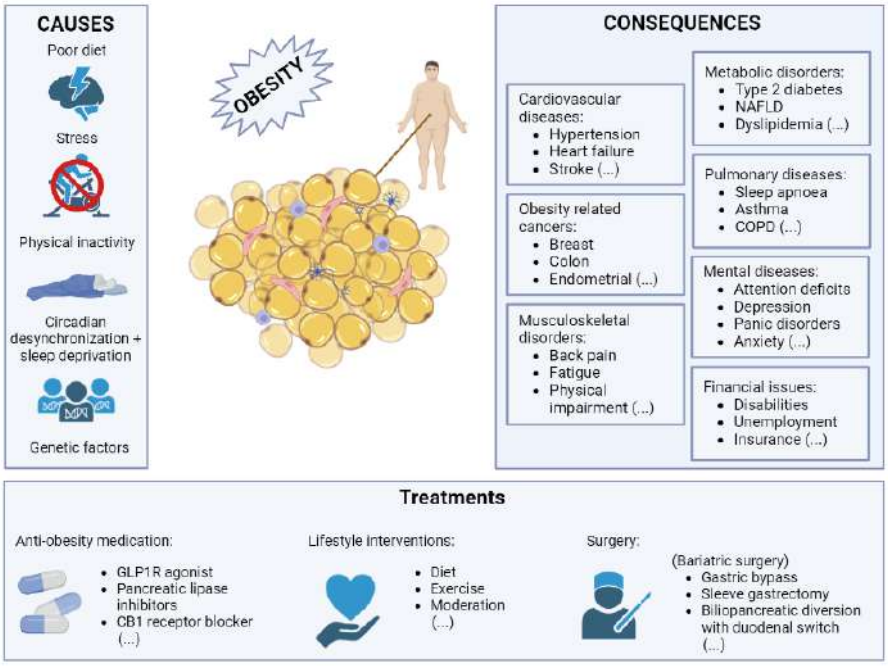


Figure 7 – Obesity summary of examples of causes, consequences, and treatments. (*Created in Biorender.com*).

Interestingly, obesity is predominantly associated with poorer outcomes, but some authors have disclosure improved survival (and better outcomes) for obese patients in certain diseases or conditions, a tendency designated as "reverse epidemiology" or "obesity paradox"[56]. A long-described paradox is tuberculosis (TB) whose risk is described to be inversely correlated with BMI. Historical data demonstrated that physicians in the XIX century encouraged TB patients into metabolic syndrome, by increasing BMI and to sustain it as long as possible to reduce TB risk[57]. Another suggested paradox concerns pneumonia patients. Several studies observed that pneumonia-related mortality is inversely correlated with BMI, conflicting with the idea that obesity impairs lung function, namely gas exchange by airway resistance[58–60]. Another reported condition where obesity revealed itself as valuable includes severe sepsis[61], Chagas disease[62], gallbladder disease[63], chronic kidney disease[64], chronic obstructive pulmonary disease[65], cardiovascular diseases (well described)[66], osteoporosis[67] among others.

Regarding cancer incidence, obesity is identified as a major risk factor in 13 types of cancer (previously described) but with no consensus regarding cancer survival. There are countless studies with contradicting results and obesity paradox is continuously the focus of discussion. An interesting study performed by Lee & Giovannucci states that the obesity paradox in most studies can be explained by a defective methodological approach like reverse causation, selection bias or confounding variables such as smoking, cancer cachexia, body composition among others[68]. Conflicting results are also found in breast cancer. For instance, a review in immunology and immunotherapy of triple negative breast cancer presented the downsides and upsides of obesity. On one hand, obesity is a risk factor to a reduced immune response and increased side effects. But on

another hand, obesity seems to ameliorate outcomes by checkpoint blockage[69]. A more in-depth insight on the effect of obesity in breast cancer is presented in the next subsection (*The observational link between breast cancer and obesity).*

1.3. The observational link between breast cancer and obesity

While the incidence rate of non-related obesity cancers has declined from 2005 to 2014, obesity associated cancers have increased[70], probably due to high and increasing incidence rates of obesity[71]. Literature associating breast cancer and obesity is extensive and scarce. A systematic literature revision was performed for a better assessment of the information. "Breast cancer", "obesity" and "observational studies" were applied as filter keywords in scientific databases (Pubmed, Mendeley, and Scopus) and inclusion and exclusion criteria were applied manually (Table 1). Figure 8 presents the flowchart of the systematic revision. Table 2 presents the results of the included articles and the main results distributed according to the scope of the study (Risk factor, Clinical features and outcomes, and Biomarkers).

Table 1 – Inclusion and exclusion criteria of the systematic review in breast cancer.

Criteria	Inclusion criteria	Exclusion criteria
Time period	Last 10 years (2013-2022)	-
Language	English	-
Geographic delimitation	No restriction	-
Population sex	Women	Men
Pathologies	Breast cancer; Obesity	Other cancers; Diabetes; Cardiovascular diseases;
Variable focus	Obesity	Race; geographic localization (rurality); Economic status; Lifestyle interventions.
Study design	Observational studies	All other study designs like reviews, meta-analysis, case reports, etc

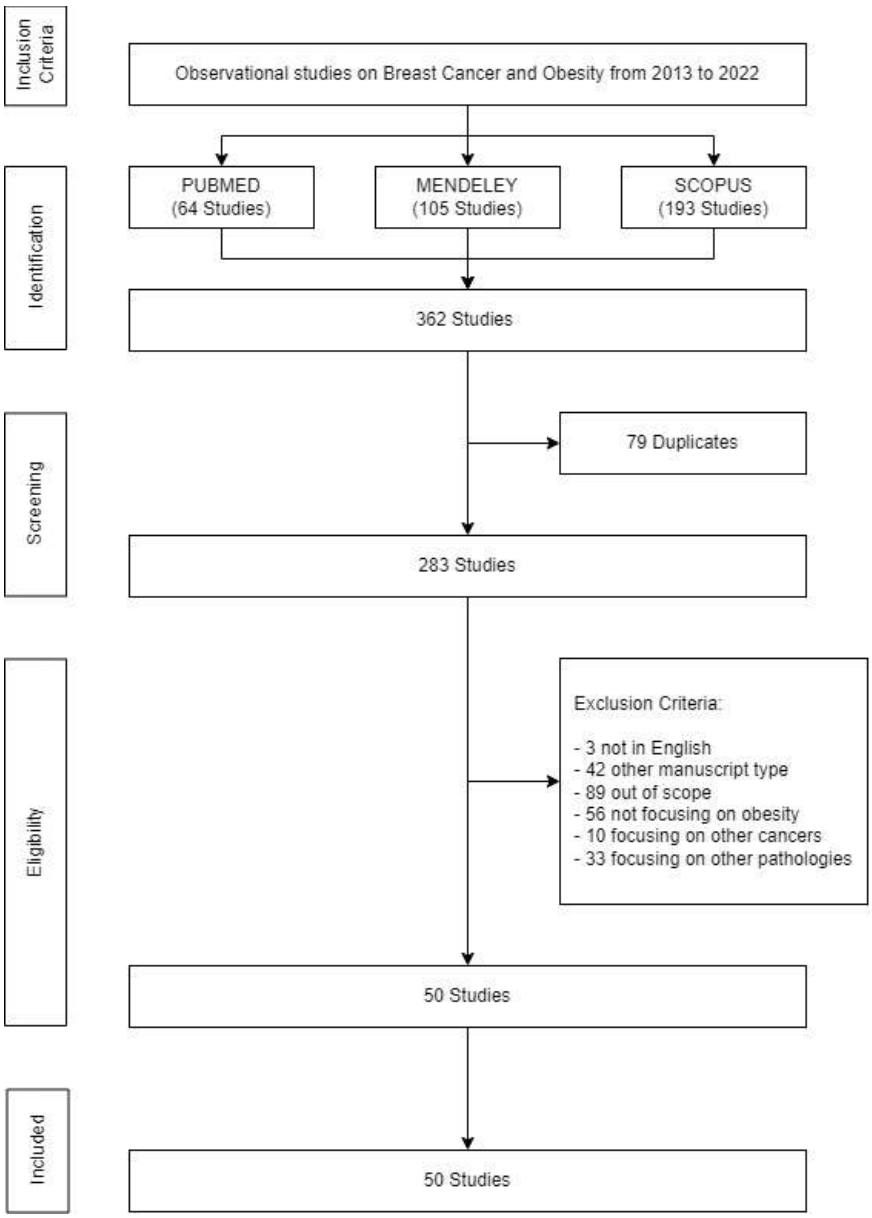


Figure 8 – Workflow of the systematic review (*Created on Draw.io*)

Table 2 – Description of the year of publication, country, design study and main results of each included study divided according to the scope of each manuscript (Risk factor, Clinical features and outcomes, and Biomarkers).

Scope	Year	Country	Cohort	Main results/conclusions	Ref
RISK FACTOR	2021	Mozambique	Patients with breast cancer (BC) (n=138) recruited for the Mozambican Breast Cancer cohort and controls (n=638) from "World Health Organization stepwise approach to surveillance of noncommunicable disease risk factor".	Higher weight and BMI were associated with an increased risk of BC among postmenopausal women, whereas premenopausal women, weight and BMI revealed a protective role, namely in hormone receptor (estrogen and progesterone) positive and HER2 negative.	72
	2021	France	Patients with metastatic breast cancer (MBC) enrolled in the "Epidemiological Strategy and Medical Economics - French metastatic breast cancer cohort" (n=12 999 patients).	In MBC, BMI was positively associated with age, menopausal status, higher rates of *de novo* MBS (metastatic condition detected within 6 months of the primary cancer), and bone metastasis (all $p < 0.001$). Underweight MBC patients were correlated with higher number of metastatic localizations and visceral metastasis. Overall survival (OS) and Progression free survival (PFS) in obese patients did not achieve significant differences, while in underweight patients presented lower OS and PFS. In triple negative cases, obesity appeared to promote a protective role.	73
	2021	Worldwide	Evaluation of BC risk of childhood obesity through the identification of 15 single-nucleotide polymorphisms (SNPs) from the Genome-Wide Association Studies, Early Growth Genetics (n= 15 249 cases vs 416 252 controls)	Childhood BMI was negatively associated with the risk of breast cancer (OR: 0.77; 95% CI: 0.64-0.94; $P < 0.01$)	74
	2021	Korea	Study of BMI and breast density in BC including 3 248 941 cancer-free premenopausal women (34 466 Cases of breast cancer detected) and 4 373 473 cancer-free postmenopausal women (30 816 Cases of breast cancer detected) in the Korean National Cancer Screening Program/National Health Insurance Service database.	NOTE: BMI was stratified according: underweight (<18.5), normal weight (18.5 to <23.0), overweight (23.0 to <27.5), and obese (>27.5). In premenopausal women, no association was found between BMI alone and breast cancer risk. Postmenopausal women revealed a significant increased breast cancer risk in all breast density categories. Results showed an additive interaction between BMI and breast density both in post and premenopausal women in the risk of BC.	75

2020	Latin America	Evaluation of anthropometry measures and body shape of women diagnosed with primary invasive breast cancer from the PRECAMA cohort (Chile, Colombia, Costa Rica, Mexico, and Brazil) (n=406 cases of premenopausal BC vs 406 matched controls selected from the general population).	In the premenopausal population was found a negative association between breast cancer risk and weight, waist circumference (WC), hip circumference (HC), and BMI. Normal weight women with central obesity presented increased BC risk than normal weight women with normal waist circumference. No association was found for height, leg length, Waist Hip Ratio (WHR), and body shape.	[76]
2019	United States	Study of the relation between BC risk and body weight alterations and BMI in postmenopausal women enrolled in the Women's Health Initiative Observational Study (n=61 335).	Postmenopausal women with intentional weight loss were more likely to presented higher BMI. Compared to women with stable weight, women with weight loss of >5% have decreased risk of invasive breast cancer. No associations were found regarding weight gain and BC risk.	[77]
2019	Japan	Assessment of BC risk and weight alterations in postmenopausal breast cancer enrolled in the Miyagi Cohort Study (17 years follow-up) and the Ohsaki Cohort Study (13 years follow up) (n=286 patients).	In women, moderate and high weight gain was significantly associated with an increased risk of postmenopausal BC.	[78]
2019	France	Assessment of obesity survival paradox in elderly women enrolled in the Physical Frailty in Elderly Cancer patients (n=433 cancer patients eligible, 79 with BC).	NOTE: BMI was stratified according: BMI<21 (Underweight), 21.0≤BMI≤24.9 (normal weight), 25.0≤BMI≤ 29.9(overweight) and BMI≥30 (obesity). Comorbidities, assessed by the Cumulative Illness Rating Scale-Geriatric (CIRS-G), and gait disorders were positively associated with obesity in BC patients. BC seems to exhibit obesity-survival paradox in elderly patients (negatively correlated with a 6-month mortality).	[79]
2019	United States	Postmenopausal women (n=58 667 eligible, 3355 invasive breast cancers) from the Women's Health Initiative Observational Study were evaluated for weight loss and cancer risk.	BC risk decreases with weight loss, regardless of the personal intentionality.	[80]
2019	India	Observational study of screened women in the National Institute of Cancer Prevention and Research under the Indian Council of Medical Research (n=6 712 women; 53 cases diagnosed).	Postmenopausal women with high blood pressure and random blood sugar were strongly associated with BC. No significant association was found between blood pressure and overweight/obesity. Both in pre and postmenopausal women, no association was found between BC and overweight and obesity.	[81]

2018	United States	Study regarding adiposity and body composition of women with nonmetastatic breast cancer diagnosed in the Kaiser Permanente Northern California and the Dana Farber Cancer Institute (n=3 241).	BMI was differentially associated with body composition. Sarcopenia feature was associated with low BMI while low skeletal muscle radiodensity (SMD) with higher BMI. Lower overall survival was found in patients with medium or high total adiposity, sarcopenia, and low SMD.	82
2017	United States	Evaluation of BC risk and weight fluctuations from the postmenopausal women of the Women's Health Initiative Observational Study (n=80 943; 5 564 women diagnosed with BC).	BMI independently analysis revealed that weight gain and weight cycling, were associated with BC risk. After BMI adjustment at baseline, weight gain remained significantly associated with BC risk.	83
2017	United States	Study on adiposity and weight alterations in childhood and late adolescence in women enrolled in the Nurses' Health Study (n=74 177; 4 965 diagnosed with invasive breast cancer).	Weight maintenance avoids BC risk increased. In hormone receptor positive cancers, adiposity in childhood and at age 18 has a protective role. Adverse effects of weight gain in postmenopausal adulthood women are more evident in hormone receptor positive cancers.	84
2016	Worldwide	Randomized study from data of 7 SNPs of birth weight, 15 SNPs of childhood BMI, 77 SNPs of adult BMI, and 14 SNPs of adult WHR included in the Genome-Wide Association Studies (n=51 537 cancer cases and 61 600 control from which 15 748/18 084 case/controls of BC).	BC risk, namely ER - cancer, was negatively correlated with childhood and adult BMI. Negative association was found between BC risk and WHR. No association found between the genetic score in BC risk and birth weight.	85
2016	Denmark	Mendelian randomisation study from genetic information of patients enrolled in the Copenhagen General Population study and the Copenhagen City Heart Study (n=1402).	Genetic studies unravelled that high number of alleles BMI associated (7–10 versus 0–4) increased BMI in 3% (approximately 0.20 kg/m^2). Allele score explained 0.4 % of BMI variation. High BMI increased the risk of postmenopausal breast cancer. No association was found between BMI and premenopausal breast cancer.	86
2016	United States	Study on the impact of adulthood overweight and obesity duration on BC risk from the observational study of the Women's Health Initiative (n=73 913 total, 3 723 postmenopausal breast cancer).	Adjusted models revealed an increased hazard ratio (HR) for BC (1.07) for every 10 years of obesity and for every 10 years of overweight (1.05). A positive correlation was found between the years of obesity and overweight and postmenopausal breast cancer risk.	87
2015	United States	Assessment of body shape index (ABSI), BMI, WC, waist circumference-to-height ratio (WHtR), and WHR from the Women's Health Initiative (n=143 901 women, 7 039 invasive postmenopausal BC).	BMI, WC, WHtR, and WHR were positively correlated with BC risk. ABSI was not associated with increased risk of BC. BMI and WC were significant predictors of BC, even in the adjusted model. ABSI was a significant predictor when compared to BMI.	88

CLINICAL FEATURES AND OUCOMES	2022	China	Study on body fat mass, visceral fat level, and skeletal muscle mass of women with BC diagnosed in the First Hospital of Jilin University (n=199).	Visceral fat was proportional higher in postmenopausal women. High visceral fat presented higher body fat mass, higher skeletal muscle mass, increased levels of minerals, proteins, triglycerides, cholesterol, and low-density lipoprotein (LDL). Normal visceral fat was associated with a more prolonged DFS.	89
	2021	Germany	Assessment of OS and PFS of BMI and medication of women diagnosed with invasive BC in the Department of Gynaecology and Obstetrics of the University Hospital Essen (n=635).	BMI was correlated with menopausal status, age, smoking, use of beta-blockers, and metformin (frequent in obese patients). Non obese patients tend to have prolonged PFS (without statistical significance). Obese beta-blockers user patients were correlated with decreased OS and PFS. No survival advantage was observed in metformin intake, regardless of BMI.	90
	2020	Italy	Nonrandomized retrospective study of HER2-Positive early BC Patients from 2 Italian University Hospitals (Università Politecnica delle Marche-Ospedali Riuniti, Ancona and Azienda Ospedaliera Universitaria Pisana, Pisa) (n=279)	Significant statistical associations were found in menopausal status, tumour size, and HR status. Obese and overweight women were more likely to be postmenopausal, and more likely to display larger tumours. No significant differences were achieved in lymph nodal status, grade, proliferation score, and adjuvant chemotherapy. Underweighted and normal weight patients have better prognosis in a 3-years follow-up, with decrease recurrence rates.	91
	2020	Spain	Observational study of the influence of trastuzumab dosage in HER2-positive nonmetastatic BC in the Complejo Hospitalario Universitario de Canarias (n=50).	Fixed dose of subcutaneous Trastuzumab revealed significant differences in the obese patients.	92
	2020	United States	Study on breast reconstruction outcomes in women submitted to breast reconstruction in 11 participating sites (n=2 259).	Obese women had increased risk of surgery associated complications and reconstructive failures. Obese women exhibited decrease satisfaction in the implant results.	93
	2020	Italy	Observation study of a patient cohort treated with pertuzumab and/or trastuzumab emtansine for HER2+ metastatic BC at 45 Italian cancer centres (n=709).	Obesity was associated with worse OS in patients treated with pertuzumab and/or trastuzumab emtansine for HER2+ metastatic BC but had no impact in PFS to first line. Evaluation of quartiles of the patients and PFS to first line revealed worse prognosis for obese patients in the first quartile.	94
	2020	United States	Assessment of patients with nonmetastatic BC who received anthracycline and taxane-based chemotherapy at Kaiser Permanente Northern California and correlation with body composition (n=1 329).	Chemotherapy treatment is influenced by obesity, higher levels of visceral and intramuscular adiposity is associated with decreased chemotherapy delivery. Adiposity (visceral, intramuscular, and subcutaneous) was associated with increased risk of discontinuing chemotherapy at early time.	95

			Visceral and intramuscular adiposity resulted in an increased risk of hospitalization during chemotherapy treatment. Higher muscle mass presented decreased risk of hematologic toxic effects.	
2019	France	Prospective study regarding the association between cardiotoxicity and BMI in early BC included in the French national multicentre prospective CANTO study of 26 French cancer centres (n=929 patients).	Obesity was associated with hypertension and diabetes. Obese and overweight patients were more likely to develop cardiotoxicity. Multivariable analysis revealed that obese patients and trastuzumab were independently associated with cardiotoxicity.	96
2019	Indonesia	Observational study on premenopausal and postmenopausal women enrolled in the oncology surgical clinic at Dr. Moewardi hospital, Surakarta (n=117).	Obesity was negatively associated with ER. Obesity and history of contraceptive method tended to be negatively associated with PR and positively associated with HER2+.	97
2018	India	Observational study on BC patients clinically followed at a tertiary cancer centre (n=476).	Postmenopausal women had higher BMI. The mean BMI was higher in luminal BC subtype (24.7), followed by Triple negative (TN) BC (23.9), and lastly by HER2+ BC (22.4). Tumour size increases with BMI. Underweight and normal presented a median tumour size of 3.99 cm; overweight, 4.13cm and obese 4.30cm (although without statistical significance).	98
2016	Turkey	Assessment of the cardiotoxicity trastuzumab in women diagnosed in Cukurova University Medical School Adana with HER2+ BC (n=111).	Obesity specifically, was significantly associated with a decrease of the left ventricular ejection fraction, a heart failure manifestation.	99
2015	United Kingdom	A prospective observational study on BC patients from 127 UK hospitals (POSH study) (n=2 843).	Tumour size was larger in obese and overweight patients. Obese and overweight patients presented more histological grade 3 cases, and lymph node positive tumours. ER negative and TN tumours were more frequent in obese patients. No association was found regarding distribution of cancer, distant metastasis, and HER2. Conservative surgery and neoadjuvant chemotherapy were higher in obese patients. Distant DFS and OS was lower in obese patients.	100
2015	Iran	Case-control study on Post-Mastectomy Pain Syndrome (PMPS) in women who underwent mastectomy surgery in Shohada Hospital (n=122).	No correlation was found for weight, height and BMI between the group who reported PMPS and the group without PMPS.	101

	2013	China	Retrospective study of women with BC, submitted to surgery, clinically followed at the General Hospital of Shenyang Military Region (n=317).	No associations were found for BMI and age, histological type, tumour size, ER/PR status, and menopausal status. BMI was associated with lymph node metastasis and smoking habits. Univariate analysis for 5-year DFS and OS revealed that BMI, tumour size, lymph node metastasis, and adjuvant endocrine therapy were statistical prognostic factors. Cox regression model achieved statistical significance for obesity as an independent prognostic factor for DFS and OS.	102
BIOMARKERS	2022	United States	BC screened participants of the National Health and Nutrition Examination Survey (n=11 460; 306 cases of BC) were correlated with triglyceride glucose (TyG) index with BC cancer risk was performed.	NOTE: TyG index= Ln [fasting triglycerides (mg/dL) X fasting glucose (mg/dL)/2]. TyG index was positively correlated with age, BMI, smoking, LDL-C, triglycerides, and fasting glucose. High levels of TyG index were associated with high prevalence rates of BC. Q4 TyG index (9 and 11.96) reveal an OR for BC of 2.25.	103
	2022	United States	Case-control study of BC cases included in the Women's Health Initiative Observational Study. Evaluation of C-reactive protein (CRP), leptin, fasting insulin, and estradiol levels (n=188).	Obese individuals are more likely to have higher levels of leptin, CRP, and fasting insulin compared to controls. Leptin, CRP, fasting insulin, and estradiol reveal a positive association with BMI (obese and overweight vs normal weight).	104
	2021	Indonesia	Cross sectional observational study correlating BMI, histopathological grading, and total cholesterol levels in BC patients followed in the Faculty of Medicine, Hasanuddin University (n=60).	No correlation was found between BMI and histological grade. Association was found between postmenopausal status and histopathological grade and histopathological grade and cholesterol levels.	105
	2021	United States	Case-control study in postmenopausal women included in the Women's Health Initiative Observational Study, associating BMI, WHR, height, BMI with androgens, twelve androgens metabolites by linear regression, and adjusted for confounder variables and stratified by hormone therapy (HT) (n=1 765).	Obese patients (whether former or current HT patients) presented higher levels of dehydroepiandrosterone sulphate (DHEAS), 5α-reduced glucuronide metabolites: androsterone-glucuronide (ADT-G), 5α-androstane-3α, 17β diol-3-glucuronide (3α-diol-3G), 3αdiol-17-glucuronide (3α-diol-17G), and DHEAS:DHEA ratio. Higher BMI levels were positively correlated with unconjugated estrone: androstenedione and unconjugated estradiol: testosterone ratios among never/former HT patients. Never/former HT patients, BMI was positively associated with estrogen-to-androgen ratios, and the proportion of total adrenal androgens and parent estrogens was lower in obese women. When stratified by BMI, never/former HT patients, WHR was positively associated with DHEA, DHEAS, androstenedione, DHTS, ADT-G, 3a-diol-3G,3a-diol-17G, and DHTS:DHT ratio in obese women.	106

			BMI at age 18 was inversely associated with adrenal androgens (DHEA, DHEAS, androstenedione, testosterone) and positively associated with estrogen-to-androgen ratios (unconjugated estrone: androstenedione, unconjugated estradiol: testosterone) among never/former HT users. Association loses statistical significance when adjusted to current BMI, except for unconjugated estrone: androstenedione in current HT users.	
2021	United States	A study from the Women's Health Initiative Database for Genotypes and Phenotypes Study evaluated a genome-wide association (GWA) gene–environment interaction (G × E) in 6 GWA for proinflammatory cytokines (n=16 088 postmenopausal women and 21 784 812 SNPs).	Women wo developed BC are more likely to be obese and have visceral obesity. In nonobese women, HNF1A-AS, HNF1A (rs2243458) and one C12orf43 (rs1169311) detected was associated with 20% decrease in chronic inflammation. In obese women, 3 SNPs in CRP/RP11–419N10.5 gene was responsible for a 70% of increased risk for chronic inflammation. In viscerally obese subgroup (WHR and Waist circumference) inflammatory patterns revealed SNPs near CRP and CRP/RP11–419N10.5 associated with 35%–50% of increased risk and SNPs near HNF1A presented decreased risk for inflammation. Biomarkers CRP and IL6 interact with obesity factors.	[107]
2021	United States	Women who underwent mastectomy for BC risk clinically followed in the Memorial Sloan Kettering Cancer Centre were evaluated for their adiposity features and BC risk (n=100).	Presence of breast adipose tissue infiltration (B-WTAi) was not correlated with WHR but was correlated with increased age, higher BMI, larger adipocytes, higher total body fat, and trunk fat. Aromatase was positively associated with BMI, B-WATi, total and trunk fat, and breast adipocyte diameter. Increasing levels of hsCRP, IL6, leptin, and insulin were increased among BMI categories and breast adipocyte diameter. Adiponectin and sex hormone-binding globulin (SHBD) were inversible related to BMI. Higher body fat was related to B-WATi, despite normal weight.	[108]
2021	Canada	Women with early BC from the Mount Sinai Hospital were characterized and evaluated for metabolic factors, BMI, and inflammation (n=119).	Crown-like structures of the breast (CLS-B) was associated with higher weight, BMI; WC, insulin, HOMA, and leptin but not with hsCRP, adiponectin, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL), cholesterol or triglycerides.	[109]
2019	United States	Evaluation between body fat and BC in postmenopausal women with normal BMI enrolled in the Women's Health Initiative (n=3 460).	BMI and WC were higher in women with high levels of trunk fat mass. Whole body fat mass (5-unit increase) was translated in a significant 28% increase in BC risk. A 5-unit increase in percentage of whole-body fat, increased BC in 19%. A 5-unit increase in trunk fat increased the risk in 46%.	[110]

			The adjusted model observed positive associations between levels of insulin, CRP, insulin resistance, IL6, white blood cells, leptin, triglycerides, and trunk fat mass. Whereas contrary association were observed for levels of HDL-C, adiponectin, and SHBG.	
2018	Italy	Observational study cross sectioning circulating testosterone and BMI in ER+ BC postmenopausal patients recruited in the Fondazione IRCSS Instituto Nazionale Tumori of Milan (n=460).	Increased testosterone levels were observed across BMI categories. Testosterone and BMI were positively associated with DFS. It was found a significant interaction between testosterone and BMI. In normal-weight women with median levels of testosterone, relapse hazard ration (HR) was increased. In overweight patients with increased testosterone levels had higher HR. In obese patients, HR decreased (not significantly) at increased testosterone concentrations. In these cases, high levels of testosterone in normal weight and overweight were associated with worse prognosis.	[111]
2018	China	Retrospective study of cases recruited from the Comprehensive Breast Health Center, Shanghai Ruijin Hospital and evaluation of obesity, metabolic syndrome features, and Neutrophil-to-lymphocyte ratio (NLR) (n=1 540 BC cases and 1 540 with benign breast disease (BBD)).	Obesity was more frequent in BC. BC also revealed higher neutrophil levels, lower lymphocyte levels, and consequent higher NLR. Impact of intermediate to high NLR levels with increased BC risk was more evident in normal weight patients or in the population without metabolic syndrome.	[112]
2018	Mexico	Association of resistin and two SNPs (RETN rs1862513CG/GG and CAP1 rs35749351GA/AA) with BC risk (n=308 controls vs 100 BC cases).	Resistin levels in BC patients were higher compared to controls. RETN rs1862513CG/GG and CAP1 rs35749351GA/AA genotypes presented an increased odd ratio (OR) of 1.61 and 2.193-fold to BC risk, respectively, compared with the CC and GG genotypes.	[113]
2017	Germany	Correlation of BRCA gene mutation carriers of patients recruited from the German Consortium of Hereditary Breast and Ovarian Cancer and association with adiposity (n=68).	BRCA mutation carriers tended to have lower BMI, whereas non-carriers revealed higher physical activity level.	[114]
2017	Turkey	Chemerin assessment in newly BC diagnosed patients (n=117).	Chemerin levels were associated with diabetes, older age, obesity, hypertension, and postmenopausal status.	[115]
2017	Italy	Patients with BC treated at University of Naples Federico II, was retrospectively assessed for vitamin D, tumour characteristics, and adiposity features (n=220).	Low levels of Vitamin D were correlated with worse prognostic factors like grade 3, lymph node positive, and higher BMI categories. BMI was correlated with postmenopausal status.	[116]
2017	United States	Data from postmenopausal women enrolled in Women's Health Initiative Observation, stratified by obesity and lifestyle modifiers, were analysed according	Seven SNPs in IGFI and IR genes were associated with BC risk and differed between non-obese and obese group, and also between oral contraception non-users and users.	[117]

		75 SNPs in genes related to insulin-like growth factor-I (IGFI), insulin resistance (IR), and signalling pathways (n=464 controls vs 539 BC cases).	Carriers of IGFIrs10860865 T and the IGFIrs978458 T mutations have increased BC risk among the non-obese patients. Carriers of the INS rs689 T allele had decreased BC risk in non-obese women. In PIK3R1 gene, carriers of 4 SNPs (rs12657050 T, rs171649 A, rs831123 G, and rs7707370 C alleles) presented increased risk of BC in obese women, and another 4 (rs706711 A, rs1664577 G, rs3730089 A, and rs251404 T alleles) in obese patients with WHR>0.85. Increased BC risk was also found in mutations carriers of the AKT1 rs2494744 T allele in non-obese women. Carriers of the AKT1 rs2494740 T SND revealed decreased risk for BC in viscerally obese (waist>88 cm). Also, carriers of rs2266966 G, rs2283791 C, and rs9610505 A alleles decreased BC risk in obese women with WHR >0.85. Carriers of rs2298432 T allele had decreased cancer risk in non-obese women with WHR≤0.85.	
2015	United States	Case-cohort analysis of circulating adipokines and inflammatory markers data, from Women's Health Initiative Observational Study (n= 875 BC cases vs 839 controls).	In HT nonusers, BMI displayed a moderate correlation with leptin, insulin, CRP, and IL-6. A moderate inverse association was found for insulin and adiponectin. The β-coefficient for obese women decreased 16%, 52%, 53%, 92%, and 115% after adjustment for estradiol, insulin, CRP, both insulin and CRP, or all three factors (estradiol, insulin, and CRP. High levels of CRP increased the risk of postmenopausal BC in women, HT nonuser. This association is independent of obesity-related pathways like hyperinsulinemia and estrogen. CRP, insulin, and estradiol seems to be a potential justification of the BMI and BC link.	[118]
2015	United States	Case-cohort study of BC risk and metabolically healthy overweight postmenopausal women based in HOMA-IR and in insulin sensitivity. Data from women enrolled in the Women's Health Initiative Observational Study (n= 497 BC cases and 2 830 participants).	Unhealthy overweight (HOMA-IR) was associated with increased BC risk. No association was observed in metabolically healthy overweight women and BC risk. Regardless of BMI, it was observed a significant association between BC risk and metabolic health.	[119]
2014	Brazil	Observational study of ER α SNPs, PvuII and XbaI in patients stratified according to BMI. Data was collected from the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (n=308 women postmenopausal).	Results revealed that PvuII mutation is associated with low BMI categories, while XbaI mutation is associated with higher BMI.	[120]

2014	Italy	Post-menopausal oestrogen-dependent breast cancer patients recruited from the Medical Oncology Units at the 'N.S. Bonaria' Hospital in San Gavino were analysed for tumour characteristics, BMI, proinflammatory cytokines, and oxidative stress biomarkers between ER + and ER - (n=216).	ER + patients presented higher BMI and increased leptin levels in patients with higher values for Tumour size (T), Nodal infiltration (N) and Metastasis (M)+ status. BMI was correlated with leptin. Both BMI and leptin were correlated with IL6 in ER + group. In ER + patients, Reactive oxygen species (ROS) levels were positively correlated with BMI, leptin and IL-6 and negatively correlated with Glutathione peroxidase (GPx), and Superoxide dismutase (SOD). ROS was also found to be correlated with TNM status. BMI, leptin, and IL-6 were associated with T and M+. Multivariate regression analysis revealed that in ER + patients, BMI, leptin, IL6, and ROS were predictive for TNM status.	121

Legend: **BC** – Breast cancer; **BMI** – Body Mass Index; **SNPs** – Single Nucleotide Polymorphisms; **ABSI** – Body Shape Index; **WHtR** – Waist Circumference-to-height Ratio; **WHR** – Waist Hip Ratio; **WC** – Waist circumference; **HC** – Hip Circumference; **SMD** – Skeletal Muscle Radiodensity; **HR** – Hormone Receptor; **ER** – Estrogen Receptor; **PR** – Progesterone Receptor; **HER2** – Human epidermal growth factor receptor-2; **TN** – Triple Negative; **OS** – Overall survival; **DFS** – Disease free survival; **PFS** – Progression Free Survival; **LDL** – Low-density lipoprotein; **PMPS** – Post-Mastectomy Pain Syndrome; **TyG** – Triglyceride glucose index; **CRP** – C-reactive protein; **HT** – Hormone therapy; **DHEAS** – Dehydroepiandrosterone sulphate; **ADT-G** – Androsterone-glucuronide; **3α-diol-3G** – 5α-androstane-3α, 17β diol-3-glucuronide; **3α-diol-17G** – 3αdiol-17-glucuronide; **B-WATi** – Breast adipose tissue infiltration; **hsCRP** – High sensitivity C-reactive protein; **SHBG** - Sex hormone-binding globulin; **CLS-B** – Crown-like structures of the breast; **HOMA** - Homeostatic model assessment of insulin resistance; **HDL** – High-density lipoprotein; **HR** – Hazard ratio; **NLR** – Neutrophil-to-lymphocyte ratio; **BBD** – Benign Breast Disease; **IGF1** – Insulin-like growth factor 1; **IR** – Insulin resistance; **PIK3R1** – Phosphatidylinositol 3-kinase regulatory subunit alpha; **AKT1** – AKT Serine/Threonine Kinase 1; **T** – Tumour size; **N** – Nodal infiltration; **M** – Metastasis; **ROS** – Reactive oxygen species; **GPx** - Glutathione peroxidase; **SOD** - Superoxide dismutase.

In summary, observational studies focusing on obesity as a risk factor unravel some contradictory results. In the majority of the included studies, BMI/obesity is positively associated with postmenopausal BC and negatively or without association with premenopausal BC. Some studies seem to support the influence of the obesity paradox namely in childhood/adolescence obesity, elderly populations, and metastatic BC. An interesting observation is that underweight and sarcopenic populations are usually associated with poorer prognosis. In the present literature review, anthropomorphic BMI-associated features were also included to analyse their influence as BC risk factors. Body Shape Index, Waist Circumference-to-height Ratio, Waist Hip Ratio, Waist circumference or Hip Circumference are obesity features that could help in establishing BC prognosis, although results did not achieve any consensual conclusion. Additionally, this systematic review included several studies in weight alterations, curiously based in the same cohort (the Women's Health Initiative). Weight loss is associated with decrease BC risk, and consequently weight gain and weight cycling are associated with increased BC risk. Moreover, duration of obesity and overweight exposure also seems to promote BC development.

In clinical features and outcomes, worse indicators in BC like tumour size, differentiation grade or metastasis are usually correlated with overweight and obesity. Some contradictory data is found regarding hormone receptors. Moreover, it is commonly found (not always with statistical significance) that overall and disease-free survival is lower in obese patients. BMI is usually correlated with the postmenopausal status of the patient. Obesity is a critical influencer in the treatment strategy. High BMI has a significant impact on chemotherapy delivery and in surgical options and outcomes. Obese patients are prone to have more complications after surgery and reconstructive failures.

Regarding biomarkers, the Women's Health Initiative Observational Study (WHI) was the database used for several studies, which could represent a significant bias in the results of this systematic review. In the included studies, biomarkers were mainly associated with adipokines, proinflammatory cytokines, and hormones. One study included oxidative stress biomarkers. As expected, there was an association between obese BC patients and adipokines such as leptin, resistin, chemerin. As for proinflammatory cytokines, obese women with BC presented higher levels of CRP, IL6, neutrophil-to-lymphocyte ratio usually associated with worse clinical features. Hormones and oxidative stress are also players in this crosstalk between obesity and BC. Discussion of biomarkers associating obesity to BC are further portrayed in the next subsection.

1.4. The obese tumour metabolism and the case of breast cancer

The complex network that links obesity and cancer include a set of diverse abnormalities consisting in an altered adipokine profile, hormone oscillations, low chronic state of inflammation, altered microbiota, extracellular matrix (ECM) remodulation of adipose tissue and metabolism dysregulation[122], as illustrated in figure 9.

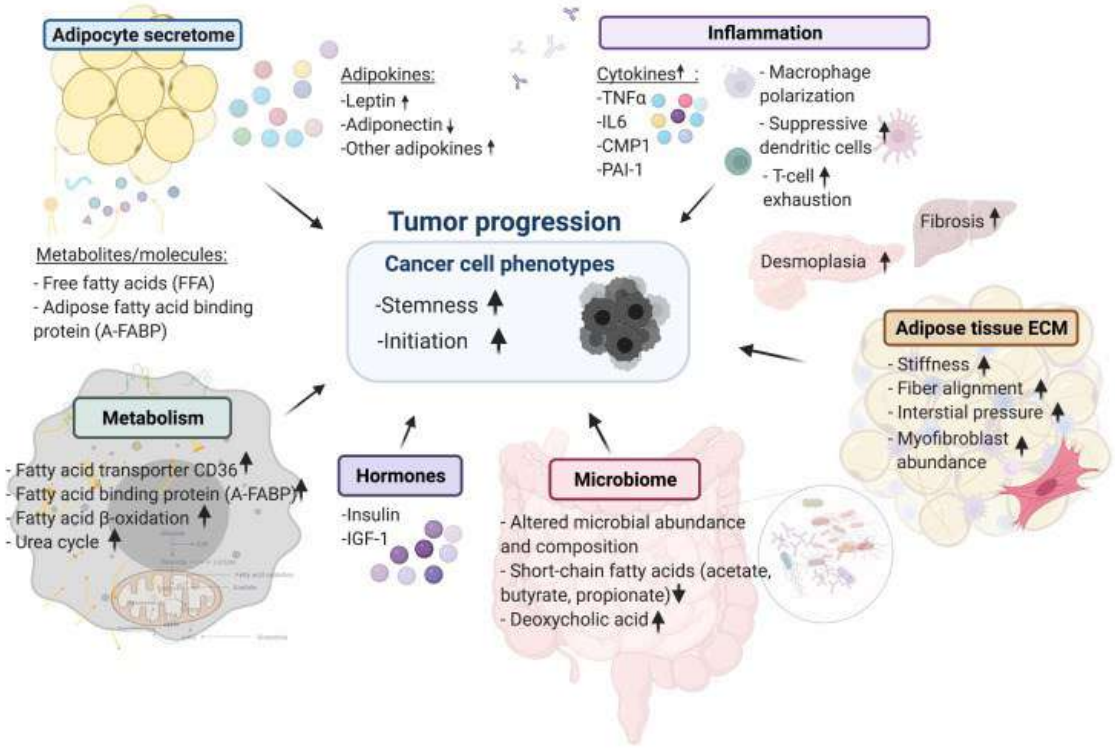


Figure 9 – Relation between obesity related characteristics and tumour initiation and progression (source: Liu, Xiao-Zheng, Line Pedersen, and Nils Halberg. "Cellular mechanisms linking cancers to obesity." Cell Stress 5.5 (2021): 55)[123].

Inflammation is critical, for it could present itself as a "hit event" in tumorigenesis and is also able to modulate tumour microenvironment by promoting a proinflammatory state[124]. Immune cells secrete several cytokines and chemokines like IL-1, IL-6, and VEGF, described to be associated with tumour growth, angiogenesis, and metastasis[125]. Extracellular matrix (ECM) components of adipose tissue remodulate stiffness and interstitial pressure enabling metastasis[126]. Microbiota is also a described player in carcinogenesis, namely in the response to therapy and immunity. Curiously, breast was until recently considered a sterile organ, but it is now known to harbour low-biomass microbial populations[127]. Obesity increases estrogen levels through aromatase activity and stimulate estrogen receptor-positive breast tumour cells[128]. Moreover, obesity can be responsible for inducing oxidative stress by increasing lipid peroxidation and reactive oxygen species (ROS). ROS accumulation may cause cellular dysfunction, altered cell signalling, genetic mutations, immune system activation and inflammation[129]. Although out of the scope of this work, it is relevant to mention that several other mechanisms, pathways, and players besides *adipofactors* have a direct or indirect modulation in obese tumour metabolism. Just to name a few, tumour microenvironment status, genetic predisposition, and epigenetic modification can also be modulated by obesity.

Each of these linking features is associated with an intricate set of molecules, which act and interplay, leading to a tumour promoting environment. Figure 10 created by Harris and colleagues presents a distinctive perspective linking *adipofactors* and the hallmarks

of cancer[130] where it is possible to observe the complex network of associations between both pathologies[131].

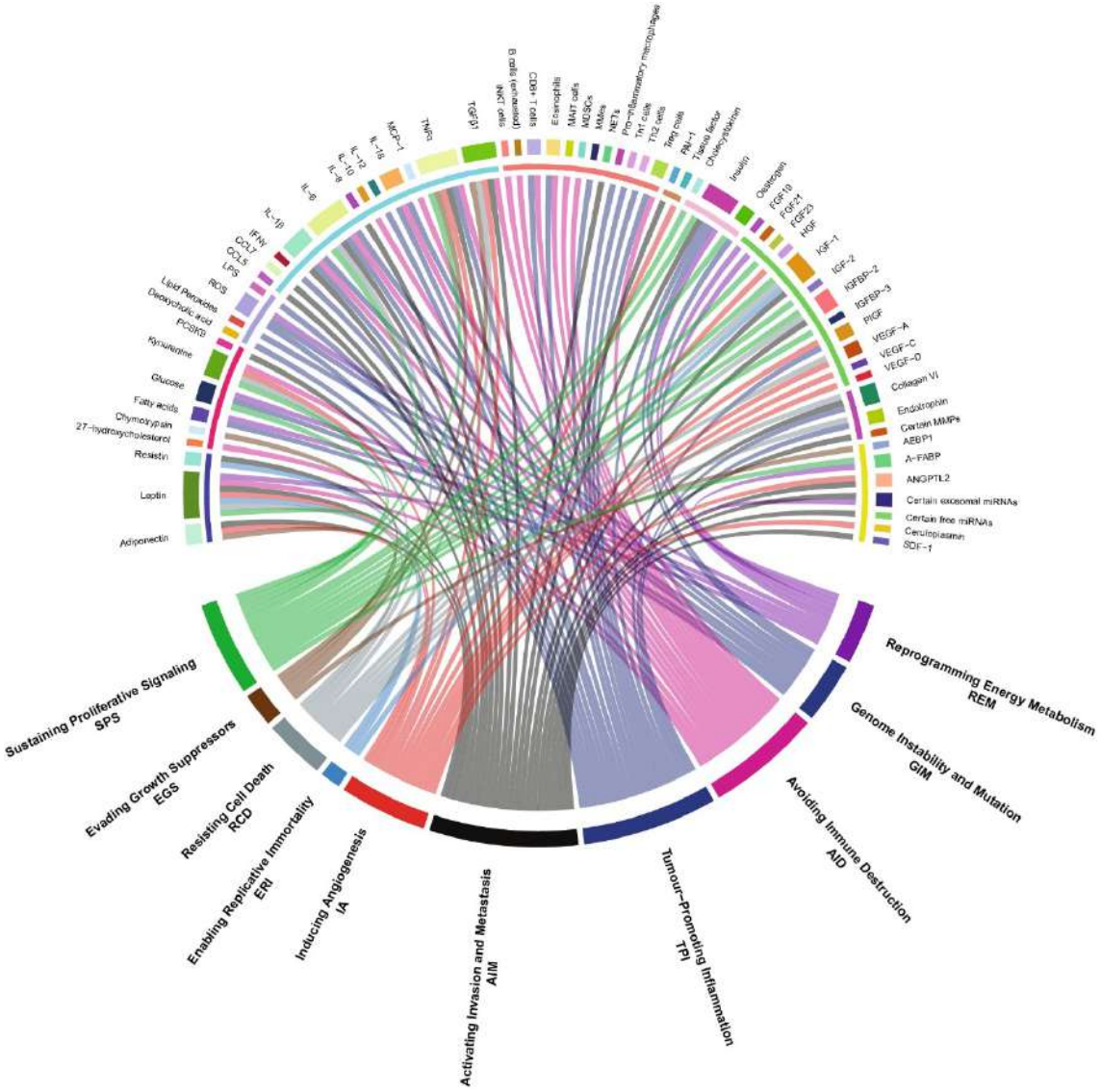


Figure 10 – Network relation between obesity-related factors and the hallmarks of cancer (source: HARRIS, Benjamin HL, et al. Obesity: A perfect storm for carcinogenesis. Cancer and Metastasis Reviews, 2022, 1-25)[131].

Activating invasion and metastasis, *tumour-promoting inflammation*, and *avoiding immune destruction* are the hallmarks that are stronger associated with obesity-related factors, which could potentially explain the worse prognosis found in obese cancer patients. As on the other side, leptin, Interleukin-6 (IL-6), Tumour necrosis factor α (TNFα), Transforming growth factor β (TGFβ1), and insulin are the obesity-related factors with association with more hallmarks. Interestingly, Leptin and IL-6 were already the focus of several observational studies as potential biomarkers in BC prognosis, as presented in the previous subsection. Regarding the aim of our study, *reprogramming energy metabolism* is associated with leptin, fatty acids, glucose, reactive oxygen species (ROS),

insulin, fibroblast growth factor 19 (FGF19), fibroblast growth factor 21 (FGF21), fatty acid binding protein A (A-FABP), and certain free miRNAs.

Reprogramming energy metabolism is an *emerging* hallmark of cancer, introduced after the first publication of Hanahan[132]. The tumour cell is known by their plasticity, namely the ability to use several energetic pathways. Such feature is an evolutionary response to their energetic and biomass demands, consequently acquiring survival advantage over normal cells. The Warburg effect, described nearly one century ago, is the upmost accepted metabolic reprogram described in tumour cells[133], although with several deviations published since[4,134]. It is well stablished that tumour cells preferably undergo aerobic glycolysis instead of oxidative phosphorylation. As so, increased glucose levels found in obesity is one described correlation between cancer-*adipofactors* in figure 10. Moreover, mitochondrial respiration is also supressed by leptin[135], and insulin stimulates glucose oxidation in obesity-associated cancers[136]. Another tumour mediator is hypoxia. Increased fat deposition enlarges the distance between tissue and vessels leading to Hypoxia-Inducible Factor 1α (HIF1α) release. Another hypoxic mechanism that reduces vascularization is the hypertrophy and hyperplasia of white adipocytes[137]. HIF1α then acts as a transcription factor in promoting a glycolytic phenotype[138]. Fundamentally, adipose factors are able to "feed" and regulate several pathways through autocrine and paracrine mechanisms. Figure 11 identifies the most pertinent factors released by the adipose tissue and describes relevant and specific actions. Many of these *adipofactors* are intrinsically associated with the central biochemical pathways, namely in glucose metabolism.

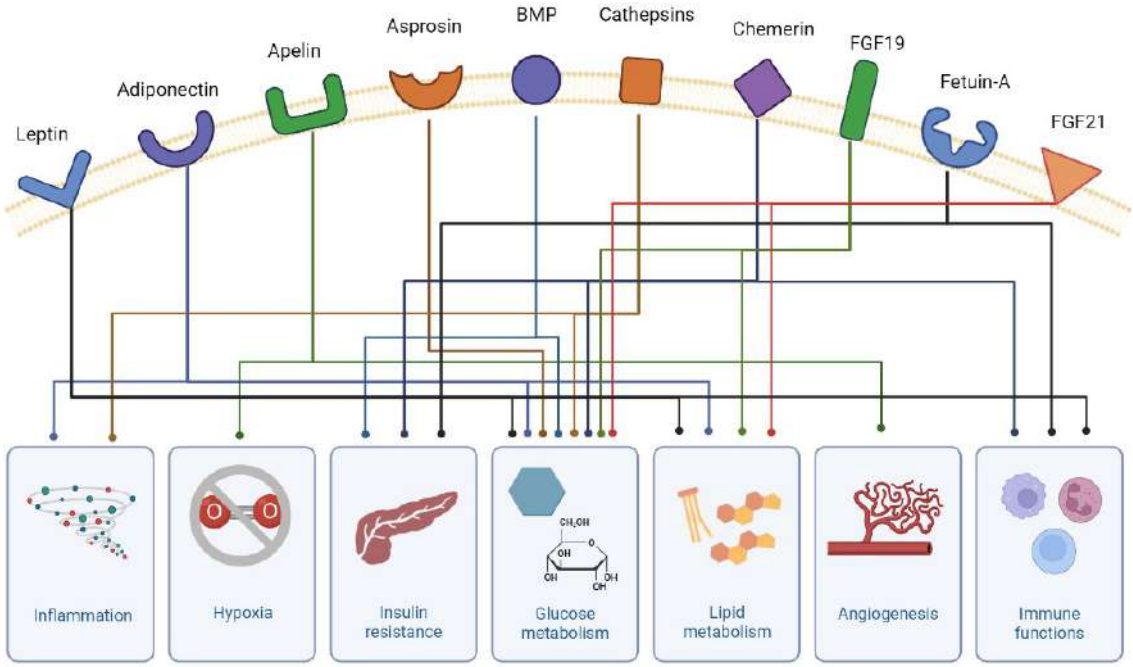


Figure 11 – Metabolic associations of *adipofactors* and their associations with different pathways (Created in *Biorender.com* with resource to information available in Rana, Mariam N., and Ian J. Neeland. "Adipose tissue inflammation and cardiovascular disease: an update." Current Diabetes Reports 22.1 (2022): 27-37. [139])

In the case of breast cancer, the influence of obesity in metabolism is of great importance. Figure 12 presents the major metabolic pathways altered in breast cancer. We will further focus on the following main mechanisms: (A) Amino acid metabolism; (B) Tricarboxylic acid cycle – TCA cycle; (C) Lipid metabolism – lipogenesis and lipolysis; (D) Carbohydrate metabolism – Pentose Phosphate Pathway – PPP; and lastly (E) Carbohydrate metabolism – glycolysis and gluconeogenesis.

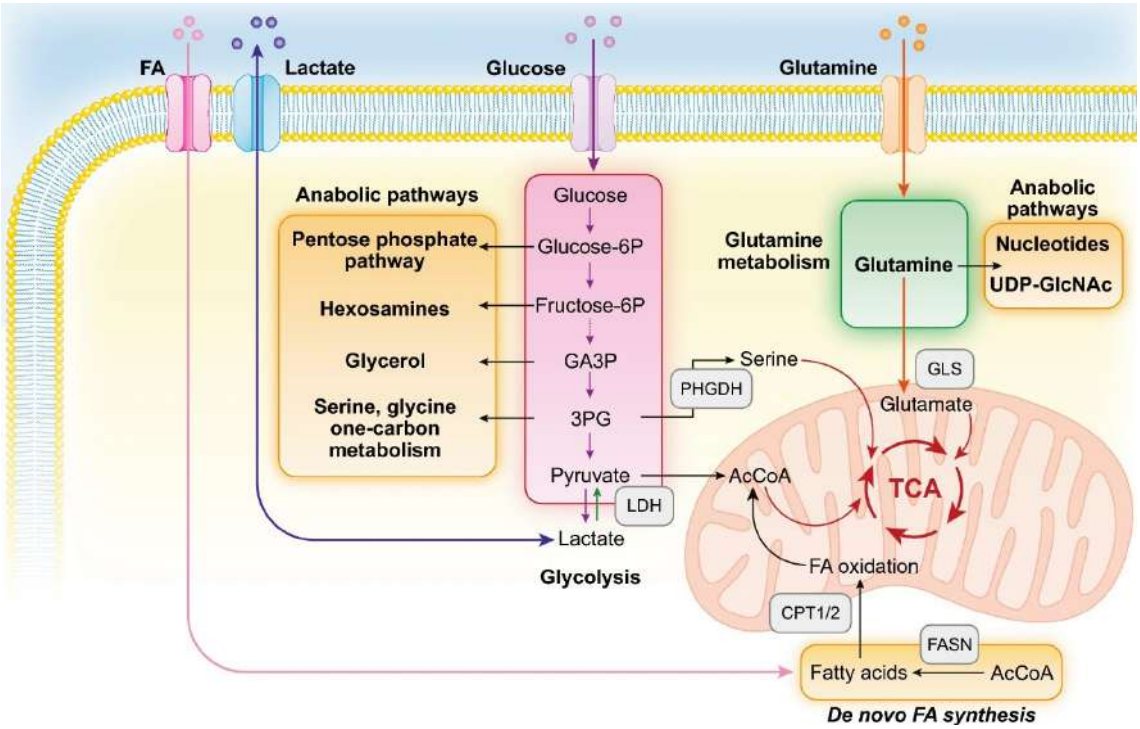


Figure 12 – Metabolic pathways altered in breast cancer (Source: Martino, F., Lupi, M., Giraudo, E. et al. Breast cancers as ecosystems: a metabolic perspective. Cell. Mol. Life Sci. 80, 244 (2023). https://doi.org/10.1007/s00018-023-04902-9)[140] Legend: FA – fatty acid; GA3P – glyceraldehyde 3-phosphate; 3PG – 3-phosphoglycerate; LDH – lactate dehydrogenase; PHGDH – phosphoglycerate dehydrogenase; AcCoA – acetyl coenzyme A; CPT1/2 – carnitine palmitoyltransferase 1 and 2; TCA – tricarboxylic acid cycle; GLS – glutaminase; FASN – fatty acid synthase; UDP-GlcNAc – uridine diphosphate N-acetylglucosamine.

(A) Of all amino acids, glutamine is the most consumed by cancer cells. Glutaminolysis is the enzymatic process in which glutamine is converted into glutamate, and derived metabolites including pyruvate, aspartate, lactate, alanine, citrate, and α-ketoglutarate. It is a pathway significantly altered in breast cancer given its importance in proliferation and progression of cancer cells. First, (1) glutamine is an important source of nitrogen, the molecular basis for nucleotides and non-essential amino acids synthesis. (2) Glutamine is also a carbon source in the TCA cycle via α-ketoglutarate. (3) It is involved in the redox homeostasis through glutathione biosynthesis. And finally (4), glutamine actives mTOR pathway[141]. A study on glutamine metabolism observed different patterns across BC molecular subtype. Triple negative breast tumours expressed lower levels of glutamate dehydrogenase (GDH), while HER-2 enriched BC reveal the highest expressed glutamine metabolic activity with high levels of GDH, stromal glutaminase-1 (GLS1), and

tumoral amino acid transporter (ASCT) [142]. Regarding the influence of obesity in cell-based assays, co-culture with adipocytes from obese individuals did not interfere in glutamine uptake in MCF-7 cell line (luminal A representative). But in MDA-MB-231 (TN representative), glutamine uptake was decreased. Additionally, no differences were found in glutamine incorporation into lipids and nucleic acids, but glutamine oxidation was increased under obese adipocyte co-culture in both cell lines. This study concluded that lipid synthesis from glutamine carbons in obesogenic environment little contributes to the overall lipid needs of BC tumour cells[143]. Concordantly, a study evaluating the impact of cholesterol, an obesity related compound, used the same cell lines (MCF-7 and MDA-MB-231) and unravel that cholesterol did not alter glutamine uptake/glutamate production although it was mediated by the expression of estrogen-related receptor alpha when comparing MCF-7 to MDA-MB-231cell lines[144].

Several amino acids play important roles in breast cancer. Serine and glycine are also important sources in nucleotide/protein synthesis and in DNA methylation[145]. Branched amino acids: isoleucine, leucine, and valine are highly taken up by breast cancer cells. Besides their support in protein and nucleotide synthesis, they also play a role in the regulation of mTOR pathway[146]. Cysteine is associated with ROS and oxidative stress responsible for genetic mutations. Choline and proline were already postulated to be potential biomarkers in metastatic BC. Asparagine in breast cancer is negatively correlated with IGF1 and estradiol[145]. Amino acid metabolism is a determining pathway in the breast cancer metabolism although it is beyond the scope of the present work.

