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Nutritional factors and metabolic biomarkers: impact in risk and survival of women with breast cancer

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Nutritional factors and metabolic biomarkers: impact in risk and survival of women with breast cancer

Corrected version

Versão corrigida

Ph. D. Thesis presented to the Graduate Program in Nutrition and Public Health at School of Public Health at University of São Paulo to obtain the degree of Doctor in Science.

Concentration area: Nutrition and Public Health

Prof. Dr. Nágila Raquel Teixeira Damasceno - Supervisor
Prof. Dr. Alfredo Carlos Simões Dornellas de Barros – Co-Supervisor

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ATA DE DEFESA

Ata de defesa de Tese do(a) Senhor(a) Isabelle Romero Novelli no Programa: Nutrição em Saúde Pública, do(a) Faculdade de Saúde Pública da Universidade de São Paulo.

Aos 16 dias do mês de dezembro de 2022, no(a) realizou-se a Defesa da Tese do(a) Senhor(a) Isabelle Romero Novelli, apresentada para a obtenção do título de Doutora intitulada:

"Fatores nutricionais e biomarcadores metabólicos: impacto no risco e sobrevida de mulheres com câncer de mama"

Após declarada aberta a sessão, o(a) Sr(a) Presidente passa a palavra ao candidato para exposição e a seguir aos examinadores para as devidas arguições que se desenvolvem nos termos regimentais. Em seguida, a Comissão Julgadora proclama o resultado:

Nome dos Participantes da Banca	Função	Sigla da CPG	Resultado
Nágila Raquel Teixeira Damasceno	Presidente	FSP - USP	Não Votante
Fernando Salvador Moreno	Titular	FCF - USP	Aprovada
Gabriela Villaça Chaves	Titular	INCA - Externo	Aprovada
Débora Levy	Suplente	HCFMUSP - Externo	Aprovada

Resultado Final: Aprovada

Parecer da Comissão Julgadora *

Eu, Maria Aparecida Mendes , lavrei a presente ata, que assino juntamente com os(as) Senhores(as). São Paulo, aos 16 dias do mês de dezembro de 2022.

Nágila Damasceno
Fernando Salvador Moreno

Nágila Damasceno
Gabriela Villaça Chaves

Nágila Damasceno
Débora Levy

Nágila Damasceno
Nágila Raquel Teixeira Damasceno
Presidente da Comissão Julgadora

To my love, Filipe

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RESUMO

NOVELLI, I. R. **Nutritional factors and metabolic biomarkers: Impact in Risk and Survival of Women with Breast Cancer**. 2022. Tese (Doutorado em Ciências, pelo Programa de Nutrição em Saúde Pública) – Faculdade de Ciências da Saúde, Universidade de São Paulo, 2022.

Câncer de mama (CM) é a causa mais comum de câncer no mundo e a principal causa de morte em mulheres. O CM é uma doença complexa e é classificada em tipos moleculares de acordo com a expressão do receptor de estrogênio (RE), receptor de progesterona (RP), *receptor tyrosine-protein kinase erbB-2* (HER2) e proteína Ki67. Embora o CM seja multifatorial, há evidências científicas que indicam que componentes nutricionais podem ser relevantes durante as diversas etapas da carcinogênese, recidiva e sobrevivência. O objetivo deste estudo foi avaliar o papel do estado nutricional e marcadores metabólicos em mulheres com CM. Trata-se de um estudo caso-controle realizado entre maio 2011 e agosto 2012. O grupo caso foi acompanhado até abril 2019, caracterizando um estudo de coorte. A partir destes grupos foram estruturados diversos manuscritos com delineamentos diferentes, segundo cada hipótese levantada, a saber: Manuscrito 1 - Coorte baseada no seguimento do grupo CM, Manuscrito 2 - Estudo transversal baseado em mulheres com CM Luminal A (RE positivo, RP positivo/negativo, HER2 negativo, Ki67 baixo) e; Manuscrito 3 - estudo transversal do tipo caso-controle. Os dados foram obtidos através de prontuários médicos, entrevista e avaliação antropométrica com uso de bioimpedância elétrica. As amostras de sangue foram coletadas após jejum de 12 horas e a partir dessas foram analisadas glicemia, hemoglobina glicada (HbA1c), insulina, fator de crescimento semelhante à insulina 1 (IGF-1), proteína 3 de ligação ao fator de crescimento semelhante a insulina (IGFBP-3), substâncias reativas ao ácido tiobarbitúrico (TBARS), ácidos graxos não esterificados (NEFA), dano oxidativo ao DNA (8-OH-dG), perfil lipídico (colesterol total – CT, colesterol associado à lipoproteína de baixa densidade - LDL-c, colesterol associado à lipoproteína de alta densidade - HDL-c e triacilgliceróis) e perfil de ácidos graxos incorporados às membranas eritrocitárias. Todos os testes estatísticos foram realizados no programa *Statistical Package for Social*