(B) TCA cycle is an amphibolic pathway already described to be altered in cancer. Metabolite leakage for anaplerotic reactions include: (1) oxaloacetate to pyrimidines and glucose; (2) Succinyl-CoA to porphyrins (including heme); (3) citrate to acetyl-CoA and oxaloacetate in the cytoplasm and into lipid biosynthesis. Amino acids, as previous presented, can be used to reload the TCA cycle[147]. TCA cycle incorporates a series of enzymatic reactions occurring in mitochondria. Many authors accept that tumour cell metabolism is reprogrammed due to mitochondrial dysfunction, given the mutations in the TCA cycle enzymes, and the excess ROS production associated with the increase TCA cycle rate[148]. Significant alterations were found in the enzymatic expression of both pyruvate dehydrogenase (PDH), Fumarate hydratase (FH), and succinate dehydrogenase (SDH) in BC[149]. PDH was correlated with decreased survival, increased glycolytic rate, and breast cancer proliferation[150]. Regarding FH, it was already described a biallelic inactivation of the gene in BC patients. Decreased FH activity is associated to increased oxidative state and impaired glutathione production. In BC, FH does not seem to play a major role in cancer development[151]. SDH has selective expression according to BC molecular subtype. While in HER2 enriched BC, SDH is overexpressed, in TNBC it is downregulated. In Luminal A subtype, low to no expression was observed[152]. Succinate is the substrate for SDH and has been recently described as a oncometabolite and SDH a tumour suppressor given its biological role on tumorigenesis and tumour development. Succinate accumulation leads to SDH downregulation, HIF1α stabilization, which

ultimately lead to tumour progression in BC cell lines[153]. Obesity, on the other hand, was previously described to deregulate certain TCA cycle enzymes such as citrate synthase[154], which in turn may provoke a metabolic dysregulation and an imbalance of the TCA cycle intermediates.

(C) Lipid metabolism is also prone to be altered in breast cancer. Lipogenesis (*de novo* synthesis of fatty acids) provides cancer cells with the necessary fatty acids for membrane synthesis, signaling molecules, and energy storage. Fatty acid synthase (FASN), a key enzyme in lipogenesis, is frequently upregulated in breast cancer and is associated with aggressive tumor behavior. Suppression of FASN in a cell-based study of MCF-7 and MDA-MB-231 cell lines caused apoptosis[155] and inhibited glycolysis[156]. Breast cancer cells may rely on endogenous and exogenous lipid uptake to fulfill their metabolic demands. Intracellular lipid trafficking and storage in lipid droplets also play vital roles in sustaining cancer cell survival and proliferation. Lipoprotein lipase (LPL) and fatty acid transport proteins (FATPs) facilitate lipids uptake from the extracellular environment.

Obviously, the impact of obesity in lipid metabolism in BC is massive, through both local and systemic actions. Systemic influence was presented in the beginning of this section. And locally by the direct crosstalk between tumour cells and adipocytes. Through lipolysis, adipocytes release fatty acids that can accumulate in non-adipose tissues, namely in the tumour microenvironment (TEM). Adipocytes (cancer-associated adipocytes – CAA) are the major cellular population in the TEM and exchange both *adipofactors* as well as fatty acids. Curiously, it is described that the impact of the fatty acid derived from CAA depends both on the fatty acid subtype and on the BC molecular subtype. i.e., oleate, a monounsaturated fatty acid, is associated with BC progression and plays a regulatory effect in the lipid metabolism[157]. This interaction is bidirectional. Not only CAA influence tumour cells metabolism but also tumour cells influence CAA. A study using biological samples of tumour and CAA observed that adipose tissue near the malignant lesion is prone to metabolic redirected towards the Pentose phosphate pathway (PPP)[158].

(D) Pentose phosphate pathway (PPP) is an alternative pathway for the phosphorylated glucose and a crucial metabolic pathway in NADPH (nicotinamide adenine dinucleotide phosphate) and ribose-5-phosphate production. NADPH is essential for maintaining the redox balance and reductive syntheses, and ribose-5-phosphate serves as a precursor for the synthesis of nucleotides and nucleic acids[159]. In breast cancer, the PPP is often dysregulated, contributing to the altered metabolic landscape that supports the aggressive behaviour of cancer cells. The PPP is activated in breast cancer cells to meet the increased demand for NADPH and building blocks required for nucleic acid synthesis during rapid cell proliferation. Several factors and molecular mechanisms contribute to the dysregulation of the PPP in breast cancer: (1) NADPH generated by the PPP is essential for maintaining the levels of reduced glutathione (GSH), a critical antioxidant enzyme that protects cells from oxidative damage; (2) Anabolic demands from the high biosynthetic demands of BC high proliferative rate, namely nucleotide synthesis; and (3) Oncogenic signalling pathways, such as the PI3K/AKT and c-Myc pathways, can stimulate glucose metabolism and,

consequently, activate the PPP in breast cancer cells. Conversely, tumor suppressor genes like p53 can negatively regulate the PPP, and their loss or mutation in breast cancer can lead to PPP activation. From the enzymes PPP associated, glucose-6-phosphate dehydrogenase (G6PD) is overexpressed in HER2 enriched BC. Suppression of G6PD decreased the tumor cell proliferation, cell survival, and increased oxidative stress as result of an increased glucose and glutamine uptake, and a decrease of lipid synthesis. Also, HER2 enriched BC and TNBC expressed higher levels of 6-phosphogluconate dehydrogenase (6PGD) when compared to luminal like BC. 6PGD inhibition, in the other hand, also lead to an increase in glucose uptake but resulted in the decrease of glutamine uptake, with no interference in the oxidative status of the MCF-7 cell line, used in a cell-based study[148]. To the best of our knowledge, no specific alterations in the PPP were directly described by the interaction of obesity and breast cancer. It is possible that the metabolic changes associated with obesity could indirectly impact the activity of the PPP through mechanisms like insulin resistance, ROS production, and TEM alterations.

(E) The Warburg effect (aerobic glycolysis) is probably the most important and regulated mechanism in the cell energetic engine. Glucose uptake, which is mediated by several factors including obesity related factors, is accomplished by a family of membrane glucose transporters recognized as GLUTs. In BC, GLUT1, 3 and 4 overexpression are associated with aggressive BC phenotypes[149]. The key limiting glycolytic enzymes are hexokinase 2 (HK2), phosphofructokinase (PFK), and pyruvate kinase (PK). HK2 also revealed to be positively correlated with overall survival, metastasis, and treatment resistance. PFK presents 3 isoforms PFKL, PFKM, PFKP. Each isoform exhibits different roles in prognosis and diagnosis, but the correlation between PFK, tumour aggressiveness and the glycolytic efficiency of BC cell lines is undisputable. Lastly, PK namely PKM2 isoform expression is associated with tumour development, autophagy inhibition, angiogenesis, and overall poor prognosis [148]. The reverse pathway, known by gluconeogenesis, is able to produce glucose from non-carbohydrate carbon substrates such as glycerol, lactate, pyruvate, and glucogenic amino acids. This mechanism can be regulated by three regulatory enzymes: Phosphoenolpyruvate carboxykinase (PEPCK), Fructose bisphosphatase (FBP), and Glucose-6-phosphatase (G6Pase). Additionally, Pyruvate carboxylase (PC) is an unidirectional enzyme in the gluconeogenesis. These enzymes performed several roles in cancer metabolism, such as regulation, signaling, proliferation, and cancer stem cell (CSC) tumor phenotype. The specific impact of *adipofactors* and their effects on glycolysis and gluconeogenesis is further presented. (1) Adiponectin is an adipokine exhibiting insulin-sensitizing properties. It enhances glucose uptake and utilization in tissues, including skeletal muscle and liver by promoting, among other mechanisms, the translocation of glucose transporter GLUT4. Adiponectin has been shown to increase glycolysis and decrease gluconeogenesis, resulting in improved glucose metabolism; (2) Leptin is a hormone primarily secreted by adipocytes. It regulates energy balance by acting on the hypothalamus to suppress appetite and increase energy expenditure. Leptin also influences glucose metabolism. It has been reported to inhibit hepatic gluconeogenesis by suppressing the expression of key enzymes involved in the gluconeogenic pathway, such as PEPCK and G6Pase. In this way, leptin promotes

glycolysis over gluconeogenesis. (3) Resistin is an adipokine that has been implicated in insulin resistance and inflammation. High levels of resistin are associated with obesity and type 2 diabetes. Resistin has been shown to impair insulin signalling and decrease glucose uptake in skeletal muscle cells, thereby affecting glycolysis. Additionally, resistin can promote gluconeogenesis in the liver by upregulating the expression of PEPCK and G6Pase; (4) Visfatin, also known as nicotinamide phosphoribosyltransferase (NAMPT), is an adipokine involved in the regulation of nicotinamide adenine dinucleotide (NAD+) biosynthesis. NAD+ is a coenzyme involved in various cellular processes, including glycolysis and gluconeogenesis. Visfatin has also been shown to enhance glycolysis by activating the glycolytic enzyme PFK-2, resulting in increased glucose uptake. It also modulates gluconeogenesis, although the mechanisms are not fully understood; (5) Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine secreted by adipose tissue and immune cells. Elevated levels of TNF-α have been observed in obesity and are associated with insulin resistance. TNF-α can inhibit insulin signalling and impair glucose uptake, leading to decreased glycolysis. It may also promote gluconeogenesis by stimulating the expression of gluconeogenic enzymes, such as PEPCK and G6Pase, in the liver[160].

Gluconeogenesis, which primarily occurs in the liver and kidney, is less well-studied in breast cancer cells. A previous study from our group observed that under adiposity conditions, breast tumour cells undergo gluconeogenesis instead of glycolysis, an unconventional condition we termed as *Warburg effect inversion*[4]. This *in vitro* study performed in a Luminal A representative cell line (MCF-7), revealed that most metabolites involved in the carbohydrate metabolism are significantly altered when exposed to condition medium (CM) from adipocyte differentiation. As illustrated in Figure 13, compared to a low glucose (LG) and high glucose (HG) environment, adiposity condition medium (CM) promotes glucose production and lactate consumption. Moreover, a high availability of acetate is observed, probably associated with increased levels of fatty acids oxidation from the CM environment. Such results were concomitant with an increased tumour cell aggressiveness, with significant increase in viability, proliferation, and migration[4].

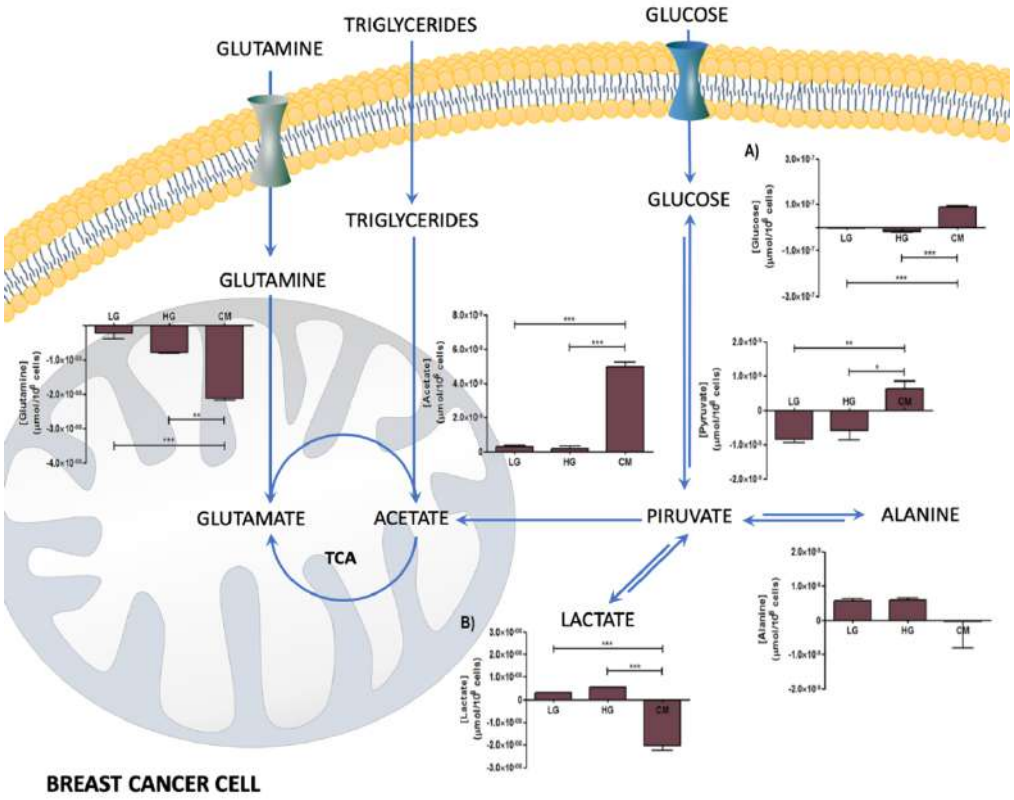


Figure 13 – Schematic representation of the metabolic pathway of the Warburg Effect Inversion (Source: Luis, Carla, et al. "Warburg Effect Inversion: Adiposity shifts central primary metabolism in MCF-7 breast cancer cells." Life Sciences 223, 2019, 38-46.)[4].

This work demonstrated that luminal A representative cell line, specifically, is able to proliferate, and even become more aggressive, when it adopts a gluconeogenic profile. Nonetheless, and aware of this study limitations, further studies are needed to better understand the impact of obesity on breast cancer, namely in different molecular subtypes, and in human subjects.

It was formerly portrayed that obesity exerts distinct effects according to molecular subtype classification. A metabolomic *in vitro* study by Willman and colleagues also observed specific fingerprints in the central metabolism across the different molecular subtypes. Compared to normal epithelial breast cells, BC cell lines presented significant alterations in the amino acid metabolism, in the TCA cycle and in glycolysis[161]. Results also revealed that each molecular subtype representative cell line had its distinct metabolic fingerprint, specifically, but not exclusively, on Luminal type due to the expression of hormone receptors and sex steroids[162]. Some authors defend that estrogens are able to act independently of its receptor. Sex steroids influence ER negative tumours systemically, resulting in increased angiogenesis and bone marrow-derived stromal cells recruitment, which are TNBC promoters[163].

By comprehensively exploring the influence of obesity on breast cancer, particularly in relation to glycolysis and gluconeogenesis, this doctoral thesis provides valuable insights into the underlying molecular mechanisms and potential therapeutic strategies. The findings presented here contribute to the understanding of the complex interplay between obesity, metabolism, and breast cancer, ultimately guiding the development of novel approaches for improved prevention, diagnosis, and treatment of this devastating disease.

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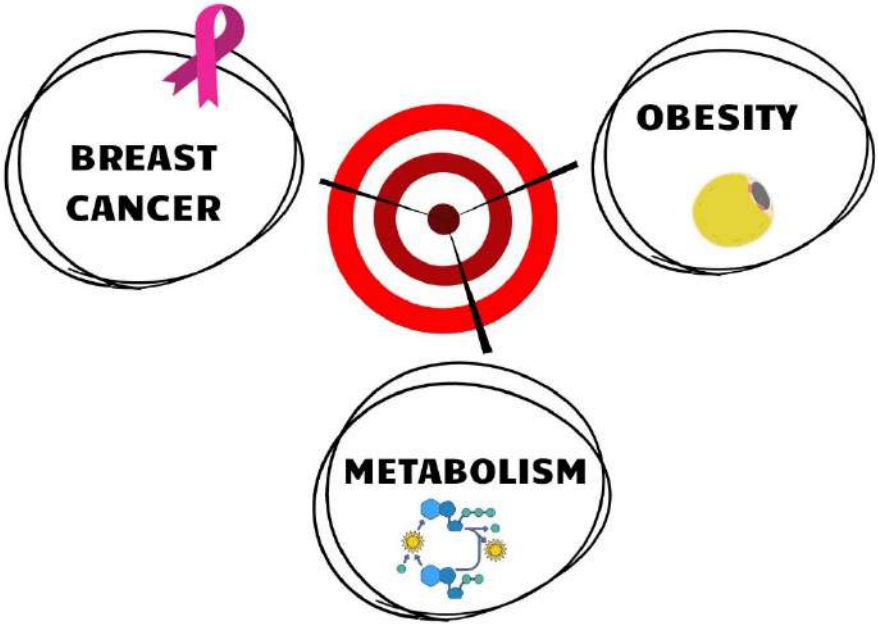
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CHAPTER II

Aims



LIST OF CONTENTS:

- General aims
- Chapter-specific aims

CHAPTER II

1. Aims

In a global landscape marked by rising incidence and mortality rates associated with both breast cancer and obesity, it is imperative to address and understand the metabolic link between both pathologies. By unravelling this intricate link, we can potentially pave the way for novel and precision targeted therapies. These therapies have the potential to significantly improve the prognosis and overall outcome for breast cancer patients grappling with obesity.

It is well stablished that obesity is associated with several releasing factors, which significantly modulate the overall metabolism and the tumour microenvironment specifically. The main association between obesity and breast cancer has been attributed to the expression of aromatase – an enzyme responsible for estrogen biosynthesis with clinical significance in hormone-related cancers. However, the knowledge linking the two pathologies is not yet completely known. Our previous study led us to conjecture the importance of both glycolysis and gluconeogenesis in breast cancer metabolism when influenced by obesity.

1.1. General aims

The general aims of this study are aligned with three main perspectives:

- Understand epidemiological trends associating BMI stratification (normal weight, overweight and obese) and clinicopathological features, tumour characteristics, and clinical outcomes.
- Evaluate the metabolic fingerprints of each molecular subtype representative cell lines when submitted to an obesogenic mimicking environment.
- Assess the enzymatic expression of glycolytic and gluconeogenic rate limiting enzymes of breast tumour tissues surgically removed from normal weight and obese patients.

To better address this issue, a working plan and related aims were outlined, presented ahead according to chapters:

1.2. Chapter III specific aims (Epidemiological approach to obesity and breast cancer)

A retrospective observational study was carried out on a series of patients with breast cancer followed at the IPO Medical Oncology department. A statistical analysis was performed stratified according to BMI and association with clinicopathological features, tumour characteristics, and clinical outcomes (including number and type of surgery) was evaluated. The main aims of this work were to:

- Identify statistically significant associations between BMI classes and tumour characteristics.
- Analyze the impact of obesity in overall survival.
- Evaluate the influence of obesity in surgical interventions (number and type) and outcomes.
- Explore the effect of obesity in bilateral breast cancer.
- Explore worldwide demographic trends to understand whether these were affected by obesity.

1.3. Chapter IV specific aims (Cell-based approach to obesity and breast cancer)

Breast tumor cell lines, representative of molecular subtypes, were subjected to treatment with obesogenic mimicking condition using adipocyte differentiation secretome. The objectives of this research were to:

- Explore the metabolic behavior of the different molecular subtypes in normal conditions and under obesogenic environment.
- Recognize potential underlying pathways associated to glycolysis and gluconeogenesis that could be promoted by obesity-related factors.
- Identify potential therapeutical targets focusing on the rate limiting enzymes.

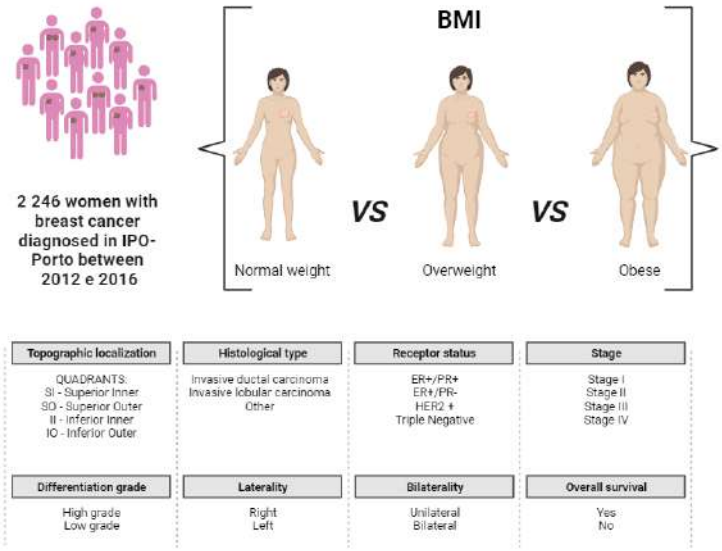
1.4. Chapter V specific aims (Tissue-based approach to obesity and breast cancer)

Samples of paraffin-embedded tumor tissue from normal weight and obese patients was obtained from the Portuguese Institute of Oncology (IPO-Porto). The samples were analyzed by histology and immunohistochemistry. Results were intersected with the patients clinicopathological features. The main goals of this study were to:

- Investigate potential features of the tumour microenvironment (adipocytes, inflammation, etc).
- Understand the effect of obesity in the enzymatic expression of glucose metabolism.
- Identify potential therapeutical targets among the enzymatic landscape of glucose metabolism for obese breast cancer patients.
- Investigate associations between obesity related comorbidities with rate limiting glucose enzymes.
- Compare the results obtained from cell culture and human tissues.

CHAPTER III

Epidemiological approach to obesity and breast cancer



DOC database - Deciphering Obesity and Cancer

LIST OF CONTENTS:

- "A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer."
- "A narrative review in why breast cancer is diagnosed later in obese women."
- "Bilateral breast cancer and the influence of body mass index in clinicopathological features and overall survival."
- "Does body mass index influence surgical options and overall survival in breast cancer patients."

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EPIDEMIOLOGY

A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer

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Abstract

Introduction Obesity and breast cancer are two major pathologies closely associated with increasing incidence and mortality rates, especially amongst women. The association between both diseases have been thoroughly discussed but much is still to uncover.

Aim The aim of this study is to analyse tumour characteristics and clinical outcomes of overweight and obese women to disclosure potential associations and better understand the impact of obesity in breast cancer.

Materials and methods Clinicopathological information of 2246 women were extracted from the institutional database of comprehensive cancer centre in Portugal diagnosed between 2012 and 2016. Women were stratified according to body mass index as normal, overweight, and obese. Patients' demographic information and tumour features (age, family history, topographic localization, laterality, histological type, and receptor status) were taken as independent variables and overall survival, tumour stage, differentiation grade and bilaterality were considered clinical outcomes.

Results The main results reveal that overweight and obesity are predominantly associated with worse outcomes in breast cancer patients. Obese patients present larger (p-value: 0.002; *OR* 1.422; 95% *CI* 1.134–1.783) and more poorly differentiated tumours (p-value: 0.002; *OR* 1.480; 95% *CI* 1.154–1.898) and tend to have lower overall survival although without statistical significance (p-value: 0.117; *OR* 1.309; 95% *CI* 0.934–1.833). Overweighted women are more likely to have bilateral breast cancer (p-value: 0.017; *OR* 3.076; 95% *CI* 1.225–7.722) than obese women. The results also reveal that overweight women present less distant metastasis (p-value: 0.024; *OR* 0.525; 95%*CI* 0.299–0.920). Topographic localization and laterality did not achieve statistical significance.

Keywords Retrospective study · Breast cancer · Obesity · BMI · Clinical outcomes · Tumour characteristics

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Introduction

Obesity is closely associated with several types of cancer, including breast cancer [1]. Strong evidence suggests that obesity-related factors such as inflammatory mediators and adipokines modulate metabolic pathways which may promote tumorigenesis and tumour progression [2].

The most common causes of obesity are a poor diet with high energy intake, lack of physical exercise and sedentary lifestyle. Obesity prevalence estimation by the World Obesity Federation calculates that in 2030, 18% of the world population will live with obesity [3]. According to the National Portuguese Health Survey, in 2019, more than half of the adult Portuguese population (53.6%) was overweight or obese [4], and prevalence in obese Portuguese women increased from 48.3 in 1999 to 51.5% in 2019 [4]. The classification of obesity is usually defined by the Body Mass Index (BMI) calculated by the Quetelet index, weight in kilograms (Kg) divided by height in squared metres (m^2). A BMI between 19 and 25 kg/m^2 is considered adequate, whereas individuals with a BMI between 25 and 29.9 kg/m^2 are classified as overweight and higher values are considered obese [5]. Adipose tissue is a metabolically active organ with diverse functions and several cell types. Besides adipocytes, adipose tissue also comprises immune and stromal cells, connective tissue matrix, vessels, and sensory neurons [6]. This cellular network is responsible for the release of several factors such as adipokines, inflammatory mediators, free fatty acids, oestrogens, hypoxia-inducible factors, and growth factors like insulin-like growth factor, that mediate several relevant metabolic pathways in tumorigenesis such as: AMP-activated protein kinase (AMPK), phosphoinositide 3-kinase (PI3K), hypoxia-inducible factor 1α (HIF1α), serine/threonine kinase (AKT), p53 amongst others [2]. Obesity can also contribute to deleterious effects in breast cancer through increased levels of local oestrogen, a product of aromatase activity [7].

Breast cancer is a complex, heterogeneous pathology with an increasing incidence since the 80 s in North America, Oceania, and Europe [8]. Female breast cancer surpassed lung cancer as the most incident cancer worldwide, with an incidence rate in 2020 of 24.5% and being responsible for 15.5% of female cancer deaths [8]. In Portugal, with a female population of roughly 5 million, in 2020, 7000 new cases of breast cancer were diagnosed, and 1800 women died from the disease [8]. Both obesity and breast cancer, share increasing trends. Epidemiological analyses cross talking obesity and breast cancer have been extensively studied and disclosed obesity as a negative prognostic factor associated with poorer outcomes [9–11]. The Women's Health Initiative Clinical Trial concluded that women with obesity grades I (BMI between 30 and 34.9 kg/m^2) and II (BMI between 35 and 39. 9 kg/m^2) had an increased risk of developing breast cancer up to 52 and 86% respectively when compared to normal BMI [12]. A meta-analysis performed by Chan and colleagues reviewed observational studies and randomized control trials associating BMI and breast cancer and concluded that obesity is associated with lower overall and disease-free survival both on pre and postmenopausal women. Comparing women with normal weight and with obesity (calculated before diagnosis) the summary relative risk of mortality was 1.41 for obese women [13].

Though, not all conclusions are consensual, there are still controversial results associating BMI and tumour features, for example, HER2 status. A previous study from Mieghem et al. HER2 was found to be inversely associated with BMI [14] but another study performed by Phipps et al. no correlation was found [15]. Additionally, population characteristics like race [16] and environmental traits like solar exposure [17] can also influence cancer evolution. Herein, we conducted a retrospective cohort study to evaluate the different tumour features and cancer outcomes of women with breast cancer stratified by BMI categories amongst the northern Portuguese population.

Materials and methods

Study population and ethical approval

This retrospective cohort study, from the "Deciphering Obesity and Cancer" (DOC) database, included patients diagnosed at Comprehensive Cancer Centre in Portugal (IPO-Porto). Clinicopathological information was extracted from the institutional database in 16, December 2021, with approval of the institutional ethics committee. Women with breast cancer diagnosed between 2012 and 2016 were identified and assessed for inclusion. Male breast cancer as well as underweight women (BMI inferior to 18.5 kg/m^2) were excluded.

Methods

BMI was calculated using the Quetelet index, weight and height were measure up to 120 days after diagnosis. BMI was grouped in three categories: normal ($18.5 \leq BMI < 25$ kg/m^2), overweight ($25 \leq BMI < 30$ kg/m^2), and obese ($BMI \geq 30$ kg/m^2) [5]. Independent variables and outcomes of interest were stratified according to BMI categorical distribution.

Age at diagnosis, family history, topographic localization, laterality, histological type, and receptor status were considered as exposure/independent variables. Outcomes of

interest was bilaterality, differentiation grade, cancer staging, and overall survival (OS). Independent variable was determined by a patient parameter able to induce an unexpected variation in a measurable outcome [18].

Family history was based on the patient referral to an oncogenetic follow-up in the host institution according to the recommendation statement found in [19].

Topographic localization was divided in inferior outer quadrant (IOQ), inferior inner quadrant (IIQ), superior outer quadrant (SOQ), superior inner quadrant (SIQ), other localizations which included nipple, skin, central quadrant, and axillary extension (Other) and overlapping lesions in different localizations (Multiple).

For histological type, cases were stratified in Invasive Ductal Carcinoma (IDC) which included Invasive Ductal Carcinoma also called no special type (NST) and IDC with other types of carcinomas; Invasive Lobular Carcinoma (ILC) which included Invasive Lobular Carcinoma with other types of carcinomas; and other types of breast cancer (Other).

Each hormonal receptor (estrogen—ER and progesterone—PR) was classified as positive or negative, cut-off of 1% of tumour cells reported as positive. HER2 status was classified according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines, a first assessment by immunohistochemistry and in equivocal results, validation by fluorescence in situ hybridization (FISH) [20].

Differentiation grade is classified as grade 1 considered well differentiated, grade 2 for moderately differentiated tumours and grade 3 in poorly differentiated ones. In the present statistical analysis, grade was grouped as High grade (Grades 1 and 2) and Low grade (Grade 3).

Cancer staging (pathologic stage) was assessed accordantly to the American Joint Committee on cancer by tumour node metastasis (TNM) system. Tumour size (T) was divided in ≤ 20 mm and > 20 mm. Lymph node involvement (N) was stratified in the presence (N+) or absence (N0) of nodal infiltration. Likewise for metastasis (M), cases were divided according to the presence or absence of distant metastasis. Overall stage classification was determined by a stage grouping process [21].

Overall survival (OS) was measured in months over 10 years, from the date of diagnosis until the date of death with censoring at the last known follow-up with a cut-off date of 16 December 2021.

Statistical analysis

Statistical analysis was performed using IBM® SPSS®, version 27 software. Descriptive statistics was used for data description in terms of absolute frequencies and valid percentages. The first approach consisted in a Pearson's Chi-squared test to analyse the association between BMI and all variables with statistical significance set for $p < 0.05$ obtained by default two-sided qui square test. Age at diagnosis was tested for normality with Shapiro–Wilk test, afterwards a One-way ANOVA and a linear correlation was performed. Independent variables were analysed in binary or multinomial logistic regression in crude and adjusted to age at diagnosis and family history. Topographic localization, histological type and receptor status were analysed with multinomial logistic regression to estimate odd ratio (*OR*) with 95% confidence interval (95% *CI*). For receptor status, each receptor was analysed in separate with a binomial logistic regression model to estimate *OR* with 95% *CI*. Bilaterality, differentiation grade, and each component of the pathological stage was accessed by binomial logistic regression in crude and adjusted to age at diagnosis, family history, histological type, topographic localization, receptor status, and laterality, to estimate *OR* with 95% *CI*. Overall survival analysis was performed by Cox proportional hazard model with a time scale of 10 years (120 months). Estimation was done in crude as well as adjusted to age at diagnosis, family history, histological type, topographic localization, receptor status and laterality, associations were expressed in hazard ratio (*HR*) with 95% *CI*.

Results

From 2012 to 2016, 2246 women with breast cancer were identified and included in this study, Fig. 1 presents a flowchart with the exclusion criteria applied from the main database.

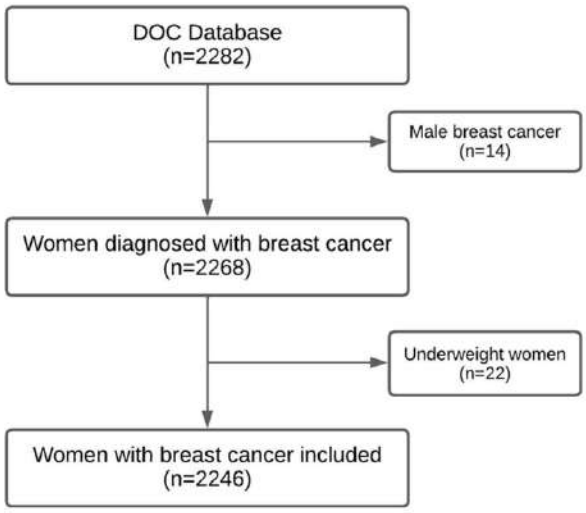


Fig. 1 Flowchart of the study inclusion/exclusion criteria Legend: DOC: "Deciphering Obesity and Cancer"

From the 2246 women, 777 (34.6%) were normal weight, 854 (38.0%) were overweight and 615 (27.4%) were obese. Table 1 presents the demographic and clinicopathological characteristics of women distributed according to BMI categories. There is a significant association between BMI and age at diagnosis, family history, progesterone receptor, receptor status, bilaterality, and tumour stage. It was also found that Invasive ductal carcinoma (IDC) was more frequent in overweight patients. No association was observed in topographic localization, laterality, estrogen receptor, HER2, differentiation grade and overall survival.

Overweight and obese patients are diagnosed later with breast cancer

The mean age of patients at diagnosis was 55.6 ± 11.5 years old with a median of 55.0 (range 24–88). Overweight (age at diagnosis 56.8 ± 11.0) and obese (age at diagnosis: 58.7 ± 10.0) patients were diagnosed later than normoponderal patients (age at diagnosis: 51.7 ± 12.0) (Fig. 2a). Age at diagnosis determined a correlation coefficient of 0.055 (Fig. 2b).

Family history is less common in obese and overweight patients

We observed a statistical association between family history and BMI categories. A descriptive analysis of our cohort observed that absence of family history is more frequent in obese patients (76.6%) when compared to overweight (72.8%) and normal weight (65.1%) subjects (Table 1, p-value < 0.001; Fig. 3).

BMI has no significant association with topographical localization and laterality

Our results did not reveal a significant association between topographic localization and BMI (Table 1, p-value $= 0.666$) and no alterations in the BMI distribution per quadrant (Fig. 4a). In a multinomial logistic regression adjusted to age at diagnosis and family history, significance remained unachievable (Supplementary Information SI1—Multinomial logistic regression for topographic localization). Association between laterality and BMI distribution did not achieve statistical significance either (Table 1, p-value $= 0.903$) as has the binary logistic regression adjusted to age at diagnosis and family history (Supplementary Information SI2—Binary logistic regression for laterality). The left to right ration (LRR) in our study was 1.09 (Fig. 4b).

Overweight women are more likely to have ductal invasive carcinoma than other histological type

IDC represents 81.1% of the breast cancer cases and ILC correspond to 8.5% of the participants. No significant association was found across BMI categories (Table 1; p value $= 0.059$). We also analysed qualitatively the distribution of all cases with all other histological types discriminated (Supplementary Information SI3—Description of all histological types stratified by BMI categories). We observed that rare breast cancers with favourable prognosis are more commonly diagnosed in patients with normal weight. For a closest insight of the statistical significance between histological type, we performed a multinomial logistic regression adjusted to age at diagnosis and family history to each reference category (ILC, IDC and Other). We observed that overweight women have increased OR for IDC (p-value $= 0.030$; *OR*: 1.457; 95% *CI* 1.038–2.047; reference: other). Additionally, ILC reference category has no statistical significance with the other groups (Table 2).

Receptor status: obese women have increased odd ratio to be progesterone receptor positive

Estrogen and progesterone receptors positivity is present in most of our cases (69.9%), HER2 is positive in 18.8% of the cases and triple negative tumours are accountable for 11.3%. Pearson's χ^2 test reported a significant association between BMI and Receptor Status (Table 1, p-value $= 0.032$).

A binary logistic regression found no significant association in ER and HER2 receptors in the crude and adjusted models, but a significant result was achieved for PR (Table 3). Obese women present an increased odd ratio of 48% (*OR* 1.481; 95% *CI* 1.163–1.887) for PR expression in comparison to women with normal weight. Women with overweight have approximately half of this odd ratio, 27%.

Overweight women are more likely to develop bilateral breast cancer than obese women

In the current study, the BBC incident rate is 1.8%, and with a significant association with BMI (Table 1; $p = 0.041$). The statistical significance persisted with a binary logistic regression analysis (Table 4). Overweight women have an increased non-significant odd ratio of 74.8% (p-value: 0.132; *OR* 1.748; 95% *CI* 0.845–3.615) to develop BBC, whereas obese women present a decreased non-significant odd ratio of 43.2% (p-value: 0.276; *OR* 0.568; 95% *CI* 0.201–1.570) comparing to normal BMI.

To better understand the significance of the adjusted model of the binary logistic regression, we performed an identical complementary analysis establishing obese as reference category and found a statistically significant p-value

Table 1 Demographic and clinicopathological characteristics of breast cancer patients stratified by BMI categories

	Normal (BMI 18.5–24.9 kg/m^2) *n* (%)	Overweight (BMI 25–29.9 kg/m^2) *n* (%)	Obese (BMI > 30 kg/m^2) *n* (%)	Total *n* (%)	*p*-value
n (%)	777 (34.6%)	854 (38.0%)	615 (27.4%)	2246 (100.0%)	–
Age at diagnosis (Median/range)	50 (24–87)	56 (27–88)	59 (33–86)	55 (24–88)	**< 0.001***
Family history					
No	506 (65.1%)	622 (72.8%)	471 (76.6%)	1599 (71.2%)	**< 0.001***
Yes	271 (34.9%)	232 (27.2%)	144 (23.4%)	647 (28.8%)	
Topographic Localization					
SIQ	68 (8.8%)	87 (10.2%)	53 (8.6%)	208 (9.3%)	0.666
SOQ	236 (30.4%)	263 (30.8%)	181 (29.4%)	680 (30.3%)	
IIQ	37 (4.8%)	35 (4.1%)	30 (4.9%)	102 (4.5%)	
IOQ	54 (6.9%)	38 (4.4%)	36 (5.9%)	128 (5.7%)	
Other	39 (5.0%)	52 (6.1%)	36 (5.9%)	127 (5.7%)	
Multiple	343 (44.1%)	379 (44.4%)	279 (45.3%)	1001 (44.5%)	
Laterality					
Right	370 (47.6%)	413 (48.5%)	292 (47.5%)	1075 (47.9%)	0.903
Left	407 (52.4%)	438 (51.5%)	323 (52.5%)	1168 (52.0%)	
Missing data	0 (0.0%)	3 (0.1%)	0 (0.0%)	3 (0.1%)	
Histological Type					
IDC	626 (80.6%)	708 (82.9%)	488 (79.3%)	1822 (81.1%)	0.059
ILC	64 (8.2%)	77 (9.0)	49 (8.0%)	190 (8.5%)	
Other	87 (11.2%)	69 (8.1%)	78 (12.7%)	234 (10.4%)	
Estrogen Receptor					
Negative	137 (17.6%)	138 (16.2%)	97 (15.8%)	372 (16.6%)	0.600
Positive	640 (82.4%)	716 (83.8%)	518 (84.2%)	1874 (83.4%)	
Progesterone Receptor					
Negative	253 (32.6%)	243 (28.5%)	158 (25.7%)	654 (29.1%)	**0.017***
Positive	524 (67.4%)	611 (71.5%)	457 (74.3%)	1592 (70.9%)	
HER2					
Negative	620 (80.9%)	669 (79.5%)	504 (83.9%)	1793 (81.2%)	0.115
Positive	146 (19.1%)	172 (20.5%)	97 (16.1%)	415 (18.8%)	
Receptor status					
ER + /PR +	443 (57.0%)	510 (59.7%)	394 (64.1%)	1347 (61.0%)	**0.032***
ER + /PR-	84 (10.8%)	69 (8.1%)	43 (7.0%)	196 (8.9%)	
HER2 +	146 (18.8%)	172 (20.2%)	97 (15.8%)	415 (18.8%)	
Triple Negative	93 (12.0%)	90 (10.5%)	67 (10.9%)	250 (11.3%)	
Missing data	11 (1.4%)	13 (1.5%)	14 (2.2%)	38 (1.7%)	
Bilaterality					
Unilateral	765 (98.5%)	831 (97.3%)	609 (99.0%)	2205 (98.2%)	**0.041***
Bilateral	12 (1.5%)	23 (2.7%)	6 (1.0%)	41 (1.8%)	
Differentiation Grade					
High grade	423 (54.4%)	468 (54.8%)	308 (50.0%)	1199 (53.4%)	0.129
Low grade	348 (44.8%)	376 (44.0%)	303 (49.3%)	1027 (45.7%)	
Missing data	6 (0.8%)	10 (1.2%)	4 (0.7%)	20 (0.9%)	
Tumor Stage					
Stage I	331 (42.6%)	359 (42.1%)	227 (36.9%)	917 (40.9%)	**0.029***
Stage II	288 (37.0%)	325 (38.1%)	241 (39.2%)	854 (38.0%)	
Stage III	124 (16.0%)	149 (17.5%)	129 (21.0%)	402 (17.9%)	
Stage IV	34 (4.4%)	20 (2.3%)	18 (2.9%)	72 (3.2%)	
Overall Survival					
No	82 (10.6%)	105 (12.3%)	71 (11.5%)	258 (11.5%)	0.544
Yes	695 (89.4%)	749 (87.7%)	544 (88.5%)	1988 (88.5%)	

IDC invasive ductal carcinoma; *ILC* invasive lobular carcinoma; *IOQ* inferior outer quadrant; *IIQ* inferior inner quadrant; *SOQ* superior outer quadrant; *SIQ* superior inner quadrant; *ER* estrogen receptor; *PR* progesterone receptor; *HER2* human epidermal growth factor receptor 2

*Statistical significance highlighted in bold ($p < 0.05$);

of 0.017 (*OR* 3.076; 95% *CI* 1.225–7.722). Comparing to obese women, women with overweight have a threefold increased likelihood to develop BBC.

differentiation grade (p-value = 0.002). Obese women have 48% more likelihood to have poorly differentiated tumours (*OR* 1.480; 95% *CI* 1.154–1.898).

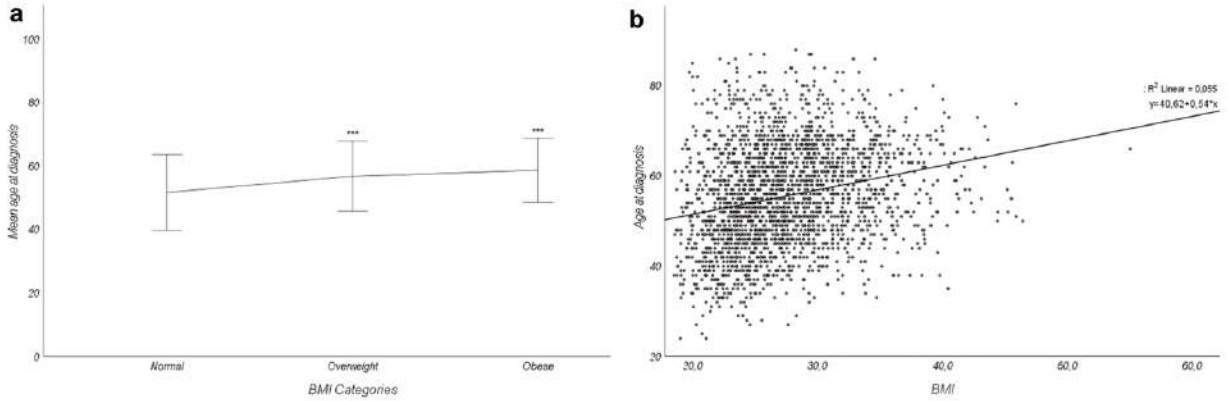


Fig. 2 **a** Graphic representation of age at diagnosis by BMI classification in mean ± standard deviation (One-way ANOVA of age at diagnosis and BMI categories, *** $p < 0.001$). **b** Scatter Plot of Age by BMI ($R^2 = 0.055$, $y = 40.62 + 0.54X$)

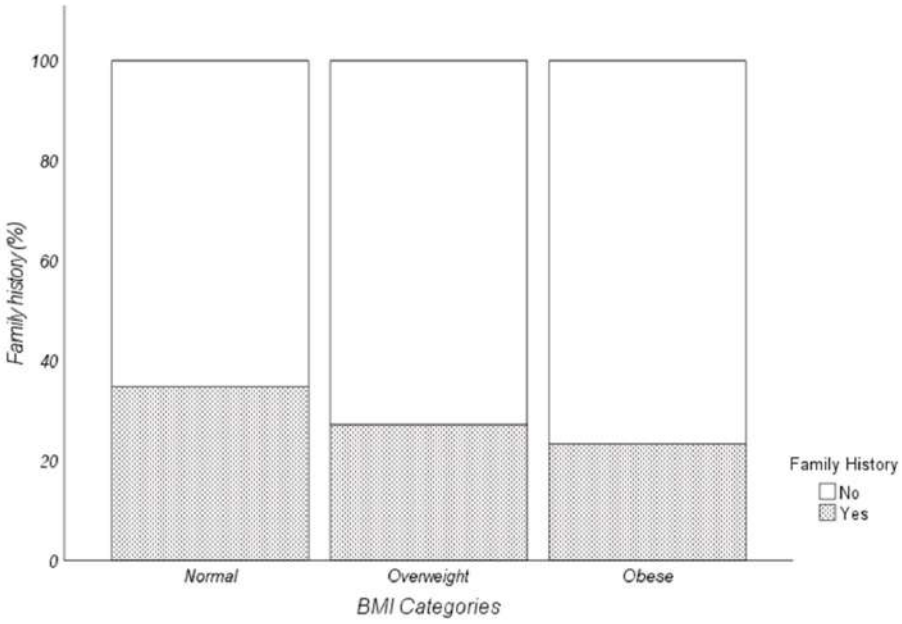


Fig. 3 Descriptive analysis of the percentage distribution according to BMI categories of patients with breast cancer and family history (Pearson's Chi-squared test p-value: < 0.001)

Obese women are more susceptible to develop poorly differentiated tumours

Herein, despite no significant association was found between BMI and differentiation grade in the Pearson's Chi-squared test (Table 1, p-value = 0.129), an adjusted binary logistic regression (Table 5) revealed that obesity is significantly associated with tumours with lower

Obesity is associated with larger primary tumours and less distant metastasis

Our results report that BMI is significant associated with pathological stage (Table 1, p-value = 0.029). As illustrated in Table 6, BMI is strongly associated with tumour size, moderately (but not significantly) associated with

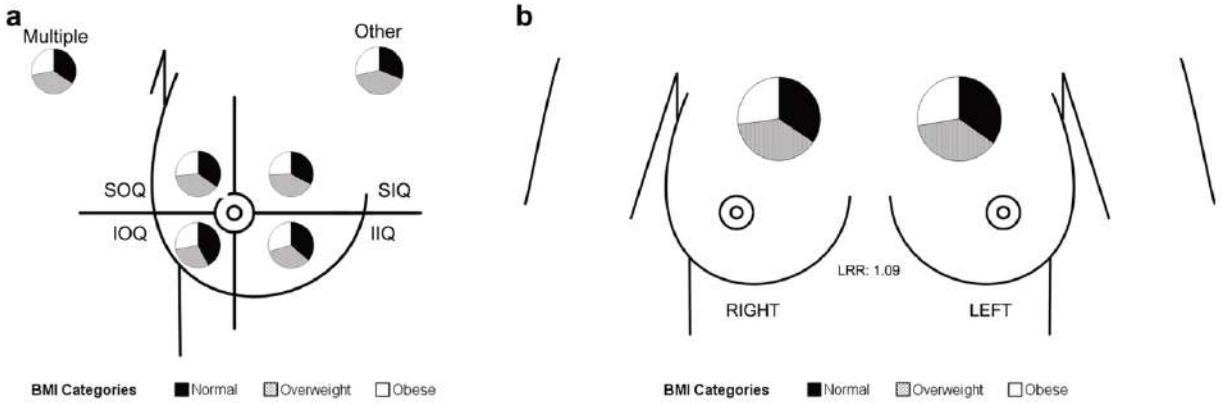


Fig. 4 **a** Pie charts of each topographic localization stratified by BMI categories **b** Pie charts of laterality stratified by BMI categories Legend: *IOQ* inferior outer quadrant; *IIQ* inferior inner quadrant; *SOQ* superior outer quadrant; *SIQ* superior inner quadrant; *LRR* left to right ration.

Table 2 Multinomial logistic regression for Histological Type

		p-value	OR	95% *CI*	
				Lower	Upper
Reference category: Other histological type					
IDC	Intercept	<0.001*	–	–	–
	Normal	–	1	–	–
	Overweight	**0.030***	**1.457**	**1.038**	**2.047**
	Obese	0.520	0.895	0.638	1.255
ILC	Intercept	0.561	–	–	–
	Normal	–	1	–	–
	Overweight	0.100	1.478	0.927	2.354
	Obese	0.439	0.822	0.501	1.349
Reference category: ILC					
IDC	Intercept	<0.001*	–	–	–
	Normal	–	1	–	–
	Overweight	0.940	0.926	0.692	1.406
	Obese	0.680	1.088	0.729	1.624
Other	Intercept	0.561	–	–	–
	Normal	–	1	–	–
	Overweight	0.100	0.677	0.425	1.078
	Obese	0.439	1.216	0.741	1.995
Reference category: IDC					
ILC	Intercept	<0.001*	–	–	–
	Normal	–	1	–	–
	Overweight	0.940	1.014	0.711	1.446
	Obese	0.680	0.919	0.616	1.372
Other	Intercept	**<0.001***	–	–	–
	Normal	–	1	–	–
	Overweight	**0.030***	**0.686**	**0.489**	**0.963**
	Obese	0.520	1.118	0.797	1.568

Adjusted to age at diagnosis and family history

CI confidence interval; *OR* odd ratio

*Statistical significance highlighted in bold (*p*-value < 0.05);

distant metastasis, and poorly associated with lymph node involvement.

After binary logistic regression for each feature, adjusted models reveal that obese patients have an 42% increased OR (95%: 1.134–1.783) to develop tumours with more than 20 mm. In the unadjusted model, there is a significant reduced OR of approximately 50% (95% *CI* 0.299–0.920) for overweight women to exhibit distant metastasis. Our results did not observe any association with lymph node involvement (Table 7).

Obese and overweight patients present lower overall survival

We observed that normal weight women have a better OS compared to women with overweight and obesity. OS of overweight women is close to women with obesity with a slight better outcome (Fig. 5). Although, statistical analysis did not achieve significant difference (Supplementary Information SI4—Cox proportional hazard models for overall survival), the HR for women with overweight and with obesity in the adjusted model was 1.224 (*p*-value = 0.182; 95% *CI* 0.909–1.648) and 1.309 (*p*-value = 95%; *CI* 0.934–1.833) respectively.

Discussion and conclusion

Breast cancer and obesity are two metabolically associated pathologies with high and increasing prevalence rates, which develop to a complex and challenging clinical concern. Our study intended to uncover associations in obese and overweight breast cancer patients within the northern Portuguese population. Integrating our results within the

Table 3 Binary logistic regression for the distinct receptors

	Crude				Adjusted[§]			
	p-value	*OR*	95% *CI*		*p*-value	*OR*	95% *CI*	
			Lower	Upper			Lower	Upper
Estrogen Receptor								
BMI	0.600	–	–	–	0.672	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.428	1.111	0.857	1.439	0.469	1.103	0.846	1.437
Obese	0.357	1.143	0.860	1.520	0.420	1.129	0.841	1.514
Progesterone Receptor								
BMI	**0.017***	–	–	–	**0.005***	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.072	1.214	0.983	1.500	**0.031***	**1.268**	**1.022**	**1.574**
Obese	**0.005***	**1.397**	**1.104**	**1.767**	**0.001***	**1.481**	**1.163**	**1.887**
HER 2								
BMI	0.116	–	–	–	0.145	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.484	1.092	0.854	1.396	0.289	1.146	0.891	1.473
Obese	0.161	0.817	0.616	1.084	0.358	0.873	0.652	1.167

[§]Adjusted to Age at diagnosis and Family history

OR odds ratio; *CI* confidence interval

*Statistical significance highlighted in bold (*p*-value < 0.05);

Table 4 Logistic binary regression for unilateral and bilateral breast cancer stratified by BMI

	Crude				Adjusted[§]			
	p-value	*OR*	95% *CI*		*p*-value	*OR*	95% *CI*	
			Lower	Upper			Lower	Upper
Reference category: Normal								
BMI	**0.049***	–	–	–	**0.037***	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.114	1.764	0.872	3.570	0.132	1.748	0.845	3.615
Obese	0.355	0.628	0.234	1.683	0.276	0.568	0.206	1.570
Reference category: Obese								
BMI	**0.049***	–	–	–	**0.037***	–	–	–
Obese	–	1	–	–	–	1	–	–
Overweight	**0.025***	**2.809**	**1.137**	**6.941**	**0.017***	**3.076**	**1.225**	**7.722**
Normal	0.355	1.592	0.594	4.267	0.276	1.759	0.637	4.859

[§]Adjusted to Age at diagnosis, Family history, laterality, Topographic localization, histological type, and Receptor status

OR odd ratio; *CI* confidence interval

* Statistical significance highlighted in bold (*p*-value < 0.05);

Portuguese population, we observed that the percentage of obese and overweight patients in the current study exceeds that of the obese/overweight female population (65.4% vs 51.5%). Our results also pointed that overweight and obese patients are diagnosed later that normal weight patients. A trend also observed in other countries, the northern Portuguese population is diagnosed later than countries such as Belgium [22], Arabian countries [23, 24], or Mexico [25], but earlier than in countries like as United States of America (USA) [26]. We postulated that this is attributed to the fact that obesity is often associated with lower social status and lower income, thus, providing patients with less health care opportunities and to a lower adhesion rate to the screening programmes of obese women [27]. Additionally, obesity is accompanied by a low-grade chronic inflammation, increased oxidative stress, hypertension,

Table 5 Logistic binary regression for differentiation grade stratified by BMI

	Crude				Adjusted[§]			
	p-value	*OR*	95% *CI*		*p*-value	*OR*	95% *CI*	
			Lower	Upper			Lower	Upper
BMI	0.129	–	–	–	**0.002***	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.813	0.977	0.802	1.188	0.860	1.021	0.814	1.279
Obese	0.100	1.196	0.967	1.479	**0.002***	**1.480**	**1.154**	**1.898**

[§]Adjusted to Age at diagnosis, Family history, laterality, Topographic localization, histological type, and Receptor status

OR odd ratio; *CI* confidence interval

*Statistical significance highlighted in bold (*p*-value < 0.05);

Table 6 Descriptive characteristics of tumour stage features by BMI Status with association analysis by Pearson's Chi-squared test

Characteristics	Normal BMI *n* (%)	Overweight *n* (%)	Obese *n* (%)	Total *n* (%)	*p*-value
Tumour size (T)					
≤ 20 mm	442 (57.2%)	472 (55.5%)	309 (50.2%)	1223 (54.6%)	**0.030***
> 20 mm	331 (42.8%)	379 (44.5%)	306 (49.8%)	1016 (45.4%)	
Distant metastasis (M)					
No	743 (95.6%)	833 (97.7%)	597 (97.1%)	2173 (96.8%)	0.060
Yes	34 (4.4%)	20 (2.3%)	18 (2.9%)	72 (3.2%)	
Lymph node involvement (N)					
N0	421 (54.4%)	486 (57.1%)	322 (52.4%)	1229 (54.9%)	0.186
N+	353 (45.6%)	365 (42.9%)	293 (47.6%)	1011 (47.6%)	

OR odd ratio; *CI* confidence interval

*Statistical significance highlighted in bold (*p*-value < 0.05)

Table 7 Binary logistic regression for the distinct features of tumour stage

	Crude				Adjusted[§]			
	p-value	*OR*	95% *CI*		*p*-value	*OR*	95% *CI*	
			Lower	Upper			Lower	Upper
Tumour size								
Normal	–	1	–	–	–	1	–	–
Overweight	0.486	1.072	0.881	1.305	0.151	1.163	0.946	1.428
Obese	**0.010***	**1.322**	**1.069**	**1.636**	**0.002***	**1.422**	**1.134**	**1.783**
Distant Metastasis								
BMI	0.065	–	–	–	0.246	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	**0.024***	**0.525**	**0.299**	**0.920**	0.094	0.611	0.343	1.088
Obese	0.160	0.659	0.368	1.178	0.498	0.811	0.442	1.488
Lymph Node involvement								
BMI	0.186	–	–	–	0.098	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.271	0.896	0.736	1.090	0.414	0.919	0.750	1.126
Obese	0.450	1.085	0.878	1.342	0.187	1.162	0.930	1.452

[§]Adjusted to Age at diagnosis, Family history, laterality, Topographic localization, histological type, and Receptor status

OR odd ratio; *CI* confidence interval

*Statistical significance highlighted in bold (*p*-value < 0.05);

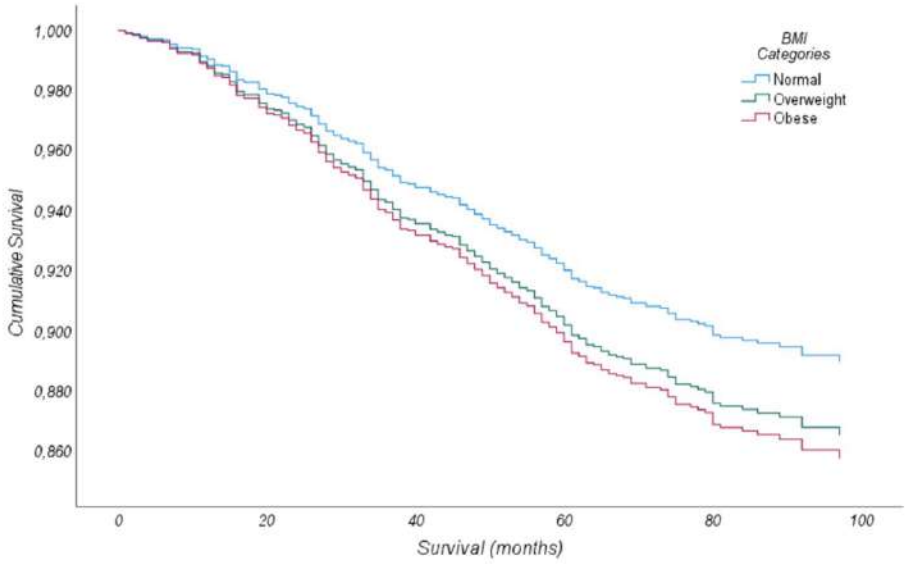


Fig. 5 Overall survival by Cox proportional hazard model stratified by BMI categories.

dyslipidaemia [28], and several other metabolic comorbidities, which may mask cancer symptoms and diagnosis. We also found that the correlation factor of age at diagnosis in our cohort is superior to the correlation found in other studies [22], indicating a stronger relation between age at diagnosis and BMI.

Breast tumorigenesis can be initiated by several factors like genetics [29], reproductive and modifiable factors. Modifiable risk factors include physical inactivity, alcohol consumption and obesity [29]. Our study revealed an association between obesity and sporadic breast cancer—lack of family history. Supporting our results, previous studies found lower family history index in both pre and postmenopausal in obese and overweight women [30].

According to the World Health Organization (WHO) there are 23 histological subtypes of breast cancer [31], the more incident ones are the IDC accountable for 70–80% of all invasive tumours and ILC diagnosed in 5–15% [32], our results are concomitant with the literature, IDC represents 81.1% and ILC corresponding to 8.5%. Our findings uncover that overweight women are more likely to have IDC than other histological type, to the best of our knowledge, no association was previously described regarding histological type and BMI [33].

Each histological type has intrinsic characteristics with different prognostic value, outcomes are modulated by several factors such as histological grade, menopausal status, therapeutical plan, or receptor positivity [32]. The association between breast cancer aetiology and steroid hormone receptors has been thoroughly studied. ER + and PR + are the most common and present a more favourable prognosis and survival advantage [34]. Though, it is suggested that postmenopausal women with overweight and obesity with ER + /PR + tumours exhibit an increased risk of developing breast cancer up to twofold [35]. HER2 + tumours account for ~ 15% of overall breast cancers. Their prognosis has shifted with the introduction of targeted therapies (such as trastuzumab). Triple negative tumours represent 10–20% of breast cancers and are the most aggressive ones, with the worst prognosis amongst breast cancers, these are usually diagnosed at a younger age with a low overall survival [36]. Previous studies have already reported an increase incidence of triple negative breast cancers in obese women, statistical significance was only achieved in premenopausal: obese premenopausal women have 42% increased risk to develop triple negative tumours [37]. Our study observed that overweight and obese women have a decreased incidence of triple negative tumours which can be associated with the menopausal status, a limitation of our study, further discussed. Obesity is the cause of increase levels of estrogen in postmenopausal women that consequently increases the risk for ER + breast cancer by estrogen-driven mechanisms [35]. Several studies already observed a statistical association between ER + breast cancers and BMI categories [38–40]. Independent HER2 expression was found to be inversely associated to BMI in postmenopausal women [14] but increase levels of HER2 in combination with PR expression, were directly correlated with BMI in postmenopausal women and inversely correlated in premenopausal women [41]. Although we do not have data regarding menopausal status, our analysis did find an association with progesterone receptor. PR + was previously associated with obesity in postmenopausal population [42] although the role of PR in obese premenopausal women is yet to be uncover. Literature states that PR is expressed in approximately

75% of ER + breast tumours [43], accordantly, our data identified 1347 women with ER + /PR + and 197 women with ER + /PR−, which represents approximately 85% of ER + with PR +. Interestingly, a study performed in animal models observed that an increased expression of progesterone receptor in obese animals is associated with increased expression of tumour glycolytic and lipogenic enzymes, and proliferation markers [44]. These results were unexpected since obesity is mainly associated with estrogen activity through aromatase expression. Previous studies concluded that PR is a critical transcription regulator and an activator of several transduction pathways connected to the proliferative index [45]. Another suggested mechanism is the mitogenic potential of progestins (form of progesterone) that are able to enhance the proliferation rate [45]. We postulate that this association could be linked to an important feature in cancer in patients with obesity that is the high proliferative status.

Tumour site has been suggested to be a prognostic factor, tumours developed in the inferior quadrants tend to have a worst prognosis [46]. To the best of our knowledge, no studies have associated BMI and topographic localization, although ours is a negative result. Regarding laterality, there is a slight ratio tendency for left sided breast tumours to be more prevalent as it was found in our cohort. Left sided tumours seem to have a worse prognosis probably associated with cardiotoxicity, a side effect from radiotherapy to the left chest wall/breast [47].

Regarding outcomes, we found a low prevalence rate of 1.8% of BBC concordant with the literature (1.4 to 11.8%) [48], and found that overweight women are more likely to develop BBC than obese women. An association between obesity and BBC was already proposed, Zhang and colleagues compared 512 BBC patients with 1024 UBC and concluded that obesity has a significant impact on the survival outcomes of patients with BBC [49].

Association studies between BMI and differentiation grade are controversial, several studies found no significant relationship between histopathology grading and BMI [50, 51], but numerous other studies found obesity to be associated with poorly differentiation tumours [52, 53]. Our results reveal that obese women have statistically significant increase likelihood to develop poorly differentiated tumours with a OR of 48%, a study from Stark and colleagues found an even higher *OR* (~ 80%) for women with obesity to develop poorly differentiated tumours with a more advanced pathological stage [52].

Epidemiological evidence agrees that patients with obesity display a poorer prognosis with a more advanced stage disease at diagnosis, which includes larger tumours. Neuhouser et al. reported that obesity present a *OR* of 2.12 for larger tumours [12], and although with a lower OR, our results are coherent with the literature. Moreover, these findings can be connected to the fact that obese patients are diagnosed later than normoponderal breast cancer patients, hence presenting larger primary tumours. In our cohort, overweight and obese women are less prone to develop metastatic cancer, we hypothesize that it could be related to a metabolic paradox found in breast cancer patients associated with the diabetic state. High levels of circulating insulin can display protective menopausal-dependent mechanisms in breast cancer [54]. Unfortunately, we were unable to confirm such hypothesis due to the study limitations (further discussed), namely the absence of information regarding insulin levels and menopausal status.

Strong evidence from epidemiological studies also supports the fact that obesity is associated with lower overall and breast free disease survival in several transversal populations [23, 24], we also observed the same result, although without statistical significance.

Nevertheless, we are conscious of our study limitations. Some considerable information was not made available for different reasons. Biomarkers that were not routinely recorded like blood insulin, estrogen, or progesterone levels. Although we had access to the date from the last clinical follow-up, we consider that some of these data could be outdated due to the constrictions derived from the pandemic condition. We also couldn't access information regarding the patient's menopausal status and other comorbidities. We cannot rule out the possibility that these patients exhibit other metabolic comorbidities that are often clustered together with obesity, including hyperglycaemia, insulin resistance, dyslipidaemia, hypertension, which are known to result in increased mortality rates.

We observed that overweight condition should be considered as an important and independent entity. Despite in, most analysis, the overweight group is closer to the obese group, the former group behaves in a distinct manner, for instances in histological type and bilaterality. We realized that our results mostly corroborate the literature, that supports the significance modulation of obesity in breast cancer. Nonetheless, our findings further show that the three patient groups unravel distinct behaviours, which represent different prognostic features and therapeutical approaches that need to be addressed in the future.

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Data availability The datasets generated during and/or analysed during the current study are not publicly available due to privacy or ethical restrictions.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Portuguese Oncology Institute of Porto (IPO-Porto) Francisco Gentil, EPE, Porto, Portugal.

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Supplementary Information:

Supplementary Information SI1 – Multinomial logistic regression for topographic localization

Reference category: Multiple		p-value	OR	95% CI	
				Lower	Upper
SIQ	Intercept	0.004	-	-	-
	Normal	-	1	-	-
	Overweight	0.366	1.178	0.825	1.683
	Obese	0.923	0.980	0.654	1.469
SOQ	Intercept	0.016	-	-	-
	Normal	-	1	-	-
	Overweight	0.728	0.960	0.760	1.211
	Obese	0.325	0.879	0.679	1.137
IIQ	Intercept	0.003			
	Normal	-	1	-	-
	Overweight	0.587	0.872	0.532	1.429
	Obese	0.940	1.020	0.604	1.723
IOQ	Intercept	0.078	-	-	-
	Normal	-	1	-	-
	Overweight	0.092	0.679	0.434	1.065
	Obese	0.641	0.895	0.560	1.429
Other	Intercept	0.000	-	-	-
	Normal	-	1	-	0
	Overweight	0.708	1.089	0.696	1.704
	Obese	0.953	0.985	0.603	1.610

Adjusted to age at diagnosis and family history; Legend: IOQ – Inferior outer quadrant; IIQ – Inferior inner quadrant; SOQ – Superior outer quadrant; SIQ – Superior inner quadrant; CI – Confidence interval; OR – Odd ratio.

Supplementary Information SI2 – Binary logistic regression for laterality

	p-value	OR	95% CI	
			Lower	Upper
BMI Categories	0.859	-	-	-
Normal	-	1	-	-
Overweight	0.589	0.947	0.776	1.154
Obese	0.861	0.981	0.788	1.220

Adjusted to age at diagnosis and family history. Legend: CI – Confidence interval; OR – Odd ratio.

Supplementary Information SI3 – Description of all histological types stratified by BMI categories

Histological type	Prognosis		BMI Categories			Total
			Normal	Overweight	Obese	
Invasive Ductal Carcinoma	Variable[1]	Count	556	617	416	1589
		% of total	24.8%	27.5%	18.5%	70.7%
Invasive Ductal Carcinoma with other types of carcinomas	Variable[1]	Count (n)	70	91	72	233
		% of total	3.1%	4.1%	3.2%	10.4%
Invasive Lobular Carcinoma	Variable[2]	Count (n)	60	72	46	178
		% of total	2.7%	3.2%	2.0%	7.9%
Invasive Ductal and Lobular Carcinoma	Variable[3]	Count (n)	58	38	49	145
		% of total	2.6%	1.7%	2.2%	6.5%
Invasive micropapillary carcinoma	Aggressive[4]	Count (n)	12	9	9	30
		% of total	0.5%	0.4%	0.4%	1.3%
Mucinous carcinoma	Favourable[1]	Count (n)	5	6	5	16
		% of total	0.2%	0.3%	0.2%	0.7%
Invasive Lobular Carcinoma with other types of carcinomas	Variable[1]	Count (n)	4	5	3	12
		% of total	0.2%	0.2%	0.1%	0.5%
Tubular adenocarcinoma	Favourable[5]	Count (n)	3	3	2	8
		% of total	0.1%	0.1%	0.1%	0.4%
Metaplasic carcinoma	Aggressive[1]	Count (n)	1	4	2	7
		% of total	0.0%	0.2%	0.1%	0.3%
Invasive intraductal papillary adenocarcinoma	Variable[4]	Count (n)	1	2	4	7
		% of total	0.0%	0.1%	0.2%	0.3%
Cribriform carcinoma	Favourable[1]	Count (n)	1	1	3	5
		% of total	0.0%	0.0%	0.1%	0.2%
Glycogen-rich carcinoma	Unfavourable[6]	Count (n)	1	0	1	2
		% of total	0.0%	0.0%	0.0%	0.1%
Adenocarcinoma with neuroendocrine differentiation	Worse long-term outcomes[7]	Count (n)	1	0	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Intracystic carcinoma	Favourable[8]	Count (n)	1	0	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Juvenile breast carcinoma	Favourable[4]	Count (n)	1	0	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Medullary carcinoma	Favourable[1]	Count (n)	1	0	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Paget's Disease and invasive breast ductal carcinoma	Variable[9]	Count (n)	1	0	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Apocrine adenocarcinoma	Poor[1]	Count (n)	0	1	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Clear cell adenocarcinoma	NA	Count (n)	0	1	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Adenocarcinoma	NA	Count (n)	0	1	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Ductular carcinoma	NA	Count (n)	0	1	0	1
		% of total	0.0%	0.0%	0.0%	0.0%
Liposarcoma	Variable[10]	Count (n)	0	1	0	1

(NA – Not available)

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Supplementary Information SI4 – Cox proportional hazard models for overall survival

	Crude				Adjusted[§]			
	p-value	HR	95% CI		p-value	HR	95% CI	
			Lower	Upper			Lower	Upper
BMI	0.186	-	-	-	0.245	-	-	-
Normal	-	1	-	-	-	1	-	-
Overweight	0.174	1.224	0.915	1.637	0.183	1.224	0.909	1.648
Obese	0.079	1.336	0.967	1.847	0.117	1.309	0.934	1.833

[§]Adjusted to Age at diagnosis, Family history, laterality, Topographic localization, histological type, and Receptor status. Legend: HR – Hazard ratio; CI – Confidence interval

REVISTA PORTUGUESA DE **ONCOLOGIA**

Artigo de revisão narrativa

A narrative review in why breast cancer is diagnosed later in obese women

Uma revisão narrativa sobre porque o cancro de mama é diagnosticado mais tarde em mulheres obesas

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Abstract

Obesity is a major risk factor in breast cancer. Both pathologies are closely associated, not only metabolically but also because they both present increasing incidence and mortalities rates tendencies.

A previous study from the DOC (Deciphering Obesity in Cancer) database observed a statistical significance increase in age at diagnosis amongst normal weight, overweight and obese women with breast cancer. An incisive view in this topic revealed a transversal result worldwide. We further addressed this issue with a survey of probable causes.

We have chosen random studies from countries of different continents with discrepant national health institutes, distinct screening programs and analysed if such external factors could influence the obese patient diagnosis. Moreover, with data from the DOC database we evaluated if a more advance age at diagnosis could be translated in more advanced disease stages. It is of main importance to better address the influence of obesity and overweight in breast cancer development and progression to improve screening and diagnosis outcomes.

Key-words: *Retrospective study; Breast cancer; Obesity; BMI; Age at diagnosis.*

Resumo

A obesidade é um importante fator de risco no cancro de mama. Ambas as patologias estão intimamente associadas, não só metabolicamente, mas também na crescente tendência das taxas de incidência e mortalidade.

Um estudo prévio baseado na base de dados DOC (*Deciphering Obesity in Cancer*) observou um aumento significativo crescente na idade ao diagnóstico entre mulheres com peso normal, excesso de peso e obesas com cancro de mama. Um estudo mais incisivo sobre o tema revelou resultados transversais em todo o mundo. Consequentemente avaliamos potenciais causas para este achado.

Para avaliar se os programas de rastreio e/ou os institutos nacionais de saúde poderiam influenciar o diagnóstico em pacientes obesas, foram analisados estudos de diferentes países em todo o mundo. Adicionalmente, recorrendo à base de dados DOC, examinamos se a obesidade poder-se-ia associar a estadios mais avançados da doença. É de grande importância abordar melhor a influência da obesidade e do excesso de peso no desenvolvimento e progressão do cancro de mama para melhorar os resultados de rastreio e diagnóstico.

Palavras-chave: Estudo retrospetivo; Cancro de Mama; Obesidade; IMC; Idade de diagnóstico.

Introduction

Breast cancer and obesity are two majors closely associated pathologies. It is well established that obesity promotes oncogenesis and increases tumour proliferation and aggressiveness leading to worse outcomes.[1] Cancer metabolism in obese patients is a deregulated complex network where obesity-related factors such as insulin growth factor, adipokines and cytokines, promote and modulate pathways like PI3K–AKT, HIF1, LKB1–AMPK and p53, key metabolic mechanisms in tumour development and progression.[2] Moreover, adipose tissue is also an estrogen producer through aromatase activity, and aromatase regulation in breast adipose cells is under the same metabolic mechanisms involving LKB1/AMPK, p53, HIF1 and PKM2.[3] Furthermore, a metabolic switch of the central biochemistry metabolism, namely in glycolysis and gluconeogenesis was already described in breast cancer cells exposed to an obesogenic environment.[4]

On an epidemiologic perspective, several retrospective studies observed significant differences in tumour characteristics and clinical outcomes of breast cancer patients with different body mass indexes.[5] The recent DOC (Deciphering Obesity and Cancer) study from our group uncovered an important and relevant issue that justifies a more incisive consideration and a call for attention to both the academic and the clinical communities.[6] We observed that overweight and obese women are diagnosed later with breast cancer than normal weight patients. This study included 2246 women diagnosed with breast cancer at the Portuguese Oncology Institute – Porto. Women were stratified according to their body mass index (BMI): normal (between 18.5 and 25 kg/m^2), overweight (between 25 and 30 kg/m^2), and obese (more than 30 kg/m^2). Results show that normoponderal patients are diagnosed at a median age of 50 (range 24-87), overweight at 56 (range 27-88) and obese women diagnosed at 59 (range 33-86) years old. These differences were statistical significance (p-value<0.001).[6] We believe that such information should be addressed accordantly to improve therapeutical approaches and ultimately outcomes.

Are external factors influencing breast cancer diagnosis in obese patients?

Given these findings, we further explored results from similar studies (**Table 1**) and observed that overweight/obese patients are diagnosed with breast cancer later that normal-weight patients worldwide. We found two surveys with a global analysis of breast cancer patients with corroborating results from different databases: The Breast International Group (BIG)[7] and the International Breast Cancer Study Group.[8] Both found statistical significance in age at diagnosis.

We observed some variations between obese and overweight patients. For instance, in Belgium, obese patients are diagnosed earlier with breast cancer than overweight patients.[9] Belgium is considered to have one of the best health systems in Europe, divided between public and private sector and is struggling with public health issues such as alcohol and tobacco consumption and obesity. Noteworthy, cancer is not the leading cause of death in Belgium, rather cerebrovascular and ischaemic heart diseases.[16]

Portugal and Spain share several similarities like culture, climate, lifestyle concerns and eating habits and have approximately the same prevalence of obesity rates in adults (Portugal 20.8% *vs* Spain 23.8%).[17] As for total (men and women) life expectancy, Spain has the highest of the European Union (EU) somewhat above Portuguese life expectancy (Portugal 81.6 *vs* Spain 83.3).[18] The leading causes of death in Spain are associated with chronic diseases, like ischaemic heart disease, stroke, and lung cancer.[18] The Spanish national health system is decentralised. Each of the 17 autonomous communities of Spain have autonomous primary jurisdiction on strategic and operational planning. The ministry of health manages national planning and regulation and the National Health system interterritorial council regulates all. Remarkably, Spanish health budget is lower than the EU average.[18] The study included in this review also reveals an increased age at diagnosis of obese patients for breast cancer.[10]

On the other hand, Iran suffered significant alterations in the country's health management since 1988 with the end of the war. Nowadays, Iran's health system is based on a primary health care model with significant discrepancies across the 31 provinces, in each province the responsibility of health service supplier and clinical formation is attributable to medical universities.[19] As a developing country, it still struggles with health issues like HIV infections and measles and risk factors such as air pollution, unsafe water, and sanitation.[20] Cancer is accountable for 16% of all deaths and obesity has a prevalence rate of 26%. We found that cancer mortality in Iran is lower than European countries[21] but breast cancer incidence is above the global mean (Iran 23.6% *vs* World 11.7%).[22,23] A study focusing on obesity and breast cancer also revealed that obese patients are diagnosed later with breast cancer, but overweight group is diagnosed somewhat earlier than normal weight patients.[11]

Curiously enough, Jordan has an even lower mortality cancer-associated than Iran, accounting for 12% of all deaths but a higher obesity prevalence rate of 33%.[21] Regarding breast cancer, Jordan has a lower incidence than industrialized countries but with an increased tendency (increased 69% in the last decade).[24] Jordan has one of the most advanced health care systems of the Arabian region and health services are provided both by public and private sectors.[24] Ayoub study uncovered statistical significance in age at diagnosis with obese patients diagnosed later in life.[12]

Focussing on the impact of obesity in breast cancer on African countries, we included a study from Egypt. Egypt has a good

Table 1. Comparative analysis of age at diagnosis between studies across different countries.

AGE AT DIAGNOSIS (MEAN AGE ± SD or MEDIAN (RANGE))								
HOST INSTITUTION, CITY, COUNTRY (POPULATION)	**UNDERWEIGHT**	**NORMAL**		**OVERWEIGHT**	**OBESE**		**TOTAL**	**P-VALUE**	**REF**
Portuguese Oncology Institute, Porto, Portugal (n=2246)	-	51.5 ± 12.1		56.5 ± 11.0	58.2 ± 10.1		55.1 ± 11.5	<0.001*	[6]
The Breast International Group (BIG), International (n=2887)	48 (27 – 70)				52 (29 – 69)		-	<0.001*	[7]
International Breast Cancer Study Group, International (n=6370)	-	48 (21 – 84)		53 (25 – 80)	55 (26 – 80)		-	<0.01*	[8]
Cliniques Universitairs Saint Luc. Brussels, Belgium (n=62)	-	49.1 ± 13.73		56.8 ± 9.96	50.5 ± 12.86		52.6 ± 12.53	0.16	[9]
University Hospital in Granada. Granada, Spain (n=524)	58.3 ± 14.10				59.3 ± 13.93		-	-	[10]
Imam Khomeini hospital and Kaviani breast disease institute; Tehran, Iran (n=646)	-	47.71 ± 12.54		46.57 ± 10.41	52.23 ± 11.34		49.62 ± 11.48	-	[11]
King Abdullah Teaching University Hospital. Ramtha, Jordan (n=348)	-	43.73±9.23		51.83±11.19	53.16±10.29		50.98±10.96	<0.001*	[12]
Kasr Al-Ainy Center. Cairo, Egipt (n=950)	48.9 ± 12.4				49.00 ± 11 (Obese)	53.06 ± 10.1 (Severely obese)	51.1 ± 11.5	<0.001*	[13]
Breast Cancer Registry (BCR), Japan (n=20090)	54.8 ±14.7	54.0 ±12.9 (Normal low)	58.6 ±21.1 (Normal high)	60.9 ±11.9	59.7 ±11.9		57.3 ±12.8	-	[14]
Kaiser Permanente Colorado and Washington. USA (n=6481)	-	60.2 ± 12.7		62.0 ± 11.9	61.4 ± 10.7		61.2 ± 11.8	-	[15]

health care system, with a wide range of providers from both the public and private sector and 95% of the Egyptian population lives within 5 km range of a health institution.[12] Cardiovascular diseases are the major cause of mortality (40%) followed by cancer (13%); obesity has a prevalence rate of 31%.[21] The study performed by Ibrahim and colleagues stratified obese patients in grade I (obese) and grade II (severely obese) and reported a statistical significance in age at diagnosis. Severely obese patients were diagnosed even later than obese patients.[13]

Japan is the third-largest developed economy with the world's highest life expectancy. Japanese population choses the health care facility covered by a universal insurance program which translates in a good quality at low-cost health care.[25] Obesity prevalence is extremely low (4%) but on the other hand, cancer is a leading cause of mortality in the country (30%), higher that cardiovascular diseases (27%).[21] Using data from the Breast Cancer registry from the Japanese Breast Cancer Society, Kawai study included 20 090 patients stratified by BMI. In this study, age at diagnosis was proportionally increasing across the BMI categories with slight variations.[14]

United States of America (US) has the world's largest national economy and possesses a unique and complex health national system. Most Americans are covered by private health insurance and the Affordable Care Act implemented by previous president Barack Obama in 2010 has yet failed to achieve national coverage[26]. Obesity is a public health issue and presents the highest incidence rate of the included countries in this review (37%).[21] Cardiovascular diseases surpass cancer in mortality rates (cardiovascular deaths 30% *vs* cancer deaths 22%) although female breast cancer is the most prevalent and the second cause of cancer-related death followed by lung cancer.[23] Feigelson study observed that overweight and obese were also diagnosed later than normoponderal patients, curiously underweight patients are approximately diagnosed at the same time as normal-low weight.[15]

The selected studies observed a trend in the pattern of obese patients' diagnosis, despite of economy, health systems, prevalence rates or environmental traits.

Are breast cancer screening programs failing in obese individuals?

In Portugal, cancer screening programs are implemented to breast, cervical, colon and rectum cancer. In 2020, Breast cancer screening covered 78% of national geographic territory, with high adhesion rate (62%). Although the number of screened women in 2020 decreased due to the Covid-19 pandemic restrictions. Breast Cancer screening program exposed 0.6% primary positive results (1053 women).[27]

Breast cancer screening in the Arabian countries is still scarce, few Arab countries (Saudi Arabia, Qatar, Egypt, and the United Arab Emirates) have implemented a genetic program for familial cancer genes. Egypt launched a screening program for breast cancer in 2018 with the main objective of the early detection of breast cancer in 28 million women.[28]

Screening program in Belgium offers women between 50 and

69 years, a free mammogram every two years, and results prove that screened tumours exhibit a more favourable prognosis than interval tumours (tumours diagnosed during the 2-year period).[29]

Developed countries such as US, Japan, and Spain have implemented breast cancer screening programs with differences regarding recommendations for age, method, or period.[30] For instance, Japan implemented an early screening age because a high incidence rate was observed in women between 45 and 49 years-old.[31]

Then, why are screening programs failing in obese women all over the world, even in developed countries with solid, implemented screening programs? A systematic review from Graham and colleagues disclosed several barriers in obese women and breast cancer screening methods, either from the patient or the professional perspectives. Overall, women have reservations regarding mammography associated with fear of pain or embarrassment, but obese women reported additional reservations with body image perception, influence on attendance and negative experiences in the screening process. From the professional point of view, difficulties are associated with the impact of body size in the safety and efficiency of mammography, the transport requirements of obese individuals, safety of the professional associated with handling heavy bodies[32], and so, women with obesity are less likely to undergo screening breast cancer methods.[33] Obese individuals struggle with constant psychosocial and physical challenges that can result in a neglected health surveillance.

Is age at diagnosis associated with a more advance tumour stage?

Given that obese patients are diagnosed later, regardless of the reason, we further speculated if obese patients are diagnosed with a more advanced stage disease. To answer this question, we used the data from the DOC database. Age at diagnosis was considered the age of the first diagnosis and used as a continuous variable. Cancer staging (pathologic stage) was assessed accordantly to the American Joint Committee on cancer by tumour node metastasis (TNM) system and divided in 4 stages. With the 615 obese women included in the DOC database, we performed a statistical analysis by One Way ANOVA of age at diagnosis stratified accordantly to tumour stage (**Fig. 1**). Curiously enough, stage IV which represents a more advance disease is associated with a lower age at diagnosis, as previously reported.[34] Interestingly, higher age at diagnosis were observed in stage I and II breast cancer, revealing a statistically significant difference from stage IV (**Fig. 1**)

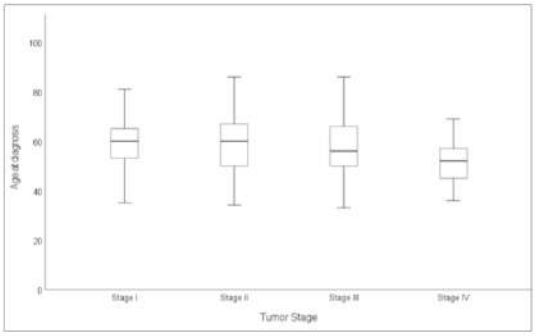


Figure 1. Boxplot representative of age at diagnosis from obese breast cancer patients stratified by tumour stage. Statistical evaluation was performed by One way ANOVA with multiple comparisons, p-value<0.05 was considered significant. Results reveal significance between Stage IV and Stage I (p-value=0.016) and between Stage IV and Stage II (p-value=0.024).

Discussion and Conclusion

To obtain a more thorough understanding on this matter, we sought for similar studies from different countries. We further analysed the economic conjuncture and health situation. Such analysis aimed to assess if lower economic status and health services deficits could be associated with the age at diagnosis of obese patients. Yet, no consistent results were observed. Countries with different economic situations present the same pattern. Also, obesity and cancer rates could be somewhat associated but again no trend was observed. Countries with different economic and health conditions exhibit identical results.

Therefore, we then evaluated national screening programs and observed that even countries with good and implemented screening programs exhibit the same results. On the other hand, obese patients are more reluctant to undergo mammographs which could mean a worst-case scenario and advanced disease, but again, that is not the case, as we observed that obese patients often develop stage I and II breast cancer.

The promptest speculation why breast cancer diagnosis occurs later in obese patients is the presence of a systemic low grade chronic inflammatory status in obese people may delay the progression of breast cancer, eventually by adapting the organism to this chronic inflammation condition. This so-called "obesity paradox" has been the focus of several studies mainly in cardiovascular diseases[35], but in oncology results are not consensual[36]. We also found literature supporting the hypothesis that other diseases related to obesity such as diabetes could further impact prognosis and survival of cancer patients.[37] In breast cancer, inflammation is being considered as a potential target for therapeutical intervention.[38]

Altogether, we weren´t able to find a clear answer supported by structured evidence to our question. We believe that further studies are needed to better understand the underlying mechanism behind these results. To the best of our knowledge, and supported on the results from DOC database, obesity seems to delay cancer development, understanding obese-cancer metabolism can highlight potential mechanisms relevant to breast oncogenesis.

We realise the limitations of this analysis and the aleatory effect of the studies choice. But we also encountered an important and transversal information worldwide. A prompt diagnosis is a cornerstone in breast cancer epidemiology and imperative in disease management.

Contributorship Statement / Declaração de Contribuição:

Carla Luís: Conceção do projeto de pesquisa; Pesquisa bibliográfica; Primeira versão do artigo; Revisão do manuscrito e aprovação final.

Rute Fernandes: Conceção do projeto de pesquisa; Pesquisa bibliográfica; Aquisição de dados; Revisão do manuscrito e aprovação final.

Deolinda Pereira: Conceção do projeto de pesquisa; Supervisão; Revisão do manuscrito e aprovação final.

Rúben Fernandes: Conceção do projeto de pesquisa; Supervisão; Revisão do manuscrito e aprovação final.

Raquel Soares: Conceção do projeto de pesquisa; Supervisão; Revisão do manuscrito e aprovação final.

Responsabilidades Éticas

Conflitos de Interesse: Os autores declaram não possuir conflitos de interesse.

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Proteção de Pessoas e Animais: As autoras declaram que os procedimentos seguidos estavam de acordo com os regulamentos estabelecidos pelos responsáveis da Comissão de Investigação Clínica e Ética e de acordo com a Declaração de Helsínquia revista em 2013. O estudo retrospetivo de onde foi extraída a base de dados "Deciphering Obesity and Cancer" (DOC) incluiu doentes diagnosticados IPO-Porto com aprovação da comissão de ética institucional.

Proveniência e Revisão por Pares: Não comissionado; revisão externa por pares.

Ethical Disclosures

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Protection of Human and Animal Subjects: The authors declare that the procedures were followed according to the regulations established by the Clinical Research and Ethics Committee and to the Helsinki Declaration of the World Medical Association updated in 2013. The retrospective study where "Deciphering Obesity and Cancer" (DOC) database was extracted included patients diagnosed at Comprehensive Cancer Centre in Portugal (IPO-Porto) with approval of the institutional ethics committee.

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Bilateral breast cancer and the influence of body mass index in clinicopathological features and overall survival

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Abstract.
BACKGROUND: Breast cancer (BC) and obesity are two closely associated pathologies with increasing incidence and mortality rates. Bilateral Breast Cancer (BBC) displays a low incidence rate within BC and obesity represents a major risk factor.
OBJECTIVE: The aim of this study is to analyzed BBC clinicopathological features distribution and determine the potential influence of obesity in BBC in these same features and overall survival.
METHODS: Clinicopathological information was obtained from 42 cases of women with BBC diagnosed in IPO-Porto. To evaluate the frequency distribution of the clinicopathological data, a chi-square goodness of fit test was performed for BBC cases. A chi-square test of independence was applied for BMI stratification. Cox regression was performed for overall survival. Statistical significance was set at p-value < 0.05.
RESULTS: Distribution of BBC clinicopathological features was found to be statistically significant in family history (p-value < 0.001), BBC type (p-value < 0.001), stage (p-value = 0.005), differentiation grade (p-value < 0.001), receptor expression (p-value < 0.001) and histological type (p-value = 0.031). In comparison to the statistical expected results, we observed an increased cases of absence of family history and less cases of metachronous BBC. Histological types between tumours of BBC were mostly concordant. All cases presented concordant receptor expression. Analysis stratified by BMI revealed that obese women were diagnosed later, although without statistical significance. All obese women presented poor differentiation grade (n = 6). Overweight patients display a tendency to a better overall survival with lower tumour stages and lower differentiation grades.
CONCLUSIONS: Our results reveal the same receptor expression between contralateral tumours. Also, most tumours share the same histological type. When stratified by BMI, we observed a tendency for overweight women to have improved overall survival.

Keywords: Bilateral breast cancer, obesity, obesity paradox, BMI, retrospective study

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1. Introduction

Bilateral breast cancer (BBC) is a rare condition of breast cancer (BC) characterized by the presence of tumours in both breasts, with an incidence rate that varies between 1.4% and 11.8% [1]. Women with one sided BC, designated Unilateral Breast Cancer (UBC) have increased risk, up to 6-fold magnitude, to develop BBC. Strong evidence suggests that BBC is a histological, molecular, biological, and genetic independent pathology different from metastatic UBC [2]. Additionally, BBC is usually associated with poorer prognosis [3]. BC risk factors include age, genetic background, alcohol, tobacco consumption, and obesity [4]. Obesity is defined by excessive accumulation of adipose tissue and is usually stratified according to the body mass

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index (BMI) [5]. The association between obesity and breast cancer evolves, among others, endogenous hormonal levels, namely estrogen with influence of aromatase, an enzyme expressed in the adipose tissue able to convert androgens into estrogen [6]. Epidemiological results regarding BBC and obesity are scarce and not always concordant but usually obesity is categorized as a major risk factor [7]. A study from Brooks and colleagues in metachronous contralateral breast cancer (diagnosed after 12 months from the first diagnosis), observed that only obese postmenopausal women with a first estrogen receptor-negative primary breast cancer presented higher risk to develop BBC comparing to normal-weight women [8]. Another study by Li et al, observed that only obese but not overweight demonstrated a significant increased risk to develop BBC [9]. Moreover, obesity negatively impacts the survival rates on BBC patients [10].

The aim of this study is to analyzed BBC clinicopathological features distribution and determine the potential influence of obesity in BBC in these same features and overall survival.

2. Materials and methods

2.1. Study population and ethical approval

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. This study was reviewed and approved by the Portuguese Institute of Oncology of Porto FG, EPE (IPO-Porto) Ethics Committee, approval number 202/020.

This study was performed with data from the "Deciphering Obesity and Cancer" (DOC) database, which included patients diagnosed at Comprehensive Cancer Centre in Portugal (IPO-Porto). Clinicopathological information was extracted from the institutional database on December 16th, 2021, including women diagnosed with breast cancer between 2012 and 2016.

2.2. Study datasets

BMI was calculated using the Quetelet index, measure up to 120 days after diagnosis and grouped in 3 categories: underweight and normal weight (BMI $< 25\ kg/m^2$), overweight ($25 \leq$ BMI $< 30\ kg/m^2$), and obese (BMI $\geq 30\ kg/m^2$) [5]. In age at diagnosis was considered the first diagnosis. Family history was based on the result of patient referral to an oncogenic follow-up according to National Comprehensive Cancer Network (NCCN) guidelines. Synchronous BBC was defined as any second cancer diagnosed within 12 months of initial diagnosis [11] and metachronous BBC with more than one year interval. Differentiation grade was classified according to the Nottingham Histologic Score System. Cancer staging (stage) was assessed accordantly to the American Joint Committee on cancer by tumor node metastasis (TNM) system [12]. Most advanced stage and higher differentiation grade were considered for analysis. It was also performed an analysis of the concordance between the tumours of both breasts.

2.3. Statistical analysis

Statistical analysis was performed using IBM® SPSS®, version 27 software. Normal distribution for age was checked using Kolmogorov-Smirnov with Lilliefors correction for each group and was applied the Kruskal-Wallis test. To evaluate the frequency distribution of the clinicopathological data, a Pearson's chi-square goodness of fit test was performed for BBC cases. A Pearson's chi-square test of independence was applied for BMI stratification. Cox proportional hazards for OS at 10 years, was performed to calculate hazard ratios with 95% confidence intervals for non-adjusted and adjusted models (Adjusted to Age at diagnosis, BBC type, Stage, Differentiation grade, and histological alteration). Statistical significance was set at p-value < 0.05.

3. Results

3.1. BBC characterization

From a total of 2282 cases with breast cancer included in the DOC database, 42 women presented BBC (1.8%). Table 1 displays the clinicopathological information of BBC cases. Synchronous BBC (sBBC) were more common than metachronous BBC (mBBC) (p-value < 0.001). BBC association with family history reach statistical significance with lower cases associated with family history (p-value < 0.001). We found a significant distribution in stage (p-value $= 0.005$) and differentiation grade (p-value < 0.001). There were also significant higher cases of estrogen receptor positive (p-value < 0.001), progesterone receptor positive (p-value < 0.001) and HER2 negative (p-value < 0.001). When comparing tumours between both breasts, we

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Table 1
Clinicopathological information of overall BBC population

	Total	*p*-value
	n (%)	
	42 (100%)	-
Age at diagnosis (median; IQR)	53.5 (47.0–68.0)	-
Family history		
No	33 (78.6%)	<0.001*
Yes	9 (21.4%)	
BBC type		
Synchronous	37 (88.1%)	<0.001*
Metachronous	5 (11.9%)	
Stage		
Stage I	10 (23.8%)	0.005*
Stage II	20 (47.6%)	
Stage III	7 (16.7%)	
Stage IV	5 (11.9%)	
Differentiation grade		
Grade 1	1 (2.4%)	<0.001*
Grade 2	20 (47.6%)	
Grade 3	21 (50.0%)	
Estrogen receptor		
Negative	3 (7.1%)	<0.001*
Positive	39 (92.9%)	
Progesterone receptor		
Negative	10 (23.8%)	<0.001*
Positive	32 (76.2%)	
HER2		
Negative	38 (90.5%)	<0.001*
Positive	4 (9.5%)	
BBC stage		
Concordant	19 (45.2%)	0.537
Discordant	23 (54.8%)	
BBC differentiation grade		
Concordant	22 (52.3%)	0.758
Discordant	20 (47.6%)	
BBC histological type		
Concordant	28 (66.7%)	0.031*
Discordant	14 (33.3%)	
BBC receptor expression		
Concordant	42 (100.0%)	#
Discordant	0 (0.0%)	

Legend: IQR – Interquartile range; *p-value < 0.05 – Statistically significant; #constant – no variation was observed.
Note: *P*-value compares observed and expected results. Expected results are distributed equally according with the number variables.

observed no significant differences in stage (p-value = 0.537) and differentiation grade (p-value = 0.758), but we found that histological type is mostly concordant between both tumours (p-value = 0.031), and receptor expression was the same in all included patients.

3.2. *Effect of BMI in BBC*

We further accessed data stratified by BMI, presented in Table 2. From the 42 cases, 14 were under/normal-weight, 22 were overweight and 6 were obese. Overweight and obese BBC cases were diagnosed later but without statistical significance (p-value = 0.230). None of the obese BBC cases presented tumours with stage I, and stage II was identified in 66.7% of obese patients, although the variable stage did not achieve statistical significance (p-value = 0.464). All obese women presented poorly differentiated tumours (p-value = 0.017).

Overweight women presented better outcomes for overall survival (OS) although without statistical significance (Fig. 1; p-value = 0.244). Hazard Ratio (HR) was calculated for each BMI categories: overweight women have a HR of 0.080 (Table 3; p-value = 0.105; 95% CI: 0.004–1.689) and obese women have HR of 1.429 (Table 3; p-value = 0.776; 95% CI: 0.123–16.635) comparing to under/normal-weight.

Considering the remarkable findings in the distribution of histological grade within different BMI categories, where a substantial proportion of overweight patients with breast cancer (BBC) were found to have grade 2 tumors, and exclusively grade 3 tumors were observed in obese BBC patients, we subsequently performed a Cox proportional hazard analysis stratified by tumor differentiation grade. However, the results did not attain statistical significance, as shown in Table 4. As anticipated, patients with grade 1 tumors demonstrated a more favorable prognosis, whereas those with grade 3 tumors exhibited the poorest prognosis.

4. Discussion

Bilateral Breast Cancer is considered an autonomous type, distinct from metastatic BC with poor prognosis by itself. But in association with major risk factors like obesity, outcomes can be much worse, resulting in a more aggressive phenotypes, lowering overall and disease-free survival and decreasing life quality of the affected patients. Our study focused on the analysis of BBC and the implications of obesity in its clinicopathological characteristics and overall survival.

Regarding the individual features of the patients, we observed that obese women are diagnosed later that under/normal-weight and overweight patients, a common finding in the literature [13,14]. Potential explanations for this result were previously discussed in

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Table 2
Clinicopathological information of BBC patients stratified by BMI

	Under/Normal-weight (BMI < 24.9 kg/m^2)	Overweight (BMI 25–29.9 kg/m^2)	Obese (BMI > 30 kg/m^2)	*p*-value
	n (%)	*n* (%)	*n* (%)	
	14 (33.3%)	22 (52.4%)	6 (14.3%)	-
Age at diagnosis (median; IQR)	51.0 (45.0–63.0)	51.5 (47.0–66.0)	70.0 (64.0–73.0)	0.230
Family history				
No	10 (71.4%)	17 (77.3%)	6 (100.0%)	0.353
Yes	4 (28.6%)	5 (22.7%)	0 (0.0%)	
BBC type				
Synchronous	13 (92.9%)	20 (90.9%)	4 (66.7%)	0.213
Metachronous	1 (7.1%)	2 (9.1%)	2 (33.3%)	
Stage				
Stage I	4 (28.6%)	6 (27.3%)	0 (0.0%)	0.464
Stage II	6 (42.9%)	10 (45.5%)	4 (66.7%)	
Stage III	1 (7.1%)	5 (22.7%)	1 (16.7%)	
Stage IV	3 (21.4%)	1 (4.5%)	1 (16.7%)	
Differentiation grade				
Grade 1	0 (0.0%)	1 (4.5%)	0 (0.0%)	0.017*
Grade 2	5 (35.7%)	15 (68.2%)	0 (0.0%)	
Grade 3	9 (64.3%)	6 (27.3%)	6 (100%)	
Estrogen receptor				
Negative	1 (7.1%)	1 (4.5%)	1 (16.7%)	0.593
Positive	13 (92.9%)	21 (95.5%)	83.3%)	
Progesterone receptor				
Negative	5 (35.7%)	3 (13.6%)	2 (33.3%)	0.266
Positive	9 (64.3%)	19 (86.4%)	4 (66.7%)	
HER2				
Negative	12 (85.7%)	21 (95.5%)	5 (83.3%)	0.508
Positive	2 (14.3%)	1 (4.5%)	1 (16.7%)	
BBC stage				
Concordant	7 (50.0%)	11 (50.0%)	1 (16.7%)	0.316
Discordant	7 (50.0%)	11 (50.0%)	5 (83.7%)	
BBC differentiation grade				
Concordant	7 (50.0%)	13 (59.1%)	2 (33.3%)	0.522
Discordant	7 (50.0%)	9 (40.9%)	4 (66.7%)	
BBC histological type				
Concordant	11 (78.6%)	12 (54.5%)	5 (83.7%)	0.213
Discordant	3 (21.4%)	10 (45.5%)	1 (16.7%)	
BBC receptor expression				
Concordant	14 (100.0%)	22 (100.0%)	6 (100.0%)	#
Discordant	0 (0.0%)	0 (0.0%)	0 (0.0%)	

Legend: IQR – Interquartile range; *p-value < 0.05 – Statistically significant; #constant – no variation was observed.

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a narrative review [15], from which we highlight the obese women reluctance to attend screening programs, the influence of inflammation obesity-related and other obesity-associated pathologies, such as diabetes.

Previous studies reported that heritability, namely BRCA1/2 mutations, is a risk factor for BBC [16]. Our results revealed a significant different distribution with lower cases of presence of family history in women with BBC. We also observed that none of the obese women included in this study presented family history.

Bilateral Breast cancer can be classified according to the time lapse between first and second diagnosis.

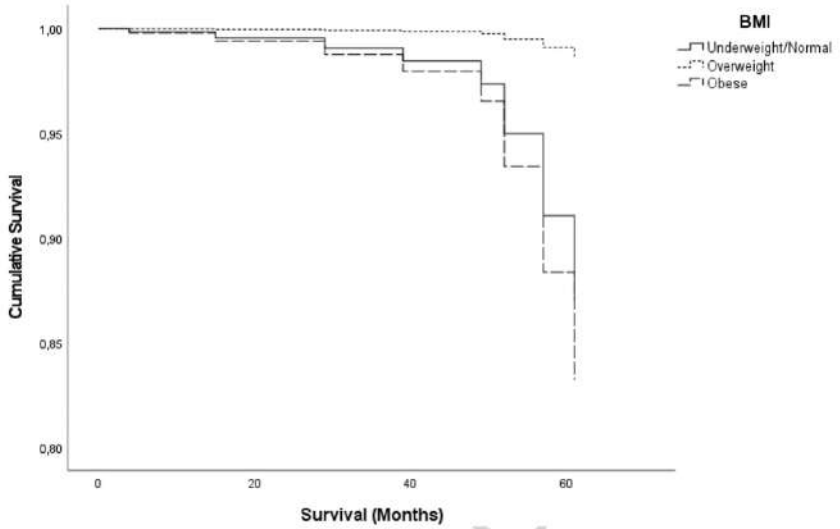


Fig. 1. Cumulative survival of BBC cases stratified by BMI using a Cox proportional-hazard adjusted model for age at diagnosis, BBC type, Stage, Differentiation grade, and histological alteration. No statistical alterations were found, nonetheless overweight patients presented a better overall survival.

Table 3
Cox proportional hazard models for overall survival stratified by BMI

	Non-adjusted				Adjusted[§]			
	p-value	HR	95% CI		*p*-value	HR	95% CI	
			Lower	Upper			Lower	Upper
BMI	0.234	-	-	-	0.244	-	-	-
Under/Normal-weight	-	1	-	-	-	1	-	-
Overweight	0.128	0.267	0.049	1.461	0.105	0.080	0.004	1.689
Obese	0.832	1.202	0.220	6.571	0.776	1.429	0.123	16.635

[§] Adjusted to Age at diagnosis, BBC type, Stage, Differentiation grade, and histological alteration. Legend: HR – Hazard ratio; CI – Confidence interval.

Table 4
Cox proportional hazard models for Overall survival stratified by differentiation grade

	Non-adjusted				Adjusted[§]			
	p-value	HR	95% CI		*p*-value	HR	95% CI	
			Lower	Upper			Lower	Upper
Differentiation grade	0.361	-	-	-	0.786	-	-	-
Grade 1	-	1	-	-	-	1	-	-
Grade 2	0.968	4724.2	0.000	9E184	0.982	34.8	0.000	2E132
Grade 3	0.964	15151.2	0.000	3E185	0.977	76.7	0.000	5E132

[§] Adjusted to Age at diagnosis, BBC type, Stage, BMI, and histological alteration. Legend: HR – Hazard ratio; CI – Confidence interval.

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Different authors classified mBBC at different temporary milestones (within 3, 6 or 12 months), this study stipulated one-year time lapse according to Al-Jurf reference [17]. From the original DOC database, we observed an incident rate of 0.002% for mBBC and 1.6% for sBBC. Although sBBC is within the range of previous reports (approximately 2%) [18,19], we found that studies in mBBC with a higher follow (15 years) incidence was considerably lower (0.002% vs 0.03–1%) [17]. Within the cases of BBC, we found a significant distribution with more sBBC cases, but when stratified by BMI no significant differences were achieved.

As for tumour characteristics, we observed a significant different distribution of stage and differentiation grade in the enrolled BBC cases. Most tumours were positive for estrogen and progesterone receptor and negative for HER2, concomitant with the literature [20] but with no further evidence when stratified by BMI. We found statistical significance in differentiation grade stratified by BMI as expected [21]; all obese patients were associated with poorly differentiated tumours. In addition, among overweight patients with BBC, a substantial proportion were diagnosed with grade 2 tumors. These findings might prompt speculation that overall survival outcomes could be closely associated with tumor differentiation grade rather than BMI status. However, subsequential analysis did not reveal any statistically significant relationship between overall survival and differentiation grade, as demonstrated in Table 4. It is worth noting that the differentiation grade reported in Table 2 represents the highest grade observed in each patient, when we assessed the concordance of differentiation grade between both tumors, we found no evidence to suggest that the bilateral occurrence is driven by early metastasis to the contralateral breast.

In a comparative analysis between tumours of both breasts, we disclosed a significant distribution in women with BBC with the same histological type and molecular subtype. All BBC cases presented the same receptor status expression, and most cases presented the same histological type. Given that both molecular and histological basis are mostly the same in BBC, we raise the controversy again whether BBC is such a detached pathology from metastatic BC. Some authors, like Chaudary, defend that contralateral breast cancer can be divided in a metastatic lesion or a primary tumour [22]. But up until now, there is no clinically implemented analysis to differentiate a relapse from a second first tumour. A genomic study on bilateral primary breast cancer was unable to find specific germline and somatic mutations in BBC [23]. Given the lack of specific biomarkers, classification usually only considers the immunohistochemistry profile and the presentation pattern. Such differentiation could be important in the implementation of the treatment strategy.

Finally, overall survival analysis revealed that overweight women presented better prognosis than obese and under/normal-weight women. This is an interesting finding since most reports associate obesity/BC with lower OS and disease-free survival [24] but controversy is implemented regarding this association with the concept of the obesity paradox. It is proposed by the obesity paradox that overweight and mild obese individuals present better outcomes due to a hypothetical protective role of obesity. Obesity paradox was already described in pathologies like cardiovascular disease [25] and some types of cancer including breast cancer [26]. Many authors propose that BMI should not be the clinical indicator for obesity since it does not translate the metabolic health of the individual. Such issue should be further addressed in the scientific community to better understand when and how obesity can display friend or foe features in cancer.

5. Conclusion

We observed interesting findings in BBC overall results and when stratified by BMI, from which we highlight overall survival and tumour characteristics. Our results show that sBBC is more common than mBBC, and statistical significance was achieved in the population sample distribution regarding tumour stage and differentiation grade. Moreover, when stratified by BMI categories, all obese BBC cases presented poorly differentiated tumours. While the distribution of tumor grades among overweight and obese BBC patients is noteworthy and prompts speculation about its impact on survival, the study's analysis did not establish a statistically significant relationship. Bilateral tumours shared the same molecular subtype and mostly the same histological type. Overweight patients tend to have better OS with lower tumour stages and lower differentiation grades. Obesity and cancer have been closely associated. Obesity is described as major risk factor in tumorigenesis and tumour progression, although this concept is highly debated. Some authors defend that the obesity paradox can have considerable influence in tumour progression [27,28] and BMI should not be the only indicator for adiposity measurement because

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it does not represent the metabolic health of the individual, moreover, the concept of "healthy obese" is nowadays more common to find between the clinical and academic communities. We acknowledge the limitations of the study regarding mainly the low number of patients enrolled, which weakens the statistical analysis and also the time follow up which can compromise the accuracy of the data with regard to the metachronous BBC.

Further research is needed to better address the role of obesity in BBC development.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability statement

Data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Author contributions

Conception: Carla Luís, Ruben Fernandes, Raquel Soares; Supervision: Ruben Fernandes, Raquel Soares; Analysis of data: Carla Luís; João Firmino-Machado; Pilar Baylina; Interpretation of data: Carla Luís, João Dias, Rute Fernandes; Deolinda Pereira; Revision for important intellectual content: All authors; The first draft of the manuscript was written by Carla Luís, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Does body mass index influence surgical options and overall survival in breast cancer patients?

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Abstract
Obesity is a relevant risk factor in breast cancer (BC), but little is known about the effects of overweight and obesity in surgical outcomes of BC patients. The aim of this study is to analyse surgical options and associated overall survival (OS) in overweight and obese women with BC. In this study, 2143 women diagnosed between 2012 and 2016 at the Portuguese Oncology Institute of Porto (IPO-Porto) were included, and the clinicopathological information was retrieved from the institutional database. Patients were stratified by body mass index (BMI). Statistical analysis included Pearson's chi-squared test with statistical significance set at $p < 0.05$. Multinomial, binary logistic regression and cox proportional-hazards model were also performed to calculate odd ratios and hazard ratios with 95% confidence intervals for adjusted and non-adjusted models. The results revealed no statistical difference in histological type, topographic localization, tumour stage and receptor status and in the number of surgical interventions. Overweight women have increased probability to be subjected to sentinel node biopsy. Obese and overweight women are more likely to be submitted to conservative surgery and contrariwise, less likely to undergo total mastectomy. Patients submitted to conservative surgery and not submitted to total mastectomy had a favourable OS although without statistical significance. No significant differences were observed in OS when stratified by BMI. Our results revealed significant variations regarding the surgical options in overweight and obese patients, but these were not translated in OS difference. More research is recommended to better address treatment options in overweight and obese BC patients.

Keywords Breast cancer · Obesity · Surgery · Mastectomy · Sentinel node biopsy · Axillary dissection

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Introduction

Breast cancer (BC) is a complex heterogeneous pathology with high incidence and mortality rates amongst women. Therapeutical strategies are associated not only with tumour biology (like molecular subtype, differentiation grade, histological type, lymphovascular invasion, etc.) but also with the extent of the disease, breast size, tumour stage, and patient preferences [1]. Treatment options includes chemo and radiotherapy, endocrine therapy with or without targeted biomarkers, however, surgery remains the cornerstone of BC treatment and is, in most cases, the number one option [1]. The evolution of BC surgery has evolved from radical mastectomy as the only surgical treatment to a more comprehensive approach with several alternative conservative methods, which comprises an aesthetic basis [2].

Mastectomies are the upmost used procedures, conservative surgery (CS), also designated partial mastectomy,

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consists in the removal of the malignant tissue and surrounding margins with satisfactory results regarding long-term survival and aesthetical effect [3]. CS is considered a safe procedure, associated with low rates of local recurrence and distant metastasis, low positive margins rate and low surgical complications [4]. The total mastectomy (TM) implies the excision of all breast tissue, skin, nipple, and areola whilst radical mastectomy (RM) includes the same procedures with additional excision of chest wall muscles and lymph node dissection. Previous results showed no survival advantage of radical mastectomy over total mastectomy with or without radiation therapy in a clinical follow-up of 25 years [5]. Another surgical alternative is mastectomy with immediate reconstruction (MR), which is associated with considerable improvement on aesthetic outcomes [6]. Surgical approaches also include sentinel node biopsy (SNB) and axillary dissection (AD). A previous study focussing on patients with BC with N1 positive sentinel nodes found no significant differences in overall and free specific survival in patients submitted to adjuvant axillary radiotherapy as compared to AD. SNB with adjuvant therapy seems to promote secure regional control [7], without the AD-associated co-morbidities [8].

Discrepancies associated with psychosocial factors [11], racial differences [9, 10], and body mass index (BMI) [11] are described as potential modulators in the selection and response to the surgical procedure. Numerous results have revealed that obesity is a depraved risk factor in BC [12]. Regarding surgical outcomes, it was already reported that obese women have more surgical complications and are less likely to be submitted to breast reconstruction [11]. Also, obese women are associated with decreased sentinel node identification due to failure in map rates [11]. To better addressed the association between surgery strategies in obese and overweight women, we performed a retrospective study associating surgery metrics and overall survival (OS) to uncover potential associations.

Materials and methods

Study population and ethical approval

This study was conducted according to the national and international ethical recommendations and approved by the Ethics Committee of the Portuguese Institute of Oncology of Porto FG, EPE (IPO-Porto). Clinicopathological and demographic information from women diagnosed in IPO-Porto between 2012 and 2016 were extracted from the institutional database of Outcomes Research Lab. Collected data was compiled in a database titled "Deciphering Obesity and Cancer" (DOC). For this study, women without surgical information were excluded from this study.

Methods

BMI was calculated using the Quetelet index measured up to 120 days after diagnosis. BMI was grouped in 3 categories: normal (BMI between 18.5 and 25 kg/m^2), overweight (BMI between 25 and 30 kg/m^2), and obese (BMI above 30 kg/m^2) [13]. Histological types were grouped in "invasive ductal carcinoma" (IDC), which included invasive ductal carcinoma with other types of carcinomas, "invasive lobular carcinoma" (ILC), which included invasive lobular carcinoma with other types of carcinomas and "other histological types". Other types of carcinomas refers to the classification of tumours of the breast by the World Health Organization (WHO) and include Apocrine adenoma, Metaplastic carcinoma, Invasive micropapillary carcinoma, amongst others [14]. Hormonal receptors were classified as positive or negative, cut-off of 1% of tumour positive cells. HER2 status was assessed by immunohistochemistry and validated by fluorescence in situ hybridization (FISH) according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines [15]. Immunohistochemistry for receptor status was performed at the pathology department according to standard protocols. Tumour stage was assessed according to the 7th edition of the American Joint Committee on cancer by tumour node metastasis (TNM) system [16]. For topographic localization, cases were divided in the four quadrants—inferior outer quadrant (IOQ), inferior inner quadrant (IIQ), superior outer quadrant (SOQ), superior inner quadrant (SIQ), other localizations including nipple, skin, central quadrant, and axillary extension (Other) and overlapping lesions (Multiple). OS was measured in months, from the date of diagnosis till death date or date of the study end (16 December 2021). The surgery analysis included 6 types of surgical intervention: Sentinel Node Biopsy (SNB), Axillary Dissection (AD), Conservative Surgery (CS), Total Mastectomy (TM), Radical Mastectomy (RM), and Mastectomy and Reconstruction (MR). Quantification of the number of surgeries consisted in the sum of surgical procedures, independently of the date.