Sciences® (SPSS), versão 21.0. Significância estatística foi considerada em p < 0,050. Os principais resultados do estudo mostram que mulheres com CM na pré-menopausa e com estadiamento clínico II e III tiveram um perfil lipídico mais aterogênico caracterizado pela diminuição do HDL-c, aumento do LDL-c, nãoHDL-c e apolipoproteína B (Apo B). Destaca-se que mulheres com CM e LDL-c e nãoHDL-c aumentados apresentaram maior chance de tumores maiores, enquanto mulheres com CM com maior HDL-c apresentaram menor risco. Mulheres com CM na pré-menopausa que apresentaram maior conteúdo de TBARS e NEFA no momento do diagnóstico tiveram menor sobrevida. Adicionalmente, mulheres com CM e tumores do tipo Luminal A tiveram maiores concentrações de glicose, IGF-1, IGFBP-3, IL1β, IL6 e menores de IL10 comparadas com o grupo controle. Mulheres com com concentrações mais elevadas de TBARS, glicose e insulina apresentaram maior risco de CM Luminal A, enquanto aquelas com concentrações mais elevadas de adiponectina apresentaram menor risco de desenvolver CM Luminal A, mesmo quando controlados pelo estado de menopausa e IMC. Mulheres com CM apresentaram alterações no eixo IGF-1/insulina que foi sustentada no sobrepeso/obesidade e no aumento da adiposidade central. Observou-se que mulheres com concentrações mais elevadas de glicose, insulina e IGF-1 tiveram maior chance de desenvolver CM. Em conclusão, os resultados demonstram o relevante impacto dos marcadores metabólicos no risco de desenvolver CM e o seu impacto na sobrevida. Este estudo reforça a relevância das estratégias de prevenção relacionadas ao estilo de vida e nutrição a fim de diminuir incidência e melhorar a sobrevida de mulheres com CM.

Palavras-chave: câncer de mama, perfil lipídico, metabolismo da glicose, estresse oxidativo, inflamação, tamanho tumoral e sobrevida.

ABSTRACT

NOVELLI, I. R. **Nutritional factors and metabolic biomarkers: Impact in Risk and Survival of Women with Breast Cancer**. 2022. Thesis. (Doctor in Sciences at the Program in Nutrition and Public Health) – School of Public Health, University of São Paulo, 2022.

Breast cancer (BC) is the most common cause of cancer and the leading cause of death in women. BC is a complex disease and distributes in distinct molecular subtypes regarding the expression of estrogen receptor (ER), progesterone receptor (PR), receptor tyrosine-protein kinase erbB-2 (HER2), and Ki67 protein status. Although BC is multifactorial evidence indicates that nutritional factors are relevant during steps of carcinogenesis, recurrence, and survival. We aim to assess the role of nutrition status and metabolic biomarkers in women with BC. This is a case-control study between May 2011 and August 2012. In the case group, there was a follow-up until April 2019, characterizing a cohort study.

From these groups, different manuscripts were structured with different study designs according to each hypothesis. Manuscript 1 - cohort based on the follow-up of the BC group, Manuscript 2 – a cross-sectional study with women with Luminal A BC (ER positive, PR positive/negative, HER2 negative, Ki67 low) and Manuscript 3 – a cross-sectional case-control study. Data were obtained by medical records, interviews and anthropometric parameters with electrical impedance. Blood samples were collected after 12-hour fasting to analyze serum glucose, glycated hemoglobin, insulin growth factor 1 (IGF-1), insulin growth factor binding protein (IGFBP-3), insulin and adipokines, thiobarbituric acid reactive substances (TBARS), non-esterified fatty acids (NEFA), DNA oxidative damage (8-OH-dG) and lipoproteins (total cholesterol – TC, Low-density lipoprotein cholesterol – LDL-c, high-density lipoprotein cholesterol - HDL-c and triacylglycerols) and fatty acid profile of erythrocyte membrane. All statistical tests were performed using Statistical Package for Social Sciences® (SPSS), version 21.0. Statistical significance was set at $p < 0.050$. The main results of the study showed that premenopausal women with BC and clinical staging (CS) between II and III had a more atherogenic lipid profile characterized by the decrease in

HDL-c, increase in LDL-c, non-HDL-C and apolipoprotein B (Apo B). We highlight that women with BC and high LDL-c and non-HDL-c had increase odd of having larger tumor size whereas HDL-c was associated with a decreased risk. Premenopausal women with BC had an increased level of TBARS and NEFA at diagnosis and had a lower survival probability. Additionally, women with Luminal A BC had higher serum levels of glucose, IGF-1, IGFBP-3, IL1**β**, IL6, and lower IL10 compared to its matching controls. Also, women with increased serum levels of TBARS, glucose, and insulin increased risk of Luminal A BC, and higher levels of adiponectin decrease the risk of developing Luminal A BC when controlled by menopause status and BMI. Women with BC presented impaired IGF-1/insulin axis, sustained by overweight/obesity and higher central adiposity. Increased levels of serum glucose, insulin, and IGF-1 showed higher odds to developing BC. In conclusion, our results demonstrate the relevant impact of metabolic biomarkers on risk of developing BC and in survival outcomes. This study reinforces the relevance to increase prevention strategies regarding lifestyle and nutrition
to decrease incidence and improve outcome of BC.

Keywords: breast cancer, lipid profile, glucose metabolism, oxidative stress, inflammation, tumor size, survival.

TABLE LIST

FIGURE LIST

LIST OF ABBREVIATIONS

8-OH-dG: 8-OH-2'-deoxyguanosine
17β-HSD: 17β-hydroxysteroid dehydrogenase
27-HC: 27-hydroxycholesterol
AA: Arachidonic acid
AJCC: American Joint Commission on Cancer
ALA: α-linolenic acid
ALS: Acid-labile subunit
AMPK: Monophosphate-activated protein kinase
Apo A-I: Apolipoprotein A-I
Apo B: Apolipoprotein B
BC: Breast Cancer
BHT: Butylated hydroxytoluene
BMI: Body mass index
BRCA: Breast Cancer gene
CI95%: Confidence interval 95%
CLS: Crown-like structure
COX: Cyclooxygenase
CS: Clinical staging
CV: Cardiovascular disease
DALY: Disability-Adjusted life years
DCIS: Ductal carcinoma in situ
DHA: Docosahexaenoic acid
E1: Estrone
E2: Estradiol
EDTA: Ethylenediaminetetra acetic acid
ELISA: Enzyme-linked immunosorbent assay
EMT: Epithelial-mesenchymal transition
EPA: Eicosapentaenoic acid
ER: Estrogen receptor
FAME: Fatty acid methyl esters
FFA: Free fatty acid
FPLC: Fast protein liquid chromatography
GH: Growth hormone
GLOBOCAN: Global Cancer Observatory
HbA1c: Glycated hemoglobin
HDI: Human development index
HDL-c: High-density lipoprotein cholesterol
HER2: Receptor tyrosine-protein kinase erbB-2
HPLC: High-performance liquid chromatography
HRT: Hormone replacement therapy
IATA: International Air Transport Association
IFN-γ: Interferon-gamma
IGF-1: Insulin Like Growth Factor 1
IGFBP-3: Insulin Like Growth Factor Binding Protein 3