Statistical analysis

Statistical analysis was performed using IBM® SPSS®, version 27 software. Descriptive statistics was used for data description. Age at diagnosis as a continuous variable was presented as mean ± standard deviation and range, tested for normality with Shapiro–Wilk test and analysed by one-way Anova. The first approach consisted in a Pearson's chi-squared test to analyse the association between BMI and variables. Significance was set at

$p < 0.05$. Binary, multinomial logistic regression and cox proportional-hazards model were used to compute odd ratios (OR) and hazard ratios (HR) with 95% confidence intervals (CI) in a non-adjusted model and adjusted for age at diagnosis, histological type, stage at diagnosis, and receptor status. Cox proportional-hazard analysis were based in the follow-up time from date of diagnosis until date of event or censoring, death, or end of follow-up

Table 1 Clinicopathological and surgical information with statistical analysis of breast cancer patients stratified by body mass index categories

	Normal-weight (BMI 18.5–24.9)	Overweight (BMI 25–29.9)	Obese (BMI > 30)	Total	*p* value
	N	*n*	*n*	*N*	
BMI distribution	734 (34.2%)	807 (37.7%)	602 (28.1%)	2143 (100%)	–
Age					
Mean ± SD	51.8 ± 11.9	56.9 ± 11.0	58.8 ± 9.9	55.7 ± 11.4	**< 0.001***
Range	24–87	27–88	33–86	24–88	
Histological type					
IDC	593 (80.8%)	670 (83.0%)	477 (79.2%)	1740 (81.2%)	0.076
ILC	59 (8.0%)	71 (8.8%)	48 (8.0%)	178 (8.3%)	
Other	82 (11.2%)	66 (8.2%)	77 (12.8%)	225 (10.5%)	
Receptor status					
ER + /PR +	426 (58.0%)	482 (59.7%)	388 (64.5%)	1296 (60.5%)	0.056
ER + /PR-	76 (10.4%)	63 (7.8%)	42 (7.0%)	181 (8.4%)	
HER2 +	138 (18.8%)	164 (20.3%)	93 (15.4%)	395 (18.5%)	
Triple negative	84 (11.4%)	85 (10.5%)	65 (10.8%)	234 (10.9%)	
Missing data	10 (1.4%)	13 (1.6%)	14 (2.3%)	37 (1.7%)	
Pathological tumour stage					
Stage I	320 (43.6%)	340 (42.1%)	226 (37.5%)	886 (41.4%)	0.137
Stage II	274 (37.3%)	311 (38.6%)	236 (39.2%)	821 (38.3%)	
Stage III	122 (16.6%)	144 (17.9%)	127 (21.1%)	393 (18.3%)	
Stage IV	18 (2.5%)	11 (1.4%)	13 (2.2%)	42 (2.0%)	
Missing data	0 (0.0%)	1 (0.0%)	0 (0.0%)	0 (0.0%)	
Topographic localization					
SIQ	66 (9.0%)	81 (10.0%)	53 (8.8%)	200 (9.3%)	0.709
SOQ	223 (30.4%)	251 (31.1%)	177 (29.4%)	651 (30.4%)	
IIQ	35 (4.8%)	34 (4.2%)	29 (4.8%)	98 (4.6%)	
IOQ	53 (7.2%)	37 (4.6%)	36 (6.0%)	126 (5.9%)	
Other	35 (4.8%)	48 (6.0%)	34 (5.7%)	117 (5.5%)	
Multiple	322 (43.8%)	356 (44.1%)	273 (45.3%)	951 (44.3%)	
Number of surgeries					
1	122 (16.6%)	126 (15.6%)	107 (17.8%)	355 (16.6%)	0.899
2	450 (61.3%)	504 (62.5%)	362 (60.1%)	1316 (61.4%)	
3	130 (17.7%)	147 (18.2%)	114 (18.9%)	391 (18.2%)	
4	28 (3.8%)	27 (3.3%)	18 (3.0%)	73 (3.4%)	
5	4 (0.6%)	3 (0.4%)	1 (0.2%)	8 (0.4%)	
Surgical procedure					
SNB	513 (67.1%)	585 (70.4%)	411 (67.5%)	1509 (68.4%)	0.213
AD	163 (21.3%)	182 (21.9%)	144 (23.6%)	489 (22.2%)	0.740
CS	337 (44.1%)	448 (53.9%)	332 (54.5%)	1117 (50.7%)	**< 0.001***
TM	293 (38.3%)	257 (30.9%)	178 (29.2%)	728 (33.0%)	**< 0.001***
RM	156 (20.4%)	154 (18.5%)	132 (21.7%)	442 (20.0%)	0.373
MR	6 (0.8%)	1 (0.1%)	1 (0.2%)	8 (0.4%)	0.051

BMI, Body Mass Index; *SD*, Standard deviation; *SNB*, Sentinel Node Biopsy; *AD*, Axillary Dissection: *CS*, Conservative Surgery; *TM*, Total Mastectomy; *RM*, Radical Mastectomy; *MR* – Mastectomy and Reconstruction

*Statistical significance highlighted in bold (p value < 0.05)

(truncated at 10 years). Additional analysis was performed to CS and TM to calculate the level of significance for surgical options and stratified by BMI.

Results

From the 2143 women included, 734 (34.2%) were normal-weighted, 807 (37.7%) were overweighted and 602 (28.1%) exhibited obesity. Table 1 displays the clinicopathological and surgical information, with statistical analysis of BC patients stratified by BMI categories. A statistically significant association was found between BMI and conservative surgery, and BMI and total mastectomy. The results of clinicopathological parameters revealed no statistical significance between BMI and tumour features like histological type, receptor status, tumour stage and topographic localization. We also observed a positive correlation between BMI and age at diagnosis.

Number of surgical interventions

We did not observe any associations between BMI and the number of surgical procedures per patient (Table 2). There was no statistical evidence that overweight and obese patients are submitted to more surgical procedures. We found some inconsistencies between the number of surgeries and number of total surgical procedures. We observed that some types of surgical approaches were performed more than once on the same patient and variable surgical procedures is presented as number of patients submitted to that specific surgery. Reconstructive procedures or reinterventions due to surgical complications were not included in the number of surgeries.

Type of surgical procedures

Regarding surgical procedures, the statistical evaluation did not achieve any significant differences in AD, RM and MR. Overweight women have increased OR to undergo SNB (p value 0.038; OR 1.317; 95% CI 1.016–1.707). Not only overweight, but also obese women are more likely to be submitted to CS and less likely to undergo total mastectomy (Table 3). Obese patients have twice the risk to be submitted to CS (p value <0.001; OR 2.059; 95% CI 1.608–2.637) and half the risk to be submitted to TM (p value <0.001; OR 0.569; 95% CI 0.447–0.725). Overweight women with BC have a 78% increased risk to be submitted to CS (p value <0.001; OR 1.780; 95% CI 1.422–2.228) and a 35.2% decreased risk to be submitted to TM (p value <0.001; OR 0.648; 95% CI 0.521–0.805).

Overall survival

To further access the impact of surgical procedures in OS, we performed a Cox proportional-hazard model stratified by BMI exclusively and by CS and TM. No significant differences were achieved and when compared to the OS reference, no significant discrepancies were observed (Table 4).

Whilst Table 3 displays the results from analysis using 'surgery' as *strata*, Fig. 1 presents the results from independent statistical analysis for each condition. No statistical significance was achieved. We observed that survival curve favours women who were submitted to conservative

Table 2 Multinomial logistic regression for number of surgeries

$N^{\#}$		P value	OR	95% CI		P value	OR	95% CI	
				Lower	Upper			Lower	Upper
2	Normal-weight	–	1	–	–	–	1	–	–
	Overweight	0.570	1.084	0.820	1.434	0.414	1.152	0.820	1.620
	Obese	0.565	0.917	0.683	1.231	0.515	1.129	0.784	1.627
3	Normal-weight	–	1	–	–	–	1	–	–
	Overweight	0.605	1.095	0.777	1.543	0.265	1.240	0.850	1.808
	Obese	0.999	1.000	0.697	1.435	0.260	1.262	0.842	1.891
4	Normal-weight	–	1	–	–	–	1	–	–
	Overweight	0.818	0.934	0.521	1.675	0.665	1.150	0.612	2.161
	Obese	0.346	0.733	0.384	1.399	0.665	1.167	0.580	2.346
5	Normal-weight	–	1	–	–	–	1	–	–
	Overweight	0.679	0.726	0.159	3.312	0.895	1.113	0.227	5.465
	Obese	0.265	0.285	0.031	2.590	0.619	0.555	0.054	5.651

N, number of surgeries; *OR*, odd ratio; *CI*, confidence interval

[#]Reference category: 1 surgical intervention

[§]Adjusted to age at diagnosis, histological type, tumour stage and receptor status

surgery (Fig. 1a) and were not submitted to total mastectomy (Fig. 1d). BMI stratification presented a similar pattern with obese women presenting a lower OS (Fig. 1b, c, e, f).

Discussion and conclusions

Obesity is an important risk factor in tumourigenesis and tumour progression for several types of cancer including BC [17]. Obese BC metabolism is associated with many different mechanisms, since adipose tissue releases several modulatory factors like adipokines, cytokines, inflammatory mediators, free fatty acids, hypoxia inducible factors, and others that activate, promote, and mediate relevant metabolic pathways closely involved in tumour biology [17].

The complex connection between both pathologies results in harshest outcomes namely more aggressive cancers, decreased overall and disease-free survival, increased and severed postoperative complications [11]. Studies reported that women with obesity have increased risk to more extensive axillary dissection interventions [18], are less likely to undergo mastectomy [19] and reconstruction after mastectomy [12]. Moreover, patients with BC and obesity have extended clinical hospitalization [19], and serious surgery-related complications, such as infections, haemorrhages, wound dehiscence, prosthetic and flap loss, venous thromboembolism, lymphoedema and pneumonia [20].

Table 3 Crude and adjusted binary logistic regression for each surgical procedure

	Crude				Adjusted[§]			
	P value	OR	95% CI		*P* value	OR	95% CI	
			Lower	Upper			Lower	Upper
Sentinel Node Biopsy								
BMI Categories	0.213	–	–	–	0.114	–	–	–
Normal-weight	–	1	–	–	–	1	–	–
Overweight	0.260	1.135	0.910	1.416	**0.038***	**1.317**	**1.016**	**1.707**
Obese	0.524	0.927	0.734	1.170	0.244	1.180	0.893	1.560
Axillary dissection								
BMI Categories	0.740	–	–	–	0.991	–	–	–
Normal-weight	–	1	–	–	–	1	–	–
Overweight	0.871	1.020	0.803	1.297	0.932	0.989	0.769	1.271
Obese	0.459	1.101	0.853	1.422	0.967	1.006	0.766	1.321
Conservative surgery								
BMI Categories	**< 0.001***	**–**	**–**	**–**	**< 0.001***	**–**	**–**	**–**
Normal-weight	–	1	–	–	–	1	–	–
Overweight	**< 0.001***	**1.470**	**1.203**	**1.797**	**< 0.001***	**1.780**	**1.422**	**2.228**
Obese	**< 0.001***	**1.449**	**1.167**	**1.799**	**< 0.001***	**2.059**	**1.608**	**2.637**
Total Mastectomy								
BMI Categories	**< 0.001***	**–**	**–**	**–**	**< 0.001***	**–**	**–**	**–**
Normal-weight	–	1	–	–	–	1	–	–
Overweight	**< 0.001***	**0.703**	**0.571**	**0.867**	**< 0.001***	**0.648**	**0.521**	**0.805**
Obese	**< 0.001***	**0.632**	**0.503**	**0.795**	**< 0.001***	**0.569**	**0.447**	**0.725**
Radical Mastectomy								
BMI Categories	0.373	–	–	–	0.486	–	–	–
Normal-weight	–	1	–	–	–	1	–	–
Overweight	0.289	0.874	0.681	1.121	0.248	0.834	0.613	1.135
Obese	0.766	1.041	0.801	1.352	0.389	0.864	0.620	1.205
Mastectomy and reconstruction								
BMI Categories	0.100	–	–	–	0.319	–	–	–
Normal-weight	–	1	–	–	–	1	–	–
Overweight	0.080	0.151	0.018	1.253	0.164	0.217	0.025	1.867
Obese	0.139	0.202	0.024	1.682	0.403	0.387	0.042	3.584

OR, Odd ratio; *CI*, Confidence interval

*Statistical significance highlighted in bold (*p* value < 0.05)

[§]Adjusted to age at diagnosis, histological type, tumour stage and receptor status

The focus of our work was to evaluate the surgical options and outcomes of patients with normal weight, overweight and with obesity. Our results disclosure that overweight and obese female patients are more likely to be submitted to conservative surgery and less likely to undergo mastectomy. Moreover, women with overweight are more likely to be submitted to sentinel node biopsy.

Conservative surgery is usually prescribed in early tumour stages and in cases presenting low tumour-breast volume ratio. Also, it is recommended that CS should be planned to include an immediate reconstruction. The total mastectomy is reserved for patients with more aggressive tumours, invasive advanced carcinomas and when breast-tumour volume ratio does not allow to conserve the breast. The finding that overweight and obese women are more likely to undergo CS led us to hypothesize that the accumulation of adipose tissue observed in overweight and obese women lead to a low tumour-breast volume ratio. CS is also prescribed in early tumour stages. However, no significant differences in tumour stage were observed, and additionally, advanced tumour stages have a lower incidence rate in the obese category when compared to normal weight category. Regarding the results for SNB, the previous studies concluded that women with obesity present higher recurrence incidence and axillary dissection failure rates [18]. However, we did not find statistically significance in obese patients, but rather in overweight women. SNB represents one of the forms of axillary lymph node involvement evaluation that is mandatory for staging (N). The evaluation can be clinical (whenever suspicious nodes are present on the physical examination) or pathological evaluation (pN), which is the parameter considered in the staging (pTNM). Women undergoing TM (with lymph node emptying) are not submitted to SNB If the disease is more invasive, it will ultimately lead to AD. It is well documented that overweight shares common outcomes with obesity [21]. Another interesting observation is the negative result in radical mastectomy. Although without significance, overweight and obese BC patients are less likely to be submitted to radical mastectomy. Considering the obesity-increased tumour aggressiveness described in the literature, an increased OR between women with obesity and radical mastectomy would be expected.

We believe that our results enclosure new information regarding treatment choices in women with obesity, although we are aware of the study limitations. OS is a variable that comprises multiple influencing factors. On one hand, different adjuvant therapy strategies, which was not included because it was beyond the scope of our work. On the other hand, the presence of obesity-associated pathologies such as diabetes or cardiovascular complications. We also considered that the available data regarding disease-free-survival could be biased due to the constrictions derived from the pandemic condition. Moreover, we believe that the menopausal status is an important trait to be addressed, but we could not access this information. Furthermore, surgical interventions may also result from some subjective factors like patient's willing and doctors' preferences, which are impractical to include in the statistical analysis.

We concluded that yes, obesity influences surgical options, but not outcomes. We agree with the authors who

Table 4 Crude and adjusted Cox proportional-hazard model for overall survival

	Crude				Adjusted[§]			
	P value	HR	95% CI		*P* value	HR	95% CI	
			Lower	Upper			Lower	Upper
BMI (reference)								
BMI	0.270	–	–	–	0.360	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.264	1.186	0.880	1.598	0.282	1.183	0.871	1.605
Obese	0.115	1.303	0.937	1.811	0.173	1.270	0.901	1.790
Conservative surgery								
BMI	0.228	–	–	–	0.296	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.224	1.204	0.893	1.625	0.235	1.204	0.886	1.637
Obese	0.095	1.325	0.952	1.843	0.137	1.300	0.920	1.837
Total Mastectomy								
BMI	0.264	–	–	–	0.329	–	–	–
Normal	–	1	–	–	–	1	–	–
Overweight	0.254	1.190	0.882	1.606	0.257	1.194	0.879	1.622
Obese	0.112	1.307	0.939	1.819	0.156	1.283	0.909	1.812

HR, Hazard Ratio; *CI*, confidence interval

[§]Adjusted to age at diagnosis, histological type, tumour stage and receptor status.

state that obesity should be addressed in BC, since it does not only modulate tumour characteristics, but also unfolds surgical interventions. Some authors also defend the inclusion of bariatric surgery in disease management. It was already documented that BC risk decreases after bariatric surgery [22] since bariatric surgery outcomes include alterations in the breast tissue composition metrics [23] by lowering the breast density, which is another BC risk factor [24].

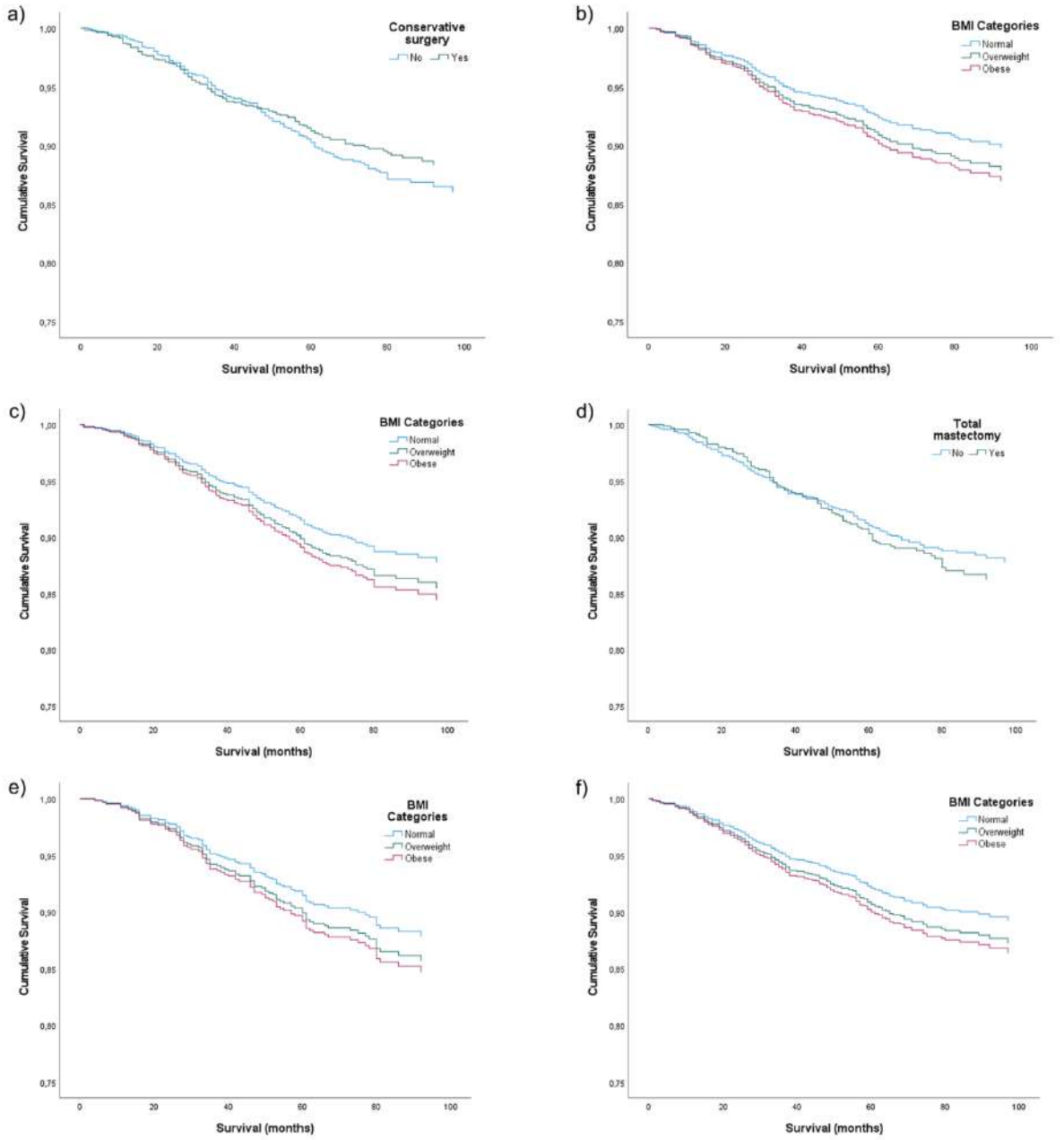


Fig. 1 Survival plot of Cox proportional-hazard model adjusted to age at diagnosis, histological type, tumour stage and receptor status. **a** Overall survival (OS) of conservative surgery intervention (p value = 0.296); **b** OS of conservative surgery intervention stratified by BMI (p value = 0.853); **c** OS of no conservative surgery stratified by BMI (p value = 0.346); **d** OS of total mastectomy intervention (p value = 0.329); **e** OS of total mastectomy intervention stratified by BMI (p value = 0.608); **f** OS of no total mastectomy intervention stratified by BMI (p value = 0.595)

Finally, we would like to stress that the implementation of targeted guidelines for patients with obesity is of upmost importance to improve recovery rates and life quality.

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Author contributions Conception and research design: CL, RF, RS; research and formal analysis: CL, JD, RF; deolinda sousa pereira; data analysis: CL; JF-M; PB; the first draft of the manuscript was written by CL, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated and/or analysed during the current study are not publicly available due to privacy or ethical restrictions.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The study was approved by the institution IPO-Porto ethical committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent Informed consent was obtained from the participants.

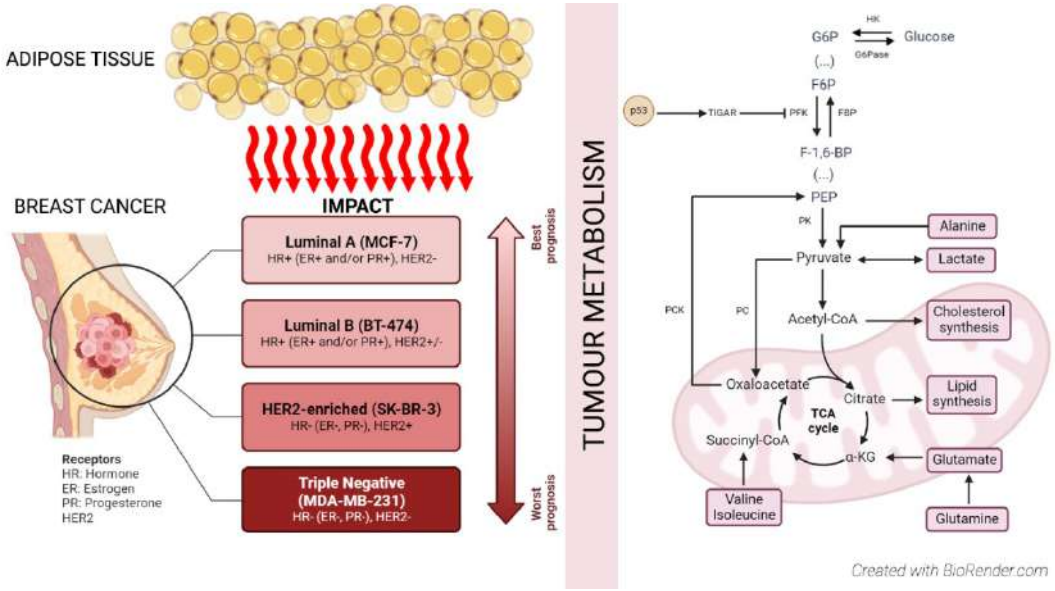
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CHAPTER IV

Cell-based approach to obesity and breast cancer



LIST OF CONTENTS:

- "The influence of adipocyte secretome on selected metabolic fingerprints of breast cancer cell lines representing the four major breast cancer subtypes."

cells

MDPI

Article

The Influence of Adipocyte Secretome on Selected Metabolic Fingerprints of Breast Cancer Cell Lines Representing the Four Major Breast Cancer Subtypes

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Abstract: Molecular subtype (MS) is one of the most used classifications of breast cancer (BC). Four MSs are widely accepted according to receptor expression of estrogen, progesterone, and HER2. The impact of adipose tissue on BC MS metabolic impairment is still unclear. The present work aims to elucidate the metabolic alterations in breast cancer cell lines representing different MSs subjected to adipocyte associated factors. Preadipocytes isolated from human subcutaneous adipose tissue were differentiated into mature adipocytes. MS representative cell lines were exposed to mature adipocyte secretome. Extracellular medium was collected for metabolomics and RNA was extracted to evaluate enzymatic expression by RT-PCR. Adipocyte secretome exposure resulted in a decrease in the Warburg effect rate and an increase in cholesterol release. HER2+ cell lines (BT-474 and SK-BR-3) exhibited a similar metabolic pattern, in contrast to luminal A (MCF-7) and triple negative (TN) (MDA-MB-231), both presenting identical metabolisms. Anaplerosis was found in luminal A and TN representative cells, whereas cataplerotic reactions were likely to occur in HER2+ cell lines. Our results indicate that adipocyte secretome affects the central metabolism distinctly in each BC MS representative cell line.

Keywords: breast cancer; obesity; molecular subtype; cancer cell metabolism; Warburg effect; cell culture

1. Introduction

Breast cancer (BC) is a complex and heterogeneous pathology with several classifications such as pathological and clinical tumor stage, histological type, differentiation grade, and molecular subtype (MS). Each of these classifications impacts prognosis and treatment strategies. One of the most influent classifications is the MS which includes the

expression assessment of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The stratification of receptor status distinguishes four main MSs of BC: luminal A, luminal B, HER2 positive (HER2+), and triple negative (TN) [1]. This is a decisive classification since treatment options highly depend on targeting the receptor. Luminal A and B share the common feature of being positive for both estrogen and progesterone receptor. Luminal A is characterized by high expression of ER and low expression of proliferation genes, conferring it a good prognosis and several therapeutical alternatives. On the other hand, luminal B usually expresses decreased levels of ER as well as PR when compared to luminal A, elevated levels of proliferation markers (such as Ki67) and of cell cycle-associated genes (such as G2/mitotic-specific cyclin B1—CCNB1, and MYB Proto-Oncogene Like 2—MYBL2) and may or not express HER2 [2]. HER2+ subtype is defined by the overexpression of HER2 (Neu/ErbB-2), which increases proliferation, modulates differentiation, and decreases apoptosis. It was previously associated with a poor prognosis, but new therapeutical agents have been introduced, improving the clinical outcomes of HER2+ patients [3]. TN subtype, also known as basal-like, does not express any of these receptors, which consequently leads to an unfavorable prognosis due to lack of targeted therapy. TN MS is usually associated with genetic instability for frequent mutations on TP53 gene and inactivation of the Rb pathways [4].

Adipose tissue accumulation is associated with ER and PR positive BCs and with worse prognosis [5]. Such accumulation leads to a metabolic impairment, responsible for a low chronic inflammation state, altered adipokine profile, extracellular matrix remodeling, and hormone oscillations [6], namely estrogen. Estrogen biosynthesis by aromatase, an enzyme expressed by the adipose tissue, is proposed to be the potential link, responsible for the increased BC risk in postmenopausal women [7]. Estrogens are responsible for several genomic and non-genomic mitogenic and mutagenic mechanisms, namely, DNA damage, oxidative stress, and high proliferative rates [5].

To accomplish these proliferative needs, cells shift their metabolism towards carbohydrate anabolic pathways and to the well-established Warburg effect, for nucleotides and NADPH biosynthesis, amino acids requirements, and basic energetics [8]. In the last decade, several deviations to the Warburg effect have been observed, namely the Warburg effect inversion [9]. This observation revealed, using cell-based and metabolomic assays, that under adipocyte secretome exposure, luminal A representative breast tumor cells (MCF-7) displayed an increased consumption of lactate and accumulation of glucose in the extracellular medium. Such an event was concomitant with an increase in cell proliferation, viability, and motility. These preliminary results paved the way to a more extensive investigation regarding the effect of adiposity conditions in BC metabolism, with all MSs included. The present study aims to analyze the effect of adipocyte secretome in BC by addressing enzymatic and metabolomic profiles of four different breast cancer cell lines representing the different MSs.

2. Materials and Methods

2.1. Ethical Approval

Adipose tissue was obtained in São João Hospital Centre, Porto, Portugal (CHUSJ) according to the protocol submitted to the Ethics committee of CHUSJ with the reference n. 226/2020. All subjects gave their informed consent. Procedures were executed in accordance with the international recommendations of Good Clinical Practice, the Declaration of Helsinki, and the applicable national legislation.

2.2. Adipocyte Differentiation

Subcutaneous adipose tissue was mechanically degraded and digested with collagenase (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 1.5 h. The stromal vascular fraction was washed and filtered to obtain isolated preadipocytes, which were cultured at a 5% CO_2 atmosphere at 37 °C on Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 17.5 mM

of glucose (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin/streptomycin (Invitrogen Life technologies, Carlsbad, CA, USA), 18 µM of pantothenic acid (Sigma-Aldrich, St. Louis, MO, USA), 100 µM of ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 16 µM of biotin (Sigma-Aldrich, St. Louis, MO, USA), and 10% newborn calf serum (NCS, Gibco, Grand Island, NY, USA). When preadipocytes reached confluency, they were differentiated into adipocytes. Cells were incubated for 3 days with DMEM/F12 medium supplemented with 17.5 mM of glucose, 1% penicillin/streptomycin, 18 µM of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin, 3% NCS, 10 µg/mL of insulin (Sanofi, Gentilly, France), 0.1 µM of dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM of 3-isobutyl-methyl-xanthine (Sigma-Aldrich, St. Louis, MO, USA), and 1 µM of rosiglitazone (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with DMEM/F12 medium supplemented with 17.5 mM of glucose, 1% penicillin/streptomycin, 18 µM of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin, 3% NCS, 10 µg/mL of insulin, and 0.1 µM of dexamethasone until the fully differentiated phenotype was reached, at day 9. Cell medium was changed every 2/3 days. The pool of adipocyte secretomes was collected from 7 assays performed from adipose tissue samples of different patients. The purpose of this approach was to control individual variability. Secretome was preserved in −80 °C until used as treatment. Photographs were taken for each time-point using Nikon TMS with attached camera Nikon D40× (Nikon, Tokyo, Japan).

2.3. Adiponectin Quantification

Adiponectin was used as an obesity-related biomarker in the differentiation of adipocytes [10]. Adiponectin was measured through a commercial ELISA kit to access differentiation grade (Cat. No. #KE00080, Proteintech, Rosemont, IL, USA). The assay was performed according to the manufacturer instructions.

2.4. Cell Culture

A representative breast cancer cell line of each molecular subtype was used according to the specifications described in Table 1. MCF-7, SK-BR-3, and MDA-MB-231 were obtained from the department cell biobank previously obtained from a commercial culture collection. BT-474 was obtained from i3S cell biobank.

Table 1. Specifications of cell lines used, representative of each molecular subtype [11].

Cell Line	Type	Molecular Subtype	Receptor Status
MCF-7	Invasive Ductal Carcinoma	Luminal A	ER+ PR+ HER2−
BT-474	Invasive Ductal Carcinoma	Luminal B	ER+ PR+ HER2+
SK-BR-3	Adenocarcinoma	HER2+	ER− PR− HER2+
MDA-MB-231	Adenocarcinoma	Triple Negative	ER− PR− HER2−

Legend: ER—estrogen receptor; PR—progesterone receptor; HER2—human epidermal growth factor receptor 2.

MCF-7 (HTB-22™, ATCC, Manassas, VA, USA), BT-474 (authenticated in i3S), SK-BR-3 (HTB-30™, ATCC, Manassas, VA, USA), and MDA-MB-231 (CRM-HTB-26™, ATCC, Manassas, VA, USA) were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen Life Technologies, Carlsbad, CA, USA) or 20% in BT-474 cell line, 1% penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA), and 3.7 g/L sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA)

and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Serum-free DMEM was used as control and treatments were performed using the secretome of differentiated adipocytes for 24 h. The nutrient composition of both cell media is presented in Supplementary Material—Table S1. Cell assays were performed in quintuplicate.

2.5. NMR (Nuclear Magnetic Resonance)-Based Metabolomics

After treatment with the secretome of differentiated adipocytes, culture media was collected and analyzed by Proton Nuclear Magnetic Resonance (^{1}H-NMR) for metabolite quantification. Samples were prepared by adding 45 µL of sodium fumarate (final concentration of 2 mM) in deuterated water solution to 180 µL of culture media. The samples were loaded into 3 mm NMR tubes (Norell, Landisville, NJ, USA) and analyzed using the NOR5X3INSB optimizer inserts (Norell, Landisville, NJ, USA). Solvent-suppressed 1D ^{1}H-NMR spectra were acquired on a Bruker Avance III HD 500 MHz spectrometer equipped with a 5-mm TXI probe (Bruker Corporation, Billerica, MA, USA), at 298 K. A zgpr pulse sequence was used and a total of 16 scans were acquired for each sample. After the acquisition, ^{1}H-NMR spectra were processed, and chosen metabolite peaks were integrated using the NUTS-Pro NMR software (Acorn NMR, Inc., Fremont, CA, USA). Sodium fumarate was used as an internal reference (6.50 ppm) for the quantification of the following metabolites (multiplicity, chemical shift): valine (doublet, 0.97 ppm), isoleucine (doublet, 1.00 ppm), alanine (doublet, 1.46 ppm), acetate (singlet, 1.90 ppm), glutamate (multiplet, 2.35 ppm), pyruvate (singlet, 2.35 ppm), glutamine (multiplet, 2.44 ppm), lactate (quartet, 4.10 ppm), and glucose (doublet, 5.22 ppm). Results were normalized to β-actin total RNA (Supplementary Material—Table S2). Metabolite consumption or release was determined for each condition by the difference between pre and post treatment.

2.6. Triglycerides and Cholesterol Quantification

Triglyceride quantification was determined by enzymatic colorimetric assay (Triglycerides reagent, Ref #OSR66118, Beckman Coulter®, Brea, CA, USA), on the AU 5800 analyzer (Beckman Coulter®, Brea, CA, USA) according to the manufacturer instructions and São João Hospital Center patronized protocol. Total cholesterol concentration was determined by an enzymatic, colorimetric method through a commercial kit (Liquick Cor-CHOL, Ref #2-205, Cormay, Varsóvia, Poland) according to the manufacturer protocol. Results were normalized to β-actin total RNA and presented as the difference between pre and post treatment.

2.7. RNA Extraction

After treatment, cells were suspended in RNA later (Invitrogen Life technologies, Carlsbad, CA, USA), and maintained at −20 °C. Afterwards, RNA later was removed by centrifugation. Cells were then washed and resuspended in 500 µL PBS with 7 µL of Poly(A) (Merck, Darmstadt, Germany). RNA extraction was performed using the Lab-Aid 824 s Nucleic Acid Extraction System (Zeesan, Xiang'an, China). Total RNA was quantified using Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA concentration was standardized to 5 µg/mL.

2.8. RT-PCR

Real time PCR was performed in sealed 96-well microplates using qTOWER^3 instrument (Analytik Jena, Jena, Germany) and a SYBR Green commercial kit (SensiFAST SYBR No-ROX One-Step MIX, Meridian Bioscience Inc., Cincinnati, OH, USA). Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) are described in Table 2, β-actin was used as housekeeping gene and enzyme isoform choice according to the literature [12]. PCR conditions were as follows: initial denaturation for 2 min at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. RT-PCR assays were performed in duplicate of each 5 cell replicates. Results were presented as fold increase of the control.

Table 2. Primer sequences used in the RT-PCR analysis.

Gene	Primer Sequence (5′-3′)
ACTB *	F—GACGACATGGAGAAAATCTG R—ATGATCTGGGTCATCTTCTC
HK-2	F—CTAAACTAGACGAGAGTTTCC R—CATCATAACCACAGGTCATC
PFK-L	F—GAAAGACACTGATTTCGAGC R—CATACTGATGCGGTATTGTG
PK-L/R	F—AAAATTGAGAACCACGAAGG R—CAATCATCATCTTCTGAGCC
PC	F—TTATGGTGCAGAATGGATTG R—TCAGTACCTTAGAGCGAAAG
PCK-2	F—GGCATTCGAGATTTTGTAGAG R—CAGCAGTTATTGTACTTGGG
FBP-1	F—CCAGATAATTCAGCTCCTTATG R—AGAAATATCCCTCCGTAGAC
G6P	F—ACTGTGCATACATGTTCATC R—TGAATGTTTTGACCTAGTGC

Legend: F—Forward; R—Reverse; *—Housekeeping gene; ACTB—β-actin; HK2—Hexokinase II; PFK-L—Phosphofructokinase—Liver; PK-L/R—Pyruvate Kinase—Liver and red blood cells; PC—Pyruvate Carboxylase; PCK-2—Phosphoenolpyruvate Carboxykinase—2; FBP-1—Fructose-1,6-bisphosphatase—1; G6P—Glucose-6-Phosphatase.

2.9. Statistical Analysis

Results were expressed as mean ± SD. Data were analyzed with GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Adipocyte differentiation was evaluated by ANOVA test. Metabolomic, lipid, and PCR results were evaluated by Student's *t*-test between controls and treatments. Significance was set at $p < 0.05$.

3. Results

3.1. Adipocyte Differentiation

Figure 1a–d displays the phenotypic alterations of adipocyte differentiation from day 3 to day 9. The adipocyte differentiation rate was quantified using adiponectin ELISA kit. Day 9 exhibited the highest differentiation rate (Figure 1e). We then used day-9 secretome in the next experiments. From day 9 onwards, no significant phenotypic differences were observed in the differentiation rate.

3.2. Carbohydrate Metabolic Profile

The levels of glucose as well as glycolysis end products, pyruvate, and their dual metabolites, lactate, and acetate are presented in Figure 2.

Under adipocyte secretome exposure, MCF-7 did not achieve significant alterations in glucose consumption nor in lactate and acetate production. Yet, our results show a decreasing trend for both lactate and acetate production when subjected to adipocyte secretome. Pyruvate variation was also not significantly affected by adiposity; however, our results point to a shift in pyruvate utilization from a consumption in the control condition, to a release under the influence of adipocyte secretome, although with a high standard deviation.

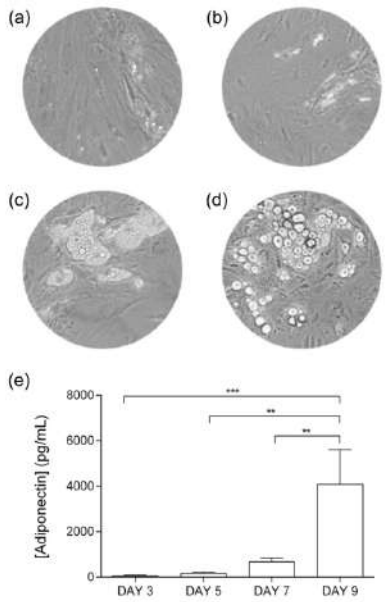


Figure 1. Phenotypic alterations of adipocyte differentiation at (**a**) day 3, (**b**) day 5, (**c**) day 7, and (**d**) day 9. Images were captured with 100x amplification. (**e**) Adiponectin quantification with a significant increase at day 9 (** p-value < 0.01; *** p < 0.001).

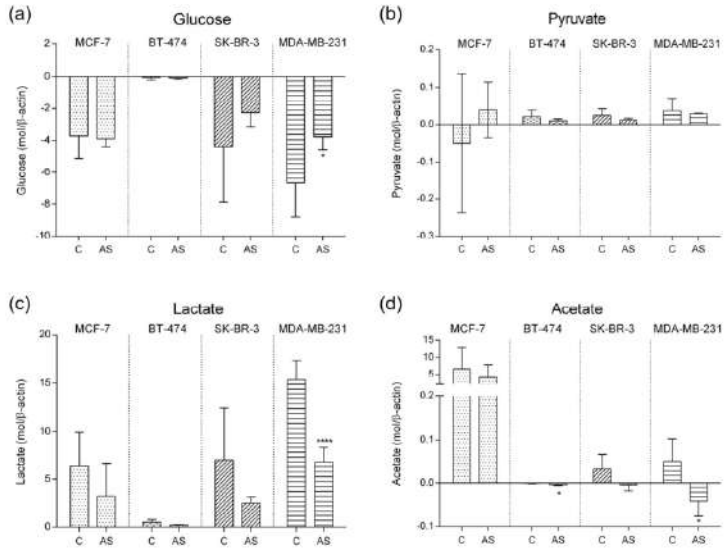


Figure 2. Metabolites concentration levels quantified in culture media by ^{1}H–NMR normalized to β-actin expression stratified by molecular subtype representative cell line. (**a**) Glucose (p-value MDA-MD-231 = 0.0213); (**b**) Pyruvate; (**c**) Lactate (p-value MDA-MD-231 < 0.0001); (**d**) Acetate (p-value BT-474 = 0.0238; p-value MDA-MD-231 = 0.0133). (* p-value < 0.05; **** p-value < 0.0001). Positive values represent metabolite production and negative values represent metabolite consumption from the media. Legend: C—Control; AS—Adipocyte secretome.

When compared to the other MS cell lines, luminal B representative cell line (BT-474) revealed lower levels of these four metabolites. Subjected to adipocyte secretome, these cells exhibited a significant increase in acetate consumption (p-value = 0.024).

Regarding SK-BR-3 cell line representing luminal B MS, no significant differences were observed in any of the metabolites, although there was a tendential decrease in glucose consumption followed by a decrease in pyruvate and lactate production under adiposity condition. The switch from acetate production, observed in the control condition, towards acetate consumption under adiposity environment was a noteworthy feature.

Finally, MDA-MB-231 cell line representing triple negative MS showed a significant decrease in both glucose consumption and lactate production under adiposity when compared to control. Under exposure to adipocyte secretome, these cells did not depend much on glucose uptake (p-value = 0.0213), resulting in a decrease in lactate production (p-value < 0.0001), implying that the Warburg effect in this aggressive cell line slowed down. A significant switch from acetate production in control conditions towards acetate consumption in adiposity conditions was also found (p-value = 0.0133). Altogether, these findings suggest that under exposure to secretome from adipocyte differentiation, the cell lines present distinct metabolic pathways.

3.3. Amino Acid Metabolic Pattern

Amino acids are used as protein building blocks as well as energy fuel in stress conditions. Figure 3 presents the profile of alanine, glutamine, glutamate, isoleucine, and valine levels in culture media.

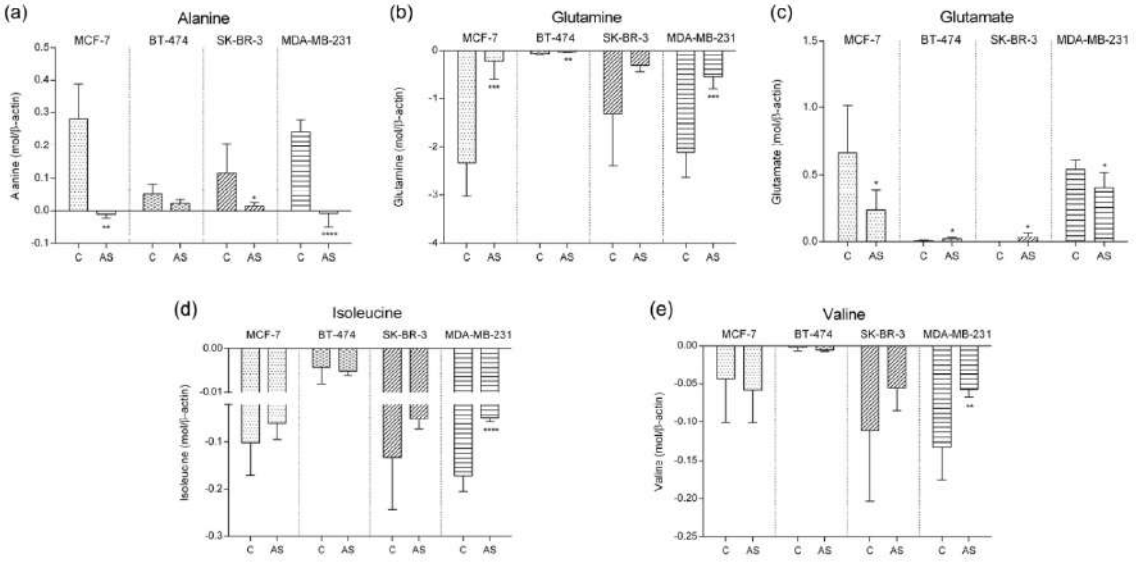


Figure 3. Amino acid concentration levels in culture media by ^{1}H–NMR normalized to β-actin expression stratified by molecular subtype representative cell line. (**a**) Alanine (p-value MCF-7 = 0.001; p-value SK-BR-3 = 0.0366; p-value MDA-MD-231 < 0.0001); (**b**) Glutamine (p-value MCF-7 = 0.0003; p-value SK-BR-3 = 0.0097; p-value MDA-MD-231 = 0.0003); (**c**) Glutamate (p-value MCF-7 = 0.0377; p-value BT-474 = 0.0167; p-value SK-BR-3 = 0.0464; p-value MDA-MD-231 = 0.0495); (**d**) Isoleucine (p-value MDA-MD-231 < 0.0001); (**e**) Valine (p-value MDA-MD-231 = 0.0052). (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001; **** p-value < 0.00001). Positive values represent metabolite production and negative values represent metabolite consumption from the media. Legend: C—control; AS—adipocyte secretome.

Alanine and pyruvate, for instance, can be directly interconverted. When exposed to adipocyte secretome, alanine metabolism was significantly altered in MCF-7, SK-BR-3, and MDA-MB-231 cell lines. Alanine shifted from production to consumption in MCF-7 (p-value = 0.0010) and MDA-MB-231 cell lines (p-value < 0.0001), while in SK-BR-3 cells (p-value = 0.0366) and BT-474, alanine release was reduced.

Glutamine consumption was significantly decreased in luminal A, luminal B, and TN representative cell lines when exposed to adipocyte secretome (p-value MCF-7 = 0.0003; p-value SK-BR-3 = 0.0097; p-value MDA-MD-231 = 0.0003). No significant difference was found in HER2+ representative cell line. This amino acid is directly converted into glutamate by glutaminase. Therefore, we next examined glutamate levels in our experiment.

Glutamate production in adipocyte secretome condition decreases significantly in luminal A (p-value = 0.0377) and TN (p-value = 0.0495), accompanying the decrease in glutamine consumption levels observed. Interestingly, glutamate release increases significantly in luminal B (p-value = 0.0167) and HER2+ (p-value = 0.0464) representative cell lines.

The role of branched amino acids, such as valine and isoleucine, is not clear in BC progression. We next investigated the level of these two amino acids in our study. No significant difference was found in any of the cell lines except for MDA-MB-231 TN cells, where these two amino acids were significantly less consumed after exposure to adipocyte secretome (p-value isoleucine < 0.0001; p-value valine = 0.0052).

3.4. Lipid Characterization

Triglycerides (TG) and cholesterol levels in culture media were then addressed (Figure 4).

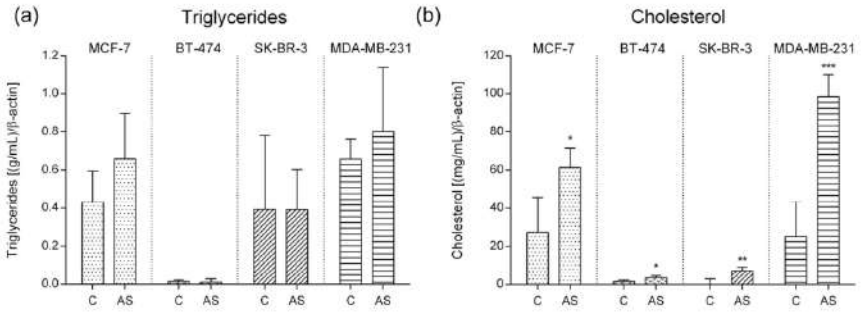


Figure 4. Lipid levels in culture media according to molecular subtype representative cell lines. (**a**) Triglycerides; (**b**) Cholesterol (p-value MCF-7 = 0.0179; p-value BT-474 = 0.0301; p-value SK-BR-3 = 0.0086; p-value MDA-MD-231 = 0.0005); (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001). Legend: C—control; AS—adipocyte secretome.

No differences were found in TG levels under adipocyte secretome exposure when compared to control, although a slight increase was observed in MCF-7 and MDA-MB-231.

In contrast, a significant increase in cholesterol release was observed in every MS representative cell line when subjected to adipocyte secretome (p-value MCF-7 = 0.0179; p-value BT-474 = 0.0301; p-value SK-BR-3 = 0.0086; p-value MDA-MD-231 = 0.0005).

3.5. Glycolytic and Gluconeogenic Enzymatic Fingerprints

To complement metabolomic data, we further examined glycolytic and gluconeogenic enzyme expression in the different cell lines subjected to adipocyte secretome (Figure 5).

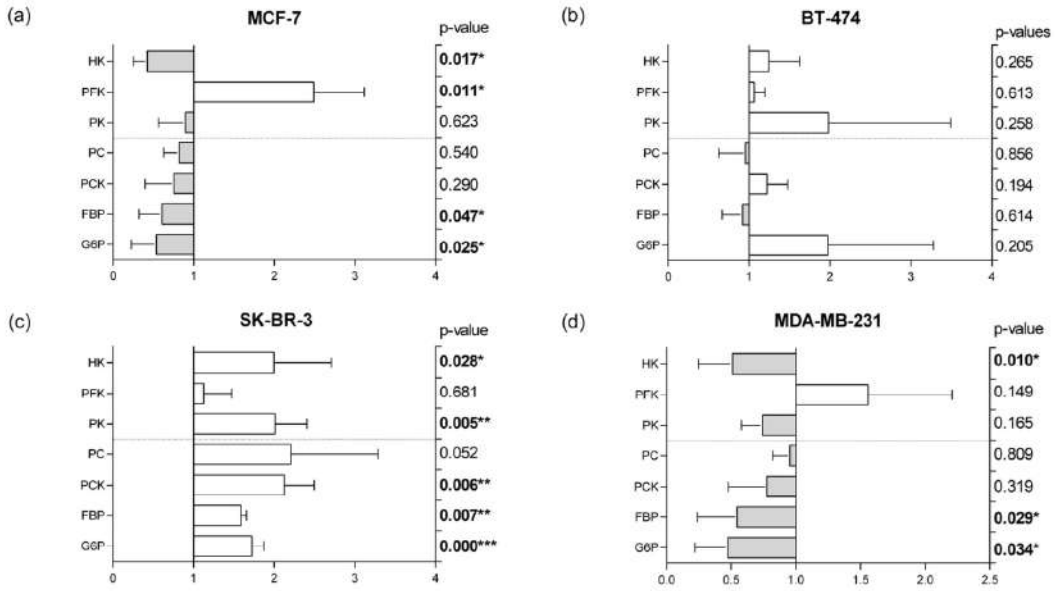


Figure 5. Enzymatic expression by RT-PCR of glycolytic and gluconeogenic patterns under adipocyte secretome exposure of the representative cell lines (**a**) MCF-7 for luminal A, (**b**) BT-474 for luminal B, (**c**) SK-BR-3 for HER2+, and (**d**) MDA-MB-231 for triple negative. Bars represent fold-increase relative to control; Positive fold-increase in white bars and negative fold-increase in grey bars. Legend: * Statistical significance highlighted in bold; Significance levels: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$; HK—Hexokinase; PFK—Phosphofructokinase; PK—Pyruvate kinase; PC—Pyruvate carboxylase; PCK—Phosphoenolpyruvate carboxykinase; FBP—Fructose-1,6-bisphosphatase; G6P—Glucose-6-phosphatase.

A distinct fingerprint for each MS can be visualized. BT-474 did not reveal any significant alteration in the expression pattern of both glycolysis and gluconeogenesis enzymes between control and treatment. In contrast, MCF-7 presented an increased expression of Phosphofructokinase (PFK) and a decreased expression of Hexokinase (HK), and of the enzymes that catalyze the late steps of gluconeogenesis: Fructose-1,6-bisphosphatase (FBP), and Glucose-6-phosphatase (G6P).

In contrast to MCF-7 fingerprint, SK-BR-3 displayed significantly increased levels of glycolytic and gluconeogenic enzymes: HK, Pyruvate kinase (PK), Phosphoenolpyruvate carboxykinase (PCK), FBP, and G6P. We also observed a tendency towards increased Pyruvate Carboxylase (PC) expression, but statistical significance was not achieved. PFK expression was not altered in SK-BR-3 cells.

Triple negative MDA-MB-231 representative cell line enzyme expression profile was similar to MCF-7, with a significant decrease in HK, FBP, and G6P and a decreasing trend in all other enzymes except PFK, which is increased under adipocyte secretome exposure.

3.6. TIGAR Expression Pattern

Given the results obtained in PFK expression, we further quantified the expression of TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR), an enzyme that modulates glycolysis by targeting PFK. Results are illustrated in Figure 6. TIGAR was significantly downregulated in MCF-7 in adiposity conditions. A similar pattern was observed in MDA-MB-231 cells, though without reaching statistical significance. Inversely, TIGAR expression was upregulated in BT-474 and in SK-BR-3, being statistically significant in SK-BR-3.

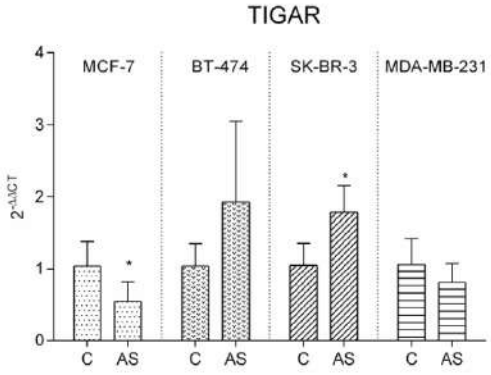


Figure 6. TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR) enzymatic expression in each representative cell lines by RT–PCR (p-value = 0.0475 for MCF-7; p-value = 0.0161 for SK-BR-3); (* p-value < 0.05). Legend: C—control; AS—adipocyte secretome.

4. Discussion

Carbohydrate metabolism and the Warburg effect in BC have been widely and extensively studied. However, the metabolic associated pathways are far from being fully understood. Tumor cell plasticity and the ability to use multiple metabolic pathways to increase survival advantage is acknowledged and recognized [13]. Recently, several deviations to the Warburg effect have been described such as the Reverse Warburg effect [14] or the Warburg effect inversion [9]. BC MSs present different metabolic signatures namely associated to the tricarboxylic acid (TCA) cycle, amino acid metabolism, and glycolysis/gluconeogenesis [15]. The present study aimed to characterize the metabolic profile alterations under adiposity conditions of cell lines representing the four MS.

Overall, we found three main similarities across the different molecular subtypes when submitted to adipocyte secretome. Firstly, there was a decrease in lactate production, which led us to postulate that the Warburg effect is attenuated by adipocyte released factors. One possible trigger for this scenario is the presence of high levels of fatty acids in the adipocyte secretome, which feed the TCA cycle, providing a robust energy source to tumor cells. A mechanism similar to the "glucose–fatty acid cycle" or "Randle cycle" seems to occur. This involves a competition between glucose and fatty acids, where glucose oxidation is able to inhibit the conversion of triacylglycerol to long chain fatty acids (LCFAs) and LCFAs are able to inhibit glucose oxidation. The absence of significant alterations of TG levels corroborates this hypothesis. Among others, PFK has an important role in the extent of glycolysis inhibition [16]. This mechanism can be modulated by mTORC1, which is activated by insulin, growth factors, and amino acids to promote protein and sphingolipid synthesis, thus securing cell proliferation [17]. Previous studies reported a potential role of the estrogen receptors in this competition between glycolysis and fatty acid oxidation [18].

Also consistent across the molecular subtypes was the decrease in glutamine consumption. The association between glutaminolysis and tumor proliferative needs is well established, glutamine being the second most used energy substrate of tumor cells [19]. Glutamine is a substrate for nitrogen and carbon atoms. Tumor cell nitrogen is essential for the synthesis of non-essential amino acids and nucleotides. In turn, carbons are the major source of catabolic, anabolic, and amphibolic pathways in the cell, such as TCA cycle metabolites and in fatty acid synthesis [20]. The lower levels of released glutamine observed after exposure to adiposity environment in the four MS representative cell lines indicate that glutamine is being redirected for these synthetic pathways.

Another common feature is the significant increase in the cholesterol levels. Redundantly, cholesterol was already described as a risk factor in BC [21]. Internal cholesterol levels derived from the consumption of acetyl-CoA, was already described to activate the mTORC1 pathway [22], intrinsically associated with the "glucose–fatty acid cycle".

Moreover, cholesterol is also required for cellular membrane biosynthesis. Therefore, the significant cholesterol release observed after incubation with adipocyte secretome is probably caused by cholesterol synthesis from acetyl-CoA. Inversely, it is also likely that the large availability of this steroid in the adipocyte secretome may be taken up by the cancer cells to a larger extent than needed, then being released to the extracellular medium.

4.1. Effect of Obesity in Luminal A Representative MCF-7 Cell Line

When exposed to adipocyte secretome, no significant alterations were found in glucose, pyruvate, lactate, acetate, isoleucine, valine, and triglyceride levels in luminal A representative cell line. Contrarily to the other cell lines, acetate levels in extracellular medium did not differ from control in MCF-7. Nevertheless, cholesterol biosynthesis is significantly enhanced, implying that part of the acetate is driven to this steroid synthesis. In addition, the slight increase in pyruvate release is probably caused by the extensive consumption of alanine, which is transaminated to this keto acid. Part of this metabolite can either be used for acetyl-CoA synthesis in the mitochondria, thus keeping TCA cycle active, or result in fatty acid, membrane phospholipids, and cholesterol synthesis. Glutamine is significantly less consumed when subjected to adipocyte associated factors. This amino acid is rapidly converted into glutamate by glutaminase. The significant decrease of this latter amino acid in the medium suggests it is metabolized to α-ketoglutarate, entering the TCA cycle through an anaplerotic reaction as PFK was the only upregulated glycolytic enzyme. PFK catalyzes the phosphorylation of Fructose-6-phosphate to Fructose-1,6-bisphosphate in glycolysis, but it is also associated with several moonlight functions such as survival, apoptosis inhibition by BAD inactivation, alteration of cell shape and motility by binding to cytoskeleton, and transcription regulation [23,24]. We suggest that the upregulation of PFK can be attributed to the detected downregulation of TIGAR expression, since the decrease in TIGAR releases PFK control. Our results reveal that when a low increase in PFK is observed, TIGAR is prone to be upregulated whereas a high increase in PFK is associated with a downregulation of TIGAR. Given the overall results, we concluded that adiposity promotes a more anaplerotic profile in luminal A representative cell line MCF-7, attenuating glycolysis.

4.2. Effect of Obesity in Luminal B Representative BT-474 Cell Line

In control conditions, we observed that BT-474 cell line exhibits both a lower secretion of lactate, pyruvate, glutamate, and alanine, and a lower uptake of glucose, glutamine, isoleucine, and valine from culture media, when compared to the other cell lines. BT-474 is characterized by its high proliferation rates, which in turn leads to higher energetic/biomass requirements. We hypothesized that, given the high proliferative state, the majority of the metabolites are used by the cell, and not released to the extracellular medium. Similar results were described previously, namely with a decrease in glucose uptake compared to other cell lines [15]. We found a significant biological difference in acetate when compared to MCF-7, because while MCF-7 releases high amounts of this metabolite to the culture media, BT-474 cells consume it. Moreover, we observed a statistical difference regarding acetate consumption from culture media between adiposity and control conditions. We hypothesize that the higher consumption of acetate under exposure to adipocyte secretome is likely to be driven by the increased cholesterol synthesis observed under this condition. We also postulate that acetate can be further used for phospholipids synthesis given the proliferative needs of this cellular line [25]. The lower consumption of glutamine and concomitant increase in glutamate release to cell medium led us to propose that BT-474, contrarily to MCF-7, has a more cataplerotic profile.

Furthermore, almost all glycolytic and gluconeogenic enzymes were upregulated, although without statistical significance. This is the case with PCK and G6P. Both PCK and G6P function are dependent on the origin tissue (gluconeogenic or non-gluconeogenic tissue). In non-gluconeogenic tissues, as the case of BC, G6P is involved in cell cycle regulation, survival, and migration. The major described function of PCK is cataplerosis by

enhancing TCA cycle intermediates' disposal that are then used in other pathways, namely pyruvate synthesis, glyceroneogenesis, pentose phosphate pathway, and most importantly glutaminolysis [26]. Glutamate availability contributes to cancer metastasis through the upregulation of Rab27-dependent recycling of MT1-MMP to promote invasive behavior leading to basement membrane disruption [27]. Taken together, our data suggest that BT-474 luminal B representative cell line is more dependent of the carbohydrate metabolic pathways such as glycolysis and gluconeogenesis.