IL1β: Interleukin 1 beta
IL6: Interleukin 6
IL10: Interleukin 10
INCA: Brazilian National Cancer Institute
IR: Insulin receptor
IRS: Insulin receptor substrate
LDL-c: Low-density lipoprotein cholesterol
LDLR: Low-density lipoprotein receptor
LOX: Lipoxygenases
LXR: Liver X receptor
MCP-1: Monocyte Chemoattractant Protein 1
NAFLD: Nonalcoholic fatty liver disease
NEFA: Non-esterified fatty acids
NF-κB: Nuclear kappa-light-chain-enhancer of activated B cells
OLR1: Oxidized LDL lecithin-like receptor 1
OPD: Orthophenylenediamnine
OR: Odds ratio
oxLDL: Oxidized LDL
PBS: Phosphate saline buffer
PI3K: Phosphoinositide 3 kinase
PMSF: Phenylmethylsulfonyl fluoride
PPAR-α: Peroxisome proliferator-activated receptor
PR: Progesterone receptor
PUFAS: Polyunsaturated fatty acids
ROS: Reactive oxygen species
SD: Standard deviation
SFA: Saturated fatty acid
SHBG: Sex hormone-binding globulin
SREBP: Steroid regulatory element-binding protein
T2DM: Type 2 diabetes mellitus
TBARS: Thiobarbituric acid reactive substance
TC: Total cholesterol
TG: Triacylglycerols
TMB: 3,3',5,5'-tetrametilbenzine
TNBC: Triple negative breast cancer
TNF-α: Tumor necrosis factor alpha
TLR: Toll-like receptors
VEGF: Vascular endothelial growth factor
VLDL-c: Very-low-density lipoprotein cholesterol
WC: Waist circumference
WHO: World Health Organization

SUMMARY

PRESENTATION

The thesis entitled “Nutritional factors and metabolic biomarkers: Impact in Risk and Survival of Women with Breast Cancer” was performed under the guidance of Prof. Dra. Nágila Raquel Teixeira Damasceno and co-guidance of Prof. Dr. Carlos Simões Dornellas de Barros. It is presented in the form of an article collection following the rules established in the second edition of 2017 “Thesis Presentation Guide of School of Public Health of University of São Paulo.”

The document is divided into:

1. Introduction – a theorical reference about what is already known about the interaction of breast cancer and nutrition and metabolic biomarkers;
2. Objectives – state the main objective;
3. Material and Methods – demonstrate the phases, data collection, and methodology of the experiments;
4. Results and Discussion – inclusion of three manuscripts that contain the main findings of the study;
5. Final considerations – contribution of the study;
6. Appendix – other manuscripts that were included to create the rational for the study, approval of the study by the ethics commission and, data collecting questionnaire.

As requested by the rules, the first manuscript, *“Unbalanced lipid and oxidative stress in premenopausal women with breast cancer: impact on clinical staging and survival,”* was submitted to the Life Science Journal. The second manuscript, *“Pivotal role of oxidative stress and inflammation increase risk to Luminal A breast cancer independent of menopause and obesity: a case-control*

study," was submitted to Breast Cancer Research and Treatment. The last manuscript, *"Insulin Growth Factor 1 (IGF-1) as an independent predictor for breast cancer in women: a case-control study,"* will be submitted after the evaluation of this committee.

In addition, as complementary material, we are attaching other submitted and published manuscripts that gather the research conducted in this thesis and help to create an important rational for this work. In the appendix section are:

- *DHA in Red Blood Cell Membrane is Associated with Lower Tumor Size in Women with Breast Cancer* (2022) – Submitted to Nutrition and Cancer.
- *Omega-3 fatty acids are associated with reduced oxidative stress and inflammation in postmenopausal women with breast cancer and ER+* – writing process.
- Nutritional Counseling Protocol for Colorectal Cancer Patients after Surgery Improves Outcome (2020) – Published at Nutrition and Cancer (doi: 10.1080/01635581.2020.1819345).
- Book chapter 1 "Dieta e Câncer" in the book Tratado de Nutrição Funcional em Oncologia – 1 ed. 2022 (ISBN: 978-65-992200-4-3).
- Book chapter 2 "Obesidade e Câncer" in the book Nutrição e Câncer: Cuidados nutricionais para a prevenção do desenvolvimento e recidiva da doença entre mulheres – unpublished.

INTRODUCTION

Breast Cancer: Epidemiological aspects and risk factors

According to the Global Cancer Observatory (GLOBOCAN), breast cancer (BC) is the most common cause of cancer worldwide with an estimated 2.3 million new cases in 2020 (SUNG et al., 2021). In the triennium statistics compiled by the Brazilian National Cancer Institute for 2020–2022 (INSTITUTO NACIONAL DE CÂNCER (INCA), 2018), there is a provision of 66,280 new BC cases per year, which is the most common cancer in women in Brazil.