4.3. Effect of Obesity in HER2+ Representative SK-BR-3 Cell Line

Interestingly, we found that the metabolic influence of mature adipocytes in SK-BR-3 is similar to the one found in BT-474. One could speculate that HER2 could be the connection. Up until now, no specific ligand has been described for HER2, although it is known that HER2 is the most favorable dimerization partner for the three other members of the EGFR/ErbB family—1, 3 and 4 [28]. Several adipose tissue related factors such as Transforming Growth Factor α (TGFα) [3] may be involved in downstream proliferative pathways requiring the metabolic pattern found in our results. We found that acetate is consumed (in a statistically dependent manner), probably replenishing cholesterol synthesis pathway. Alanine release is significantly decreased, and glutamate is significantly increased. Since there is no difference in glutamine levels, glutamate comes from α-ketoglutarate in a cataplerotic reaction. Valine and isoleucine do not seem to play a significant role in these cells under the adipocyte secretome condition, presenting a moderate consumption.

SK-BR-3 was the only cell line overexpressing all glycolytic and gluconeogenic enzymes when exposed to adipocyte secretome, which is in agreement with the high proliferative profile of this cell line. PFK expression is somewhat increased, attributed to the TIGAR upregulation, as previously discussed.

4.4. Effect of Obesity in Triple Negative Representative MDA-MB-231 Cell Line

Triple negative representative cell line, MDA-MB-231, exhibited significant alterations in most metabolites studied. Glucose uptake and lactate production were decreased when exposed to adipocyte secretome, revealing a diminished Warburg effect. MDA-MB-231 also revealed the most accentuated acetate consumption correlated with a heightened cholesterol production. Alanine switched from release in control to uptake in adiposity mimicking conditions. The conversion of this amino acid into pyruvate is likely to occur, which then suffers oxidative decarboxylation originating acetylCoA. Upon adipocyte secretome exposure, this cell line does not depend on glutaminolysis, as we observed by the lower consumption of glutamine. Glutamate, in turn, originates a-ketoglutarate entering the TCA cycle by an anaplerotic reaction. Valine and isoleucine are significantly decreased in the MDA-MB-231 cell line. Elevated levels of these branched-chain amino acids (BCAA) are associated with body mass index, although no consensus has yet been reached regarding the potential risk in breast cancer [29]. Metabolically, both BCAA were already associated with glucose and lipid metabolism [30].

Concordant with the results obtained from BT-474 and SK-BR-3, MDA-MB-231 has an enzymatic pattern identical to MCF-7. Only PFK is increased, although without statistical significance, corroborating the unchanged TIGAR expression. HK, FBP, and G6P are significantly downregulated in accordance with the proposed attenuated dependence on glycolytic and gluconeogenic pathways.

Lastly, we would like to mention the study limitations. We are aware that cell culture medium from control and treatment are two different references with different nutrient composition. We overcome this by presenting the results as the difference between pre and post treatment. Additionally, we would like to highlight that: (1) we could not change the required medium for adipocyte differentiation since alterations would affect the protocol, and (2) we did not have access to adipose tissue from normal weight individuals. Moreover, we would like to mention that the use of only one representative line is not sufficient to extrapolate the results.

5. Conclusions

Our findings suggest that adipocyte-related factors impact breast tumor metabolism beyond estrogen/progesterone receptors. HER2 receptor seems to play a major role in central metabolism since molecular HER2 receptor positivity subtypes share common features that differ from those with HER2 negative receptors. Metabolic imbalance is more evident in triple negative BC representative cell line MDA-MB-231.

Overall, our results highlight the metabolic differences among molecular BC subtypes, relative to one representative cell line, but also the influence of the secretome of differentiated adipocytes on the central biochemical metabolism in each BC molecular subtype representative cell line. Further studies are needed regarding the importance of adipocyte-related factors in breast cancer development, which ultimately will provide new therapeutic guidelines.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12172123/s1, Table S1: Nutrient composition of cell medium of control (DMEM) and treatment (secretome); Table S2: Expression of beta actin on mRNA level from the performed RT-PCR.

Author Contributions: Conceptualization, R.S., R.F. and C.L.; Methodology, R.S., R.F., M.G.A. and C.L.; Validation, R.S., R.F., M.G.A. and C.L.; Formal Analysis, B.G.-C., P.C.B., C.G., E.P., M.G.A., R.F., R.S. and C.L.; Resources, R.S., E.P., C.G., B.G.-C., P.C.B. and M.G.A.; Writing—Original Draft Preparation, C.L.; Writing—Review, Editing and Validation, B.G.-C., P.C.B., C.G., E.P., M.G.A., R.F. and R.S.; Supervision, R.F. and R.S.; Project Administration, R.F., R.S. and C.L.; Funding Acquisition, R.F., R.S. and C.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of São João Hospital Centre (protocol code 226/2020-23/07/2020).

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

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary materials:

Table S1 – Nutrient composition of cell medium of control (DMEM) and treatment (secretome).

Metabolite	Control (DMEM)	Treatment (secretome)
Glucose (mM)	20	15
Pyruvate (mM)	0.3	0.03
Lactate (mM)	0	1.2
Acetate (mM)	0.09	0.2
Alanine (mM)	0	0.6
Glutamine (mM)	6	1.6
Glutamate (mM)	0	0
Isoleucine (mM)	0.7	0.4
Valine (mM)	0.7	0.4
Triglycerides (mg/dL)	1	53
Cholesterol (mg/dL)	21	24

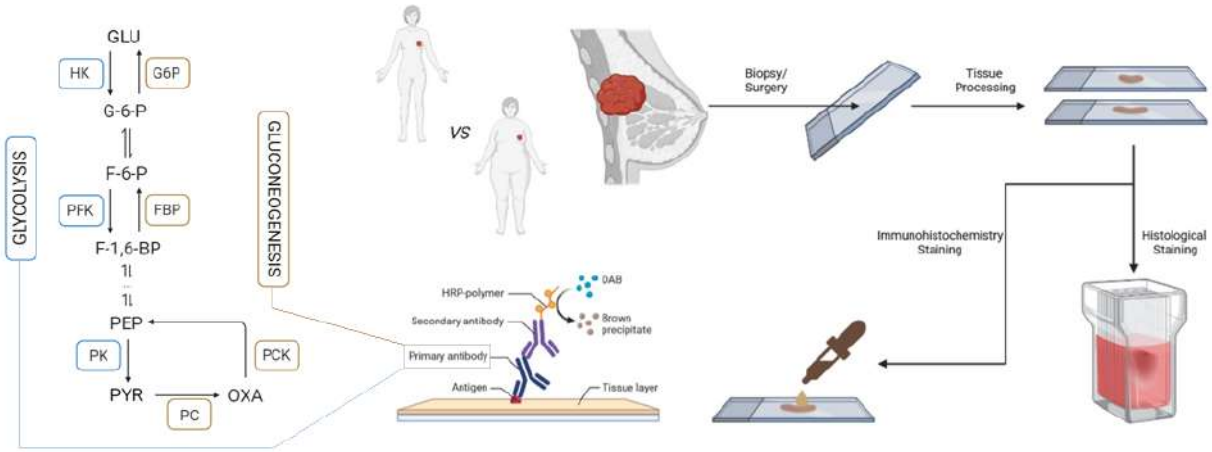
Table S1 – Expression of beta actin on mRNA level from the performed RT-PCR.

	CT PCR1	CT PCR2	CT PCR3	Final CT	2^(-CT)
MCF7 C1	18,3377	18,77367	18,41137	18,50758	2,68327E-06
MCF7 C2	18,3295	19,17043	18,61358	18,7045	2,34091E-06
MCF7 C3	18,38988	19,68531	18,46581	18,847	2,12074E-06
MCF7 C4	18,58482	19,69935	18,74795	19,0107	1,89325E-06
MCF7 C5	18,85521	19,73949	19,01118	19,20196	1,65819E-06
MDA 231 C1	18,43274	19,1138	18,64384	18,73013	2,29969E-06
MDA 231 C2	18,41857	18,8613	18,42292	18,56759	2,57393E-06
MDA 231 C3	18,5863	19,12144	18,63262	18,78012	2,22136E-06
MDA 231 C4	17,51715	18,99463	18,2226	18,2448	3,21936E-06
MDA 231 C5	18,24021	19,19319	18,39148	18,60829	2,50234E-06
BT474 C2	13,68	13,235	13,205	13,37333	9,42379E-05
BT474 C3	13,58	13,31	13,25	13,38	9,38034E-05
BT474 C4	14,14	13,77	13,65	13,85333	6,75665E-05
BT474 C5	13,56	13,33	13,37	13,42	9,12384E-05
SKBR3 C1	17,98981	18,4534		18,22161	3,27151E-06
SKBR3 C2	15,33128	15,44609	15,89798	15,55845	2,07224E-05
SKBR3 C3	18,10436	17,99487	17,98436	18,02786	3,74173E-06
SKBR3 C4	16,38149	16,77188	16,65473	16,6027	1,00482E-05
SKBR3 C5	15,78928	15,90693	15,97893	15,89171	1,64482E-05
MCF7 T1	17,32354	19,47494	17,88758	18,22869	3,25551E-06
MCF7 T2	18,04393	18,9345	18,11221	18,36355	2,96498E-06
MCF7 T3	17,96866	19,3034	17,58455	18,28554	3,12972E-06
MCFT T4	18,14206	19,25178	17,62466	18,3395	3,01481E-06
MCFT T5	17,68686	19,05613	17,23485	17,99261	3,83428E-06
MDA 231 T1	17,34028	19,38313	18,26016	18,32785	3,03925E-06
MDA 231 T2	18,36129	18,75279	18,47408	18,52939	2,64301E-06
MDA 231 T3	17,95972	18,79696	18,27936	18,34535	3,00262E-06
MDA 231 T4	17,76845	18,79349	18,21941	18,26045	3,18462E-06
MDA 231 T5	17,08262	18,42048	17,96621	17,82311	4,31231E-06
BT474 T2	13,69	13,56	13,45	13,56667	8,24188E-05
BT474 T3	14,2	14,005	13,86	14,02167	6,01254E-05
BT474 T4	13,285	13,2	13,26	13,24833	0,000102767
BT474 T5	14,39	14,085	13,99	14,155	5,48176E-05
SKBR3 T1	17,25593	17,45463	17,36785	17,35947	5,94674E-06
SKBR3 T2	16,27944	16,80253	16,74227	16,60808	1,00108E-05
SKBR3 T3	17,21542	17,43576	17,28724	17,31281	6,14221E-06
SKBR3 T4	17,68618	17,21191	17,1729	17,357	5,95694E-06
SKBR3 T5	16,33717	16,3909	16,52253	16,41687	1,14296E-05

NOTE: Average of the assays was taken in account to calculate 2^(-CT).

CHAPTER V

Tissue based approach to obesity and breast cancer



LIST OF CONTENTS:

- "Breast Cancer Molecular Subtypes Differentially Express Gluconeogenic Rate-Limiting Enzymes—Obesity as a Crucial Player"

cancers

MDPI

Article

Breast Cancer Molecular Subtypes Differentially Express Gluconeogenic Rate-Limiting Enzymes—Obesity as a Crucial Player

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Simple Summary: Breast cancer is a complex pathology characterized by several features including molecular subtype (MS). Immunohistochemistry assays were used to investigate the expression of enzymes involved in glycolysis and gluconeogenesis. The analysis involved stratifying the data based on MS, body mass index (BMI), and the combination of BMI with MS (mBMI). This study revealed significant differences in the expression of three specific enzymes—pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK), and fructose-1,6-bisphosphatase (FBP)—among tumor cells when stratified by MS and mBMI. Moreover, the expression levels of these enzymes were found to be closely related to hormonal receptor and HER2 status, as well as correlated pathological stage and histological grade. Obesity appeared to have an impact on these differences, particularly in the expression of PC. However, it was observed that these differences were not influenced by the presence of adipocyte deposition or inflammatory infiltration within the tumor microenvironment. Nevertheless, the expression of PCK and FBP was also influenced by the presence of obesity-related conditions such as diabetes, hypertension, and dyslipidemia. In summary, this study highlights the existence of distinct metabolic profiles for breast cancer based on its molecular subtypes, and how these profiles are affected by obesity and related health conditions.

Abstract: Breast cancer is a heterogeneous entity, where different molecular subtypes (MS) exhibit distinct prognostic and therapeutic responses. A series of 62 breast cancer samples stratified by MS was obtained from the tumor biobank of IPO-Porto. The expression of glycolysis and gluconeogenesis-regulating enzymes was investigated by immunohistochemistry. Data analysis included stratification according to MS, body mass index (BMI), and BMI with MS (mBMI). We observed significant differences in pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK), and fructose-1,6-bisphosphatase (FBP) tumor cell expression when stratified by MS and mBMI. The expression

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of these enzymes was also statistically dependent on hormonal receptors and HER2 status and correlated with pathological stage and histological grade. Obesity tended to attenuate these differences, particularly in PC expression, although these were not affected by adipocyte deposition or inflammatory infiltration at the tumor microenvironment. Nonetheless, PCK and FBP expression was also modified by the presence of obesity-associated disorders like diabetes, hypertension, and dyslipidemia. Taken together, these findings identify metabolic fingerprints for breast cancer as distinct histological types, which are affected by the presence of obesity and obesity-associated conditions. Despite the biological role of the differential expression of enzymes remaining unknown, the current study highlights the need to identify the expression of gluconeogenic-regulating enzymes as a tool for personalized medicine.

Keywords: breast cancer; molecular subtype; obesity; cancer cell metabolism; Warburg effect; glycolysis; gluconeogenesis

1. Introduction

Cancer and obesity are two noncommunicable diseases considered a global epidemic with a high impact on health outcomes [1]. Obesity is associated with several types of cancer including breast cancer (BC) [2], a complex and heterogeneous disease, regarding tumor classification, prognostic factors, therapeutical strategies, and clinical outcomes [3]. Since both diseases present increasing incidence rates [4,5], it is of paramount importance to understand how obesity affects breast cancer.

The obesity–breast cancer link has been extensively studied. Several mechanisms have been proposed to contribute to this connection. Among them, the local and systemic effect of the low chronic state of inflammation characteristic of obese patients; the dysregulated concentrations of adipokines like leptin and adiponectin; the disrupted insulin/IGF signaling pathway; and the most straightforward association, the expression of aromatase in the adipose tissue, which is able to convert androgens into estrogen, are the most established ones [6]. High estrogen levels were found to contribute to tumorigenesis and tumor progression by its capacity to induce DNA damage and promote angiogenesis and cell proliferation in hormone-dependent breast cancers [7]. Adipocytokines, responsible for metabolic homeostasis, are also found dysregulated in obesity. and this dysregulation has been associated with metabolic syndrome conditions like diabetes, hypertension, and dyslipidemia [8].

Tumor cells may respond to the obese stimulation factors through the expression of particular receptors, including hormone receptors. Molecular subtype (MS) identifies breast cancer according to hormone receptors (estrogen and progesterone) and human epidermal growth factor receptor 2 (HER2) expression. Four MS: luminal A, luminal B, HER2-enriched, and triple negative are widely accepted. This classification is of extreme importance both for BC prognosis as well as therapeutic targets, namely endocrine therapy for luminal A and B or anti-HER2 monoclonal antibodies against HER2+ breast cancer [9].

Reprogramming energy metabolism has been considered a hallmark of cancer since 2011 [10]. It reflects the tumor cell ability to modify or reprogram the metabolism to most efficiently support its proliferation [10]. As first described by Otto Warburg, tumor cells reprogram their glucose metabolism to an aerobic glycolysis, hence surpassing oxidative phosphorylation even in the presence of oxygen, the well-known *Warburg effect* [11]. Several variations and observations of this mechanism have been uncovered ever since. Previous studies identified two metabolic different populations, working symbiotically in the tumor microenvironment: one population secreting lactate, and the other consuming lactate [12]. Another recent proposition describes that cancer-associated fibroblasts consume lactate and pyruvate, producing glucose. In turn, this glucose is captured by tumor cells, thus promoting an efficient production of essential energy to meet the proliferative needs of both cells. This self-sustaining "Cori cycle" between the microenvironment stroma and the

tumor cell is called the *reverse Warburg effect* [13]. In 2019, our group unraveled another metabolic reprograming in breast cancer cell lines. Under obesity-mimicking conditions, we observed a switch in the central metabolic pathway, which we termed *Warburg effect inversion*. MCF-7 breast cancer cells in the presence of adipocyte-secreted medium consumed lactate-originating glucose as observed by metabolomic assays. This inversion of tumor cell metabolism was concomitant with increased tumor cell aggressiveness in vitro [14].

The present work aims to explore glycolysis and gluconeogenesis rate-limiting enzymes in a human breast cancer series. The expression of glycolysis and gluconeogenesis rate-limiting enzymes, catalyzing irreversible reactions, was quantified in tumor samples from different molecular subtypes. The results were correlated with body mass index (BMI) and obese-associated characteristics such as clinical parameters, adipose tissue infiltration, nonspecific inflammation status, and metabolic syndrome conditions.

2. Materials and Methods

2.1. Study Population and Ethical Approval

The study was performed according to the principles of the Declaration of Helsinki. Ethical approval was obtained by the institutional ethics committee of the Portuguese Oncology Institute of Porto (IPO-Porto) Francisco Gentil, Porto, Portugal. All participants gave their informed consent.

Tumor samples of female patients diagnosed in IPO-Porto were obtained from the IPO biobank. All patients were submitted to conservative surgery or mastectomy between 2017 and 2019. Inclusion criteria included no neoadjuvant treatment, tumors with no special type (NST—Invasive ductal carcinoma), the pathological stage between I-III, and with PROSIGNA/PAM50 genetic study performed for molecular subtypes.

2.2. Sample Characteristics

Body mass index (BMI) was calculated according to the Quetelet index [15] and samples ranked in normal weight ($18.5 \leq$ BMI < 25 kg/m^2) and obese (BMI ≥ 30 kg/m^2), as calculated before surgical intervention. PROSIGNA/PAM50 genetic study was performed to assess the MS. Tumors were classified according to their hormonal and HER2 status as positive or negative. Cancer staging (pathologic stage) was assessed accordantly to the *American Joint Committee* on Xancer by the tumor node metastasis (TNM) system [16]. Histological differentiation grade was classified according to the *Nottingham histological grade*, a combination of nuclear grade, mitotic rate, and tubule formation [17]. Topographic localization was divided into quadrants: the inferior outer quadrant (IOQ), inferior inner quadrant (IIQ), superior outer quadrant (SOQ), superior inner quadrant (SIQ), central quadrant (CQ), multiple (present in more than one quadrant), or other (axillary and nipple). Diabetes, hypertension, and dyslipidemia were assessed at the time of the first medical appointment in IPO-Porto. Ki67 proliferation biomarker was quantified by immunohistochemistry according to internal protocols, stratified in three groups (<15%; 15–30%; >30%).

2.3. Hematoxylin–Eosin Staining

Hematoxylin and eosin (H&E) (Hematoxylin: Cat. 72704; Thermo Fisher Scientific, Waltham, MA, USA; Eosin: Cat. 71211; Thermo Fisher Scientific, Waltham, MA, USA) staining was performed. Adipose tissue infiltration and nonspecific inflammation were quantified anonymously and independently by three researchers. Each trait was scored as follows: 0. absent; 1. scarce; 2. moderate; and 3. abundant.

2.4. Immunohistochemistry Assays

Paraffin-embedded sections were deparaffinized and hydrated. Sections were submitted to heat antigenic recovery in citrate buffer. Endogenous peroxidase activity and nonspecific binding were blocked. Tissue sections were allowed to react with primary/secondary pairing antibodies. Immunoreactivity was visualized with avidin–biotin reaction (Avidin/Biotin Blocking Kit #SP-2001, Vector Laboratories, Newark, NJ, USA) and revelation was

performed using DAB substrate (Pierce™ DAB Substrate Kit #34002, Thermo Fisher Scientific, Waltham, MA, USA) counterstained with haematoxylin (#72704; Thermo Fisher Scientific, Waltham, MA, USA).

The isoforms under analysis were hexokinase (HK)—HK II; phosphofructokinase (PFK)—PFK L; pyruvate Kinase (PK)—PK M; pyruvate carboxylase (PC)—PC B; phosphoenolpyruvate carboxykinase (PCK)—PCK 2; fructose-1,6-bisphosphatase (FBP)—FBP 1; glucose-6-Phosphatase (G6P)—G6P alpha. Specifications of each assay are described in the Supplementary Material (Table S1) and results from the positive control can be visualized in the Supplementary Material (Figure S1). A negative control was performed with incubation with a blocking solution (without primary antibody).

All stained specimens were visualized under a Nikon Eclipse 501 optical microscope (Nikon, Tokio, Japan). Quantification regarding enzymatic expression in tumor cells was anonymous and independently scored by three researchers. The area was classified according to the percentage of positive cells, as follows: 0. unstained; 1. less than 5%; 2. from 5 to 50%; and 3. over 50% stained. Intensity was scored as: 0. negative; 1. weak; 2. moderate and 3. strong. The score was obtained by multiplying the area and intensity on a scale of 0 to 9. The final score was categorized as follows: No expression = score of 0; low expression = score of 1, 2, and 3, and high for scores of 4, 6, and 9.

2.5. Statistical Analysis

The analysis included stratification according to BMI (Normal weight vs. Obese patients), MS (luminal A vs. luminal B vs. triple negative vs. HER2+), and BMI with MS, further presented as mBMI (luminal A normal weight vs. luminal A obese vs. luminal B normal weight vs. luminal B obese vs. triple-negative normal weight vs. triple-negative obese vs. HER2+ normal weight vs. HER2+ obese).

Statistical analysis was performed using IBM® SPSS® version 27 software (IBM, Endicott, NY, USA). Continuous variables were tested for normality by the Kolmogorov–Smirnov test. Descriptive statistics was used for data description in terms of absolute frequencies and valid percentages. Continuous variables were presented as mean values and standard deviation. The chi-square (χ^2) test was used to analyze enzymatic expression, adipose tissue infiltrations, and nonspecific inflammation with BMI, MS, and mBMI. The correlation between tumor characteristics/obese-related features and enzymatic expression was also examined using Spearman's correlation coefficient (r) with two tails. The p-value inferior to 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinicopathological Data

A total of 62 samples were included in this study, 32 from normal-weight patients (8 luminal A; 8 luminal B; 8 triple negative; 8 HER2+) and 30 from obese patients (8 luminal A; 8 luminal B; 8 triple negative; 6 HER2+). Table 1 displays the demographic and clinicopathological information from the patients and the p-value from chi-square analysis of MS, BMI, and mBMI stratification.

As expected, a significant association was found between the pathological stage (p-value = 0.037), histological grade (p-value: <0.001), and MS, which was also evident with mBMI. Luminal cases are frequently associated with lower pathological stages and histological grades. Luminal A specifically expresses lower levels of proliferation genes which translates to a good prognosis [18]. MS stratification further revealed a significantly higher percentage of carcinoma in situ presenting necrosis (p-value: 0.042), which is lost in mBMI stratification. Apart from histological grade, none of the included features was significantly different in obese versus nonobese patients within each MS. A more in-depth insight from the results of MS, BMI, and mBMI stratification can be consulted in Supplementary Material—Table S2.

Table 1. Demographic and cancer-associated clinicopathological data from the breast cancer patient series stratified by BMI, MS, or both (mBMI).

n (%)	Total 62 (100.0%)	*p*-Value BMI Analysis	*p*-Value MS Analysis	*p*-Value mBMI Analysis
Age at diagnosis				
(Mean ± SD)	57.48 ± 10.53	0.480	0.218	0.604
BMI categories				
Normal weight	32 (51.6%)	-	0.974	-
Obese	30 (48.4%)			
Molecular subtypes				
Luminal A	16 (25.8%)	0.974	-	-
Luminal B	16 (25.8%)			
Triple negative	16 (25.8%)			
HER2+	14 (22.6%)			
Topographic localization				
CQ	3 (4.8%)	0.680	0.052	0.075
IOQ	4 (6.5%)			
SOQ	21 (33.9%)			
SIQ	4 (6.5%)			
Multiple	27 (43.5%)			
Other	3 (4.8%)			
Laterality				
Right	32 (51.6%)	0.806	0.632	0.075
Left	30 (48.4%)			
Pathological stage				
Stage I	36 (59.0%)	0.321	**0.037** *	0.051
Stage II	22 (36.1%)			
Stage III	3 (4.9%)			
Histological grade				
Grade I	0 (0%)	0.227	**<0.001** *	**<0.001** *
Grade II	37 (59.7%)			
Grade III	25 (40.3%)			
Carcinoma in situ				
Presence	24 (38.7%)	0.469	0.282	0.357
Size. cm (mean ± SD)	1.35 ± 2.47	0.649	0.798	0.463
Extensive	20 (32.3%)	0.472	0.367	0.253
Microcalcifications	28 (45.2%)	0.818	0.236	0.333
Necrosis	39 (62.9%)	0.946	**0.042** *	0.126
Invasive carcinoma				
Size. cm (mean ± SD)	1.76 ± 0.75	0.465	0.197	0.210
Multifocal	14 (22.6%)	0.891	0.564	0.656
Invasion	19 (30.6%)	0.511	0.661	0.783
Metastatic nodes				
0	41 (66.2%)	0.364	0.144	0.082
1–3	18 (29.0%)			
4–9	1 (1.6%)			
>10	2 (3.2%)			
Size cm (mean ± SD) (Larger metastasis)	11.48 ± 31.49	0.764	0.056	0.340

Legend: SD—standard deviation; CQ—central quadrant; IOQ—inferior outer quadrant; SOQ—superior outer quadrant; SIQ—superior inner quadrant; * statistical significance highlighted in bold (p-value < 0.05).

3.2. Expression of Glycolysis and Gluconeogenesis Rate-Limiting Enzymes

The tumor cell expression of enzymes catalyzing the irreversible reactions of glycolysis (HK, PFK, and PK) and gluconeogenesis (PC, PCK, FBP, and G6P) was investigated by immunohistochemistry. Examples of absence, low expression, and high expression patterns are illustrated in Supplementary Material—Figure S2.

It is interesting to note that no significant difference was observed in any of the glycolytic enzymes studied regarding MS stratification. HK expression was rather reduced for every MS, being absent in luminal B cases. Inversely, there were significant differences in most gluconeogenic enzymes, namely in PC, PCK, and FBP. Triple-negative subtypes did not express FBP in contrast to luminal A and B, where a significant number of cases overexpressed FBP but not PC and PCK. HER2+ cases displayed higher expression of PC and PCK.

When stratified by BMI, we observed a slight decreased expression in the three glycolytic enzymes in breast cancer tissue of obese individuals (although without statistical significance). Regarding gluconeogenic enzyme expression, there was a switch in the expression pattern in FBP and G6P. An increased number of obese patients overexpressed both these enzymes in tumors (again without statistical differences) in contrast to PC and PCK. A tendency towards downregulation of PC expression was observed in obese cases ($p = 0.065$) relative to normal-weight subjects.

We next investigated whether BMI influenced the metabolic characteristics of MS. Statistical differences observed in MS analysis were maintained although with lower statistical power, probably due to the smaller size of the sample. In most cases, PFK and PK glycolytic enzyme expression tended to decrease in obese patients of the different MS, except in triple-negative cases. Further, in comparison to normoponderal cases with the same MS, obese patients presented PC downregulation, and an upregulation of FBP (again, in this latter, with the exception of triple-negative cases) (Figure 1).

In summary, glycolytic enzyme expression did not differ either between MS or between BMI groups. PFK and PK expression was more abundant than HK. Expression of gluconeogenic enzymes (PC, PCK, FBP, and G6P) varied significantly with MS. A tendency to decrease PC was observed in breast cancer from obese patients.

3.3. Correlation between Rate-Limiting Enzymes Expression and Clinicopathological Features

We further assessed the correlation between the enzymatic score (0–9) and cancer-associated clinicopathological features. The results are shown in Table 2. HK was positively correlated with the HER2 receptor status and with the presence of vascular invasion. PK was solely correlated with the pathological stage. PC was negatively correlated with BMI, ER, and PR and positively correlated with the pathological stage, histological grade (including histological grade mitosis assessment), and HER2. PCK was negatively correlated with ER and PR and positively correlated with HER2. An additional positive correlation was found with histological grade mitosis assessment. Inversely, FBP was positively correlated with hormonal receptors and negatively correlated with histological grade (including nuclear grade and mitosis of histological grade assessment).

These findings indicate that rate-limiting enzymes of the gluconeogenic pathway are potential prognostic factors in breast cancer, FBP a marker of good prognosis, and PC and PCK markers of bad prognosis.

3.4. Intratumor Adipose Tissue Deposition

Adipocytes influence the central metabolism by releasing adipokines, cytokines, fatty acids, and growth factors, among others [19]. We next investigated whether the presence of tumor microenvironment (TME) adipocytes accompanied the observed differences in enzymatic expression in tumor cells. No statistical significance was achieved in the number of adipose tissue depots when stratified either by BMI, MS, or mBMI (Figure 2).

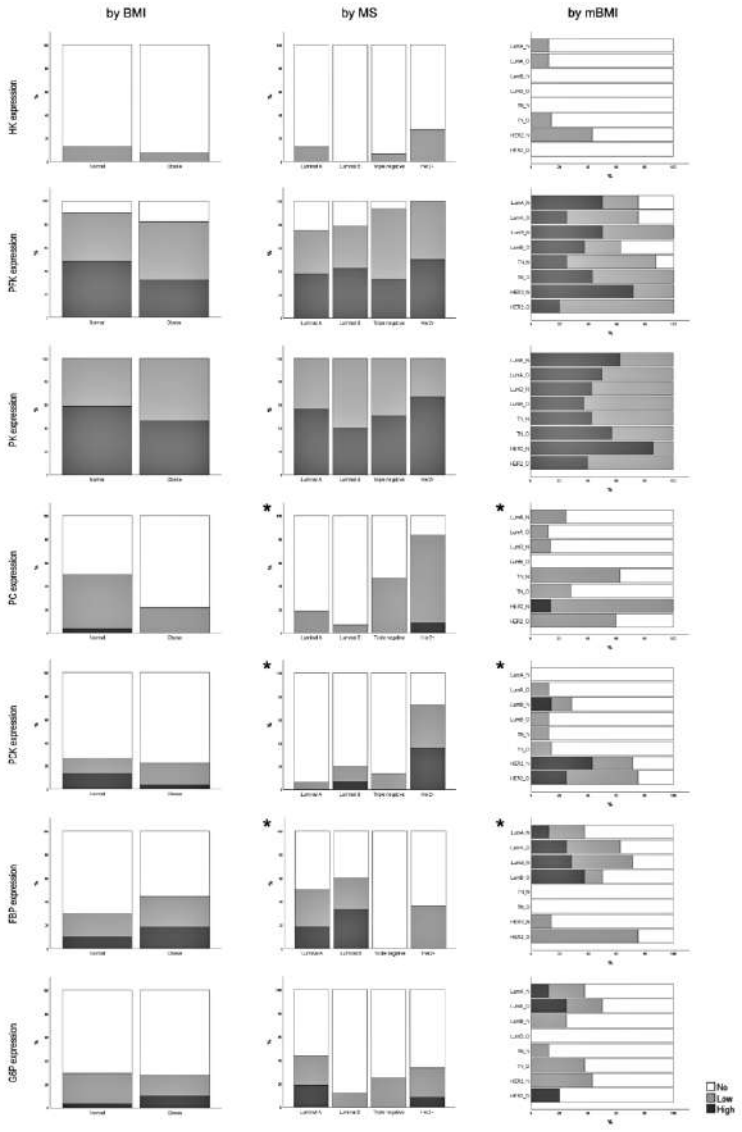


Figure 1. Glycolytic and gluconeogenic rate-limiting enzyme expression assessed by immunohistochemistry stratified by BMI, MS, and mBMI. No significant differences between normal weight and obese patients were observed. [HK χ^2 test *p*-value: 0.467; PFK χ^2 test *p*-value: 0.422; PK χ^2 test *p*-value: 0.357; PC χ^2 test *p*-value: 0.065; PCK χ^2 test *p*-value: 0.410; FBP χ^2 test *p*-value: 0.491; G6P χ^2 test *p*-value: 0.438]; significant differences between molecular subtypes were observed in PC, PCK, and FBP. [HK χ^2 test *p*-value: 0.148; PFK χ^2 test *p*-value: 0.428; PK χ^2 test *p*-value: 0.566; PC χ^2 test *p*-value: 0.001 *; PCK χ^2 test *p*-value: 0.002 *; FBP χ^2 test *p*-value: 0.007 *; G6P χ^2 test *p*-value: 0.262]; significant differences between molecular subtypes were observed in PC, PCK, and FBP; [HK χ^2 test *p*-value: 0.134; PFK χ^2 test *p*-value: 0.345; PK χ^2 test *p*-value: 0.653; PC χ^2 test *p*-value: 0.010 *; PCK χ^2 test *p*-value: 0.047 *; FBP χ^2 test *p*-value: 0.023 *; G6P χ^2 test *p*-value: 0.304]; legend: HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6 phosphatase.

Table 2. Spearman correlation between enzymatic score and demographic and clinicopathological features.

	HK		PFK		PK		PC		PCK		FBP		G6P	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
BMI	−0.147	0.275	−0.162	0.229	−0.059	0.650	**−0.303**	**0.021** *	−0.138	0.305	0.138	0.305	0.047	0.720
ER	−0.151	0.268	−0.177	0.192	−0.168	0.196	**−0.514**	**<0.001** *	**−0.284**	**0.034** *	**0.463**	**<0.001** *	0.020	0.881
PR	−0.151	0.268	−0.177	0.192	−0.168	0.196	**−0.514**	**<0.001** *	**−0.284**	**0.034** *	**0.463**	**<0.001** *	0.020	0.881
HER2	**0.277**	**0.039** *	0.259	0.054	0.139	0.285	**0.503**	**<0.001** *	**0.569**	**<0.001** *	−0.075	0.580	0.059	0.658
Ki67	−0.045	0.799	0.122	0.486	0.123	0.463	0.089	0.604	0.288	0.093	−0.038	0.829	0.009	0.956
TL	0.043	0.752	0.185	0.169	0.132	0.306	0.105	0.433	−0.141	0.296	−0.209	0.119	−0.209	0.110
LAT	−0.086	0.524	0.113	0.401	0.131	0.312	−0.084	0.529	−0.028	0.834	0.156	0.246	−0.046	0.726
pST	0.053	0.698	0.138	0.310	**0.263**	**0.041** *	**0.437**	**<0.001** *	0.185	0.172	−0.215	0.111	0.154	0.246
HG	0.078	0.566	0.106	0.434	0.120	0.353	**0.452**	**<0.001** *	0.238	0.075	**−0.396**	**0.002** *	0.014	0.918
HGnc	0.117	0.384	0.198	0.139	0.137	0.287	0.249	0.060	0.193	0.149	**−0.304**	**0.021** *	0.059	0.656
HGm	0.050	0.711	0.032	0.812	0.034	0.796	**0.392**	**0.002** *	**0.272**	**0.041** *	**−0.320**	**0.015** *	−0.007	0.955
HGtf	−0.083	0.541	−0.021	0.875	0.090	0.489	0.243	0.066	0.036	0.793	−0.144	0.286	−0.020	0.881
ISs	−0.016	0.906	−0.120	0.373	−0.094	0.469	−0.071	0.595	−0.151	0.263	0.088	0.514	0.230	0.077
ISext	0.026	0.846	−0.027	0.840	−0.063	0.624	−0.166	0.212	−0.173	0.198	0.142	0.293	0.212	0.194
ISmc	0.122	0.365	−0.082	0.546	−0.187	0.146	−0.148	0.267	−0.143	0.289	0.183	0.172	0.063	0.631
ISnec	0.154	0.254	0.098	0.468	−0.091	0.483	0.196	0.139	0.025	0.854	0.032	0.813	0.026	0.842
INVs	−0.016	0.907	0.043	0.753	0.155	0.229	0.242	0.067	0.055	0.683	−0.237	0.075	0.210	0.108
INVm	0.234	0.080	−0.137	0.309	−0.053	0.680	−0.031	0.816	0.014	0.918	−0.014	0.920	0.109	0.407
INVvi	**0.269**	**0.043** *	0.160	0.234	0.094	0.467	0.077	0.567	−0.027	0.840	−0.012	0.930	0.059	0.656
METs	−0.090	0.504	0.089	0.509	−0.093	0.474	0.048	0.720	−0.154	0.253	0.009	0.947	0.076	0.564

Legend: r—spearman correlation factor; *p*—*p*-value; HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6-phosphatase; BMI—body mass index; ER—estrogen receptor; PR—progesterone receptor; HER2—human epidermal growth factor receptor 2; TL—topographic localization; LAT—laterality; pST—pathological stage; HG—histological grade; HGng—nuclear grade for histological classification; HGm—mitosis for histological classification; HG—tubule formation for histological classification; ISs—in situ size; ISext—in situ extensive; ISm—in situ microcalcifications; ISnec—in situ necrosis; INVs—invasive size; INVmi—invasive carcinoma multifocality; INVvi—vascular invasion; METs—metastatic size; * statistical significance highlighted in bold (*p*-value < 0.05).

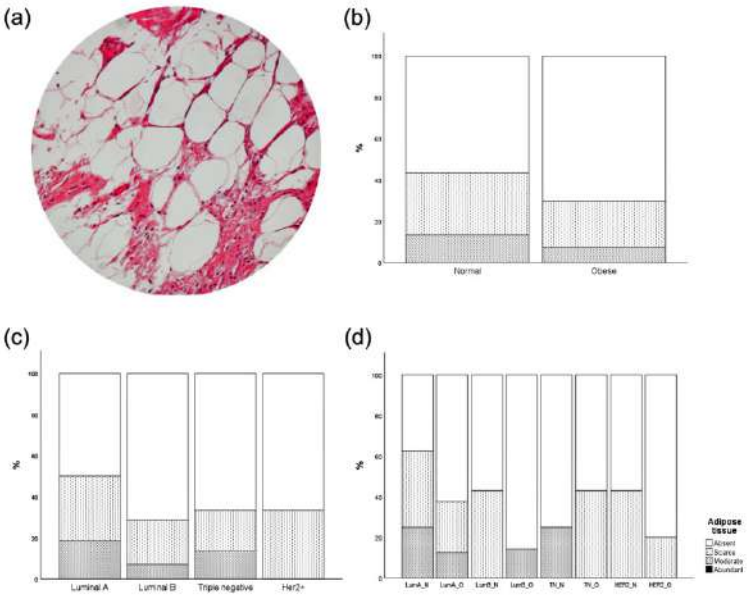


Figure 2. Quantification of intratumor adipose tissue deposition according to BMI, MS, or both (mBMI). No statistical differences were found. (**a**) Representative image of adipose tissue infiltration

(amplification: 100X); (**b**) stratification by BMI, χ^2 test *p*-value: 0.542; (**c**) stratification by MS χ^2 test *p*-value: 0.687; (**d**) stratification by mBMI χ^2 test *p*-value: 0.409; legend: LumA_N—luminal A normal weight; LumA_O—luminal A obese; LumB_N—luminal B normal weight; LumB_O—luminal B obese; TN_N—triple-negative normal weight; TN_O—triple-negative obese; HER2_N—HER2+ normal weight; HER2_O—HER2+ obese.

Adipose tissue infiltration was not associated with tumor cells' enzymatic expression (Table 3). Interestingly, we found PC expression to be the only enzyme with a positive correlation with adipose tissue infiltration within TME.

Table 3. Chi-square analysis and spearman correlation between adipose tissue infiltration and enzymatic expression.

Enzymes	χ^2 Test	Spearman Correlation	
	p-Value	R	*p*-Value
HK	0.635	−0.038	0.784
PFK	0.509	−0.054	0.690
PK	0.318	−0.113	0.403
PC	0.580	0.035	0.796
PCK	0.590	−0.111	0.415
FBP	0.222	−0.190	0.160
G6P	0.569	−0.049	0.718

Legend: r—spearman correlation factor; HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6-phosphatase.

3.5. Inflammatory Infiltration

Obesity is associated with increased inflammation. Thus, we next examined the intratumoral presence of inflammatory infiltration in our series of breast cancer tissue (Figure 3). No difference was observed regarding the extent of inflammatory infiltration in breast cancer series upon BMI, MS, or mBMI stratification. Triple-negative and HER2+ cases exhibit more abundant inflammatory infiltration than luminal A and B, still without significant differences. Comparison between BMI and normal weight for each MS (Figure 3d) revealed that a more abundant inflammatory infiltration was observed in luminal B and Her2+ normal weight cases, and in obese triple-negative cases.

Statistical results between inflammation and enzymatic expression are displayed in Table 4. Tumor inflammatory infiltration was statistically associated with PC upregulation in tumor cells. Spearman correlation revealed a tendency to a positive correlation between inflammation and PC expression.

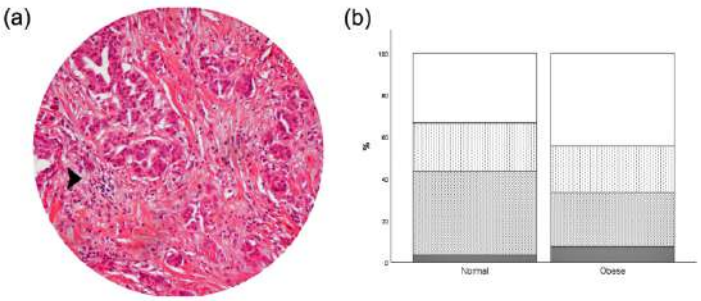


Figure 3. *Cont.*

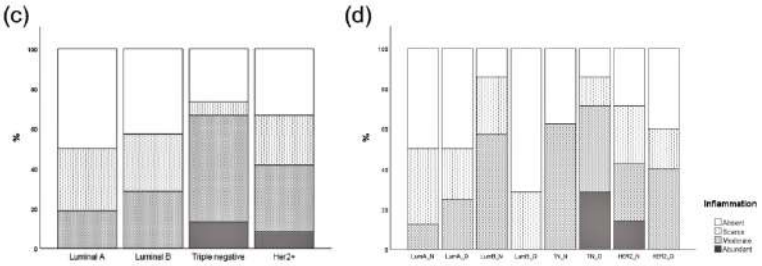


Figure 3. Quantification of inflammatory infiltration according to BMI, MS, or both (mBMI). No statistical differences were found. (**a**) Representative image of inflammatory infiltration (amplification: 100X). Black arrow indicates an example of inflammatory infiltration; (**b**) stratification by BMI, χ^2 test *p*-value: 0.625; (**c**) stratification by MS χ^2 test *p*-value: 0.330; (**d**) stratification by mBMI χ^2 test *p*-value: 0.221; legend: LumA_N—luminal A normal weight; LumA_O—luminal A obese; LumB_N—luminal B normal weight; LumB_O—luminal B obese; TN_N—triple-negative normal weight; TN_O—triple-negative obese; HER2_N—HER2+ normal weight; HER2_O—HER2+ obese.

Table 4. Chi-square analysis and Spearman correlation between inflammatory infiltration and enzymatic expression.

Enzymes	χ^2 Test *p*-Value	Spearman Correlation	
		r	*p*-Value
HK	0.411	0.209	0.126
PFK	0.739	0.117	0.391
PK	0.264	−0.017	0.900
PC	**<0.001** *	0.248	0.062
PCK	0.411	0.179	0.186
FBP	0.465	−0.071	0.604
G6P	0.938	0.097	0.471

Legend: r—spearman correlation factor; HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6-phosphatase. * Statistical significance highlighted in bold (*p*-value < 0.05).

3.6. Correlation between Tumor Cell Enzymatic Expression and Obesity-Associated Conditions

Diabetes, hypertension, and dyslipidemia cluster together with obesity, under the umbrella of metabolic syndrome. Table 5 illustrates the presence of diabetes, hypertension, and dyslipidemia in breast cancer patient series. The only statistically significant association was found between BMI and hypertension. Of the 25 individuals with hypertension, 17 were obese. Detailed data from this analysis can be consulted in Supplementary Materials—Table S3.

Table 5. Chi-square test of obesity-associated pathologies (diabetes, hypertension, and dyslipidemia) stratified according to BMI, MS, and mBMI.

n (%)	Total 62 (100.0%)	*p*-Value BMI Analysis	*p*-Value MS Analysis	*p*-Value mBMI Analysis
Diabetes				
Yes	7 (11.5%)	0.589	0.883	0.791
No	54 (88.5%)			
Hypertension				
Yes	25 (41.0%)	**0.008** *	0.949	0.088
No	36 (59.0%)			
Dyslipidemia				
Yes	39 (63.9%)	0.175	0.495	0.549
No	22 (36.1%)			

Note: Data presented in valid percentage (missing data from 1 patient). * Statistical significance highlighted in bold (*p*-value < 0.05).

We further assessed the correlation between enzyme expression and obesity-related conditions. A positive correlation was observed between PCK and diabetes and between FBP and both hypertension and dyslipidemia (Table 6).

Table 6. Spearman correlation between enzymatic score and obesity related features (diabetes, hypertension, and dyslipidemia).

Enzymes	Diabetes		Hypertension		Dyslipidemia	
	r	*p*-Value	r	*p*-Value	r	*p*-Value
HK	−0.131	0.337	−0.065	0.635	0.079	0.564
PFK	0.070	0.607	0.177	0.187	0.180	0.180
PK	−0.099	0.447	0.025	0.849	0.029	0.822
PC	−0.035	0.794	−0.142	0.287	0.040	0.768
PCK	**0.304**	**0.022** *	0.189	0.159	0.157	0.244
FBP	0.057	0.676	**0.320**	**0.015** *	**0.328**	**0.013** *
G6P	0.240	0.065	0.082	0.531	0.149	0.257

Legend: r—spearman correlation factor; HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6-phosphatase. * Statistical significance highlighted in bold (p-value < 0.05).

4. Discussion

To our knowledge, this is the first study addressing the expression of glycolytic and gluconeogenic rate-limiting enzymes in the molecular subtypes of breast cancer, and the potential influence of obesity on the expression of these enzymes.

As expected, a significant association was found between pathological stage, histological grade, and MS. In fact, luminal cases are associated with lower pathological stages and histological grades [18]. Moreover, with the exception of histological grade, none of these parameters was significantly different in obese versus nonobese patients within each MS (mBMI), being the difference in the histological grade being attributed to the molecular subtype itself. Additionally, the presence of necrotic tissue within in situ carcinoma was also statistically associated with molecular subtypes, in agreement with previous results [20].

We observed a reduction in glycolytic enzyme expression in obese cases, although without statistical significance. Recent evidence suggests that glucose in highly proliferating cells undergoes glycolysis to provide building blocks for anabolic pathways [21], such as the pentose phosphate pathway (PPP) that produces NADPH and ribose, hexosamines, glycerol-3-phosphate, and nonessential amino acids (Figure 4). Therefore, the decrease in glycolysis observed in obese patients with luminal and HER2+ breast cancers may be compensated by an increase in specific enzymes of the gluconeogenic pathway. That can be the case of FBP, an enzyme converting fructose-1,6-bisphosphate into fructose-6-phosphate, which then isomerizes to glucose-6-phosphate, the substrate of the PPP. Remarkably, obese triple-negative breast cancer patients behave differently. These tumors depend primordially on glycolysis for metabolism, and do not express FBP at all, either in obese or in nonobese patients. Similar findings have been reported in prior studies, with TNBC exhibiting either low FBP expression or the complete absence of expression [22]. It has been suggested that p53 deleterious mutations may play a role in suppressing FBP1 expression within this molecular subtype [23]. However, it is worth noting that there are studies presenting conflicting results, indicating that FBP1 expression levels in TNBC can be comparable to those observed in HER2+ [24]. We also found that FBP correlates positively with hormone receptors and negatively with histological grades.

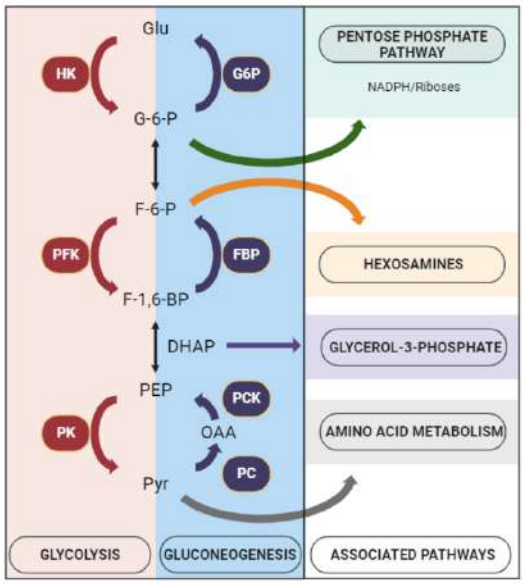


Figure 4. Glycolysis, gluconeogenesis, and associated pathways. Legend: Glu—glucose; G-6-P—glucose-6 phosphate; F-6-P—fructose 6 phosphate; F-1,6-BP—fructose 1,6 bisphosphate; DHAP—dihydroxyacetone phosphate; PEP—phosphoenolpyruvate; Pyr—pyruvate; OAA—oxaloacetate; HK—hexokinase; PFK—phosphofructokinase; PK—pyruvate kinase; PC—pyruvate carboxylase; PCK—phosphoenolpyruvate carboxykinase; FBP—fructose-1,6-bisphosphatase; G6P—glucose-6-phosphatase.

It is interesting to note that PC, another gluconeogenic enzyme, does not follow the same trend; rather, it is significantly decreased in obese cases in every luminal, TN, and HER2+ breast cancer. The expression of PC in breast cancer can vary based on the molecular subtype. For instance, the HER2+ highly invasive cell line (MDA-MB-435) exhibits the highest levels of PC expression, which aligns with the findings of our study [25]. PC catalyzes the conversion of pyruvate into oxalacetate, the initial step of gluconeogenesis. The downregulation of this mitochondrial enzyme may be due to impaired mitochondrial or pyruvate metabolism, common features in tumor cells [26]. This enzyme is probably underestimated, given its correlation with other poor prognostic clinicopathological features. PC is negatively correlated with hormone receptors and positively with HER2. It is also associated with mitosis. Moreover, PC is the only enzyme that is significantly associated with tumor inflammatory infiltration. PCK expression is significantly increased in HER2+ cases from our cohort, contradicting previous results. In a study involving 177 breast cancer patients, stratified based on receptor status, a significant association was observed solely with the estrogen receptor and not with HER2 [27]. It was previously described that PCK leads to increased glucose production, promoting hyperglycemia and worsening the diabetes condition [28]. These findings corroborate the positive correlation between diabetes and PCK described in Table 6. PCK achieves statistical significance when stratified both by MS and mBMI, being also statistically positively correlated with HER2 and negatively with hormonal receptors and mitosis (similar to PC).

Our findings suggest that overexpression of PC and/or PCK is associated with poor prognosis. On the other hand, FBP is a marker of good prognosis in breast cancer. Altogether, the current study further provides evidence that gluconeogenic enzymes present prognostic value.

Obese and nonobese patients present similar rates of inflammatory infiltration and adipocytes within the tumor. These results were rather striking, since it is well established

that obese people often display systemic low-grade chronic inflammation [29]. Nevertheless, this systemic inflammatory environment does not seem to play a crucial role in the local inflammation of the neoplastic tissue in the current analysis. In fact, comparing the different MS, the percentage of tumors lacking adipocytes was always lower in obese patients than in normal-weight ones, except in the case of triple-negative tumors. This decrease in the number of adipocytes within tumor tissue in obese subjects may just be a consequence of an excess of fat in normal adjacent breast tissue that plays a paracrine role. Previous studies observed that adipocytes can undergo phenotypic alterations when exposed to tumor cells, termed cancer-associated adipocytes (CAA) [30]. CAA is mostly associated with increased catabolism, with the release of metabolites such as pyruvate, lactate, free fatty acids, and ketone bodies. We recognized that our adipose tissue deposition results could be, to some extent, limited, given the adipocyte dedifferentiation to a fibroblast-like phenotype, which could be confused with other stromal cells. Nonetheless, the importance of differentiated adipocytes in the TME should not be underestimated. However, several questions remain to be clarified. Given the well-established metabolic, inflammatory, and endocrine role of adipose tissue [31], it is important to know whether this switch in cancer cell metabolism is caused by inflammatory cytokines, adipokines (leptin, adiponectin, resistin) or a mere enhanced energy availability, as adipocyte-derived fatty acids may be used as energy source in tumor cells.

We addressed the expression of glycolysis and gluconeogenesis-regulating enzymes in breast cancer-stratified cases by MS and by BMI. Nonetheless, there are certainly several other players in this crosstalk. Adipose tissue-releasing hormones, adipocytokines, and oxidative stress are also of paramount importance [19,32]. Moreover, menopausal status is also a crucial player when the sample population is stratified by BMI. It is previously described that obesity may be associated with a reduced risk of breast cancer before menopause and with increased risk in postmenopausal women, associated with hormonal receptors and MS [33]. Although relevant, such features are beyond the scope of the current study. Additionally, we cannot rule out the effect of oncogenes and tumor suppressor genes that control metabolic cues within the tumor cell. For instance, TIGAR is a glycolysis regulator that is controlled by p53 [34]. A deleterious mutation of this tumor suppressor gene interferes with metabolism, ultimately resulting in cell behavior changes.

We are aware of the study's limitations. We believe that an increased number of samples could improve the statistical strength of the study and uncover additional differences. Moreover, immunohistochemistry is a limited technique, prone to human errors and lacking standardized protocols for research. Nonetheless, we believe that this study brought new insights regarding the expression of enzymes of two main metabolic pathways in human breast cancer, paving the way towards a precise medicine. Further studies are needed to deeper highlight the precise role of these enzymes in breast tumor cells, and the potential therapeutic targets.

5. Conclusions

This study reveals that each molecular subtype of breast cancer holds a particular gluconeogenic fingerprint, which ultimately adapts metabolism to the needs of the tumor cell. PC and PCK are overexpressed in HER2+ cases, in contrast to the other MS. On the other hand, FBP was overexpressed in luminal groups, and absent in triple-negative ones.

In obese patients, however, an increase in FBP expression has been observed in luminal A, B, and HER2+ MS. PC expression was decreased upon obesity, but this trend was not found in PCK. These results are not associated with variations in TME inflammation or adipocyte depots, since no changes were observed in obese vs. nonobese patients for any MS.

In addition, gluconeogenic enzymes such as FBP, PC, and PCK correlated differently with pathological stage, histological grade, HER2, and hormonal receptors, suggesting a potential prognostic value of these enzymes. Additionally, PCK is significantly correlated with diabetes and FBP is significantly correlated with hypertension and dyslipidemia,

implying that not only obesity, but rather other obesity-associated disorders must be taken into account in breast cancer prognosis and treatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15204936/s1, Figure S1: Representative images of positive controls of each assay. (a) Kidney, HK—Hexokinase; (b) liver, PFK—phosphofructokinase; (c) umbilical cord, PK—pyruvate kinase; (d) liver, PC—pyruvate carboxylase; (e) kidney, PCK—phosphoenolpyruvate carboxykinase; (f) liver, FBP—fructose bisphosphatase; (d) kidney, G6P—glucose-6-phosphatase; Figure S2: Representative images of expression patterns for (a) no expression; (b) low expression; and (c) high expression, in the example case of pyruvate kinase. Amplification: 100X; Table S1: Immunohistochemistry specifications; Table S2: Demographic and clinicopathological information cancer-associated, with the patients included in the study stratified by (a) body mass index (BMI), molecular subtype (MS), and (b) BMI and molecular subtype (mBMI); Table S3: Pathological information of obesity-associated pathologies—diabetes, hypertension, and dyslipidemia—from the patients included in the study stratified by (a) body mass index (BMI), molecular subtype (MS), and (b) BMI and molecular subtype (mBMI).

Author Contributions: Conceptualization: C.L., R.F. (Rúben Fernandes) and R.S.; methodology: C.L., R.F. (Rúben Fernandes) and R.S.; validation: C.L., R.F. (Rúben Fernandes) and R.S.; formal analysis: C.L., J.R., R.S. and F.S.; resources: N.C., P.D., R.F. (Rute Fernandes) and D.L.; writing—original draft preparation: C.L.; writing—review, editing, and validation: C.L., F.S., R.F. (Rute Fernandes), N.C., J.R., P.D., D.L., R.F. (Rúben Fernandes) and R.S.; supervision: R.F. (Rúben Fernandes) and R.S.; project Administration: C.L., R.F. (Rúben Fernandes) and R.S.; funding acquisition: C.L., R.F. (Rúben Fernandes) and R.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Portuguese Oncology Institute of Porto (IPO-Porto) Francisco Gentil, EPE, Porto, Portugal (Ref. CES. 261/020, date of approval: 3 September 2020).

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

Data Availability Statement: The datasets generated during and/or analyzed during the current study are not publicly available due to privacy or ethical restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary material – Figure S1

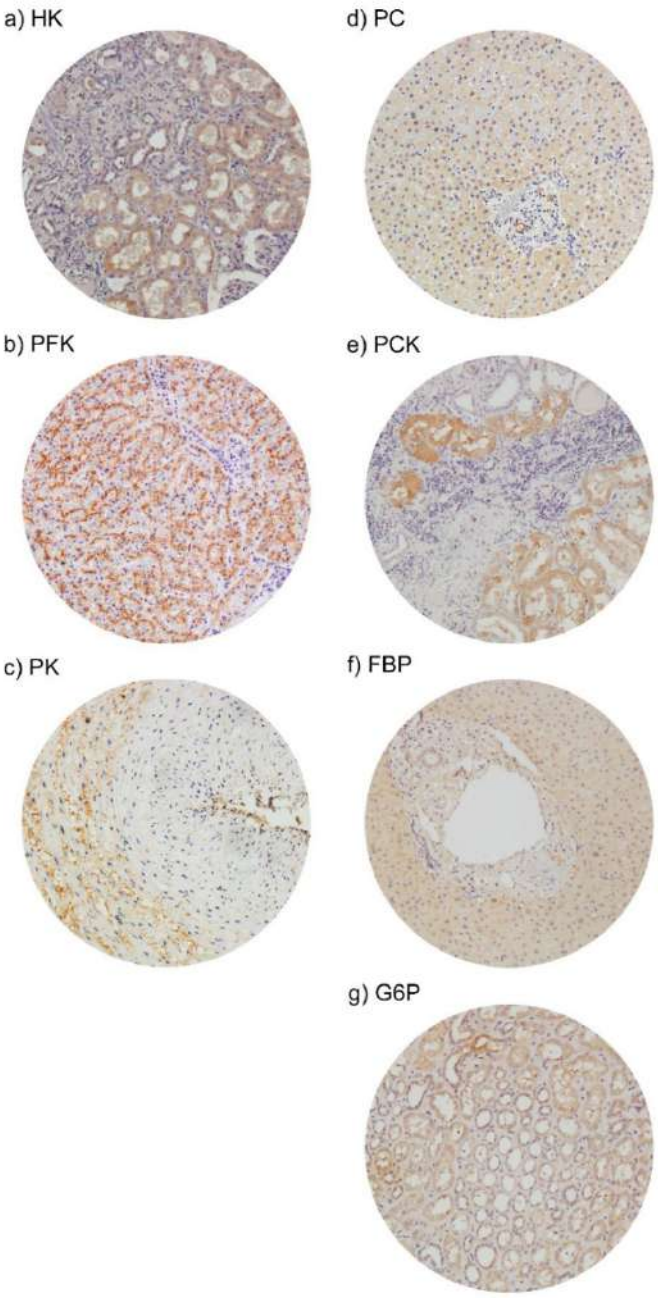


Figure S1 – Representative images of positive controls of each assay. a) Kidney, HK – Hexokinase; b) Liver, PFK – Phosphofructokinase; c) Umbilical cord, PK – Pyruvate kinase; d) Liver, PC – Pyruvate carboxylase; e) Kidney, PCK – Phosphoenolpyruvate carboxykinase; f) Liver, FBP – Fructose bisphosphatase; d) Kidney, G6P – Glucose-6-phosphatase.

Supplementary material – Figure S2

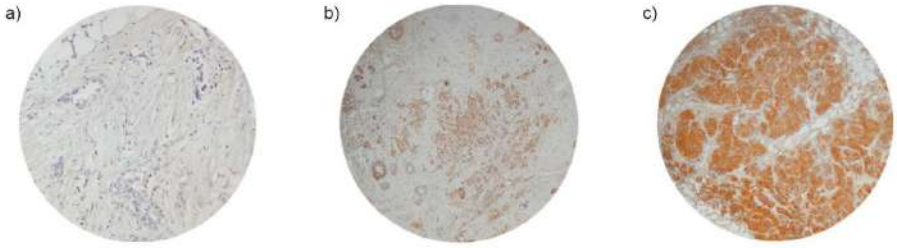


Figure S2 – Representative images of expression patterns for a) no expression; b) low expression; and c) high expression, in the example case of pyruvate kinase. Amplification: 100x.

Supplemental Material – Table S1

Table S1 – Immunohistochemistry specifications.

Enzyme	Peroxidase blockage	Blocking solution	Primary antibody		Secondary antibody		Positive Control
			Ref./Man.	Dilution	Ref./ Man.	Dilution	
HK	30min@RT 4% H_2O_2 Methanol	1h@37°C 0.2% BSA. 10% NSS in PBS	ab104836/ abcam	1:300	ab97021/ abcam	1:200	Kidney
PFK	30min@RT 4% H_2O_2 in PBS	1h@37°C 4% BSA. 10% HS in PBS	sc-393713/ Santa Cruz Biotechnology	1:400	ab97021/ abcam	1:600	Liver
PK	30min@RT 4% H_2O_2 in PBS	1h@37°C 4% BSA. 10% HS in PBS	sc-365684/ Santa Cruz Biotechnology	1:400	ab97021/ abcam	1:600	Umbilical cord
PC	30min@RT 4% H_2O_2 in PBS	1h@37°C 4% BSA. 10% HS in PBS	sc-365673/ Santa Cruz Biotechnology	1:300	ab97021/ abcam	1:400	Liver
PCK	30min@RT 4% H_2O_2 in PBS	1h@37°C 4% BSA. 10% HS in PBS	sc-271029/ Santa Cruz Biotechnology	1:200	ab97021/ abcam	1:200	Kidney
FBP	30min@RT 4% H_2O_2 in PBS	1h@37°C 4% BSA. 10% HS in PBS	ab196556/ abcam	1:200	ab97021/ abcam	1:200	Liver
G6P	30min@RT 4% H_2O_2 Methanol	1h@37°C 4% BSA. 10% HS in PBS	ab243319/ abcam	1:2500	ab97021/ abcam	1:200	Kidney

Footnotes: Ref. – Reference; Man. – Manufacturer; HK – Hexokinase; PFK – Phosphofructokinase; PK – Pyruvate Kinase; PC – Pyruvate Carboxylase; PCK – Phosphoenolpyruvate Carboxykinase; FBP – Fructose-1,6-bisphosphatase; G6P – Glucose-6-Phosphatase; PBS – Phosphate-buffered saline; RT – Room temperature; H_2O_2 – Hydrogen Peroxide; BSA – Bovine Serum Albumin; NSS – Normal Swine Serum; HS – Human serum.

Reagents specifications:

H_2O_2: Cat. 3323; Alfa Aesar, Haverhill, Mass, EUA.

Methanol: Cat. 179337-2.5L, Merck, Darmstadt, Germany.

PBS: NaCl (Merck, Darmstadt, Germany): 137 mM; KCl (Merck, Darmstadt, Germany): 2.7 mM; Na2HPO4 (Merck, Darmstadt, Germany): 10 mM; KH2PO4 (Merck, Darmstadt, Germany): 1.8 mM in water.

BSA: Cat. A23016, Sigma, Darmstadt, Germany.

NSS: Cat. S-4000-20, Vector laboratories, Newark, NJ, USA.

Antibodies: Santa Cruz Biotechnology, Texas, USA; abcam, Cambridge, United Kingdom.

Hematoxylin: Cat. 72704; Thermo Fisher Scientific, Waltham, Mass, EUA.

Supplementary material – Table S2

Table S2 - Demographic and clinicopathological information cancer associated, from the patients included in the study stratified by (A) body mass index (BMI), molecular subtype (MS), and (B) BMI and molecular subtype (mBMI).

(A)

	Normal weight (18.5 – 24.9 Kg/m²)	Obese (> 30.0 Kg/m²)	Total	p-value	Luminal A	Luminal B	Triple Negative	HER2+	Total	p-value
n (%)	32 (51.6%)	30 (48.4%)	62 (100.0%)		16 (25.8%)	16 (25.8%)	16 (25.8%)	14 (22.6%)	62 (100%)	
Age at diagnosis										
(Mean ± SD)	56.88 ± 11.15	58.13 ± 9.98	57.48 ± 10.53	0.480	53.44 ± 8.76	58.38 ± 9.11	57.19 ± 13.07	61.43 ± 10.06	57.48 ± 10.53	0.218
Topographic localization										
CQ	2 (6.3%)	1 (3.3%)	3 (4.8%)		0 (0.0%)	3 (18.7%)	0 (0.0%)	0 (0.0%)	3 (4.8%)	
IOQ	1 (3.1%)	3 (10.0%)	4 (6.5%)		0 (0.0%)	1 (6.3%)	2 (12.5%)	1 (7.1%)	4 (6.5%)	
SOQ	13 (40.6%)	8 (26.7%)	21 (33.9%)	0.680	5 (31.2%)	1 (6.3%)	9 (56.3%)	6 (42.9%)	21 (33.9%)	0.052
SIQ	2 (6.3%)	2 (6.7%)	4 (6.5%)		0 (0.0%)	2 (12.5%)	1 (6.3%)	1 (7.1%)	4 (6.5%)	
Multiple	12 (37.5%)	15 (50.0%)	27 (43.5%)		11 (68.8%)	8 (50.0%)	3 (18.7%)	5 (35.7%)	27 (43.5%)	
Other	2 (6.2%)	1 (3.3%)	3 (4.8%)		0 (0.0%)	1 (6.3%)	1 (6.3%)	1 (7.1%)	3 (4.8%)	
Laterality										
Right	17 (53.1%)	15 (50.0%)	32 (51.6%)	0.806	10 (62.5%)	7 (43.8%)	7 (43.8%)	8 (57.1%)	32 (51.6%)	0.632
Left	15 (46.9%)	15 (50.0%)	30 (48.4%)		6 (37.5%)	9 (56.2%)	9 (56.2%)	6 (42.9%)	30 (48.4%)	
Pathological stage										
Stage I	16 (50.0%)	20 (69.0%)	36 (59.0%)		11 (68.8%)	14 (87.5%)	6 (40.0%)	5 (35.7%)	36 (59.0%)	
Stage II	14 (43.8%)	8 (27.6%)	22 (36.1%)	0.321	5 (31.2%)	2 (12.5%)	8 (53.3%)	7 (50.0%)	22 (36.1%)	**0.037***
Stage II	2 (6.2%)	1 (3.4%)	3 (4.9%)		0 (0.0%)	0 (0.0%)	1 (6.7%)	2 (14.3%)	3 (4.9%)	
Histological grade										
Grade II	17 (53.1%)	20 (66.7%)	37 (59.7%)	0.227	16 (100.0%)	16 (100.0%)	2 (12.5%)	3 (21.4%)	37 (59.7%)	**<0.001***
Grade III	15 (46.9%)	10 (33.3%)	25 (40.3%)		0 (0.0%)	0 (0.0%)	14 (87.5%)	11 (78.6%)	25 (40.3%)	
Carcinoma *in situ*										
Presence	11 (34.4%)	13 (43.3%)	24 (38.7%)	0.469	9 (56.3%)	4 (25.0%)	5 (31.3%)	6 (42.9%)	24 (38.7%)	0.282
Size. Cm (Mean ± SD)	1.16 ± 2.02	1.55 ± 2.91	1.35 ± 2.47	0.649	1.70 ± 1.99	0.99 ± 1.95	1.61 ± 3.79	1.04 ± 1.63	1.35 ± 2.47	0.798
Extensive	9 (28.1%)	11 (36.7%)	20 (32.3%)	0.472	8 (50.0%)	4 (25.0%)	4 (25.0%)	4 (28.6%)	20 (32.3%)	0.367
Microcalcifications	14 (43.8%)	14 (46.7%)	28 (45.2%)	0.818	9 (56.3%)	9 (56.3%)	4 (25.0%)	6 (42.9%)	28 (45.2%)	0.236
Necrosis	20 (62.5%)	19 (63.3%)	39 (62.9%)	0.946	10 (62.5%)	9 (56.3%)	7 (43.8%)	13 (92.9%)	39 (62.9%)	**0.042***
Invasive carcinoma										
Size. Cm (Mean ± SD)	1.94 ± 0.71	1.57 ± 0.75	1.76 ± 0.75	0.465	1.54 ± 0.34	1.58 ± 0.45	1.97 ± 0.78	1.97 ± 1.16	1.76 ± 0.75	0.197
Multifocality	7 (21.9%)	7 (23.3%)	14 (22.6%)	0.891	5 (31.3%)	3 (18.8%)	2 (12.5%)	4 (28.6%)	14 (22.6%)	0.564
Invasion	11 (34.4%)	8 (26.7%)	19 (30.6%)	0.511	5 (31.3%)	6 (37.5%)	3 (18.8%)	5 (35.7%)	19 (30.6%)	0.661
Metastatic nodes										
0	20 (62.5%)	21 (70.0%)	41 (66.2%)		9 (56.3%)	11 (68.8%)	13 (81.3%)	8 (57.1%)	41 (66.2%)	
1-3	10 (31.3%)	8 (26.7%)	18 (29.0%)	0.364	7 (43.7%)	5 (31.2%)	2 (12.5%)	4 (28.6%)	18 (29.0%)	0.144
4-9	0 (0.0%)	1 (3.3%)	1 (1.6%)		0 (0.0%)	0 (0.0%)	1 (6.2%)	0 (0.0%)	1 (1.6%)	
>10	2 (6.2%)	0 (0.0%)	2 (3.2%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (14.3%)	2 (3.2%)	
Size cm (Mean ± SD) (Larger metastasis)	12.66 ± 33.16	10.23 ± 30.11	11.48 ± 31.49	0.764	18.83 ± 39.78	24.77 ± 44.26	0.54 ± 1.22	0.41 ± 0.64	11.48 ± 31.49	0.056

(B)

	Luminal A Normal weight	Luminal A Obese	Luminal B Normal weight	Luminal B Obese	Triple Negative Normal weight	Triple negative Obese	HER2+ Normal weight	HER2+ Obese	Total	p-value
n (%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	6 (9.7%)	62 (100%)	
Age at diagnosis										
(Mean ± SD)	52.13 ± 8.71	54.75 ± 9.19	59.25 ± 10.51	57.50 ± 8.09	56.88 ± 14.93	57.50 ± 11.95	59.25 ± 10.24	64.33 ± 10.09	57.48 ± 10.53	0.604
Topographic localization										
CQ	0 (0.0%)	0 (0.0%)	2 (25.0%)	1 (12.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (4.8%)	
IOQ	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	2 (25.0%)	1 (12.5%)	0 (0.0%)	4 (6.5%)	
SOQ	3 (37.5%)	2 (25.0%)	0 (0.0%)	1 (12.5%)	4 (50.0%)	5 (62.5%)	6 (75.0%)	0 (0.0%)	21 (33.9%)	0.075
SIQ	0 (0.0%)	0 (0.0%)	1 (12.5%)	1 (12.5%)	1 (12.5%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	4 (6.5%)	
Multiple	5 (62.5%)	6 (75.0%)	4 (50.0%)	4 50.0%)	2 (25.0%)	1 (12.5%)	1 (12.5%)	4 (66.7%)	27 (43.5%)	
Other	0 (0.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	3 (4.8%)	
Laterality										
Right	4 (50.0%)	6 (75.0%)	3 (37.5%)	4 (50.0%)	5 (62.5%)	2 (25.0%)	5 (62.5%)	3 (50.0%)	32 (51.6%)	0.606
Left	4 (50.0%)	2 (25.0%)	5 (62.5%)	4 (50.0%)	3 (37.5%)	6 (75.0%)	3 (37.5%)	3 (50.0%)	30 (48.4%)	
Pathological stage										
Stage I	6 (75.0%)	5 (62.5%)	6 (75.0%)	8 (100.0%)	3 (37.5%)	3 (42.9%)	1 (12.5%)	4 (66.7%)	36 (59.0%)	0.051

Stage II	2 (25.0%)	3 (37.5%)	2 (25.0%)	0 (0.0%)	5 (62.5%)	3 (42.9%)	5 (62.5%)	2 (33.3%)	22 (36.1%)	
Stage II	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	2 (25.0%)	0 (0.0%)	3 (4.9%)	
Histological grade										
Grade II	8 (100.0%)	8 (100.0%)	8 (100.0%)	8 (100.0%)	0 (0.0%)	2 (25.0%)	1 (12.5%)	2 (33.3%)	37 (59.7%)	**<0.001***
Grade III	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (100.0%)	6 (75.0%)	7 (87.5%)	4 (66.7%)	25 (40.3%)	
Carcinoma *in situ*										
Presence	4 (50.0%)	5 (62.5%)	3 (37.5%)	1 (12.5%)	1 (12.5%)	4 (50.0%)	3 (37.5%)	3 (50.0%)	24 (38.7%)	0.357
Size, cm (Mean ± SD)	1.79 ± 2.48	1.61 ± 1.53	1.69 ± 2.52	0.30 ± 0.85	0.33 ± 0.92	2.9 ± 5.12	0.83 ± 1.73	1.33 ± 1.60	1.35 ± 2.47	0.463
Extensive	4 (50.0%)	4 (50.0%)	3 (37.5%)	1 (12.5%)	0 (0.0%)	4 (50.0%)	2 (25.0%)	2 (33.3%)	20 (32.3%)	0.253
Microcalcifications	4 (50.0%)	5 (62.5%)	6 (75.0%)	3 (37.5%)	1 (12.5%)	3 (37.5%)	3 (37.5%)	3 (50.0%)	28 (45.2%)	0.333
Necrosis	5 (62.5%)	5 (62.5%)	5 (62.5%)	4 (50.0%)	2 (25.0%)	5 (62.5%)	8 (100.0%)	5 (83.3%)	39 (62.9%)	0.126
Invasive carcinoma										
Size, cm (Mean ± SD)	1.56 ± 0.28	1.53 ± 0.40	1.74 ± 0.50	1.41 ± 0.36	2.2 ± 0.60	1.74 ± 0.92	2.25 ± 1.07	1.60 ± 1.27	1.76 ± 0.75	0.210
Multifocality	3 (37.5%)	2 (25.0%)	1 (12.5%)	2 (25.0%)	0 (0.0%)	2 (25.0%)	3 (37.5%)	1 (16.7%)	14 (22.6%)	0.656
Invasion	3 (37.5%)	2 (25.0%)	3 (37.5%)	3 (37.5%)	1 (12.5%)	2 (25.0%)	4 (50.0%)	1 (16.7%)	19 (30.6%)	0.783
Metastatic nodes										
0	5 (62.5%)	4 (50.0%)	6 (75.0%)	5 (62.5%)	7 (87.5%)	6 (75.0%)	2 (25.0%)	6 (100.0%)	41 (66.2%)	0.082
1-3	3 (37.5%)	4 (50.0%)	2 (25.0%)	3 (37.5%)	1 (12.5%)	1 (12.5%)	4 (50.0%)	0 (0.0%)	18 (29.0%)	
4-9	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (12.5%)	0 (0.0%)	0 (0.0%)	1 (1.6%)	
>10	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (25.0%)	0 (0.0%)	2 (3.2%)	
Size cm (Mean ± SD) (Larger metastasis)	24.93 ± 45.72	12.74 ± 34.86	24.75 ± 45.83	24.79 ± 45.81	0.24 ± 0.67	0.84 ± 1.59	0.73 ± 0.70	0.00 ± 0.00	11.48 ± 31.49	0.340

Legend: SD – Standard Deviation; CQ – Central Quadrant; IOQ – Inferior Outer Quadrant; SOQ – Superior Outer Quadrant; SIQ – Superior Inner Quadrant; Statistical significance highlighted in bold (p-value<0.05).

Supplementary material – Table S3

Table S3 – Pathological information of obesity associated pathologies – diabetes, hypertension, and dyslipidemia – from the patients included in the study stratified by (A) body mass index (BMI), molecular subtype (MS), and (B) BMI and molecular subtype (mBMI).

(A)

	Normal weight (18.5 – 24.9 Kg/m²)	Obese (> 30.0 Kg/m²)	Total	p-value	Luminal A	Luminal B	Triple Negative	HER2+	Total	p-value
n (%)	32 (51.6%)	30 (48.4%)	62 (100.0%)		16 (25.8%)	16 (25.8%)	16 (25.8%)	14 (22.6%)	62 (100%)	
Diabetes										
Yes	3 (9.4%%)	4 (13.3%)	7 (11.3%)		1 (6.3%)	2 (12.5%)	2 (12.5%)	2 (14.3%)	7 (11.3%)	
No	29 (90.6%)	25 (83.3%)	54 (87.1%)	0.589	15 (93.7%)	14 (87.5%)	14 (87.5%)	11 (78.6%)	54 (87.1%)	0.883
Missing data	0 (0.0%)	1 (3.4%)	1 (1.6%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (7.1%)	1 (1.6%)	
Hypertension										
Yes	8 (25.0%)	17 (56.6%)	25 (40.3%)		6 (37.5%)	7 (43.8%)	6 (37.5%)	6 (42.9%)	25 (40.3%)	
No	24 (75.0%)	12 (40.0%)	36 (58.1%)	**0.008***	10 (62.5%)	9 (56.2%)	10 (62.5%)	7 (50.0%)	36 (58.1%)	0.949
Missing data	0 (0.0%)	1 (3.4%)	1 (1.6%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (7.1%)	1 (1.6%)	
Dyslipidemia										
Yes	9 (28.1%)	13 (43.3%)	22 (35.5%)		5 (31.2%)	8 (50.0%)	4 (25.0%)	5 (35.7%)	22 (35.5%)	
No	23 (71.9%)	16 (53.3%)	39 (62.9%)	0.175	11 (68.8%)	8 (50.0%)	12 (75.0%)	8 (57.2%)	39 (62.9%)	0.495
Missing data	0 (0.0%)	1 (3.4%)	1 (1.6%)		0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (7.1%)	1 (1.6%)	

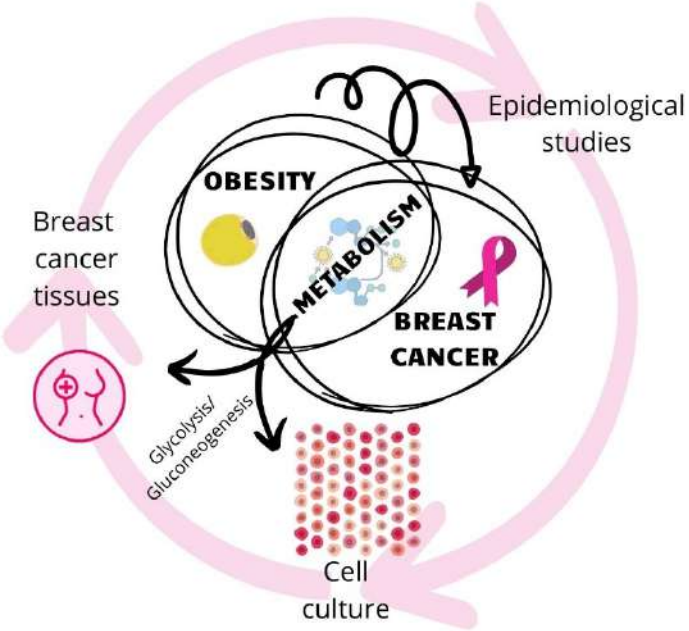
(B)

	Luminal A Normal weight	Luminal A Obese	Luminal B Normal weight	Luminal B Obese	Triple Negative Normal weight	Triple negative Obese	HER2+ Normal weight	HER2+ Obese	Total	p-value
n (%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	8 (12.9%)	6 (9.7%)	62 (100%)	
Diabetes										
Yes	1 (12.5%)	0 (0.0%)	1 (12.5%)	1 (12.5%)	0 (0.0%)	2 (25.0%)	1 (12.5%)	1 (16.7%)	7 (11.3%)	
No	7 (87.5%)	8 (100.0%)	7 (87.5%)	7 (87.5%)	8 (100.0%	6 (75.0%)	7 (87.5%)	4 (66.6%)	54 (87.1%)	0.791
Missing data	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	1 (1.6%)	
Hypertension										
Yes	2 (25.0%)	4 (50.0%)	3 (37.5%)	4 (50.0%)	2 (25.0%)	4 (50.0%)	1 (12.5%)	5 (83.3%)	25 (40.3%)	
No	6 (75.0%)	4 (50.0%)	5 (62.5%)	4 (50.0%)	6 (75.0%)	4 (50.0%)	7 (87.5%)	0 (0.0%)	36 (58.1%)	0.088
Missing data	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	1 (1.6%)	
Dyslipidemia										
Yes	1 (12.5%)	4 (50.0%)	4 (50.0%)	4 (50.0%)	1 (12.5%)	3 (37.5%)	3 (37.5%)	2 (33.3%)	22 (35.5%)	
No	7 (87.5%)	4 (50.0%)	4 (50.0%)	4 (50.0%)	7 (87.5%)	5 (62.5%)	5 (62.5%)	3 (50.0%)	39 (62.9%)	0.549
Missing data	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	1 (1.6%)	

Legend: Statistical significance highlighted in bold (p-value<0.05).

CHAPTER VI

General discussion



LIST OF CONTENTS:

- A comprehensive discussion of the research findings linking obesity and breast cancer, exploring the connections from both an epidemiological and metabolic point of view.
    - Epidemiological studies in obesity and breast cancer.
    - Glucose metabolism differences in *in situ* and *in vitro* assays in breast cancer exposed to obesity/obesogenic environment.

CHAPTER VI

1. General discussion

The metabolic complexity that links obesity and breast cancer has already been established. The present work aimed to deconstruct such intricacy by examining the connection between both pathologies from three different perspectives: 1) epidemiology studies, complemented with 2) *in vitro* assays and 3) human sample *in situ* assays. In the development of this work, national and international ethical recommendations were followed, and the study was approved by the Ethics Committee of the institutions involved (see Appendix A).

In the epidemiological studies performed, several notable trends and associations between normal weight and overweight/obese breast cancer patients were observed. Obesity was associated with delayed breast cancer diagnosis, reduced association with cancer family history, progesterone receptor overexpression, greater tumor aggressiveness, and an interesting relationship between tumor size and metastasis. Overweight and obese patients also exhibit lower overall survival rates when compared to normal weight patients.

In breast cancer tissues, we identified differential expression of rate-limiting enzymes for glycolysis and gluconeogenesis when stratified by molecular subtype and BMI, which correlated with clinicopathological features and metabolic syndrome conditions. Moreover, expression of rate limiting enzymes resulted in some controversial results between cell culture and BC tissues. Our findings in cell cultures indicated that different molecular subtypes displayed varying metabolic profiles in response to an obesogenic environment. Notably, the presence of HER2 receptor seemed to play a pivotal role in influencing central metabolic processes. Additionally, triple-negative BC subtype exhibited a more pronounced metabolic imbalance. The tumor microenvironment played a role in enzymatic expression alterations, with implications in various metabolic pathways.

1.1. Epidemiological studies in obesity and breast cancer

The epidemiological studies are often crucial for the decision-making process and can result in the development of programs, policies, and interventions to improve public health. Such is of upmost importance especially when the focus of the work is on two major pathologies with high incidence and mortality rates associated with a huge burden to the national healthcare systems.

Our epidemiological study focused on breast cancer patients diagnosed at IPO-Porto and involved 2282 clinical records (surgery information included) of individuals with varying BMI categories. From this database, referred to as the "DOC – Deciphering Obesity and Cancer" database, we conducted individual case analyses and established

specific exclusion criteria for each study outlined in Chapter III. A total of 14 cases of male breast cancer was identified and excluded from all subsequent analysis and studies. Male breast cancer is a rare condition and considered a different entity from female breast cancer. It is accountable for less than 1% of diagnosed breast cancers although with increasing rates in the last decades[1] According to data from the Surveillance, Epidemiology, and End Results (SEER), the men breast cancer rate witnessed a 40% increase between 1975 and 2015[2]. Given the rarity of this disease, available studies are limited and scarce, making its exact etiology an ongoing challenge. Risk factors for male breast cancer included family history – genetic predisposition, liver disease, testicular abnormalities, obesity, with advanced age being the most common[1]. From the 14 cases male breast cancer cases (corresponding to 0.6% of the target population under study) included in the DOC database, we observed 4 normal-weighted, 9 overweighted and 1 obese. Given the reduced number of cases and the fact that their etiopathogenesis differs from the female breast cancer ones, these cases were excluded from our study.

The retrospective study regarding the analysis of tumor characteristics and clinical outcomes of overweight and obese women with breast cancer included 2246 cases and excluded both male breast cancer and underweight women (n=22). It can be somewhat challenging to evaluate clinicopathological information for underweight individuals since it is often grouped together with those of normal weight[3,4]. However, studies with separated BMI categories, observed that underweight women prognosis is distinct from normal weight. In metastatic cancer, underweight patients presented higher metastatic rates (in number of metastatic localizations and in visceral metastasis) associated with lower overall survival (OS) and disease-free survival (DFS)[5]. Also, underweight women tend to exhibit a less favorable prognosis and experience worse outcomes[6,7]. In certain studies, their outcomes are comparable to those of individuals with morbid obesity[8]. Considering these conclusions, the aim of our study, and the limited number of underweight patients, we chose not to include this BMI category in the analysis that culminated in the manuscript titled "A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer" (Chapter III).

This study focused on the northern Portuguese population where obesity presents a high prevalence rate. According to the latest update of INE (*Instituto Nacional de Estatística* - Statistics National Institute) from data collected in 2019, the northern Portuguese population proportion of overweight and obese adults reached 53.7%, with a higher occurrence in women (58.1%)[9]. Among our breast cancer series, a higher proportion of overweight and obese patients was observed. The combined percentage of overweight and obese women amounted to 65.4%, while those of normal weight comprised 34.6% of the cohort. These findings led us to assume that obese patients are more prone to develop BC, which is corroborated by the literature[10]. Overall results from this study further revealed that overweight and obese patients are mostly associated with worst outcomes. These results are consistent with the supporting evidence provided in Table 2 – Chapter I. A limitation of our study is the lack of data regarding menopausal status. Table 2 also disclosures that obesity increases the risk of postmenopausal BC and is either negatively associated or not associated with premenopausal breast cancer. An elevated

risk of breast cancer is largely attributed to increased estrogen exposure, which is associated with a later age at menopause[11].

From the aforementioned study, as previously reported, overall results revealed worst outcomes for obese and overweight women, which includes poorly differentiated tumours, larger primary tumours, and lower overall survival. Distinctly, breast cancer in obese women had progesterone receptor overexpression, while overweight women had increased risk of developing ductal invasive carcinoma. Both overweight and obese women were less likely to have BC family history and were diagnosed later with breast cancer than normal weight women.

Confronted with the outcome (overweight and obese patients were diagnosed at a later age), we were prompted to investigate whether this delayed diagnosis correlated with a more advanced tumor stage. This study resulted in the narrative review titled "A narrative review in why breast cancer is diagnosed later in obese women" (Chapter 3). Interestingly, a subsequent analysis of the DOC database, disclosed that age at diagnosis was inversely correlated to tumour stage. A more advanced disease (stage IV) was found in younger ages, while stages I and II exhibited significant older age at diagnosis. We speculated that this could be associated with triple negative BC cases. TNBC is an aggressive and clinically challenging type of BC due to lack of therapeutical options. Also, TNBC is more frequently diagnosed in younger women[12], which could provide an explanation for our findings. Nonetheless, we conducted an in-depth analysis encompassing various factors such as lifestyle, culture, climate, economic conditions, healthcare systems, and screening programs to explore additional explanations for this phenomenon. In conclusion, while we could not pinpoint a definitive cause supported by structured evidence, obesity appears to delay cancer development. A consistent and widespread global trend. This analysis underscored the significance of raising awareness about the impact of obesity on breast cancer within the clinical community and among healthcare decision-makers. We deliberately selected a national journal, edited by a national society (*Sociedade Portuguesa de Oncologia*), to ensure that this awareness is effectively conveyed to professionals in the field.

Revisiting the initial study on tumour characteristics and clinical outcomes, another relevant finding arose, overweight women are more likely to develop bilateral breast cancer (BBC) that obese woman, and so we performed a subsequent analysis in BBC. Despite the extensive research on obesity and breast cancer, the specific relationship between obesity and BBC has received limited attention. The presence of tumours in both breasts is considered to be a distinct type of metastatic breast cancer. Being a relatively rare condition, DOC database included only 42 women with BBC, from which 14 women with normal weight and underweight, 22 with overweight and 6 with obesity. The study resulted in the publication of the manuscript entitled "Bilateral breast cancer and the influence of body mass index in clinicopathological features and overall survival", where we aimed to analyze BBC as an independent disease and the potential implication of obesity in the tumours clinical features. A population analysis revealed that overweight and obese women represented 66.6% of the population, approximately the same

proportion as previously observed in the initial study. The clinicopathological characterization of BBC cases in this study revealed several noteworthy findings. Synchronous BBC cases (tumours diagnosed within one year interval) were more common than metachronous BBC, but no significant differences were found when stratified by BMI. Furthermore, it was observed that BBC cases were less likely to be associated with genetic predisposition, and notably, there were no association with family history among obese patients. The distribution of tumor stage and differentiation grade exhibited notable variations in the analysis of BBC cases. A significant distribution of tumour stage was observed, where stage 2 emerged as the most prevalent, accounting for roughly 50% of the cases. Only one case was categorized as grade 1, and it belonged to an overweight woman. Remarkably, all obese women displayed poorly differentiated tumors. The histological type was often concordant between both breast tumors when no BMI stratification was applied. All cases exhibited concordance in terms of receptor status. The most prevalent was luminal subtype with expression for both estrogen and progesterone receptor and negative for HER2. When stratified by BMI, no statistical significance was achieved. The analysis of overall survival unveiled that overweight BBC patients tend to experience an extended OS, although without statistical significance. This inconsistency is worth highlighting. Overweight women are more susceptible to develop BBC, yet obese women display a worst outcome, as expected. One could speculate that the extended OS in the overweight group may be influenced by the declined survival rate in the normal weight group due to the inclusion of underweight women. Moreover, overweight patients usually present a healthier phenotype when compared to those who are obese, who generally display metabolic pathologies.

As mentioned before, underweight group may present some challenging metabolic profiles that could result in a worst prognosis. In this study, we merged underweight and normal weight women in the same group to improve statistical strength (as observed in other related studies) and to include all BBC cases. We now hypothesize that this might have introduced bias into our results. A review of the literature on underweight women and bilateral breast cancer yielded no findings related to tumor characteristics and overall survival. However, three studies were identified regarding bilateral reconstruction surgery[13-15]. Underweight women are a challenging group for breast reconstruction because they usually present minimal subcutaneous fat and refuse to consider breast implants. All three studies focused on surgical options for underweight and normal weight women with low subcutaneous fat, aiming to achieve a better outcome with fewer surgical complications.

The study presented in Chapter III entitled "Does body mass index influence surgical options and overall survival in breast cancer patients?" did not include underweight women. A statistical analysis was performed focusing on the number and type of surgical interventions in our cohort and overall survival. The maximum number of surgery procedures was 5 and type of surgery included: sentinel node biopsy, axillary dissection, conservative surgery, total mastectomy, radical mastectomy, and mastectomy with reconstruction. No statistical association was found between BMI and number of surgical procedures. Overweight women have an increased likelihood to be submitted to sentinel

node biopsy, while overweight and obese women are more likely to be submitted to conservative surgery and less likely to undergo total mastectomy, eventually being attributed to increased subcutaneous fat. As expected, patients who underwent conservative surgery tended to experience improved OS. On the other hand, women not submitted to total mastectomy also exhibited improved OS. We postulated that this result could be correlated with the extension of the disease. When stratified by BMI, all analysis conducted revealed that obese patients presented a lower OS, as observed in the study "A retrospective study in tumour characteristics and clinical outcomes of overweight and obese women with breast cancer". Unfortunately, we were unable to access information concerning adjuvant therapy and surgical complications. We believe that an analysis of these factors would provide a more comprehensive understanding of the detrimental effects of obesity on tumour surgery, particularly in terms of postsurgical infections and increased resistance to adjuvant therapy[16].

In light of the discussed findings and the association between obesity and a lower incidence of distant metastasis (not yet mentioned), we suggest that the implications of obesity may not be entirely detrimental. Our results also disclosured that overweight women have less distant metastasis (in a non-adjusted statistical model). It is plausible that the concept of the "obesity paradox" implies a more nuanced perspective, where the situation is not strictly dichotomous but rather manifests with varying complexity. The term "obesity paradox" is often used to describe the phenomenon where individuals with obesity, despite having a higher body mass, may exhibit better health outcomes or survival rates in certain medical conditions or situations and may present themselves as metabolically healthy[17]. The definition of metabolically healthy/unhealthy obesity remains a subject of ongoing discussion, over than 30 different definitions have already been recognized[18]. Up till now, four major obesity subtypes have been identified: metabolically healthy obese; metabolically unhealthy obese; normal weight metabolically obese; and sarcopenic obese[19]. Previous studies of the literature review accessible in Chapter I have noted that BMI could play a protective role in breast cancer[20,21]. It is possible that some individuals in our cohort may fit into these groups, but for a more accurate identification, it is important to establish clear criteria for defining the characteristics of healthy obesity. This led us to conduct an additional study aimed at refining the classification of obese women into healthy and unhealthy categories through an analysis of routine clinical biomarkers (see Appendix B). We believe that a range of employed routine features could identify the metabolic pattern of obese patients and result in the improvement of breast cancer treatment program in obese women.

In summary, our epidemiological study examined breast cancer patients in the northern Portuguese population, highlighting the complex relationship between obesity, breast cancer regarding clinical outcomes. The implications of the epidemiological results regarding obesity on breast cancer involved a metabolic perspective of both pathologies. The intrinsic metabolic connection is discussed further on the following subsection.

1.2. Glucose metabolism differences in in situ and in vitro assays in breast cancer exposed to obesity/obesogenic environment.

To better understand the epidemiological results, we further concentrated on the metabolic pattern of glycolysis and gluconeogenesis pathways in cell cultures exposed to an obesogenic environment and tissue samples from normal weight and obese women with breast cancer. Among the several metabolic pathways previously described in the literature as altered in the obese condition, we concentrated our efforts in the carbohydrate metabolism. In particular, in glycolysis and gluconeogenesis based on prior findings from our research group[22]. As documented in Chapter I, our group developed a preliminary study in the influence of obesity in BC cell lines. The findings implied that under obesogenic conditions, BC MCF-7 cell line triggered the activation of gluconeogenic pathways, contrary to the anticipated Warburg effect. This observation was termed "Warburg effect inversion". These findings were the foundation of the current research plan and paved the way for other parallel studies, namely in other types of cancer.

Our current findings did not corroborate the former ones regarding the Warburg effect inversion. We speculate that the activation of the gluconeogenic pathway occurred as a consequence of cell mutations or was influenced by the varying compositions of obesogenic secretome during the adipocyte differentiation attempts. Previous studies also observed a downregulation of the Warburg effect, despite not associated with obesity. For example, a study conducted post - "Warburg effect inversion", observed a similar metabolic system inversion in malignant neuroblastoma cell lines, pHe dependent[23].

Nonetheless, our present findings were able to underscore the intricate complexity of obesity's impact on the overall metabolism of breast cancer. Specifically, these results highlighted distinct influences leading to a specific metabolic profile for each molecular subtype. We also found distinct results in BC cell lines comparing to BC tissues.

In the context of cell-based assays, our investigation extended beyond the examination of glycolysis and gluconeogenesis rate-limiting enzymes expression to include a comprehensive analysis of the associated metabolites in cell lines representing the four major molecular subtypes. This study culminated in the published manuscript "The influence of adipocyte secretome on selected metabolic fingerprints of breast cancer cell lines representing the four major breast cancer subtypes". A comprehensive examination of glycolysis rates in BC tissues presented in Chapter V in the paper "Breast Cancer Molecular Subtypes Differentially Express Gluconeogenic Rate-Limiting Enzymes—Obesity as a Crucial Player" indicates a downregulation of most glycolytic enzymes, consistent with the observations in BC cell lines. The coherence between glucose consumption and lactate production aligns with the enzymatic expression in cell-based assays, particularly in the triple-negative molecular subtype, where a notable decrease in glucose consumption is evident, as well as the expression of most glycolytic rate limiting enzymes.

In BC cell lines, we observed a similar pattern in Luminal A and triple negative molecular subtypes. Both exhibited an increased expression of PFK and a decreased expression of all remaining enzymes when exposed to obesogenic environment. On the other hand, in BC tissues stratified by molecular subtype and obesity, the number of cases with PFK high expression was higher in triple negative but not in luminal A. Regarding PK expression in BC tissues, luminal and HER2 positive share a common decrease in obese women, although not observed in cell culture, the pattern between Luminal B and Her2+ is similar in cell-based assays. Interestingly, HK expression is nearly absent in BC tissues.

Regarding gluconeogenesis enzymatic rates in BC tissues, we noted a significant variation in the expression of PC, PCK, and FBP. However, this difference is most likely related to the enzymatic pattern of molecular subtypes than to alterations induced by obesity. All molecular subtypes presented a decreased number of cases with PC expression, mostly coherent with cell-based assays where PC is exclusively upregulated in HER2 positive representative cell line. PCK has opposite expression rates in BC cell lines in comparison with BC tissues. We disclosure an interesting result for FBP expression, none of the *in-situ* tumors in the triple-negative molecular subtype expressed FBP. In cell lines there was a downregulation of the FBP expression in nearly all molecular subtype except in HER2 positive, mirroring the observed pattern in PC. In the final rate-limiting step of gluconeogenesis, the expression of G6Pase showed a opposite pattern between BC tissues and BC cell lines, discrepancy previously observed in PCK expression.

Gluconeogenesis seems to be upregulated in Luminal B and Her2 positive cell lines, articulated with increased expression of most gluconeogenic rate limiting enzymes. In BC tissues, when stratified by molecular subtype and obesity, there is no clear evidence of the metabolic imbalance promoted by obesity in gluconeogenesis pathway.

Cell lines exposed to the obesogenic environment expressed different enzymatic patterns aligned with specific metabolomic profiles. This prompted us to acknowledge that glycolysis/gluconeogenesis pathways are intrinsically associated with several other pathways such as citric acid cycle, pentose phosphate pathway, amino acid, and lipid metabolism. This standpoint has motivated us to support glycolysis as an anabolic pathway, challenging the prevailing notion of its primarily catabolic role. Glycolytic and gluconeogenic rate limiting enzymes play pivotal roles in energy metabolism, and alterations in their activities can have profound effects on cellular functions, not limited to the glucose metabolism.

In the initial stage of glycolysis, glucose undergoes phosphorylation to form G6P and is subsequently isomerized to F6P. These two metabolic intermediates, G6P and F6P, have the potential to participate in the PPP. These pathways play a crucial role in supporting the anabolic processes observed in cancer cells. Depending on the cell requirements, the oxidative or the nonoxidative phase of the PPP can be activated. The oxidative phase originates NADPH, which is essential for reductive syntheses like fatty acid, cholesterol, and certain amino acids. When the cell needs nucleic acid synthesis only, the non-oxidative phase will be activated rendering ribose-5-phosphate as a substrate for nucleotide assembly. Both glycolysis/gluconeogenesis and PPP are intrinsically

connected. Gluconeogenic enzymes such as PCK was already described as a potential mediator in PPP and promotes building blocks synthesis to boost tumour growth[23,25]. Our results reveal opposite results regarding this enzyme. In luminal B and Her2 positive BC cell lines, PCK is overexpressed in obesogenic conditions, whereas in BC tissues from obese patients, the molecular subtype with most evident overexpression of PCK is luminal A and the molecular subtype with most evident downregulation of PCK is luminal B.

Glycolytic and gluconeogenic enzymes also play a role in influencing amino acid metabolism as part of their functions. Our findings from cellular cultures demonstrate a substantial impact of obesity on amino acid metabolism. The reduction in glycolytic intermediates observed in our study may suggest the conversion of these metabolites into amino acid metabolism. Glycolytic intermediates, like phosphoenolpyruvate (and associated PK activity), can be diverted into pathways that contribute to the biosynthesis of amino acids like serine[26]. Curiously enough, it was also described that the BC molecular subtype can influence serine metabolism[27]. Another example is alanine, which can be generated through transamination reactions involving pyruvate. Glutamine plays a vital role in BC metabolism. The well-established link between glutaminolysis and the proliferative requirements of tumors is emphasized, with glutamine serving as the second most utilized substrate for tumor cells regarding both nitrogen and carbon atoms demands[28]. The nitrogen is indispensable for tumor cell synthesis of non-essential amino acids and nucleotides for instances. Simultaneously, carbons play a pivotal role as the primary source for various cellular pathways, including the TCA cycle metabolites and fatty acid synthesis[29].

Lipid metabolism is also influenced by glycolysis and enzymes of the glycolytic pathway. PK, for instance, provides substrates like pyruvate that is metabolized into acetyl-CoA which is also originated from amino acids and used as fatty acids substrate. Additionally, gluconeogenic intermediates form glycerol, which is a precursor for complex lipid synthesis. Fatty acids and lipid metabolism play important roles in cancer, namely in breast cancer[30]. Our cellular based results reveal that PK is upregulated in luminal B and HER2 positive BC subtypes attenuating the triglycerides levels in comparison to luminal A and triple negative subtypes, which express higher levels of triglycerides in the obesogenic condition. Moreover, all molecular subtypes present high amounts of cholesterol in the obesity mimicking condition.

TCA cycle is closely connected to glycolysis and gluconeogenesis. Pyruvate generated from glycolysis is a key substrate for the TCA cycle, which shares several intermediates with gluconeogenic metabolites like oxaloacetate. The TCA cycle functions as a central hub, seamlessly integrating diverse molecules and metabolic pathways, encompassing carbohydrates, fatty acids, amino acids, and ketone bodies[31]. It operates as an amphibolic pathway, serving dual roles in both catabolic and anabolic processes. Anaplerotic reactions include reloading escaping TCA cycle intermediates. Oxaloacetate often escapes from this pathway for pyrimidines and glucose biosynthesis. Succinyl-CoA escape may lead to the formation of porphyrins. Several other intermediates might also break from TCA cycle for protein synthesis. Citrate is able to escape to the cytoplasm to

form acetyl-CoA and oxaloacetate (OAA). Acetyl-CoA may then participate in lipid biosynthesis. Amino acids represent an additional source that can be used to fill the leaks in the TCA cycle[32]. Most of these available molecules may be derived from the tumour microenvironment.

The tumor microenvironment (TME) plays a crucial role in breast cancer progression, influencing various aspects of the pathways involved on its complex metabolism. The TME consists of a complex network of cellular and non-cellular components, including immune cells, fibroblasts, blood vessel cells, extracellular matrix, and signaling molecules and could also be modulated by obesity-related factors. Immune cells within the TME, such as tumor-associated macrophages (TAMs), T lymphocytes, and regulatory T cells (Tregs), present a relevant impact in breast cancer tumorigenesis and progression. Previous studies observed a decrease in the prevalence of infiltrating CD8+ T cells and a diminished M1/M2 macrophage ratio. These outcomes suggest a compromised anti-tumoral immune response. Moreover, it was also observed that tumour signaling factors can also modulate the immune macroenvironment by a regulatory crosstalk. So, M1/M2 ratio is also altered in visceral adipose tissue, correlated with an increase level of Tregs and FoxP3[33]. Cancer-associated fibroblasts and Extracellular Matrix (ECM) contribute to the physical structure of the TME. Aberrant interactions between cancer cells and the ECM can facilitate invasion and metastasis[34]. Alternatively, adipose-derived stem/stromal cells have the potential to differentiate into carcinoma-associated fibroblasts (CAFs) in response to tumor-released factors, with this differentiation occurring more swiftly under obesity conditions[35]. CAFs are able to increase tumorigenesis and tumour progression, with higher likelihood for tumour recurrence and therapeutical resistance[36]. TME is also able to influence angiogenesis, factors like VEGF and HIF-1α play key roles in regulating angiogenesis[37]. Hormonal receptors, such as estrogen and progesterone receptors, are crucial in breast cancer. The TME can modulate hormone receptor signaling, affecting not only tumor response to endocrine therapies but also the tumour development[38]. Obesity, as previously presented, is able to regulate estrogen levels through the enzyme aromatase and therefore modulate TME[39].

Overall, the TME is modulated by obesity-related factors, such as inflammation, which results in a substantial impact in tumour initiation, proliferation, angiogenesis, metastasis, and response to therapy. Obesity leads to an augmented infiltration of proinflammatory cytokines, adipokines, adipocytes, immune cells, and tumor cells within the expanded adipose tissue. The intricate networks of cellular-molecular crosstalk undergo complex changes, influencing crucial pathways, orchestrating reprogramming in metabolic and immune functions[40]. In tumour tissues, we quantified the presence of adipocytes, fibrosis, and inflammation, which could highlight potential TME alterations, but no significant alterations were observed in cases stratified according to BMI. Nonetheless, it is important to highlight the correlations observed between enzymatic expression, tumor characteristics, and patient comorbidities. Gluconeogenic enzymes like FBP, PC, and PCK exhibited distinct correlations with pathological stage, histological grade, HER2, and hormonal receptors, indicating a potential prognostic significance for these enzymes. Notably, PCK demonstrated a significant correlation with diabetes, while FBP showed a

significant association with hypertension and dyslipidemia. This suggests that in the context of breast cancer prognosis and treatment, considerations should extend beyond obesity alone to encompass other obesity-related disorders.

Regarding the patients characteristics in the development of BC, menopausal status represents a pivotal turning point. Unfortunately, we were unable to obtain information on the menopausal status of the women included in the study, an acknowledged limitation of the study. As highlighted in Chapter I, menopause distinctly affects metabolic status of patients and their susceptibility to breast cancer development[41].

Another limitation we acknowledged in both studies relates to the selection of enzymatic isoforms. A brief revision of the literature in this matter reveals the variability in isoform expression within the glycolytic/gluconeogenic pathways[42,43]. To overcome this limitation, we have additionally conducted a quantification of enzymatic isoforms within the glycolytic/gluconeogenic pathways using RT-PCR and compared the results to those of breast cancer tissues publicly available on the *GEPIA* platform[44]. Our comparative study centers on the expression of each isoenzyme in a tumor cell line (MCF-7) and tumor tissue, juxtaposed with an epithelial cell line (MCF-10A) or adjacent normal breast tissue. Consistent with the previously presented results, we observe opposite expression patterns in the majority of isoforms (Appendix C).

Obesity in the context of breast cancer introduces a complex and detrimental metabolic imbalance. Its complexity is transversal to several pathways and may be driven by TME signaling factors. Moreover, BC molecular subtypes seems to be a crucial characterization and metabolic influencing factor. We intend to continue our research on obese BC patients to better understand the metabolic impairment promoted by obesity. Future perspectives can be consulted in the next Chapter.

Based on our findings, we emphasize the significance of personalized medicine in addressing each patient as a distinct entity, with specific metabolic features. This approach recognizes the unique molecular and metabolic characteristics of individuals, paving the way for more targeted and effective medical interventions tailored to specific patient needs.

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CHAPTER VII

Main conclusions and future perspectives



LIST OF CONTENTS:

- Synopsized results and main conclusions
- SWOT analysis
- Future perspectives

CHAPTER VII

1. Synopsized results and main conclusions

This doctoral thesis aimed to provide a comprehensive analysis of the intricate relationship between breast cancer, obesity, and metabolism through the analysis of three different perspectives. Through epidemiological studies, addressing in different ways how obesity influence in breast cancer as well as original research in both cell lines and human tissues, we believe that the integration of these multi-disciplinary perspectives significantly contributed to the understanding of this critical health issue.

The overall findings demonstrated that obesity is a complex and multifaceted risk factor for breast cancer development. We have established that the association between obesity and breast cancer is not uniform across all molecular subtypes of the disease and that specific molecular mechanisms underlie this connection. The implications of this research may not be limited to the realms of clinical practice. They carry profound public health significance, emphasizing the need for comprehensive prevention strategies that address the obesity epidemics and its impact on breast cancer incidence and prognosis.

In the epidemiological studies, several noteworthy trends and associations related to breast cancer among overweight and obese patients have emerged:

- Firstly, the research highlights that those patients with overweight and obesity, presenting unilateral or bilateral breast cancer, tend to be diagnosed at a later age. This delayed diagnosis could have implications for treatment options and outcomes. A coextensive article in this matter strengthens this result (Chapter III - A narrative review in why breast cancer is diagnosed later in obese women"): Obese patients are diagnosed later worldwide.
- Family history of breast cancer (according to previous literature, mainly associated with BRCA1 and BRCA2 mutations) is less common among overweight and obese patients, suggesting that tumorigenesis in obese women is sporadic, mainly due to environmental/ lifestyle factors.
- Obesity is associated with a higher likelihood of having progesterone receptor-positive breast cancer. This finding deserves further study since this association may potentially result in preventive/prognostic/therapeutical strategy. There are few studies addressing the importance of the progesterone receptor in breast cancer patients, specifically in obese individuals.
- Obese individuals are more susceptible to poorly differentiated tumors.
- Furthermore, obesity is associated with the presence of larger primary tumors, although paradoxically, with fewer cases of distant metastasis. This complex relationship between tumor size and metastasis warrants further investigation disclosing a potential obesity paradox in breast cancer.
- As expected, overweight and obese patients generally present lower overall survival rates.

- There is no statistical evidence suggesting that overweight and obese patients undergo a greater number of surgical procedures.
- In terms of type of surgery, overweight women have an increased likelihood of undergoing sentinel node biopsy, while overweight and obese women are more likely to be submitted to conservative surgery and less likely to undergo total mastectomy.
- In bilateral breast cancer, the findings reveal that all obese women presented poorly differentiated tumors. Intriguingly, overweight women demonstrated better overall survival, although without statistical significance.

It is our understanding that the epidemiological findings are the result of the metabolic complexity between obesity and breast cancer. To better address this, we focused on the glucose central metabolism. We evaluated the glycolytic and gluconeogenic enzymatic expression both *in vitro* and in breast cancer tissues. Interestingly, we observed different results in cultured cells and in human tissues. Perceptibly, the tumour microenvironment potentially plays a significant role on the central metabolic carbohydrate pathway. Regarding the *in vitro* research, our study unravels:

- All molecular subtypes representative cell lines based their metabolic profile in the Warburg effect, with lactate production and glucose consumption. Notably, the triple negative cell line displayed a significant reduction in both metabolites.
- With the exception of the Luminal, the remaining subtypes switched from consumption to production of acetate in the adiposity mimicking condition.
- In the adiposity environment, all molecular subtypes exhibit decreased glutamine, valine, and isoleucine consumption, with the triple negative cell line showing a particularly significant reduction.
- The metabolic profile of alanine shifts from production to consumption in Luminal A and triple negative cell lines when exposed to obesogenic conditions.
- An increased lipids consumption was also observed in all subtypes.
- All glycolytic and gluconeogenic rate-limiting enzymes were elevated in HER2+.
- PFK was the only enzyme with increased expression in both Luminal A and Triple negative cell lines.
- TP53-Induced Glycolysis Regulatory Phosphatase (TIGAR) was reduced in the Luminal A and Triple negative cell lines but increased in the Luminal B and HER2+ cell lines.
- Comparative analysis with the control group revealed similar patterns in glycolytic and gluconeogenic enzymes of obese cases within the group with HER2 positive (Luminal B and HER2+) and the group with HER2 negative (Luminal A and Triple negative).

Our research findings provide deeper insights into the complex relationship between breast tumour metabolism and adipocyte-related factors. One prominent observation is the pivotal role played by the HER2 receptor in influencing central metabolic processes. Molecular subtypes with HER2 positivity exhibit distinct metabolic features that set them apart from those with HER2 negative. This suggests that the HER2 receptor status is a

critical determinant of metabolic behaviour in breast tumours. Our investigation also highlights a more pronounced metabolic imbalance in the triple-negative breast cancer MDA-MB-231 cell line. This subtype lacks expression of estrogen, progesterone, and HER2 receptors and is associated with more aggressive and challenging-to-treat breast cancer. Moreover, and as expected, *adipofactors* play a significant role not only on the carbohydrate, but also in the lipid and amino acid metabolism, which in turn is also distinguished across the molecular subtype.

To further elucidate the role of adipocyte-related factors in breast cancer glycolysis and gluconeogenesis rate-limiting enzymes, additional immunohistochemistry studies were performed in a breast cancer tissue cohort integrating normal weight and obese patients. We further highlight the main findings:

- A statistical association between the pathological stage, histological grade and molecular subtype was identified. This pattern was also observed with respect to molecular subtype and body mass index combined. Notably, luminal cases frequently exhibited lower pathological stages and histological grades. Within the Luminal A subtype, downregulation of proliferation genes was observed, which is indicative of a more favourable prognosis.
- When stratified by molecular subtype, results achieved significant alterations on. Significant levels of PC, PCK, and FBP in tumour cells were maintained when population were stratified by molecular subtype and body mass index combined.
- Also, PC, PCK, and FBP correlated significantly with patients clinicopathological features such as receptor status and histological grade.
- Obese women with triple negative molecular subtype tend to express high levels of all glycolytic enzymes, lead us to speculate that this subtype might present an increased glycolytic rate.
- Infiltration of adipose tissue did not show any association with the enzymatic expression. Intriguingly, we uncovered that PC expression was the sole enzyme exhibiting a positive correlation with adipose tissue infiltration within the tumour microenvironment. On the other hand, PC was also significantly correlated with inflammatory infiltrate.
- Regarding metabolic syndrome conditions (diabetes, hypertension, and dyslipidemia), a positive correlation was observed between PCK and diabetes and between FBP and both hypertension and dyslipidemia.

Our observations revealed contrasting results between the expression of rate-limiting enzymes of glycolysis and gluconeogenesis when comparing cell-based assays with results obtained from breast cancer tissues. In cell culture, the enzyme that exhibited a more pronounced difference was PFK, a glycolytic enzyme. In contrast, in breast tissues, the enzymes PC, PCK, and FBP, gluconeogenesis pathway, were identified as differentially expressed and correlated with various features obesity related. Furthermore, we hypothesize that the tumour microenvironment in obese breast cancer patients differs from that in normal-weight individuals, although we did not observe statistical differences in adipocyte and inflammatory infiltration. We believe that these enzymatic

alterations have implications in other pathways such as the pentose phosphate pathway, amino acid metabolism, hexosamines, and glycerol-3-phosphate metabolism.

This thesis underscores the importance of continued research regarding the importance of addressing the glucose metabolism to better understand the complex interplay between breast cancer and obesity. Gluconeogenesis, specifically, seems to play a critical role in reshaping the metabolic landscape within the tumour, influencing its behavior, particularly in obesity-associated breast cancer.

2. SWOT analysis

For the purpose of methodically examining our study and its potential subsequent research (from conclusions to future perspectives) a SWOT analysis was conducted. The acronym SWOT stands for: S: Strengths; W: Weaknesses; O: Opportunities; T: Threats. Strengths are internal attributes and resources that can use as an advantage. Weaknesses are internal factors that hinder the ability to achieve the goals. Opportunities are external conditions or trends that can be capitalized to achieve the goals. Threats are external factors that could potentially harm the continuation of the research.

It is a strategic planning tool, originally employed in the realm of general management, but now adapted for various contexts, including education, health, and healthcare. Figure 1 presents the SWOT analysis of the present work.

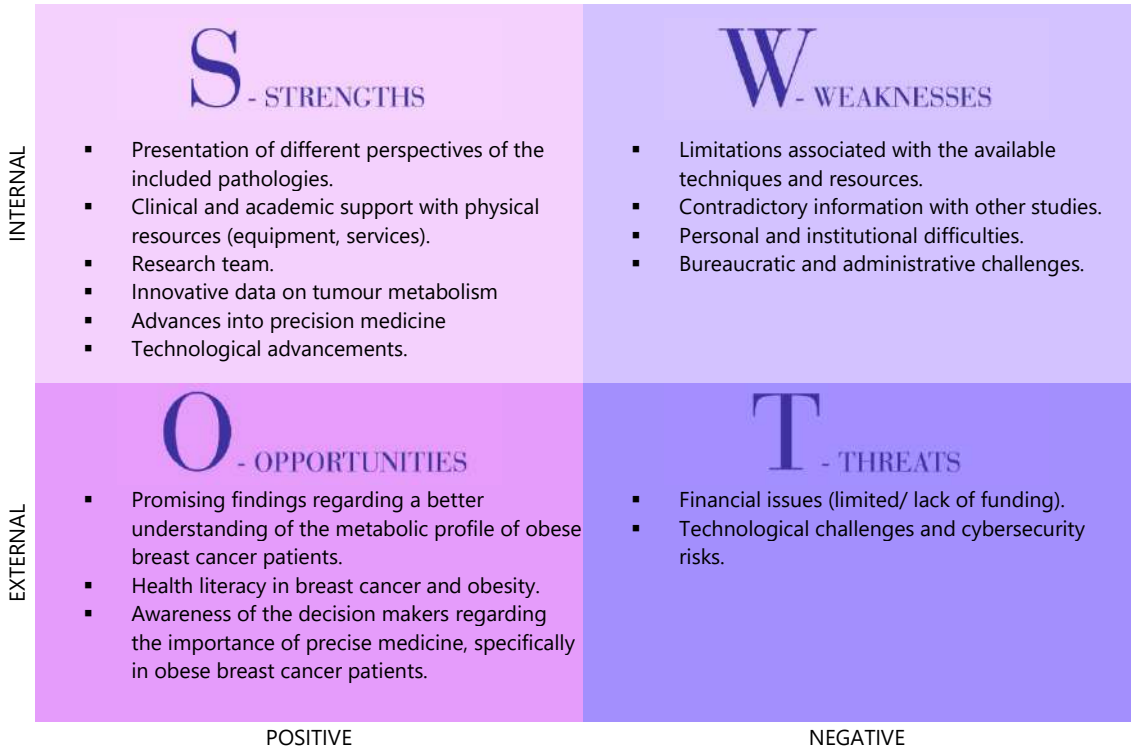


Figure 1 – SWOT analysis of the presented work.

The positive outputs of this study expose excellent back up resources for continuing the work. The collaboration between relevant national institutions, FMUP (Faculdade de Medicina da Universidade do Porto), i3S (Instituto de Investigação e Inovação em Saúde), and IPO-Porto (Instituto Português de Oncologia, Porto) associated with a research team with excellent expertise in both oncology and metabolic diseases.

Moreover, in the work development, we had the opportunity to publish the results in renowned scientific journals and share the information in international congresses, targeted to the scientific community. Results were also shared to the general population with the promotion of casual talks (FNAC talks, Pint of science) in the awareness of obesity and breast cancer.

On the other hand, the downfalls of this work are determinant. Some difficulties were found in the applied materials and methods, such as, a standard protocol for adipocyte differentiation is yet to be developed and generally accepted, or the lack of immunohistochemistry protocols for basic research. Last, but not least, funding is a major threat for advancing this work.

3. Future perspectives

From the promising results achieved with this work, it is planned to advance in several lines of investigation. If feasible, we preferably plan to use clinical data and human biological samples for conducting the subsequent studies:

- Metabolomic study of blood samples of normal weight and obesity breast cancer patients (ongoing work).

This study became possible due to the collaboration with IPO-Porto and a grant awarded by Liga Portuguesa contra o Cancro (LPCC) and AUSONIA. The request for ethical approval was submitted on July 18, 2022, but unfortunately, approval was deferred only on February 23rd, 2023, leading to a substantial delay, and preventing to deliver results within the present thesis.

This study focused on comparing the metabolomic signature of 60 samples: 20 normal weight breast cancer patients, 20 obese breast cancer patients; and 20 obese individuals. Inclusion criteria of breast cancer patients include post-menopausal women without previous therapeutic intervention with invasive ductal carcinoma with behavior 3, luminal A molecular subtype. Untargeted metabolomics service is already foreseen from Creative Proteomics. Presently, we are assembling the biological samples.

- The influence of the progesterone receptor in breast cancer patients (ongoing work).

Given the results attained in the epidemiological studies, we wish to understand the impact of progesterone receptor in breast cancer patients. The main aims of this work

are (1) Correlate progesterone receptor with clinical features of breast tumours (namely pathological stage – size, lymph node status, distant metastasis, differentiation grade, among others) as well as pathophysiological data (therapeutic strategies, disease free survival, overall survival); and (2) Investigate the expression and role of nuclear and mitochondrial progesterone receptor isoforms in different subtypes of breast cancer cell lines.

- Development of predictive models of breast cancer and obesity by artificial intelligence.

This collaborative effort will involve working alongside with Astrophysics Institute in Porto to leverage the data obtained from immunohistochemistry results. Our objective is to harness the power of artificial intelligence of deep learning and transfer learning to construct predictive models. These models will help us gain deeper insights into the complex relationship between enzyme expression, breast cancer and obesity, aiding in the development of innovative tools for prognosis and treatment decision-making.

- Understand the impact of gluconeogenesis pathway in breast cancer.

This intends to be a preliminary study, and as such, we intend to employ both *in vitro* and *in vivo* experiments to assess the consequences of deactivating rate-limiting enzymes involved in gluconeogenesis on breast cancer. Our research plan comprises two research strategies: (1) We will undertake a cell-based approach, using different molecular subtypes of breast cancer cells, and independently inhibit PC, PCK, and FBP, as well as collectively inhibit all three enzymes. (2) For the second line of research, knockout breast cancer cells for these enzymes will be inoculated into normal weight and obese mice. Tumour progression, metastasis, and other associated tumour features will be subsequently evaluated.

Future perspectives include integrating more institutions such as HFP (Hospital-Escola Fernando Pessoa), CHUSJ (Centro Hospitalar e Universitário São João), Astrophysics Institute of Porto, and pursue financial support to advance with our endeavors. We wish to maintain the dissemination of our work by publishing in renowned scientific journals and with public initiatives for general population. Furthermore, we intend to share our findings with key stakeholders once more robust and conclusive results are obtained.

The knowledge gained from this work could present an instrument in shaping healthcare policies, clinical guidelines, and research priorities aimed to mitigate the impact of breast cancer, particularly in the context of the global obesity crisis. Ultimately, the integration of these findings into clinical practice could have a tangible impact on obese patient outcomes.

APPENDIX A

Ethical approvals



LIST OF CONTENTS:

- Ethical approval granted by the Ethics Committee of São João University Hospital Centre.
- Ethical approval granted by the Ethics Committee of IPO – Porto.
- Ethical approval granted by the Ethics Committee of IPO – Porto (addendum 1).
- Ethical approval granted by the Ethics Committee of IPO – Porto (addendum 2).

Unidade de Investigação

Tomei conhecimento. Nada a opor. À DC.

22 de Julho de 2020

A Coordenadora da Unidade de Investigação

(Prof.ª Doutora Ana Azevedo)

n.º 226 / 2020

DIRECÇÃO CLÍNICA
23/7/2020

SÃO JOÃO

PEDIDO DE AUTORIZAÇÃO

Realização de Investigação

Exmo. Senhor Presidente do Conselho de Administração
do Centro Hospitalar de São João

Nome do Investigador Principal:

Carla Sofia Martins Luís

Título da Investigação:

Compreender os mecanismos da inversão do efeito Warburg em células tumorais em condições de obesidade

Pretendendo realizar no(s) Serviço(s) de:

Centro de Responsabilidade Integrada (CRI) de Obesidade

a investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efetivação.

Para o efeito, anexo toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto respeitante à investigação, à qual enderecei pedido de apreciação e parecer.

Com os melhores cumprimentos. *O Investigador/ Promotor*

Porto, 28 *de* Maio *de* 2020 . Carla Sofia Martins Luís

assinatura

• Centro Hospitalar São João •
Centro de Epidemiologia Hospitalar
21/7/2020

CES-IM005-0

Parecer da Comissão de Ética do

Centro Hospitalar Universitário de São João / Faculdade de Medicina da Universidade do Porto

Título do projeto: Compreender os mecanismos da inversão do efeito Warburg em células tumorais em condições de obesidade.

Nome da Investigadora Principal: Dra. Carla Sofia Martins Luís.

Onde decorre o estudo: No Centro de Responsabilidade Integrada de Obesidade do CHUSJ. Apresentou declaração do Dr. John Preto, que será o profissional de ligação.

Objetivos do estudo: Identificar as enzimas da glicólise que se encontram alteradas em obesidade em células tumorais. Compreender as alterações do metabolismo energético tumoral em condições de obesidade. Estudo realizado no âmbito do Programa Doutoral em Metabolismo da FMUP, sob orientação da Prof.ª Doutora Raquel Soares.

Conceção e pertinência do estudo: Um estudo preliminar sobre o metabolismo da célula tumoral revelou que, em obesidade, a célula tumoral da mama apresenta um aumento de crescimento e de agressividade com alteração do metabolismo energético basal. A célula tumoral da mama, ao contrário do efeito Warburg apresenta produção de níveis de glicose em vez do seu consumo. O presente projeto pretende investigar este mecanismo a nível celular e bioquímico com o tratamento de células tumorais de mama com tecido adiposo/adipócitos provenientes de pacientes obesos. Os resultados poderão melhorar o diagnóstico e a terapêutica associada a pacientes obesos. O tecido adiposo será obtido de pacientes propostos para cirurgia, sem riscos acrescidos para o paciente.

Benefício/risco: Benefício potencial para a sociedade através da evolução do conhecimento científico neste domínio.

Confidencialidade dos dados: O investigador não terá acesso a qualquer tipo de dado clínico do paciente.

Respeito pela liberdade e autonomia do sujeito de ensaio: Dispõe de uma adequada informação ao participante e de modelo de consentimento informado do CHUSJ.

***Curriculum* da investigadora**: Adequado à investigação.

Data previsível da conclusão do estudo: Junho de 2021

Conclusão: Proponho um parecer favorável à realização deste projeto de investigação no pressuposto de que após a conclusão do estudo os produtos biológicos colhidos sejam destruídos ou, em alternativa, incorporados num biobanco legalmente constituído.

Porto, 3 de julho de 2020

O Relator da CE, Prof. Doutor Rui Nunes

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

SÃO JOÃO

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

Questionário para submissão de Investigação

Exmo. Sr. Presidente da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto,

Pretendendo realizar a investigação infracitada, solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer. Para o efeito, anexo toda a documentação requerida.

IDENTIFICAÇÃO DO ESTUDO

Título da investigação: Compreender os mecanismos da inversão do efeito Warburg em células tumorais em condições de obesidade

Nome do investigador: Carla Sofia Martins Luís

Endereço eletrónico: carlaluis@med.up.pt **Contacto telefónico:** 911812883

Caracterização da investigação:

☐ Estudo retrospetivo ☐ Estudo observacional ☐ Estudo prospetivo

☐ Inquérito ☒ Outro. Qual? Investigação básica

Tipo de investigação:

☐ Com intervenção ☒ Sem intervenção

Formação do investigador em boas práticas clínicas (GCP): ☐ Sim ☒ Não

Promotor *(se aplicável)*: Não aplicável

Nome do orientador de dissertação/tese *(se aplicável)*: Raquel Soares

Endereço eletrónico: raqsoa@med.up.pt

Local/locais onde se realiza a investigação: Centro de Investigação Médica - Departamento de Biomedicina

Data prevista para início: 15 / 06 / 2020 Data prevista para o término: 15 / 06 / 2021

PROTOCOLO DO ESTUDO

Síntese dos objetivos:

Identificar as enzimas da glicólise que se encontram alteradas em obesidade em células tumorais;

Compreender as alterações do metabolismo energético tumoral em condições de obesidade.

Fundamentação ética (ganhos em conhecimento/inovação; ponderação benefícios/riscos):

Um estudo preliminar realizado pelo nosso grupo sobre o metabolismo da célula tumoral revelou que em obesidade, a célula tumoral de mama apresenta um aumento de crescimento e de agressividade com alteração do metabolismo energético basal. A célula tumoral de mama, ao contrário do efeito Warburg apresenta produção de níveis de glicose em vez do seu consumo.

O presente projeto propõe-se a investigar este mecanismo a nível celular e bioquímico com o tratamento de células tumorais de mama com tecido adiposo/adipócitos provenientes de pacientes obesos. Os seus resultados poderão melhorar o diagnóstico e a terapêutica associada a pacientes obesos.

O tecido adiposo será obtido de pacientes propostos para cirurgia, não acarretando riscos extra para o participante.

CES-IM007-0

CONFIDENCIALIDADE

De que forma é garantida a anonimização dos dados recolhidos de toda a informação?

O investigador não terá acesso a qualquer tipo de dado clínico do participante.

O investigador necessita ter acesso a dados do processo clínico? ☐ Sim ☒ Não

Está previsto o registo de imagem ou som dos participantes? ☐ Sim ☒ Não

Se sim, está prevista a destruição deste registo após o sua utilização? ☐ Sim ☒ Não

CONSENTIMENTO

O estudo implica recrutamento de:

Doentes: ☒ Sim ☐ Não Voluntários saudáveis: ☐ Sim ☒ Não

Menores de 18 anos: ☐ Sim ☒ Não

Outras pessoas sem capacidade do exercício de autonomia: ☐ Sim ☒ Não

A investigação prevê a obtenção de Consentimento Informado: ☒ Sim ☐ Não

Se não, referir qual o fundamento para a isenção:

Existe informação escrita aos participantes: ☒ Sim ☐ Não

PROPRIEDADE DOS DADOS

A investigação e os seus resultados são propriedade intelectual de:

☒ Investigador ☐ Promotor ☐ Ambos ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

BENEFÍCIOS, RISCOS E CONTRAPARTIDAS PARA OS PARTICIPANTES

Benefícios previsíveis:

Não existem benefícios para o participante.

Riscos/incómodos previsíveis:

Não existem riscos adicionais para os participantes.

São dadas contrapartidas aos participantes:

- *pela participação* ☐ Sim ☒ Não ☐ Não aplicável
- *pelas deslocações* ☐ Sim ☒ Não ☐ Não aplicável
- *pelas faltas ao emprego* ☐ Sim ☒ Não ☐ Não aplicável
- *por outras perdas e danos* ☐ Sim ☒ Não ☐ Não aplicável

CUSTOS / PLANO FINANCEIRO

Os custos da investigação são suportados por:

☒ Investigador ☐ Promotor ☐ Serviço onde é realizado

☐ Não aplicável Outro: _____

Existe protocolo financeiro? ☐ Sim ☒ Não

LISTA DE DOCUMENTOS ANEXOS

- [x] Pedido de autorização ao Presidente do Conselho de Administração do Centro Hospitalar de São João *(se aplicável)*
- [ ] Pedido de autorização à Diretora da Faculdade de Medicina da Universidade do Porto *(se aplicável)*
- [x] Protocolo do estudo
- [x] Declaração do Diretor de Serviço onde decorre o estudo
  (sendo um estudo na área de enfermagem deve anexar também a concordância da chefia de enfermagem)
- [x] Profissional de ligação
- [x] Informação dos orientadores
- [x] Informação ao participante
- [x] Modelo de consentimento
- [ ] Instrumentos a utilizar *(inquéritos, questionários, escalas, p.ex.)*: _____
- [x] Curriculum Vitae abreviado *(máx. 3 páginas)*
- [ ] Protocolo financeiro
- [x] Outros:

Fluxograma do processo de recolha de amostras

COMPROMISSO DE HONRA E DECLARAÇÃO DE INTERESSES

Declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (1960 e respetivas emendas), e da Organização Mundial da Saúde, Convenção de Oviedo e das "Boas Práticas Clínicas" (GCP/ICH) no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo, nos últimos três meses. Comprometo-me a entregar à CES o relatório final da investigação, assim que concluído.

Porto, 28 de Maio de 2020

Nome legível: Carla Sofia Martins Luís

Carla Sofia Martins Luís
assinatura

Parecer da Comissão de Ética do Centro Hospitalar de São João/ FMUP Emitido na reunião plenária da CE de 03 / 07 / 2020

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exma. Senhora,
Prof.ª Doutora Maria José Bento
Diretora da Escola Portuguesa de Oncologia do Porto (EPOP)
IPO Porto FG EPE

Ref. CES. 202/020
Porto, 04 de junho de 2020
Assunto: **Avaliação de Pedido de Parecer**

Exma. Sra. Prof.ª Doutora Maria José Bento,

Cumpre-me remeter a V/Exa. o pedido de parecer dirigido a esta CES sobre o pedido de realização de projeto de investigação intitulado **"Unraveling the Warburg Effect Inversion – A new insight in the primary biochemistry metabolism"**, tendo como Investigador Principal a **Dra. Carla Sofia Martins Luís,** foi avaliado em reunião ordinária da Comissão de Ética a 04 de junho de 2020, emitindo-se o parecer anexo.

Respeitosos cumprimentos,

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Parecer CES IPO: 202/020
Assunto: Avaliação do pedido realização de projeto de investigação intitulado **"Unraveling the Warburg Effect Inversion – A new insight in the primary biochemistry metabolism"**
Investigador Principal: **Dra. Carla Sofia Martins Luís**
Data: 04 de junho de 2020

PARECER

É parecer desta CES, não existir impedimento de natureza ética ao desenvolvimento do referido estudo de Investigação.

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exma. Senhora,
Profª. Doutora Maria José Bento
Diretora da Escola Portuguesa de Oncologia do Porto (EPOP)
IPO Porto FG EPE

Ref. CES. 261/020
Porto, 03 de setembro de 2020
Assunto: **Adenda ao Pedido de Parecer**

Exma. Sra. Prof.ª Doutora Maria José Bento,

Cumpre-me remeter a V/Exa. o pedido de parecer dirigido a esta CES sobre o Adenda ao pedido de Projeto de Investigação intitulado **"Unraveling the Warburg Effect Inversion – A new insight in the primary biochemistry metabolism"**, tendo como Investigadora Principal a **Drª. Carla Sofia Martins Luís,** foi avaliado em reunião ordinária da Comissão de Ética a 03 de setembro de 2020, emitindo-se o parecer anexo.

Respeitosos cumprimentos,

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

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INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Parecer CES IPO: 261/020
Assunto: Adenda ao pedido de realização de Projeto de Investigação **"Unraveling the Warburg Effect Inversion – A new insight in the primary biochemistry metabolism"**
Investigadora Principal: **Drª. Carla Sofia Martins Luís**
Data: 03 de setembro de 2020

PARECER

É parecer desta CES, não existir impedimento de natureza ética ao desenvolvimento do referido estudo de Investigação.

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exma. Senhora,
Profª. Doutora Maria José Bento
Diretora da Escola Portuguesa de Oncologia do Porto (EPOP)
IPO Porto FG EPE

Ref. CES. 221R/022
Porto, 23 de Fevereiro de 2023
Assunto: **Reavaliação de Pedido de Parecer a Adenda (202/020)**

Exma. Senhora Profª. Doutora Maria José Bento,

Cumpre-me remeter a V/Exa. o pedido reavaliação do pedido de parecer ao protocolo dirigido a esta CES sobre o pedido de realização de Estudo Observacional Transversal intitulado ***"Unraveling the Warburg Effect Inversion - A new insight in the primary biochemistry metabolim - Desvendando a Inversão do Efeito Warburg - Uma nova visão do metabolismo bioquímico primário"***, tendo como Investigadora Principal a **Dra. Carla Sofia Martins Luís,** foi avaliado em reunião ordinária da Comissão de Ética a 23 de fevereiro de 2023, emitindo-se o parecer anexo.

Respeitosos cumprimentos,

Enf.º José Carlos Pimentel
Vice-Presidente da CES – IPO Porto EPE

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Parecer CES. 221R/022

Assunto: Avaliação do pedido de realização do Estudo Observacional Transversal intitulado ***"Unraveling the Warburg Effect Inversion - A new insight in the primary biochemistry metabolim - Desvendando a Inversão do Efeito Warburg - Uma nova visão do metabolismo bioquímico primário".*** Investigadora Principal: **Dra. Carla Sofia Martins Luís**

23 de Fevereiro de 2023

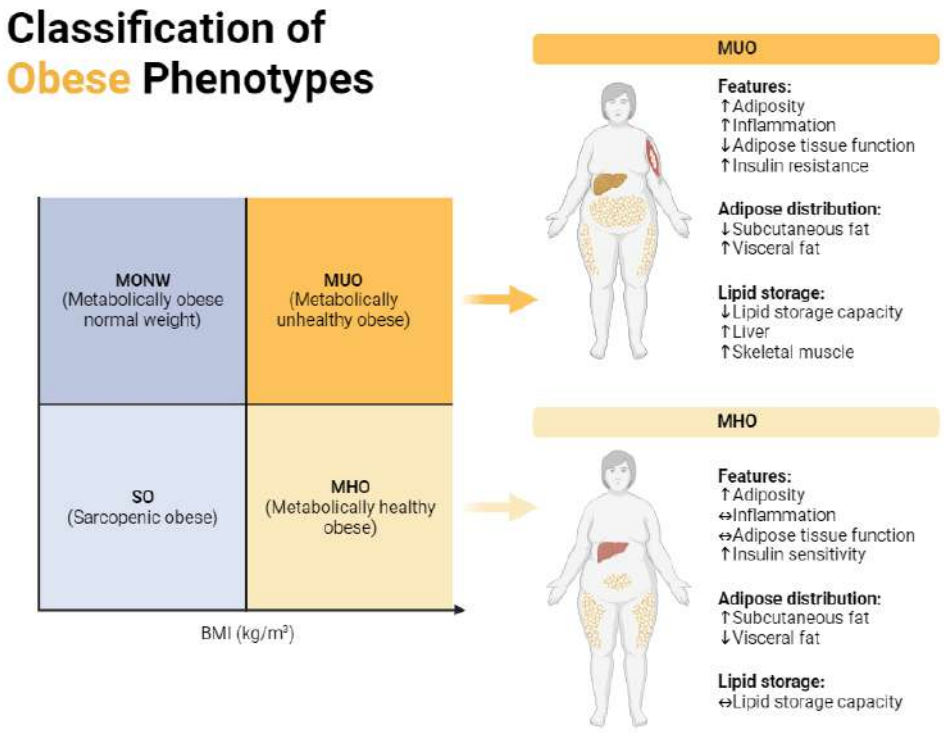
PARECER

É parecer desta CES não existir impedimento de natureza ética à continuidade deste estudo.

Enf.º José Carlos Pimentel
Vice-Presidente da CES – IPO Porto EPE

APPENDIX B

Clinical approach on Metabolically Healthy Obese



LIST OF CONTENTS:

- "Distinguishing Metabolically (Un)Healthy Obese Women. Metabolic stratification of health parameters"

Society for Endocrinology
JOURNAL OF ENDOCRINOLOGY

Distinguishing Metabolically (Un)Healthy Obese Women

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Manuscript Type:	Research Paper
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Complete List of Authors:	Mendonça, Fernando; CHUSJ, Endocrinology, Diabetes and Metabolism Soares, Pietra; FMUP, Biomedicine Moreno, Telma; CHUSJ, Endocrinology, Diabetes and Metabolism Freitas, Paula; Centro Hospitalar Universitário de São João, Endocrinology Rodrigues, Ilda; Faculty of Medicine, University of Porto, Biomedicine - Biochemistry Unit Festas, Diana; CHUSJ, Endocrinology, Diabetes and Metabolism Pedro, Jorge ; Centro Hospitalar Universitário de São João, Endocrinology, Diabetes and Metabolism Varela, Ana; CHUSJ, Endocrinology, Diabetes and Metabolism Fernandes, Ana; CHUSJ, Nuclear Medicine Department Fernandes, Ruben; Universidade Fernando Pessoa, Faculdade Ciências da Saúde Soares, Raquel; Universidade do Porto Faculdade de Medicina, Biomedicine Costa, Eduardo; CHUSJ, CRIO group – Centro de Responsabilidade Integrado de Obesidade Centro de Responsabilidade Integrado de Obesidade, CRIO group; CHUSJ, CRIO group – Centro de Responsabilidade Integrado de Obesidade Luis, Carla; FMUP, Biomedicine; i3S - Instituto de Investigação e Inovação em Saúde, Metabesity
Keywords:	Obesity, Obesity paradox, Metabolism, Metabolically Healthy Obese, Metabolically Unhealthy Obese

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Distinguishing Metabolically (Un)Healthy Obese Women

Short title: Metabolic stratification of health parameters

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25 **Keywords:**

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27 Unhealthy Obese.

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Distinguishing Metabolically (Un)Healthy Obese Women

Short title: Metabolic stratification of health parameters

Abstract:

Obesity represents a global health crisis, yet a dichotomy is evident, classifying individuals into two groups based on their metabolic state: metabolically healthy obese (MHO) and metabolically unhealthy obese (MUO). This study aimed to identify distinctive health-related parameters between MHO individuals and the broader obese population, employing a comprehensive comparative analysis of 50 biomarkers. The study included 182 women diagnosed with obesity referred for bariatric surgery at the Endocrinology, Diabetes, and Metabolism Service of São João Hospital and University Centre in Portugal. Patients were stratified based on the presence or absence of metabolic disorders, including diabetes, hypertension, or dyslipidemia. MUO was identified in individuals presenting with at least one of these metabolic disorders. Significantly divergent health-related parameters were observed between MHO and MUO patients. Notable differences included: albumin (40.1±2.2 vs 40,98±2.6, p-value=0.017), triglycerides (110.7±51.1 vs 137.57±82.6, p-value=0.008), glucose (99.49±13.0 vs 119.17±38.9, p-value<0.001), glycated hemoglobin (5.58±0.4 vs 6.15±1.0, p-value<0.001), urea (31.40±10.0 vs 34.61±10.2, p-value=0.014), total calcium (4.64±0.15 vs 4.74±0.17, p-value<0.001), ferritin (100.04±129.1 vs 128.55±102.1, p-value=0.005), chloride (104.68±1.5 vs 103.04±2.6, p-value <0.001), prolactin (13.57±6.3 vs 12.47±7.1, p-value=0.041), insulin (20.36±24.4 vs 23.87±19.6, p-value=0.021), c

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peptide (3.78±1.8 vs 4.28±1.7, p-value=0.003), albumin/creatinine ratio (15.41±31.0 vs 48.12±158.7, p-value=0.015), and whole-body mineral density (1.27±0.1 vs 1.23±0.1, p-value=0.016). In summary, our findings reveal noteworthy significant alterations in health parameters, suggesting that MHO may represent a transitional phase preceding the development of the MUO phenotype.

Introduction

Over the past few decades, the global prevalence of obesity has increased and has emerged as a global health crisis(Purnell 2023). Within the heterogeneous spectrum of obesity, an enigmatic and clinically relevant dichotomy has emerged. While obesity traditionally correlates with an elevated risk of metabolic complications, such as type 2 diabetes, cardiovascular disease, and hypertension(Kinlen *et al.* 2018), it has become evident that not all subjects with excess body weight share the same metabolic profile. This has resulted in the stratification of obese individuals into two distinct subgroups: "Metabolically Healthy Obese" (MHO) and "Metabolically Unhealthy Obese" (MUO)(Blüher 2020).

The MHO phenotype represents a paradoxical group of individuals with obesity who, despite carrying excess mass of adipose tissue, exhibit a metabolic profile of normal healthy body weight subjects. In contrast, the MUO phenotype is characterized by a clustering of metabolic disturbances, including insulin resistance, dyslipidemia, and inflammation, mirroring the classic complications of obesity. Understanding the heterogeneity of obesity is crucial for developing targeted prevention and treatment strategies(Blüher 2020).

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While numerous studies have explored the MHO phenotype(Phillips 2013; Stefan *et al.* 2013; Smith *et al.* 2019; Blüher 2020), a standardized definition of MHO has yet to be universally agreed upon. Criteria such as body mass index (BMI) equal or superior to 30 kg/m^2 associated with the absence of type 2 diabetes, hypertension or dyslipidemia have been proposed(Mayoral *et al.* 2020). Some authors advocate the inclusion of biomarker thresholds, such as a triglyceride concentration below 150 mg/dL or a high-density lipoprotein (HDL) concentration above 40 mg/dL in men, or 50 mg/dL in women(Blüher 2020). Previous analysis in studies including metabolically healthy individuals with obesity have reported more than 30 different definitions(Rey-López *et al.* 2014).

The objective of this study is to explore the complex metabolic profile that distinguishes MHO and MUO phenotypes through a comparative analysis of health-related parameters in both groups and assessing their potential contribution to defining the MHO phenotype.

Materials and Methods

Study population and ethical approval

This study was conducted according to the international ethical recommendations, complied by the Declaration of Helsinki. Informed consent was obtained from all participants, and confidentiality was ensured through anonymization of all collected data.

Clinicopathological and demographic data were collected from the Endocrinology, Diabetes and Metabolism Service of São João Hospital and University Centre in Portugal and integrated 182 cases of women with obesity (BMI≥30kg/m^2) referred for

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bariatric surgery. Exclusion criteria involved age under 40 years, weight superior to 120kg (due to equipment limitation), previous treatment with estrogens, glucocorticoids, hyperthyroidism, and intestinal disease.

Methods

Demographic information was obtained from patient records. The study population was stratified into two groups: MHO and MUO, based on the presence or absence of metabolic disorders, specifically diabetes, hypertension, or dyslipidemia. Individuals with at least one of these metabolic disorders were classified as MUO.

BMI was calculated according the Quetelet index before surgical intervention(Jelliffe & Jelliffe 1979) and stratified according to three categories: mild obesity (BMI between 30 to 34.9 kg/m^2), moderate obesity (range of 35 to 39.9 kg/m^2) and morbid obesity (BMI of 40 kg/m^2 or higher). During clinical consultations, 7 body densitometric measurements were calculated with resource to GE Lunar iDXA densitometer. Blood and urine samples were collected and processed to measure 39 blood serum biomarkers and 4 urine biomarkers using the AU 5800 analyzer by Beckman Coulter®, following manufacturer's instructions and São João Hospital Center's protocol.

Statistical analysis

Statistical analysis was performed using IBM® SPSS®, version 27 software. For continuous variables, Kolmogorov-Smirnov normality test was performed to evaluate normal distribution individually. To assess variance equality, it was applied Levene's test. A first assessment of the demographic data was performed by the Pearson's chi-squared and one-way analysis of variance (ANOVA). Comparison between MHO and MUO was assessed by independent t-student test or by Mann-Whitney U Test,

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according to the result of the Kolmogorov-Smirnov normality test. Significance was set at p-value<0.05.

Results

Detailed demographic and clinical information of included women stratified as MHO and MUO, is presented in Table 1. Out of the 182 participants, 34.6% (63 women) were categorized as MHO, while 65.4% (119 individuals) were classified as MUO. Statistical analysis revealed a significant age difference between the two groups, with the unhealthy obese exhibiting a higher mean age of 51.78±7.2, while healthy obese displayed a mean age of 46.59±6.8 (p-value<0.001). Weight, height, and BMI did not achieve statistically significance.

Intriguingly, when stratified by BMI categories, we observed a progressive increase in the number of individuals with metabolically unhealthy obesity as one moved from the category of mild obesity to morbid obesity (Figure 1a). The distribution of menopausal status also showed significance (p-value=0.003) (Figure 1b), with postmenopausal women being more prominent in the MUO group. As expected, diabetes, hypertension, and dyslipidemia displayed statistically significance, as they were considered during the group distribution.

Table 1 – Demographic and clinical information for individuals with Metabolically Healthy Obesity (MHO) and Metabolically Unhealthy Obesity (MUO).

	MHO	MUO	Total	p-value
n (%)	63 (34.6%)	119 (65.4%)	182 (100.0%)	-
Age [Mean±SD (Range)]	46.59±6.8 (39-66)	51.78±7.2 (38-70)	49.98±7.5 (38-70)	**<0.001***

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Weight (kg) (Mean±SD)	109.31±14.2	107.34±12.4	108.02±13.0	0.332
Height (m) (Mean±SD)	1.61±0.06	1.60±0.06	1.60±0.06	0.079
BMI (kg/m^2) (Mean±SD)	42.0±5.2	42.2±4.4	42.1±4.7	0.866
BMI categories [n (%)]				
Mild obesity	2 (3.2%)	2 (1.7%)	4 (2.2%)	
Moderate obesity	22 (34.9%)	39 (32.8%)	61 (33.5%)	0.756
Morbid obesity	39 (61.9%)	78 (65.5%)	117 (64.3%)	
Menopause status [n (%)]				
No	40 (63.5%)	48 (40.3%)	88 (48.4%)	
Yes	23 (36.5%)	70 (58.9%)	93 (51.1%)	**0.003***
Missing data	0 (0.0%)	1 (0.8%)	1 (0.5%)	
Alcohol consumption [n (%)]				
No	63 (100.0%)	118 (99.2%)	181 (99.5%)	0.466
Yes	0 (0.0%)	1 (0.8%)	1 (0.5%)	
Physical exercise [n (%)]				
No	43 (68.3%)	84 (70.6%)	127 (69.8%)	
Yes	19 (30.2%)	35 (29.4%)	54 (29.7%)	0.863
Missing data	1 (1.5%)	0 (0.0%)	1 (0.5%)	
Diabetes [n (%)]				
No	63 (100.0%)	67 (56.3%)	130 (71.4%)	**<0.001***
Yes	0 (0.0%)	52 (43.7%)	52 (28.6%)	
Hypertension [n (%)]				
No	63 (100.0%)	17 (14.3%)	80 (44.0%)	**<0.001***
Yes	0 (0.0%)	102 (85.3%)	102 (56.0%)	
Dyslipidaemia [n (%)]				
No	63 (100.0%)	59 (49.6%)	122 (67.0%)	**<0.001***
Yes	0 (0.0%)	60 (50.4%)	60 (33.0%)	

Legend: Statistical significance highlighted in bold; * p-value<0.05.

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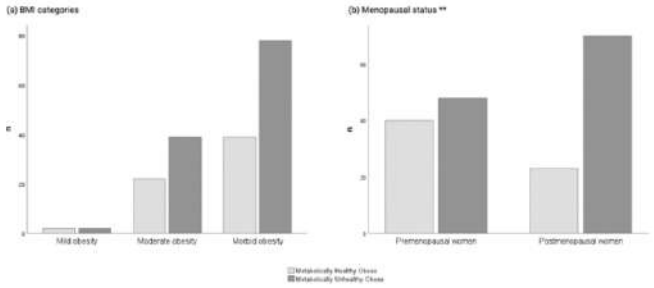


Figure 1 – Graphic representation of the distribution of MHO and MUO according to BMI categories (a) and menopausal status (b). Legend: ** p-value<0.01.

Results showed statistically significant differences between the MHO and MUO groups in several health-related parameters: albumin, triglycerides, glucose, glycated hemoglobin (HbA1c), urea, total calcium, ferritin, chloride, prolactin, insulin, and c peptide in serum blood samples, as well as the albumin/creatinine ratio in urine samples. Additionally, a statistically significance was observed in whole-body mineral density. Detailed findings can be consulted in Table 2.

Table 2 – Description of health-related parameters with reference values and statistical analysis between patients with metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO) with result range, mean±SD, and p-value associated.

Designation	Reference values	MHO Range Mean±SD	MUO Range Mean±SD	p-value
Blood sample				
Hemoglobin (g/dL)	12-16	11.6 – 15.9	11.6 – 16	0.746

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		13.55±0.9	13.60±0.99	
Leucocytes (x 10^9/L)	4 -11	4.03 – 10.56 7.39±1.6	4.43 – 12.15 7.70±1.9	0.259
Platelets (x 10^9/L)	150 - 400	194 – 499 270.06±59.1	183 – 398 263.42±50.2	0.654
Total Proteins (g/L)	64 - 83	63.2 – 79.2 69.95±3.6	61.3 – 85.2 71.17±4.7	0.073
Albumin (g/L)	38 - 51	35.1 – 45.5 40.05±2.2	34.8 – 45.6 40.98±2.6	0.017*
Aspartate aminotransferase (U/L)	10 - 31	13 – 42 22.79±9.5	11 – 70 23.36±9.6	0.512
Alanine aminotransferase (U/L)	10 – 31	9 – 52 22.25±9.5	10 – 89 25.19±12.5	0.119
Gamma-glutamyl transferase (U/L)	7 – 32	12 – 119 28.86±17.3	12 – 101 37.68±62.8	0.235
Phosphatase alkaline (U/L)	30 - 120	38 – 113 79.68±20.5	43 – 158 80.74±21.9	0.850
Total cholesterol (mg/dL)	< 200	117 – 279 186.08±35.8	130 – 480 192.41±48.1	0.551
High density lipoproteins (mg/dL)	> 60	36 – 71 51.17±10.1	27 – 91 51.50±11.8	0.870
Low density lipoproteins (mg/dL)	< 150	51 – 176 112.70±28.8	55 – 324 113.96±37.9	0.866
Triglycerides (mg/dL)	< 150	34 – 291 110.7±51.1	28 – 753 137.57±82.6	0.008*
Glucose (mg/dL)	75 – 110	69 – 151 99.49±13.0	10 – 345 119.17±38.9	<0.001*
Glycated Haemoglobin (%)	4 – 6	4.3 – 6.6	4.8 – 11.5	<0.001*

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		5.58±0.4	6.15±1.0	
Urea (mg/dL)	10 – 50	2 – 72 31.40±10.0	3.3 – 66 34.61±10.2	0.014*
Creatinine (mg/dL)	0.51 – 0.95	0.43 – 1.19 0.64±0.1	0.4 – 1.67 0.66±0.2	0.878
Uric acid (mg/dL)	2.3 – 6.1	2.7 – 8 5.1±1.1	2.7 – 10.4 5.3±1.3	0.262
Total Calcium (mEq/L)	4.1 – 5.2	4.4 – 5 4.64±0.1	4.5 – 5.1 4.74±0.2	<0.001*
Ionized calcium (mEq/L)	2.26 – 2.64	2.3 – 2.88 2.52±0.1	2.4 – 2.74 2.52±0.1	0.751
Inorganic Phosphorus (mg/dL)	2.7 – 4.5	2.1 – 4.5 3.25±0.5	2.5 – 4.5 3.39±0.5	0.107
Magnesium (mg/dL)	1.55 – 2.05	1.29 – 1.86 1.60±0.1	1.21 – 1.87 1.57±0.1	0.132
Iron (ug/dL)	49 – 151	25 – 145 76.89±30.9	31 – 137 75.05±26.0	0.954
Transferrin (mg/dL)	200 – 360	217 – 411 280.45±40.4	191 – 392 273.32±43.0	0.365
Ferritin (ng/mL)	10 - 120 (PreM) 10 – 250 (PostM)	7.8 – 907.2 100.04±129.1	12 – 415.8 128.55±102.1	0.005*
Sodium (mEq/L)	135 - 147	127 – 144 139.10±2.3	41 – 144 139.12±10.4	0.084
Potassium (mEq/L)	3.5 - 5.1	3.5 – 5.1 4.34±0.3	3.5 – 5.2 4.31±0.4	0.620
Chloride (mEq/L)	101 - 109	102 – 108 104.68±1.5	95 – 109 103.04±2.6	<0.001*
C reactive protein (mg/L)	< 3	1.2 – 46.4	1 – 61.9	0.886

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		9.78±8.2	9.41±8.2	
Folic acid (ng/mL)	2.2 – 17.5	2.4 – 28 6.42±4.2	1.8 – 28.7 6.72±3.6	0.151
Vitamin B12 (pg/mL)	187 – 883	173 – 815 368.73±121.3	83 – 1545 394.37±229.9	0.876
Vitamin D (ng/mL)	> 30	7 – 31 17.40±5.4	4 – 45 19.13±8.3	0.304
Thyroxine (T4) (ng/dL)	0.7 – 1.48	0.76 – 1.13 0.94±0.1	0.61 – 1.31 0.97±0.1	0.162
Thyroid Stimulating Hormone (uUI/mL)	0.35 – 4.94	0.64 – 17.46 1.92±2.1	0.28 – 5.2 1.96±1.9	0.715
Parathyroid hormone (pg/mL)	10 – 65	27.2 – 94.7 55.91±17.4	13.3 – 141.3 55.34±22.5	0.528
Prolactin (ng/mL)	4.8 – 23.3	6 – 29.3 13.57±6.3	4 – 36.6 12.47±7.1	0.041*
Total testosterone (ng/mL)	0.06 – 0.82	0.03 - 0.57 0.25±0.1	0.03 – 10.19 0.31±1.0	0.162
Insulin (uU/mL)	2.6 – 24.9	1.5 – 195.5 20.36±24.4	5.6 – 170.6 23.87±19.6	0.021*
C Peptide (ng/mL)	1.1 – 4.4	1.56 – 12.7 3.78±1.8	1.93 – 13.3 4.28±1.7	0.003*
Urine samples				
Creatinine 24h urine (mg/24h)	600 – 1800	592 – 2445 1416.22±436.7	555 – 2360 1341.69±352.4	0.260
Cortisol 24h urine (ug/dia)	36 – 137	5.7 – 85.99 39.21±20.0	5.15 – 118.76 38.95±23.1	0.443
Microalbumin (mg/L)	< 20	1.2 – 240.1 20.63±42.3	1.2 – 577.2 438.67±4228.2	0.057

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Albumin/creatinine ratio (mg/g creatinine)	< 30	1.2 – 205.2 15.41±31.0	1.2 – 443.7 48.12±158.7	0.015*
Body densitometry				
Whole body mineral density (g/cm2)	NA	1.058 – 1.547 1.27±0.1	0.953 – 1.521 1.23±0.1	0.016*
Total body mass (kg)	NA	82.1 – 129.7 107.29±13.4	69.2 – 133.3 105.7±12.38	0.426
Total fat mass (kg)	NA	37.99 – 72.25 54.57±9.1	39.568 – 75.585 54.1±8.1	0.731
Total lean mass (kg)	NA	40.819 – 61.421 49.88±5.7	37.37 – 71.387 49.16±5.6	0.411
% leg fat (kg)	NA	34.3 – 61.5 50.3±5.3	33 – 59.4 48.83±5.3	0.073
% trunk fat (kg)	NA	42.4 – 64.2 55.28±4.5	47.5 – 63.8 56.23±3.4	0.116
% total fat (kg)	NA	42.5 – 58.8 52.1±3.8	43.2 – 60.2 52.3±3.3	0.731

Legend: Statistical significance highlighted in bold; * p-value<0.05. NA – Not applicable. PreM – Premenopausal status. PostM – Postmenopausal status.

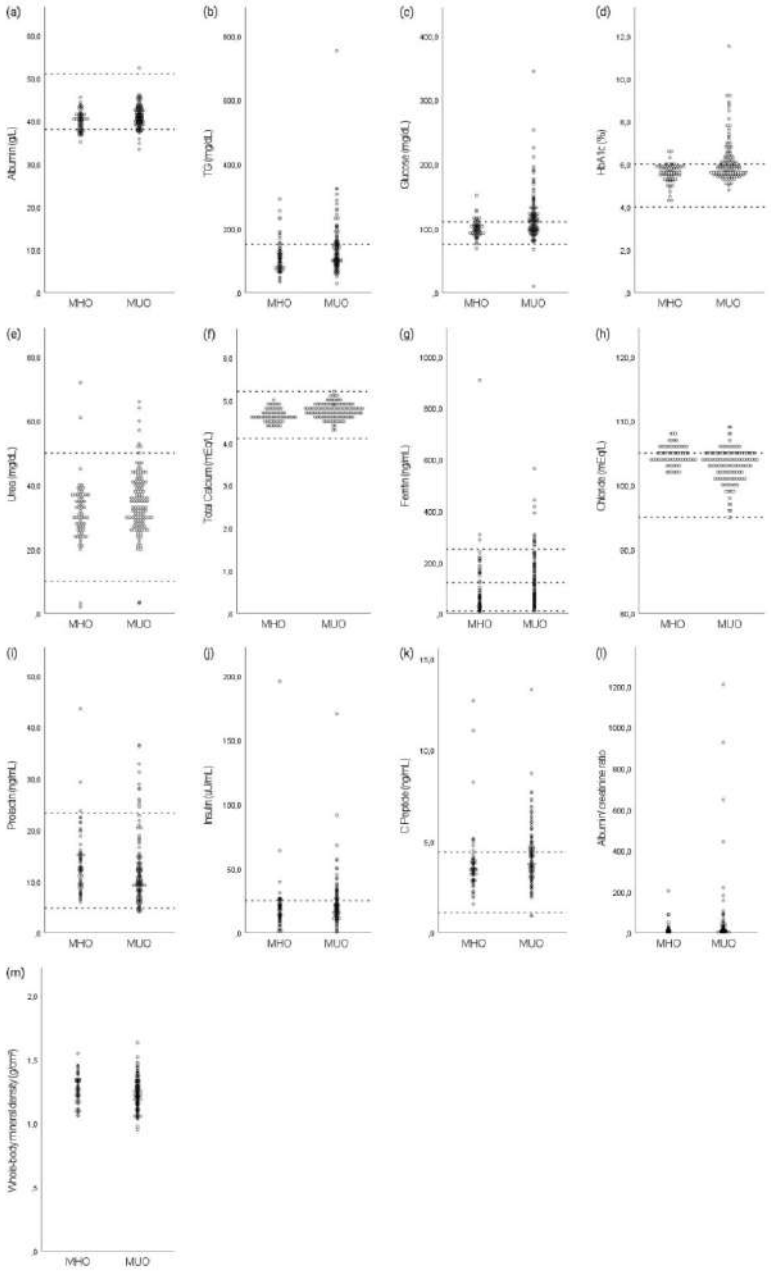
Among the health parameters that were found to be significant, we conducted additional comparisons with the reference ranges and assessed their compliance (Figure 2). Overall, we observed that MUO have a broader range of results, with an observational tendency towards higher values compared to reference values.

None of the patients with healthy obesity presented albumin levels above the reference range, and the same trend was observed for total calcium. Moreover, there are fewer individuals with metabolically healthy obesity exhibiting values above the reference

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range for triglycerides, glucose, glycated hemoglobin, urea, ferritin, prolactin, insulin, and C-peptide. Overall, chloride levels in MHO group is higher than in MUO. In urine samples, the albumin/creatinine ratio mean was higher in MUO women. MUO women exhibited a wider range of values and lower whole-body mineral density.

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(a)
Albumin(g/L)
(b)
TG(mg/dL)
(c)
Glucose(mg/dL)
(d)
HbA1c (%)
(e)
Urea(mg/dL)
(f)
Total Calcium(mEq/L)
(g)
Ferritin(ng/mL)
(h)
Chloride (mEq/L)
(i)
Protein(ng/mL)
(j)
Insulin (µU/mL)
(k)
C Peptide (ng/mL)
(l)
Albumin/Creatinine ratio
(m)
Whole-body mineral density (g/cm²)
MHO
MUO

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Figure 2 – Graphical representation of the statistically significant parameters between MHO and MUO with identification of reference values available. (a) Albumin p-value=0.017; (b) Triglycerides, TG p-value=0.008; (c) Glucose p-value<0.001; (d) Glycated hemoglobin p-value<0.001; (e) Urea p-value=0.014; (f) Total calcium p-value<0.001; (g) Ferritin p-value=0.005; (h) Chloride p-value<0.001; (i) Prolactin p-value=0.041; (j) Insulin p-value=0.021; (k) C peptide p-value=0.003; (l) Albumin/creatinine ratio p-value=0.015; (m) Whole-body mineral density p-value=0.016.

Discussion

Obesity is a complex pathology, intrinsically associated with several other diseases under the umbrella metabolic syndrome. Nonetheless, it has been increasingly debated the existence of individuals with obesity who present a metabolic healthy profile and exhibit obesity related outcomes different from the remaining population with this disease.

The identification of MHO remains inadequately established; consensus remains in considering BMI equal or above 30 kg/m^2. A previous study has described more than 30 different definitions for MHO(Rey-López *et al.* 2014), which included parameters related to metabolic disorders such as type 2 diabetes, dyslipidemia, hypertension, and cardiovascular disease(Blüher 2020). Moreover, biomarkers thresholds are also a subject of ongoing discussion and debate. Some authors defend a more restricted criteria for MHO classification (Lavie *et al.* 2018) whereas others acknowledge the presence of a less strict definition(van Vliet-Ostaptchouk *et al.* 2014). It is our understanding that, given the complexity of obesity pathology, an array of parameters should be defined to better address and identify metabolic unhealthy obesity and act to prevent the before mentioned diseases. We conceptualized this preliminary study with the aim of

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identifying potential parameters that could potentially distinguish healthy individuals from the obese population.

This study included 182 women with obesity referred to bariatric surgery and a comparative analysis of health-related parameters. Our sample population yielded 34.6% of individuals classified as MHO, which represented a higher proportion compared to the prevalence of the MHO phenotype reported in the literature, ranging from 1.1% to 28.5%(Liu *et al.* 2019). Notably, the MUO group consisted of significantly older postmenopausal women, supporting the hypothesis that MHO may be a transient state(Hamer *et al.* 2015).

Of the 50 parameters analyzed (43 biomarkers commonly employed in clinical practice and 7 body measurements), 13 displayed significant differences between MHO - a predefined group demonstrating the absence of diabetes, hypertension, and dyslipidemia (commonly accepted criteria for metabolic health) and MUO. Identified statistically significant parameters included: albumin, triglycerides, glucose, glycated hemoglobin (HbA1c), urea, total calcium, ferritin, chloride, prolactin, insulin, c peptide, Albumin/creatinine ratio, and whole-body mineral density.

The presence of diabetes-associated factors like glucose, insulin, and glycated hemoglobin could introduce bias into the statistical significance found in the results. We believe that a more restricted criteria (as previously mentioned) should be implemented for a more accurate classification. This bias may also be applicable to triglycerides.

Albumin is the most common protein found in blood serum and also a main carrier of fatty acids(Fanali *et al.* 2012). Additionally, glycated albumin was previously described

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as a potential biomarker in prediabetes alongside with ferritin(Luís, C.; Baylina, P.; Soares, R.; Fernandes 2021). Iron metabolism appears to be linked to the status of uncontrolled diabetes and higher ferritin levels are indicative of an increased risk of developing diabetes(Kunutsor *et al.* 2013). Serum urea has been used as a biomarker for renal function and plays an important role in metabolic changes observed in diabetes(Zhang *et al.* 2023). We observed an increased number of MUO with higher levels of serum albumin, ferritin, and urea, potentially indicating the predisposition for diabetic development.

We speculate that total calcium, prolactin, and whole-body mineral density results could be influenced by the menopausal status of the included women given that more than half of the included women were in a postmenopausal state. Total calcium levels were all within the reference range. It is uncertain whether these patients can be externally controlled for calcium levels. Prolactin plays a significant role in regulating fat storage and release. Outside of pregnancy, body weight alterations influences prolactin secretion, it appears that MUO tend to have less serum prolactin(Kopelman 2000) but again, prolactin levels results can also be influenced by the menopausal status(Tanner *et al.* 2011). Obesity was previously associated with whole-body mineral density. A systematic revision on this matter observed that obesity was associated with a lower risk of fracture in postmenopausal with no association in premenopausal women (Turcotte *et al.* 2021).

Chloride levels plays a vital role in acid-base balance, blood pressure, kidney, and gastrointestinal function. Levels above reference range could lead to hyperchloremic metabolic acidosis and promote several metabolic alterations(Pfortmueller *et al.* 2018).

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Our findings show that MHO women have chloride levels near the upper threshold, with a greater number of individuals displaying higher levels. This is an interesting result, contradictory to what was expected. C peptide is important in insulin biosynthesis and so is closely associated with diabetic progression. It improves the body's capacity to process glucose and regulates metabolic functions more effectively(Hills & Brunskill 2009). Interestedly, the graphic pattern found in C peptide is similar to the one found in insulin.

Albumin/creatinine ratio was the statistically significant parameter found in urine sample. Albumin/creatinine ratio is considered a risk factor for both renal and cardiovascular diseases. Elevated levels of albumin in the urine can be indicative of underlying kidney dysfunction and are associated with an increased risk of developing cardiovascular complications(Rossi *et al.* 2010), also is closely associated with obesity(Du *et al.* 2014). Concordantly, we observed that MHO have lower levels of Albumin/creatinine ratio.

This could reaffirm the concept that MHO may represent a transitional phase before the onset of MUO. Despite obtaining significant results, it is important to acknowledge the limitations of the study, namely the small sample size and the influence of menopausal status in the results. Additionally, we would like to acknowledge the existence of two other previously described groups: "Normal weight metabolically obese" and "Sarcopenic obese"(Mayoral *et al.* 2020). While these groups fall beyond the scope of the present study, we recognize the importance of addressing them in future research.

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We believe that our study has paved the way for more extensive research on the importance of identifying metabolically unhealthy individuals. It is imperative to enhance the focus on treatment and awareness in order to prevent and address the health issues faced by these individuals in a more effective manner. This approach is necessary to strike the right balance and avoid the onset of associated diseases among the patients with metabolically healthy obesity.

Conflicts of Interest: The authors declare no conflict of interest.

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Author Contributions:

Conceptualization: F.M., C.L.; Methodology: F.M., C.L.; Validation: R.F., R.S. E.L.C.; Formal Analysis: F.M., C.L.; Resources and methodology: P.S.; T.M.; P.F.; I.R.; D.F.; J.P.; A.V.; A.R.; Supervision: P.F.; R.F., R.S. E.L.C. ; Writing – Original Draft Preparation: C.L.; Writing – Review, Editing and Validation: F.M.; P.S.; T.M.; P.F.; I.R.; D.F.; J.P.; A.V.; A.R.; R.F.; R.S.; E.L.C.; C.L. All authors have read and agreed to the published version of the manuscript.

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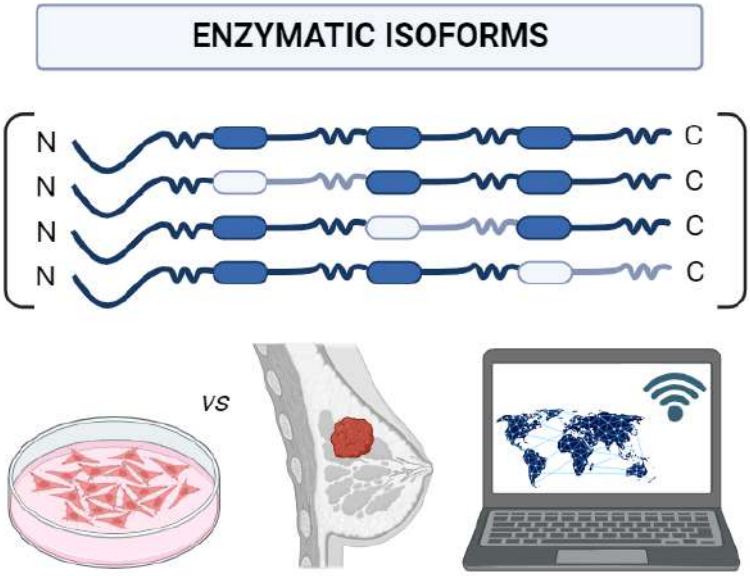
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APPENDIX C

Integrative approach on enzymatic isoforms



LIST OF CONTENTS:

- "Glycolytic and Gluconeogenic Enzymes Isoforms: Implications in Breast Cancer Metabolism"

Carbohydrate Research

Glycolytic and Gluconeogenic Enzymes isoforms: Implications in Breast Cancer Metabolism

--Manuscript Draft--

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Corresponding Author:	Carla Luís University of Porto Faculty of Medicine PORTUGAL
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	Rúben Fernandes
	Raquel Soares
Abstract:	It is well established that tumour cells undergo metabolic changes to acquire biological advantage over normal cells with activation of the glycolytic pathway, a process termed "Warburg effect". Enzyme isoforms are alternative enzymatic forms with the same function but with different biochemical or epigenetic features. Moreover, isoforms may have varying impacts on different metabolic pathways. We challenge ourselves to analyse the glycolytic and gluconeogenic enzymes isoforms in breast cancer, a complex and heterogeneous pathology, associated with high incidence and mortality rates especially among women. We analysed epithelial and tumour cell lines by RT-PCR and compared values to a publicly available database for the expression profile of normal and tumour tissues (Gepia) of enzymatic isoforms from glycolytic and gluconeogenic pathways. Additionally, GeneMANIA was used to evaluate interactions, pathways, and attributes of each glycolytic/gluconeogenic steps. The findings reveal that the enzymatic isoforms expressed in cell culture were different from those in breast tissue. We propose that the tumor microenvironment plays a crucial role in the expression of glycolytic and gluconeogenic enzymes in tumour cells, without necessarily implying significant changes in the core metabolic pathways. Nonetheless, these isoforms not only participate in glycolytic and gluconeogenic enzymatic activities but may also influence other pathways, such as the Pentose-Phosphate-Pathway, TCA cycle, as well as other carbohydrate, lipid, and amino acid metabolism.
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Porto, 7th December 2023

Dear Editor-in-Chief of *Carbohydrate Research*

We are most honored to submit our work entitled "**Glycolytic and Gluconeogenic Enzymes Isoforms: Implications in Breast Cancer Metabolism**" to the *Carbohydrate Research* Journal.

We believe to have followed the policies for manuscript preparation required:

- We confirm that our manuscript has not and will not be submitted elsewhere;
- All authors have contributed significantly and equally in the design and revision of the manuscript;
- We further confirm that all authors listed in the manuscript agree with the manuscript publication;
- We wish to confirm that there are no conflicts of interest associated with this publication, and there was no significant financial support for this work that could have influenced its outcome.

For all the reasons above, we believe to contribute to the quality standards of *Carbohydrate Research*.

Looking forward to hearing from you.
With my best regards,



Carla Luis

Highlights

- Enzymatic isoforms may have varying impacts on different metabolic pathways.
- Enzymatic isoforms expressed differently in cell culture from breast cancer tissue.
- Glycolysis and gluconeogenic enzymatic isoforms present a distinct expression

Glycolytic and Gluconeogenic Enzymes isoforms: Implications in Breast Cancer Metabolism

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Abstract

It is well established that tumour cells undergo metabolic changes to acquire biological advantage over normal cells with activation of the glycolytic pathway, a process termed "Warburg effect". Enzyme isoforms are alternative enzymatic forms with the same function but with different biochemical or epigenetic features. Moreover, isoforms may have varying impacts on different metabolic pathways. We challenge ourselves to analyse the glycolytic and gluconeogenic enzymes isoforms in breast cancer, a complex and heterogeneous pathology, associated with high incidence and mortality rates especially among women.

We analysed epithelial and tumour cell lines by RT-PCR and compared values to a publicly available database for the expression profile of normal and tumour tissues (*Gepia*) of enzymatic isoforms from glycolytic and gluconeogenic pathways. Additionally, *GeneMANIA* was used to evaluate interactions, pathways, and attributes of each glycolytic/gluconeogenic steps.

The findings reveal that the enzymatic isoforms expressed in cell culture were different from those in breast tissue. We propose that the tumor microenvironment plays a crucial role in the expression of glycolytic and gluconeogenic enzymes in tumour cells, without necessarily implying significant changes in the core metabolic pathways. Nonetheless, these isoforms not only participate in glycolytic and gluconeogenic enzymatic activities but may also influence other pathways, such as the Pentose-Phosphate-Pathway, TCA cycle, as well as other carbohydrate, lipid, and amino acid metabolism.

Keywords

Breast cancer; Metabolism; Glycolysis; Gluconeogenesis; Enzymes; Isoforms.

1. Introduction

Breast cancer (BC) is a complex and heterogeneous pathology and the most common cancer in women worldwide with high incidence and mortality rates[1]. The intricate network between genetic, epigenetic, and environmental factors contributes to the diverse molecular landscape of breast cancer, influencing disease progression and therapeutic outcomes. Among the multifaceted alterations occurring within cancer cells, metabolic reprogramming has emerged as a hallmark feature that supports their relentless growth and survival[2]. To fulfil the high demands of energy and biosynthesis requirements, tumour cells undergo metabolic switch with glycolysis activation in detriment to oxidative phosphorylation, the Warburg Effect[3]. This mechanism has been the focus of several studies for the last decades, namely in breast cancer metabolism and therapeutics[4,5].

Glycolysis, namely the Warburg effect allows cancer cells to produce energy despite the oxygen-rich environment. Consequently, glycolysis rate-limiting enzymes become attractive targets for developing novel therapeutic interventions[6]. Gluconeogenesis, on the other hand, is primarily associated with glucose production from non-carbohydrate sources, particularly in the liver, to maintain glucose homeostasis during fasting or energy-demanding situations. In the context of cancer, gluconeogenesis has received comparatively less attention. However, emerging evidence suggests that cancer cells can hijack gluconeogenic pathway enzymes to support their anabolic demands and contribute to the biosynthesis of macromolecules essential for growth[7].

Critical to the regulation of both glycolysis and gluconeogenesis are the various enzyme isoforms involved in these pathways. Isoforms are distinct forms of enzymes encoded by different genes, often displaying differential expression patterns in various tissues and cellular contexts, and present different biochemical and epigenetic features[8]. Their presence adds an additional layer of complexity to the metabolic landscape of cancer. Glycolytic and gluconeogenic enzyme isoforms expressed only in tumour cells can exhibit altered expression levels, catalytic activities, and regulatory mechanisms in breast cancer cells compared to normal cells, thereby influencing the metabolic phenotype of cancer cells and their response to therapy. Questions are raised about the evolutionary significance of enzymatic isoforms and their biological implications are not fully comprehended. Previous studies observed that significant alterations in glycolytic enzyme isoforms in cancer cells triggered by transcription factors (like HIF1α) can influence pathways like apoptosis (e.g., hexokinase-2, HK2) and migration (e.g., enolase-1, ENO1)[9]. In breast cancer, isoforms modulation can be critical in cancer histological type[10] and glycolytic efficiency, which in turn, translates in tumour aggressiveness[11].

This study aims to explore the current understanding of glycolytic and gluconeogenic enzymes isoforms in breast cancer. By examining the specific isoform alterations that occur in breast cancer cells and tissues in comparison to their healthy counterparts, we gain insights into the molecular mechanisms underpinning the metabolic rewiring of cancer cells.

2. Results and discussion

We observed different expression levels of glycolytic/gluconeogenic enzymatic isoforms in breast cancer cell culture assays and tissue expression analysis for the majority of isoforms, occasionally suggesting a transition between isozymes. Isoforms not only play a role in glycolysis and gluconeogenesis, but also be associated with different protein families and pathways. Pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, galactose, fructose, and mannose metabolism are the most common mechanisms influenced by these isoforms, although correlations between isoenzymes and amino acid metabolism, glutaminolysis, and Cori cycle were also found. Results are further presented and discussed independently.

Hexokinase (isoforms: HK1, HK2, HK3, HK4)

Hexokinase catalyses glucose phosphorylation into glucose-6-phosphate, The first glycolytic step. Results from cell-based assays and *Gepia* bioinformatics analysis of HK isoforms are presented in Figure 1.

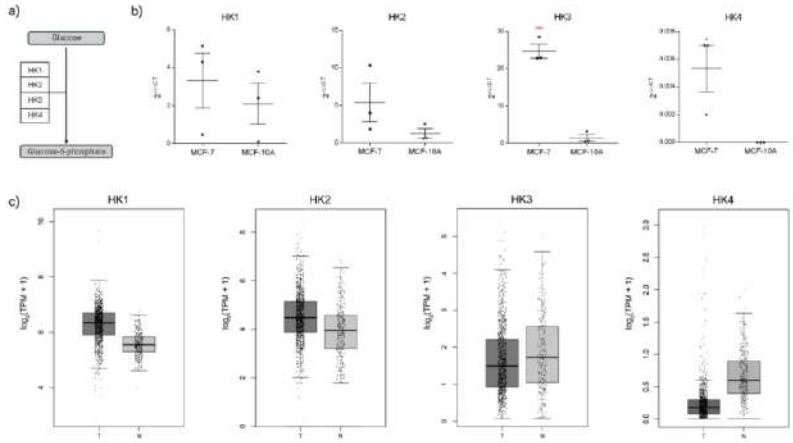


Figure 1 a) Rate limiting step from glycolysis of Hexokinase isoforms catalysing the conversion of glucose into glucose-6-phosphate; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of HK isoforms; c) Plots of HK isoforms gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; *** p-value<0.001; T – Tumour tissue; N – Normal breast tissue.

We observed an increased expression in both tumour cell line and cancer tissue of HK1. According to *Gepia*, the HK1 isoform presents the higher levels of expression, although the most described HK isoform in cancer is isoform 2, which is mostly associated with

tumorigenesis and tumour progression. HK1 is a broad expressed isoenzyme whose functions are still poorly understood. Some studies correlated HK1 function with its localization and the apoptotic potential through TNFα pathway[12]. Curiously enough, is HK3 the HK isoform with the higher and significant expression in tumour breast cell line (p-value<0.001), with no expression in breast epithelial cell. Interestingly, in BC tissue, HK3 is downregulated. Interestingly, in colorectal cancer, HK3 induces apoptosis and endoplasmic reticulum stress[13]. Therefore, these findings indicate that HK3 is probably a marker of stress in cell cultures, whereas in BC tissue microenvironment may attenuate/reverse this aggressiveness marker. HK4, also known as Glucokinase (GCK), is closely associated with the insulin pathways, and is expressed in pancreatic beta cells and hepatocytes[14]. In BC, its expression reveals residual values, both in tissues [$\log_2$ (TPM + 1) <1] and in BC cell line ($2^{-\Delta\Delta CT}$<0.01). Regarding HK2, both cell-based assays as well as tissue enzymatic assessment revealed an increased expression in cancer. This isozyme can be expressed in the cytosol and in the mitochondria, being its location a major determinant of its catalytic and regulatory character. HK2 located in the cytoplasm facilitates anabolic processes (PPP and glycogen synthesis) while in the mitochondria potentiates glycolysis, reduces reactive oxygen species production, blocks pro-apoptotic Bax and Bak enrolment, among others. HK2 has a distinct advantage compared to other isoforms as it possesses two catalytic sites for glucose phosphorylation, in contrast to other hexokinases, which have only one[15].

The *GeneMANIA* reports reveals that Hexokinase family is one of the most glycolytic investigated enzymes. They are described to interfere with other pathways like fructose, galactose, and mannose metabolism, but each isoenzyme has specific roles. HK1 shows the exclusively interaction with VDAC, HK1-VDAC binding promotes apoptosis in tumour cells[12] and has been proposed to be a potential therapeutical target in breast cancer[16]. HK3 evidences itself by its interaction with the PI3K/AKT/mTOR signalling pathway and its association with the nucleoporins (NUP) superfamily. NUP are a family of proteinic components of the nuclear pore complex accountable for the transport of macromolecules from the cytoplasm to the nucleus[17]. Interestingly enough, the NUP superfamily will also be associated with the gluconeogenic corresponding step in Glucose-6-phosphatase. HK4 (GCK) presents a more functional spectrum role, mainly as an insulin metabolism modulator.

Phosphoglucose isomerase (GPI)

GPI catalyses the reversible reaction between glucose-6-phosphate and fructose-6-phosphate. Figure 2 presents the results obtained from cell-based RT-PCR assays and *Gepia* bioinformatics analysis of GPI.

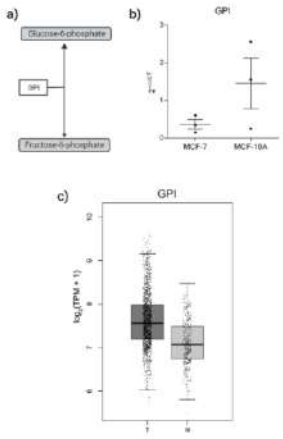


Figure 2 a) Reversible step from glycolysis/gluconeogenesis pathways of glucose-6-phosphate isomerase; b) Results from the cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of GPI; c) Plot of gene expression of GPI in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: T – Tumour tissue; N – Normal breast tissue.

The results obtained in BC cell lines and in BC tissues are contradictory regarding GPI expression. While in BC cells a decrease in GPI expression was observed when compared to the epithelial breast line, GPI expression in BC tissues was higher than in adjacent healthy breast tissues. One potential explanation for this result, is the correlation of GPI expression with immune cell infiltration, a typical characteristic of the tumour microenvironment[18]. The literature on GPI expression in breast cell lines is extremely rare, leading to speculate that this enzyme might be underestimated. Even though, GPI is associated with different pathways. As expected, it is associated with both glycolytic and gluconeogenic pathways, but also with the PPP enzyme, transketolase (TKT), and with SLC2A1 (GLUT1) – a major glucose transporter. The most described non-glycolytic function of GPI is cell motility and invasion as GPI is also an autocrine motility factor (AMF). A study in PGI/AMF silencing in MDA-MB-231, a highly aggressive BC cell line, found that it reversed epithelial to mesenchymal transition (EMT)[19]. Also, as AMF, GPI has affinity to AMF Receptor. It is internalized and exerts several functions in different pathways such as small GTPase RhoA, lipoxygenase, and protein kinase C[20].

Phosphofructokinase (isoforms: PFKP, PFKL, PFKM)

Results from *Gepia* bioinformatics analysis and cell-based RT-PCR assays of PFK are presented in Figure 3.

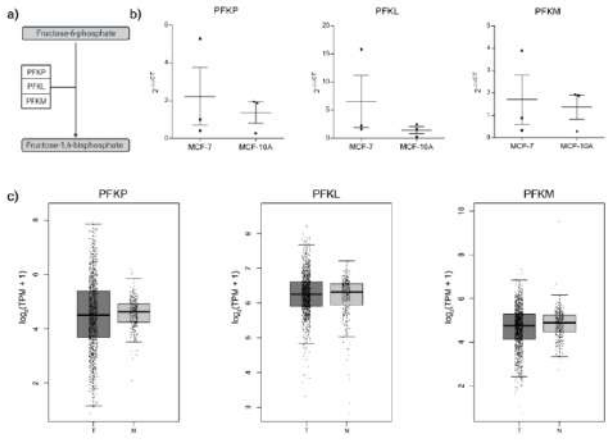


Figure 3 Phosphofructokinase isoforms, a glycolytic rate-limiting enzyme that catalyses the conversion of fructose-6-phosphate into fructose-1,6-bisphosphate. a) schematic diagram of glycolytic pathway; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PFK isoforms; c) Plot of PFK isoforms gene expression in tumour tissue (n=1085) and normal breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: T – Tumour tissue; N – Normal breast tissue.

No noteworthy isoform variation of PFK was observed in BC tissues. In BC cell lines, PFK isoforms expression increased slightly. Higher expression levels were found for PFKL isoform. Previous cell-based assays of breast cancer cell lines revealed a correlation between PFKL and cell line aggressiveness, PFKL expression in triple negative cell line (MDA-MB-231) was increased when compared to MCF7 and MCF-10A (non-tumorigenic)[21]. Literature associates PFK to many non-glucogenic functions such as survival, apoptosis inhibition by BAD inactivation (PFKP), cell shape and motility by binding to cytoskeleton and transcription regulation (PFKM)[21,22]. Nonetheless, further studies are needed to clarify the involvement of each PFK isoform in breast cancer since *GeneMANIA* report didn't highlight any other pathway involvement besides glycolysis and Cori cycle. Cori cycle can be considered a shuttle between extrahepatic tissue and liver but intriguingly, *GeneMANIA* associated the Cori cycle with the Platelet (PFKP) isoform in opposition to the Liver (PFKL) and Muscle (PFKM) isoform.

Aldolase (isoforms: ALDOA, ALDOB, ALDOC)

Figure 4 displays the outcomes derived from cell-based RT-PCR assays and the *Gepia* bioinformatics analysis of ALDO isoforms.

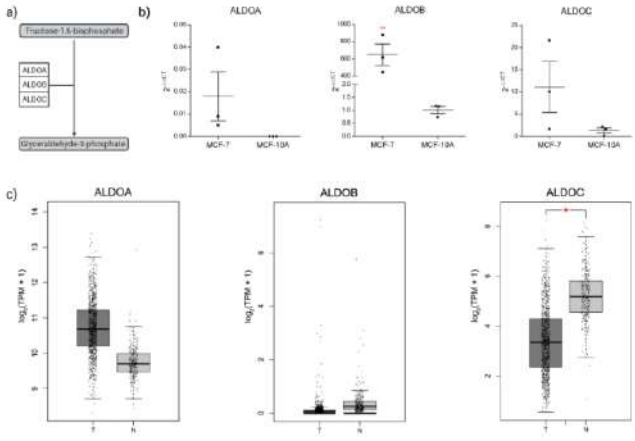


Figure 4 Aldolase isoforms catalyse the conversion of fructose-1,6-biphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate and vice-versa. a) Schematic diagram of glycolysis and gluconeogenesis pathways; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of ALDO isoforms; c) Plot of ALDO isoforms gene expression in tumour tissue (n=1085) and normal breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; ** p-value<0.01; T – Tumour tissue; N – Normal breast tissue.

ALDOA and ALDOC are the isoenzymes with more representative expression with ALDOA presenting increased levels and ALDO C showing decrease levels in BC tissues. Regarding cell culture, MCF-7 (tumour cell line) expressed increased levels of all isoforms of ALDO comparing to MCF-10A. Residual but significant in ALDOA (p-value<0.05), highly increased in ALDOB (p-value<0.01) and increased in ALDOC.

GeneMANIA reports on the ALDO enzymatic family have shown shared associations with pathways such as glycolysis, fructose metabolism, and galactose metabolism across all isoforms. Regarding moonlight functions, ALDOA has been thoroughly associated with several non-glycolytic functions by its ability to interact with multiple factors and co-operators with relevant roles in maintaining cytoskeleton and promoting proliferation. Moreover, as a HIF1α target, it accelerates glycolysis and promotes fatty acid and nucleotide biosynthesis. It is speculated that such functions are just a small part of ALDOA biological purposes[23]. ALDOA is the predominant isozyme found, and while it has been previously reported to be expressed in normal tissues, our RT-PCR results did not align with this finding in breast tissue[23]. ALDOB is primarily found in the liver and in some other tissues, but it is generally not highly expressed in breast or in BC cells, and again, results from RT-PCR analysis reveal a contradictory significant high expression (p-value<0.001) of ALDOB. ALDOC is mostly expressed in the central nervous system and is suppressed by STAT3, a transcription factor which responds to several cytokines and

growth factors (IL5, IL6, BMP2 IFNs, EGF, HGF, LIF)[23], inflammation is a common feature in cancer and a critical factor in tumour progression [23], which can modulate glucose metabolism.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Cell-based RT-PCR assays and Gepia bioinformatics analysis of GAPDH are illustrated in Figure 5.

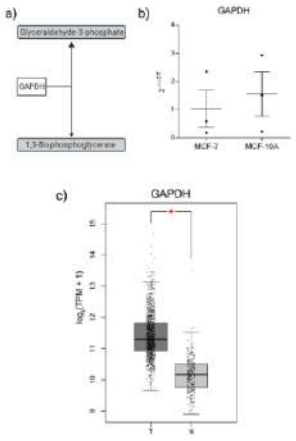


Figure 5 a) Glyceraldehyde-3-phosphate dehydrogenase catalyses the reversible conversion of glyceraldehyde-3-phosphate into 1,3-biphosphoglycerate; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of GAPDH; c) Plot of GAPDH gene expression in tumour tissue (n=1085) and normal breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; T – Tumour tissue; N – Normal breast tissue.

GAPDH is significantly increased in BC tissue and is decreased in MCF-7 cell line. GAPDH was previously described to be deregulated in different types of cancer, namely in BC. Estradiol, IGF1 and FGF can promote the expression of GAPDH[24], as observed in tumour tissue.

GAPDH is associated with Glycolysis, Cori cycle, Tyrosine kinase family (ERBB4), Adenosine-5-diphosphoribose, HIF1α, and PPARδ regulation of glycolysis. GAPDH has been promoted to be a housekeeping gene, but novel studies demonstrate that GAPDH can be a key regulator of central metabolism [25,26].

Additionally, several non-glycolytic functions have been associated with this enzyme, including transcription regulation and apoptosis. Moreover, GAPDH seems to be implicated in several mechanisms such as phosphorylation, tubulin bundling, and membrane fusion and nuclear RNA export, DNA repair and interaction with cellular components, including nitric oxide, dinucleoside polyphosphate, and RNA[20] and with

the immune metabolism[27]. Due to the multiple implications, we are uncertain about the role GAPDH and whether it is a friend or a foe in cancer metabolism.

Phosphoglycerate kinase (isoforms: PGK1, PGK2)

PGK isoforms result from cell-based RT-PCR assays and *Gepia* bioinformatics analysis can be visualized in Figure 6.

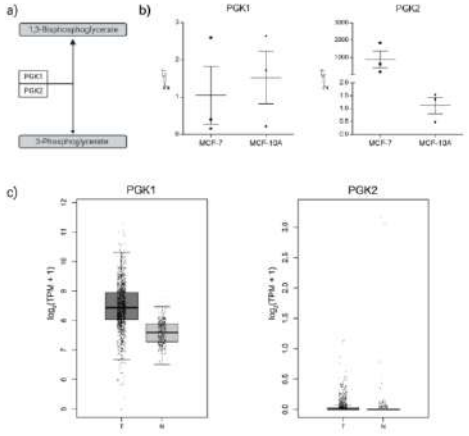


Figure 6 Phosphoglycerate kinase isoforms catalyse the reversible conversion of 1,3-biphosphoglycerate into 3-phosphoglycerate. a) Schematic diagram of its pathway; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PGK isoforms; c) Plot of PGK gene expression isoforms in tumour tissue (n=1085) and normal breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: T – Tumour tissue; N – Normal breast tissue.

PGK1 is overexpressed and PGK2 presents residual expression in BC tissues as previously observed[28]. PGK1 presented lower expression and PGK2 was overexpressed in BC cell line MCF-7. Results from previous studies unravel that cellular expression of PGK varied among different cell lines[29].

GeneMANIA reveals that PGK2 relates with gluconeogenesis and PGK1 is not only associated with glucose metabolism and cancer but also with other metabolic pathologies like microbial infection. PGK1 plays a controlling role as one of the two enzymes involved in ATP production in glycolysis. This enzyme was previously proposed as an unfavourable prognostic factor in BC and is involved in numerous non-glycolytic mechanisms like angiogenesis, DNA repair and autophagy. Moreover, its ubiquitous functions are dependent of its location. According to our literature revision, extracellular PGK1 reveals anti-tumour capacity by decreasing angiogenesis. Intracellular PGK1 can be considered an oncogene by interacting with several pathways that promote tumorigenesis and proliferation like HIF1α, MYC, CXCR4/CXCL12, AKT/mTOR[30].

Phosphoglycerate mutase (isoforms: PGAM1, PGAM2, PGAM5)

Figure 7 highlights the outcomes stemming from cell-based RT-PCR assays and *Gepia* bioinformatics analysis of PGAM isoforms.

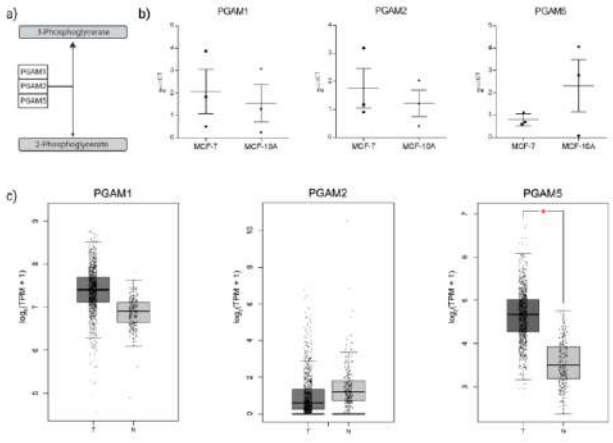


Figure 7 Phosphoglycerate mutase isoforms catalyse the conversion of 3-phosphoglycerate into 2-phosphoglycerate and vice-versa. A) Schematic diagram of its pathway; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PGAM isoforms expression; c) Plot of PGAM isoforms gene expression in tumour tissue (n=1085) and normal breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; T – Tumour tissue; N – Normal breast tissue.

PGAM expression in BC is heterogeneous. PGAM4 (not presented) has a residual expression associated with specific cell types such as melanocytes, keratinocytes, basal respiratory cells, or Langerhans cells. PGAM3 was considered by *GEPIA* as a pseudogene, a DNA sequence structurally identical to a gene but uncapable to code a protein.

Our results disclosure higher levels of PGAM1 on both BC cell line and tissue. PGAM2 and PGAM5 present opposing results for cell and tissue BC analysis. PGAM2 has increased while PGAM5 has decreased levels of expression in BC cell line MCF-7.

PGAM1 overexpression is associated with multiple cancers, including BC, due to its intervention in tumour growth, survival, and migration pathways. Its metastatic capacity is related to the interaction with α-smooth muscle actin (ACTA2) but PGAM1 has a complex network and interacts in several pathways such as HIF1α, AKT, mTOR, PI3K, S1P or Hippo. It was also proposed as an unfavourable prognostic factor in BC[31]. PGAM2 interacts with Ras signalling pathways and CDK1 to enhance tumour growth. Moreover, IGF1/PI3K leads to PGAM2 nuclear accumulation and induce RNA synthesis[32]. PGAM5 although belonging to the PGAM family, lacks the mutase activity due to its poorly conserved phosphohistidine motif. However, it can regulate mitochondria dynamics and

programmed necrosis. The current study shows that PGAM5 is significantly overexpressed in BC tissue, which is in accordance with its presence in hepatocellular carcinoma. But in colorectal cancer, PGAM5 expression was decreased[33] correlating with the cellular assays findings. It is described that under stress promoted by different signals (TNF, oxidative stress, or calcium ionophore) PGAM5 induces apoptosis[14,33].

Enolase (isoforms: (isoforms: ENO1, ENO2, ENO3, and ENO4)

Figure 8 presents cell-based RT-PCR assays and *Gepia* bioinformatics analysis of ENO isoforms.

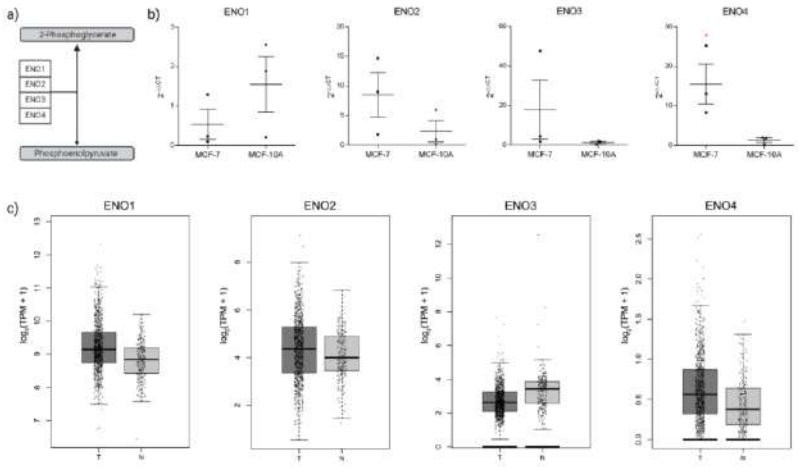


Figure 8 a) Enolase isoforms catalyse the conversion of 2-phosphoglycerate into phosphoenolpyruvate and vice-versa; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of ENO isoforms expression; c) Plot of ENO isoforms gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; T – Tumour tissue; N – Normal breast tissue.

ENO2 and ENO4 share the same enzymatic pattern in both BC cells and tissue. ENO1 and ENO3 expressed opposite results. Lower levels of ENO1 are observed in MCF-7 while in BC tissue there is a slight increase. ENO3 is overexpressed in BC cell line (p-value<0.05) and a decrease in BC tissues. ENO4 is highly expressed in MCF-7 but presents residual expression in BC tissues, since it is mainly associated in cilium organization of ciliated cells[14].

Their main correlations are associated with the glucogenic pathway, except for ENO4 that disclosures widespread functions. ENO1 and ENO2 are the most expressed isoforms in BC tissues, and besides their role in glucose metabolism, both have additional functions and can interact with different metabolic players. ENO1 interest has been increasing due to the multiple pathophysiological functions associated, ENO1 acts as a receptor and

activates plasminogen, which promotes migration and invasion and can also act as a regulator in gene transcription and translation from its binding capacity to DNA and RNA[34]. It also interacts with several pathways namely in immune response, viral replication, and autoimmune diseases[32]. ENO2 is mostly associated to central nervous system and displays neurotrophic and neuroprotective properties, promoting cell survival in neurons[14]. In breast cancer, ENO2 is used to identify neuroendocrine differentiation, and is considered a pro-survival factor by triggering PI3K/Akt and MAPK/ERK signalling pathways[35]. ENO3 is other isoenzyme underexpressed in BC tissues associated with the muscle. Chen study in colorectal cancer observed that ENO3 is increased in tumour samples, and ENO3 knockdown was associated with proliferation and migration suppression in cell-based assays[36]. ENO4 is correlated with mitochondrial transport and enzymes, and also with Notch signalling pathway (MYCBP), Heat Shock Protein Beta-2 (HSPB2), Calcium-Binding Tyrosine Phosphorylation-Regulated Protein (CABYR), IL-17 Family signalling pathways, a proinflammatory cytokine family.

Pyruvate kinase (isoforms; PKLR, PKM)

Cell-based RT-PCR assays and *Gepia* bioinformatics analysis of PK isoforms can be consulted in Figure 9.

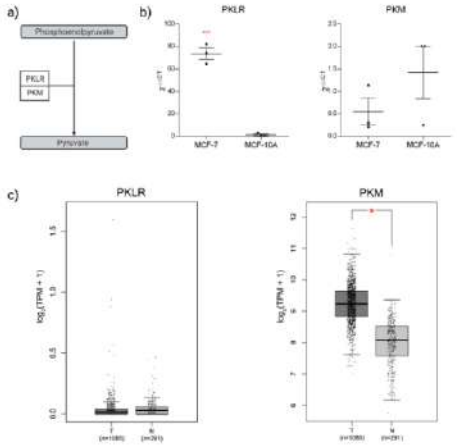


Figure 9 a) Glycolytic rate limiting step of Pyruvate kinase isoforms, catalysing the conversion of phosphoenolpyruvate into pyruvate; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PK isoforms expression; c) Plot of PK isoforms gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; *** p-value<0.001; T – Tumour tissue; N – Normal breast tissue.

Along with PGK, PK is another enzyme involved in ATP production. PKLR gene encodes two isoenzymes, PKL and PKR while PKM encodes PKM1 and PKM2. Similar to what has been observed in other isoforms, there seems to be a transition from PKLR to PKM in breast cancer cells compared to breast cancer tissue. PKLR is significantly increased in MCF-7 cell line (p-value<0.001) while in BC tissue, the higher levels are found in PKM (p-value<0.05).

PKLR gene in tissue seems to have no direct influence on breast cancer with residual expression although it was previously reported that it could be the driver for liver metastasis of colorectal cancer[37]. Moreover, in gallbladder cancer, PKLR regulation by circFOXP1 promotes tumour progression. Both are strictly associated with glucose and pyruvate metabolism according to *GeneMANIA*.

PKM1 is expressed in most adult tissues and seems to play a relevant role in drug resistance. PKM2 isozyme is expressed in embryonic tissues and usually overexpressed in cancer with poorer outcomes. PKM2 enhances cell cycle progression, tumour growth, migration, and invasion. Although our results were not able to distinguish PKM1 and PKM2, we found that together, both display pro-cancer functions.

Pyruvate Carboxykinase (PC)

Figure 9 presents the cell-based RT-PCR assays and the *Gepia* bioinformatics analysis of PC expression.

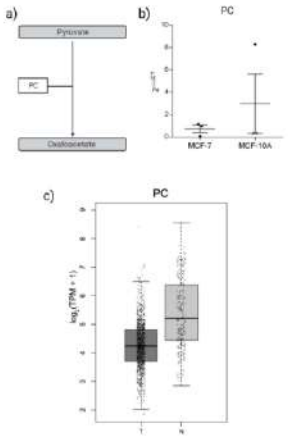


Figure 10 a) Gluconeogenic rate limiting step pyruvate carboxylase enzyme, catalysing the conversion of pyruvate into oxaloacetate; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PC; c) Plot of PC gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: T – Tumour tissue; N – Normal breast tissue.

PC is a gluconeogenic specific enzyme and is decreased in both BC cells and tissue. Its associated interactions, co-expression, attributes, and pathways are numerous and extensive. PC was previously described as involved in several pathways besides gluconeogenesis, such as amino acid metabolism, ketogenesis, TCA and urea cycle, lipogenesis, insulin secretion and synthesis of glutamate neurotransmitter [14], additionally, PC confers protection against oxidative stress[38]. Furthermore, PC expression knockdown in cell-based assays resulted in decreased proliferation, migration, and invasion in breast cancer cell lines[39]. PC presents no isoforms and is correlated with diverse signalling pathways such as biotin proteins associated pathways, arginine biosynthesis, malate-aspartate shuttle and ERBB2. Previous studies already observed intrinsic correlations of PC with ERBB2 expression in the metabolic fingerprint of different molecular cell line subtypes. Results show that HER2 positive cell lines shared common patterns in glycolytic/gluconeogenic enzymatic expression under obesity mimicking conditions[40].

Phosphoenolpyruvate Carboxykinase (isoforms: PCK1, PCK2)

Figure 11 displays cell-based RT-PCR assays and the *Gepia* bioinformatics analysis of PCK isoforms findings.

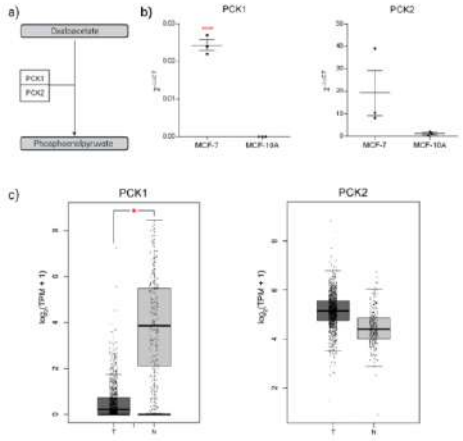


Figure 11 a) Gluconeogenic rate limiting step Phosphoenolpyruvate carboxykinase isoforms, catalysing the conversion of oxaloacetate into phosphoenolpyruvate; b) Results from the cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of PCK isoforms; c) Plot of PCK isoforms gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; **** p-value<0.0001; T – Tumour tissue; N – Normal breast tissue.

PCK gene integrates 2 isoforms (1 and 2), PCK1 has a significant decreased expression in BC tissue (p-value<0.05) while in cell culture presented a significant increased (p-

value<0.0001). PCK2 has increased expression levels in BC tissues concordant with the results from the cell-based RT-PCR. It was previously described that PCK expression differs in tumours from gluconeogenic and non-gluconeogenic tissues. The major described function of PCK is anaplerosis enhancing TCA cycle and promoting neighbouring metabolic pathways namely glutaminolysis, pyruvate synthesis, glyceroneogenesis, and PPP[7]. Furthermore, there is a close association of this isoform with obesity through PPAR signalling pathway and adipogenesis regulation. Overexpression of mitochondrial PCK2 will avoid malate transport to the cytosol, maximizing energy consumption. On the other hand, PCK1 expression was decreased, although it was already observed to be overexpressed in melanoma and colorectal cancer[7]. In brief, PCK is associated with tumour progression by fuelling the energy metabolism.

Fructose 1,6 Bisphosphatase (isoforms: FBP1, FBP2)

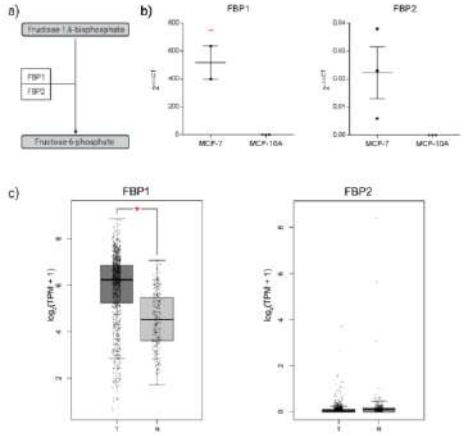


Figure 12 a) Gluconeogenesis rate limiting step Fructose-1,6-Bisphosphatase isoforms expression, catalysing the conversion of fructose-1,6-bisphosphate into fructose-6-phosphate; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of FBP isoforms; c) Plot of FBP isoforms gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; ** p-value<0.01; T – Tumour tissue; N – Normal breast tissue.

Fructose bisphosphatase contemplates 2 isoforms, though FBP2 usually has residual expression in BC. FBP2 is the muscle isoform present in myocytes, gastric mucus-secreting cells, and Spermatogonia[14]. The results between cell-based assays and BC tissues only diverge in the expression of FBP2 in MCF-7 cell line, although values are also residual ($2^{-\Delta\Delta CT}$<0.04).

Regarding *GeneMANIA* results, no relevant differences were seen between both isoenzymes. FBP1 is expressed mainly in the liver and is described to act as a tumour suppressor gene by supressing HIF1α, enhancing OXPHOS and consequently increasing ROS in several types of cancer including breast cancer[7]. It is a glycolytic suppressor, and by associated means, it also supresses the neighbouring pathways, decreasing metabolites biosynthesis of serine, glyceroneogenesis, and PPP[7].

Glucose 6 Phosphatase (Isoforms: G6PC1, G6PC2, G6PC3)

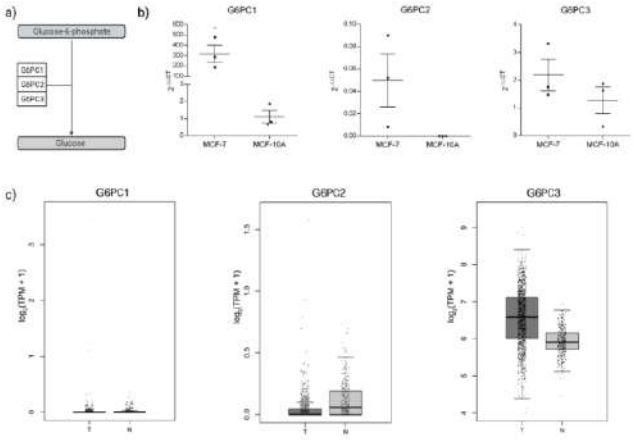


Figure 13 a) Gluconeogenic rate limiting step glucose-6-phosphatase catalytic subunits, catalysing the conversion of glucose-6-phosphate into glucose; b) Cell-based RT-PCR of MCF-7 (tumour cell line, n=3) and MCF-10A (epithelial cell line, n=3) of G6P catalytic subunits; c) Plot of G6P catalytic subunits gene expression in tumour tissue (n=1085) and breast tissue (n=291) extracted from *Gepia* publicly available database. Legend: * p-value<0.05; T – Tumour tissue; N – Normal breast tissue.

Glucose-6-phosphatase is an enzyme that includes several integral membrane proteins, the catalytic subunit (G6PC) and transporters for phosphate, glucose, and glucose-6-phosphate. The G6PC gene family consists of three members (G6PC1, G6PC2, and G6PC3), resulting in the recognition of three isoforms of this gluconeogenic enzyme[41].

In BC tissue, the catalytic unit with the most prominent expression is G6PC3, which exhibits increased expression. On the other hand, in the MCF-7 cell line, G6PC1 stands out. In cell-based assays, all catalytic units are upregulated in MCF-7 cells, whereas the expression of G6Pase in MCF-10A cells is either low or absent.

However, with shared correlations, each isoform demonstrated specific functions. G6PC1 is associated with gene regulations while G6PC2 in galactose metabolism and carbohydrate digestion and absorption and G6PC3 is correlated with RNA transport. Like PCK, G6Pase expression differs in gluconeogenic/non-gluconeogenic tissues. For non-

gluconeogenic tissues, G6PC is involved in cell cycle regulation, survival, and migration[7]. In ovarian and glioma cancers, G6PC also promotes glycogen degradation to avoid metabolic stress[7].

Summary results

Summarized results can be visualized in Table 2. Reports from *GeneMANIA* were presented in Supplemental material (Supplemental Material – Report S1).

Table 2 Summarize results from cell-based assays and the bioinformatics analysis (including outcomes from *Gepia* and *GeneMANIA*). Expression is presented as increased/decreased expression of breast cancer in comparison to normal breast.

Enzyme	Pathway	Isoform	Expression in BC cell lines	Expression in BC tissues	Interactions, co-expressions, attributes, and associated pathways
HK	Glycolysis	HK1	+	+++	Glycolysis Fructose metabolism Galactose metabolism Mannose metabolism Glycogen biosynthesis Butirosin and neomycin biosynthesis Pentose phosphate pathway Mitochondrial transport (VDAC)
		HK2	+	++	Glycolysis Fructose metabolism Galactose metabolism Butirosin and neomycin biosynthesis Pentose phosphate pathway
		HK3	+++(***)	-	Glycolysis Galactose metabolism Fructose metabolism Mannose metabolism Glucose transport (Solute Carrier family; NUP) PI3K/AKT/mTOR pathway
		HK4	+ (*)®	®	Insulin metabolism Glycolysis Glucose/G1P degradation Butirosin and neomycin biosynthesis GDP-glucose biosynthesis UDP-N-acetyl-D-galactosamine biosynthesis Glycogen metabolism Glucose transport Regulation of gene expression AKT pathways
GPI	Glycolysis/ Gluconeogenesis	GPI	-	++	Glycolysis Gluconeogenesis Glucose transport (Solute Carrier family) Pentose phosphate pathway UDP-N-acetyl-D-galactosamine biosynthesis
PFK	Glycolysis	PFKP	+	=	Glycolysis Cori cycle
		PFKL	++	= =	Glycolysis
		PFKM	+	=	Glycolysis
ALDO	Glycolysis/ Gluconeogenesis	ALDOA	+®	+++	Glycolysis Fructose metabolism Galactose metabolism Cori cycle Sucrose degradation

		ALDOB	+++ (**)	®	Glycolysis Fructose metabolism Galactose metabolism Sucrose degradation
		ALDOC	++	- - (*)	Glycolysis Gluconeogenesis Fructose metabolism Galactose metabolism Mannose metabolism Pentose phosphate pathway
GAPDH	Glycolysis/ Gluconeogenesis	GAPDH	-	+++ (*)	Glycolysis Cori cycle Tyrosine kinase family (ERBB4) Adenosine-5-diphosphoribose HIF1α and PPARδ regulation of glycolysis
PGK	Glycolysis/ Gluconeogenesis	PGK1	-	+++	Glycolysis Cori cycle Response of MTB to phagocytosis Adenosine-5'-βδ-methylene triphosphate
		PGK2	+++	®	Glycolysis Gluconeogenesis Cori cycle Glucose metabolism Spermatogenesis
PGAM	Glycolysis/ Gluconeogenesis	PGAM1	+	++	Glycolysis Gluconeogenesis Cori cycle
		PGAM2	+	-	Glycolysis
		PGAM5	-	+++ (*)	Glycolysis Pentose phosphate pathway Heparin-induced thrombocytopenia pathway TCR signaling modulation and T cell activation MAPK pathway Cell cycle regulation KEAP1-NFE2L2 pathway Oxidative stress NRF2-ARE regulation
ENO	Glycolysis/ Gluconeogenesis	ENO1	-	+	Glycolysis
		ENO2	++	+	Glycolysis
		ENO3	+++	-	Glycolysis Gluconeogenesis
		ENO4	+++ (*)	+®	Glycolysis Mitochondrial transport (SLC25A1) Mitochondrial enzymes (HSD17B10) Notch signaling pathway (MYCBP) Heat shock protein beta 2 Calcium-binding tyrosine phosphorylation-regulated protein IL-17 family signaling pathways
PK	Glycolysis	PKLR	+++(***)	®	Glycolysis Pyruvate metabolism
		PKM	-	+++(*)	Glycolysis Pyruvate metabolism
PC	Gluconeogenesis	PC	-	-	Gluconeogenesis Amino acid metabolism Fatty acid metabolism Ketogenesis ERK signaling Pyruvate transport Biotin proteins pathways TCA cycle Urea cycle

					Metabolism of water-soluble vitamins and cofactors Malate-aspartate shuttle Pyrimidine biosynthesis Arginine biosynthetic pathway ERBB2 signaling pathway
PCK	Gluconeogenesis	PCK1	+(****)®	- - - (*)	Gluconeogenesis Estrogen receptor pathway TCA cycle Pyruvate metabolism Regulation of gene expression Malate-aspartate shuttle Abacavir metabolism and transport
		PCK2	+++	+	Gluconeogenesis TCA cycle Mitochondrial transport (SLC25A1) PPAR signalling pathway Adipogenesis regulation
FBP	Gluconeogenesis	FBP1	+++(**)	+++ (*)	Gluconeogenesis Fructose metabolism Mannose metabolism TCA cycle Pentose phosphate pathway
		FBP2	+®	®	Gluconeogenesis Fructose metabolism Mannose metabolism Pentose phosphate Pathway
G6Pase	Gluconeogenesis	G6PC1	+++(*)	®	Gluconeogenesis Glucose transport Galactose metabolism Glycogen metabolism Gene regulation (Oxidative stress, metabolic and neuronal)
		G6PC2	+®	®	Gluconeogenesis Hexose uptake Glucose transport (Solute Carrier family; NUP) Galactose metabolism Carbohydrate digestion and absorption
		G6PC3	+	+	Gluconeogenesis Hexose uptake Glucose transport Glycogen metabolism RNA transport (viral mechanisms)

LEGEND: (Enzymes/isoforms) HK1 – Hexokinase I; HK2 – Hexokinase II; HK3 – Hexokinase III; HK4 – Hexokinase IV ; GPI – Glucose 6 phosphate isomerase; PFKP – Phosphofructokinase Platelet; PFKL – Phosphofructokinase Liver; PFKM – Phosphofructokinase Muscle; ALDOA – Aldolase A; ALDOB – Aldolase B; ALDOC – Aldolase C; GAPDH – Glyceraldehyde 3 phosphate dehydrogenase; PGK1 – Phosphoglycerate kinase 1; PGK2 – Phosphoglycerate kinase 2; PGAM1 – Phosphoglycerate mutase 1; PGAM2 – Phosphoglycerate mutase 2; PGAM5 – Phosphoglycerate mutase 5; ENO1 – Enolase 1; ENO2 – Enolase 2; ENO3 – Enolase 3; ENO4 – Enolase 4; PKL/R – Pyruvate Kinase Liver and red blood cells; PKM – Pyruvate Kinase Muscle; PC – Pyruvate Carboxylase; PCK1 – Phosphoenolpyruvate Carboxykinase 1; PCK2 – Phosphoenolpyruvate Carboxykinase 2; FBP1 – Fructose 1,6 bisphosphatase 1; FBP2 – Fructose 1,6 bisphosphatase 2; G6PC1 – Glucose-6-Phosphatase catalytic subunit 1; G6PC2 – Glucose-6-Phosphatase catalytic subunit 2; G6PC3 – Glucose-6-Phosphatase catalytic subunit 3. (Expression) + increase at low levels; ++ increase at medium levels; +++ increase at high levels; - decrease at low levels; - - decrease at medium levels; - - - decrease at high levels; ® Residual levels: $\log_2(TPM + 1) < 1$; * p-value<0.05; ** p-value<0.01; *** p-value<0.001; **** p-value<0.0001. (Others) VDAC – voltage-dependent anion channel; NUP – Nucleoporin; PI3K – Phosphoinositide 3-kinases; mTOR – mammalian target of rapamycin; G1P – Glucose-1-phosphatase; F6P – Fructose-6-phosphate; GDP – Guanosine diphosphate; UDP – Uridine diphosphate; AKT – Protein kinase B; ERBB - erb-b2 receptor tyrosine kinase; HIF – Hypoxia inducible factor; PPAR – Peroxisome proliferator-activated receptor; MTB – Mycobacterium tuberculosis; TCR – T-cell receptor; KEAP – Kelch like ECH associated protein; NFE2L2 – Nuclear factor erythroid 2-related factor 2; NRF – Nuclear respiratory factor; SLC25A1 - Solute Carrier Family 25 Member 1; DSH17B - hydroxysteroid 17-beta dehydrogenase; MYCBP - MYC proto-oncogene binding protein; TCA – Tricarboxylic acid.

The metabolic network complexity is one major challenge in cancer and the enzymatic evaluation of the central biochemistry metabolism reveals the high plasticity capacity of tumour cells to insure their own survival. Our results unravel the importance of the tumour microenvironment (TME), which we conjectured to the implicated in the differences observed between cell and tissue expression patterns. The TME is a complex, and dynamic ecosystem within the tumour. It is composed by several cell types able to release multiple signalling factors, which can modulate the tumour development, progression, and response to treatment. Cell and surrounding diversity include immune cells, stromal cells, adipocytes, blood vessels, extracellular matrix, hypoxia, among others.

It is observed in some cases like PGK and PK, there is a switch of the enzymatic isoform from cell culture to breast tissue. Such deviations could not only be influencing the core function of glycolysis and gluconeogenesis enzymes but also modulating other metabolic pathways. Moreover, it is established that tumour metabolism is associated with certain enzymatic isoforms. Nonetheless, we hypothesized that the expression patterns of the remaining isoforms might unravel relevant moonlight functions tumour associated.

The literature revision performed to better understand our results disclosure that in most isoforms, information is minimal and scarce. Nonetheless, it is described that the associated neighbouring pathways (Pentose Phosphate Pathway, Fatty Acid Synthesis, Hexosamine biosynthesis, glutaminolysis, fructose metabolism, mannose metabolism, galactose metabolism, etc) can be modulated by the central biochemical metabolism. Such metabolic complexity could be the cornerstone in cancer comprehension and disease control.

We acknowledge the limitations of our study associated with the limited information of *GEPIA* platform, regarding not only controls but also breast cancer etiology, individual characteristics, and sample collection methods. Moreover, we would also like to highlight the difference between the expression units used in cell and tissue assays. Nevertheless, it is our understanding, that such limitations were not fatal concerning the focus of our study, which was the evaluation of enzymatic isoforms in breast cancer.

Our results overall reveal that we still find ourselves very distant from understanding the complex metabolism of breast cancer. A better understanding of the central biochemical metabolism may perhaps uncover new therapeutical targets and boost precision medicine.

3. Experimental

3.1. In vitro assays

Non-tumorigenic epithelial MCF-10A cell line (ATCC CRL-10317, purchased to American Tissue and Cell Culture, USA), was cultured in DMEM/F12 medium (Gibco Life technologies, Netherlands) containing 1% penicillin/streptomycin (Invitrogen Life

technologies, USA), 20 ng/mL epidermal growth factor (Sigma-Aldrich, USA), 100 ng/mL Cholera toxin (Sigma–Aldrich, USA), 10 µg/mL insulin (Sigma-Aldrich, USA), 0.5 mg/mL hydrocortisone (Sigma-Aldrich, USA), and 10% horse serum (Pan Biotech, Germany).

Human breast carcinoma cells MCF-7 (ATCC HTB22, purchased to American Tissue and Cell Culture, USA) were cultured in DMEM high glucose (Sigma–Aldrich, USA), supplemented with 1% penicillin/streptomycin (Invitrogen Life technologies, USA), 3.7 g/L sodium bicarbonate (Merck, Germany), and 10% Fetal Bovine Serum (Invitrogen Life technologies, USA).

Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3.2. RNA extraction

Culture cells were allowed to reach confluency in a 10 cm^2 cell culture dish. To establish a baseline metabolic state, cells were cultured in serum-free cell media for 24 hours. Cells were then lysed by adding 1 mL of NZYol (Nzytech, Portugal). Phase separation included the addition of 200 µL of chloroform and centrifugation at 12,000g for 15 minutes. For RNA precipitation, the aqueous phase was mixed with 500 µL of isopropyl alcohol, incubated at -20°C for 10 minutes and centrifugated at 12,000g for 10 minutes. Afterwards, RNA was washed with 1 mL of 75% ethanol, centrifuged at 12,000g for 5 minutes and pellet was air dried for 10 minutes.

RNA concentration and purity levels were quantified in the Varioskan™ LUX multimode microplate reader (ThermoFischer Scientific, USA).

3.3. cDNA synthesis reaction

cDNA synthesis reaction was performed using NZY M-MuLV First-strand cDNA synthesis kit (Nzytech, Portugal) following the manufacturer's protocol. Briefly, 5 µg of total RNA was mixed with 10 µL NZYRT 2x Master Mix, 2 µL of NZYM-MuLV RT Enzyme mix, and 3 µL of DEPC-treated H_2O (Sigma–Aldrich, USA) to complete a final volume of 20 µL. The mixture was incubated at 25°C for 10 minutes, followed by an incubation of 50 minutes at 37°C and finalized by heating inactivation reaction of 85°C for 5 minutes.

To remove potential bonds between RNA and cDNA, an additional step was included consisting in the addition of 1 µL of NZY RNase H and incubation at 37°C for 20 minutes.

3.4. RT-PCR

Real time PCR was performed in sealed 96-well microplates using BioQuant-96 Real-time PCR Detection System (Biosan, USA) and the NZYSupreme qPCR Green Master mix (nzytech, Portugal) according to the manufactures instructions. Briefly, 10 µL of NZYSupreme qPCR green master mix, 0.8 µL of forward and 0.8 µL of reverse primers, 4.4 µL of DEPC-treated H_2O (Sigma–Aldrich, USA), and 4 µL of cDNA was mixed for each sample. PCR conditions were as follows: initial denaturation for 2 minutes at 95°C, 40 cycles of 5 seconds at 95 C, and 30 seconds at 60°C. Additional melting curve analysis was performed.

Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) are described in Table 1, β-actin was used as housekeeping gene. RT-PCR assays were performed in duplicate of each 3 cell replicates.

Table 1. Primer sequences used in the RT-PCR.

Gene	Primer Sequence	Gene	Primer Sequence
ACTB *	F—GACGACATGGAGAAAATCTG R—ATGATCTGGGTCATCTTCTC	**PAGM2**	F—CTACTACAACTCCATTAGCAAG R—TTGATCTGGGGAACAATCTC
HK1	F—AGGTATGAGAAGATGATCAGTG R—GAGAAACTTGGTCTCAAAGATG	**PGAM5**	F—AAATCGTCCATTCGTCTATG R—CTTCGTAATACACAGCTTCC
HK2	F—CTAAACTAGACGAGAGTTTCC R—CATCATAACCACAGGTCATC	**ENO1**	F—TTACCACAACCTGAAGAATG R—TCCAGGCCTTCTTATTCTC
HK3	F—AGCAGAGGTTTGAAAAGATG R—GAACTTGGTCTTGAAGATGTC	**ENO2**	F—ATGTCCATAGAGAAGATCTGG R—GACACCTTTGCCTAAGTAAC
HK4	F—GGACTTTGAAATGGATGTGG R—ACTGATGGTCTTCGTAGTAG	**ENO3**	F—CAACATCCTGGAGAACAATG R—ATTGCGATAGAACTCAGATG
GPI	F—CCAACTCTATTGTGTTCACC R—CGAAGATCTTGTGCTCATAC	**ENO4**	F—GTAAGAAGCACATCACTGTC R—TTCTTTGAAACCCAGTGTTC
PFKP	F—ATTGTGTGCTGGGAATAAG R—GGAATCCTGTGCTCAAAATC	**PKL/R**	F—AAAATTGAGAACCACGAAGG R—CAATCATCATCTTCTGAGCC
PFKL	F—GAAAGACACTGATTTCGAGC R—CATACTGATGCGGTATTGTG	**PKM**	F—ATGTTGATATGGTGTTTGCG R—ATTTCATCAAACCTCCGAAC
PFKM	F—AGGACCAGACAGATTTTGAG R—GATCAGGTAATCTATTCCCCTC	**PC**	F—TTATGGTGCAGAATGGATTG R—TCAGTACCTTAGAGCGAAAG
ALDOA	F—AGTATGTGACCGAGAAGG R—TGAGAAAACTTCTGAGTGC	**PCK1**	F—ATTCTGGGTATAACCAACCC R—GTTGATGGCCCTTAAATGAC
ALDOB	F—AGAATGGACTGGTACCTATTG R—GTAACATACTGGCAGTGTTC	**PCK2**	F—GGCATTCGAGATTTTGTAGAG R—CAGCAGTTATTGTACTTGGG
ALDOC	F—CCAATACAAGAAGGATGGTG R—CAGGCAATATTTCAGGTTCC	**FBP1**	F—CCAGATAATTCAGCTCCTTATG R—AGAAATATCCCTCCGTAGAC
GAPDH	F—TCGGAGTCAACGGATTTG R—CAACAATATCCACTTTACCAGAG	**FBP2**	F—AAAGAAATTCCCTGAGGATG R—GGATTGCATTCATACAGGAG
PGK1	F—CTCAACAACATGGAGATTGG R—CTTTGGACATTAGGTCTTTGAC	**G6PC1**	F—ACTGTGCATACATGTTCATC R—TGAATGTTTTGACCTAGTGC
PGK2	F—GAACTCTTAATATCAGCACAGC R—TTTCAGTTTCTTCAGCTCAC	**G6PC2**	F—CCGAGCTTACTACACTTTTC R—GGTCGATGACCAAATAATATCC
PGAM1	F—CTACAGCAACATCAGTAAGG R—GGGAACTATTTCTTCATTCCAG	**G6PC3**	F—CAAGATCCTCTTTCTGTTCTAC R—TTCACTTGAAGATGAGGTTG

Legend: F – Forward; R – Reverse; * Housekeeping gene; ACTB – β-actin; HK1 – Hexokinase I; HK2 – Hexokinase II; HK3 – Hexokinase III; HK4 – Hexokinase IV ; GPI – Glucose 6 phosphate isomerase; PFKP – Phosphofructokinase Platelet; PFKL – Phosphofructokinase Liver; PFKM – Phosphofructokinase Muscle; ALDOA – Aldolase A; ALDOB – Aldolase B; ALDOC – Aldolase C; GAPDH – Glyceraldehyde 3 phosphate dehydrogenase; PGK1 – Phosphoglycerate kinase 1; PGK2 – Phosphoglycerate kinase 2; PGAM1 – Phosphoglycerate mutase 1; PGAM2 – Phosphoglycerate mutase 2; PGAM5 – Phosphoglycerate mutase 5; ENO1 – Enolase 1; ENO2 – Enolase 2; ENO3 – Enolase 3; ENO4 – Enolase 4; PKL/R – Pyruvate Kinase Liver and red blood cells; PKM – Pyruvate Kinase Muscle; PC – Pyruvate Carboxylase; PCK1 – Phosphoenolpyruvate Carboxykinase 1; PCK2 – Phosphoenolpyruvate Carboxykinase 2; FBP1 – Fructose 1,6 bisphosphatase 1; FBP2 – Fructose 1,6 bisphosphatase 2; G6PC1 – Glucose-6-Phosphatase catalytic subunit 1; G6PC2 – Glucose-6-Phosphatase catalytic subunit 2; G6PC3 – Glucose-6-Phosphatase catalytic subunit 3.

3.5. Bioinformatics analysis of expression patterns of the glycolytic and gluconeogenic isoforms by Gene Expression Profiling Interactive Analysis Dataset (GEPIA)

Gene Expression Profiling Interactive Analysis Dataset (GEPIA)[42] was used to conduct a comparative assessment of gene expression among the glycolytic and gluconeogenic

isoforms using samples derived from both breast cancer and normal breast tissue. A total of 1085 cases of tumour tissue and 291 cases of normal breast tissue were included.

3.6. Bioinformatic analysis of associated interactions by GeneMANIA

We employed *GeneMANIA*[43], a publicly accessible platform that integrates genomic and proteomic data (Application version 3.6.0), to explore genetic and physical interactions, co-expression patterns, as well as associated pathways. Additional supplementary data pertaining to interactions, co-expressions, and attributes were obtained from *GeneCards* - the human gene database[44] .

3.7. Statistical analysis

RT-PCR results were statistical analysed using unpair t-student test. Significance was set at p-value<0.05. Results were presented as $2^{-\Delta\Delta CT}$ mean with standard error of the mean (SEM). Statistical analysis in *Gepia* was performed in the online platform, using ANOVA test, cut-off fold was set at 1, and p-value <0.05 was considered significant. Results were presented in the corresponding plot, extracted from the database.

Supplemental materials

Supplemental materials – Report S1: Reports from *GeneMANIA*

Author Contributions:

Conceptualization, C.L., R.F. and R.S.; methodology, C.L., R.F. and R.S.; validation, R.F. and R.S.; formal analysis, C.L., R.F. and R.S.; investigation, C.L.; resources, C.L., R.F. and R.S.; writing—original draft preparation, C.L.; writing—review and editing, C.L., R.F. and R.S.; supervision, R.F. and R.S.; funding acquisition, C.L., R.F. and R.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, C.L., upon reasonable request.

Conflicts of interest

The authors declare no conflict of interest.

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