On the global landscape, BC is also the leading cause of cancer death in women and responsible for 684,996 deaths (SUNG et al., 2021). In Brazil, the mortality rate of BC between 1992 and 2012 more than doubled (CECILIO et al., 2015). This increase could be related to socioeconomic characteristics. When dividing countries into four levels of Human Development Index (HDI), we found the direct impact of this index in incidence and mortality rate (high HDI: 45.0% incidence of BC and 33.0% mortality; low HDI: 18.4% incidence of BC and 30.1% mortality) (LEI et al., 2021) (Figure 1). Therefore, Ginsburg et al. (2017) concluded that women who develop BC in high-income countries have a better chance of surviving than women in middle or low-income countries. Thus, the increase in life expectancy related to BC can be directly correlated with important socioeconomic parameters, such as a better health care system and investments in health public policy.

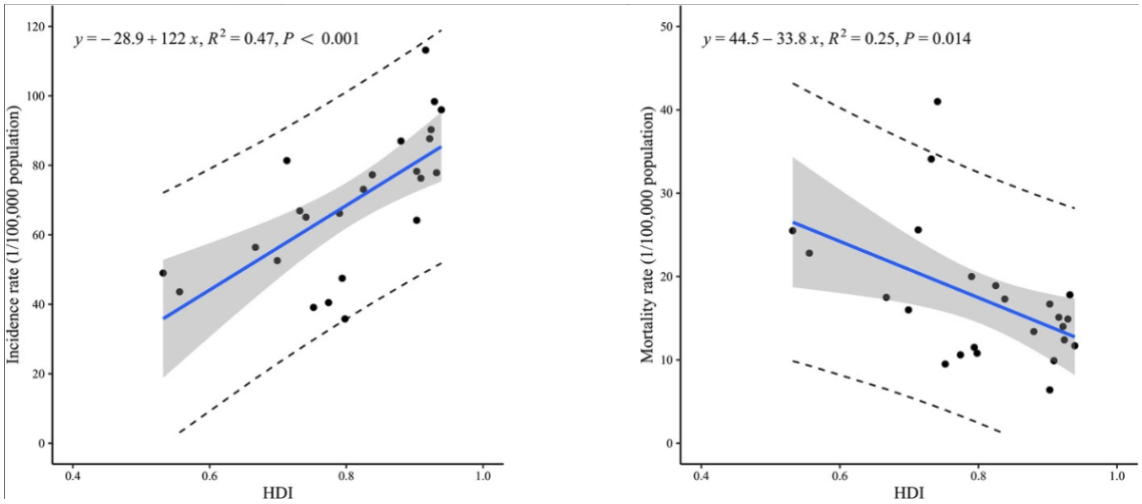

Figure 1. Association of HDI with incidence and mortality of breast cancer. Adapted from Lei et al. (2021)

The global burden of cancer is rising fast, the projections are that by 2040 there will be a 47% increase in cases compared to 2020, with a possibility of a greater increase in middle and low-income countries due to westernization of lifestyle (SUNG et al., 2021).

There is a clear need for further understanding of the factors that contribute to breast carcinogenesis to improve prevention strategies. The multifactorial aspect of risk factors for BC is a result of both modifiable and non-modifiable aspects, which generate a heterogenous disease. They can be divided into non-modifiable risk factors and modifiable risk factors. The first is due to mainly hereditary genetic factors, age, race, and reproductive history; the second is related to behavioral and lifestyle (ŁUKASIEWICZ et al., 2021) (Table 1).

In a recent study by Gomes et al. (2022) with a Brazilian population from the Northeast, a combination of family history, previous contraceptive use, obesity, and alcohol consumption had an impact in increasing BC risk. According to Rezende et al. (2018), 3.8% of new cases of cancer in Brazil are related to a body mass index (BMI) higher than 25.0 kg/m^2 (overweight/obesity).

Table 1. Modifiable and non-modifiable risk factors for breast cancer

Non-modifiable Factors	*Modifiable Factors*
Female sex	
Age	Hormonal replacement therapy
Family history (breast and/or ovarian cancer)	Physical activity
	Overweight/obesity
Genetic mutations	Alcohol intake
Race / ethnicity	Smoking
Pregnancy and breastfeeding	Insufficient vitamin supplementation
Menstrual period and menopause	Intake of processed food
Density of breast tissue	Exposure to chemicals
Previous history of breast cancer	
Non-cancerous breast disease	
Previous radiation therapy	

Adapt from Łukasiewicz et al. (2021)

Menopause is one of the most relevant risk factors for BC risk. In a guideline provided by the World Health Organization (WHO), natural menopause is defined as the permanent cessation of menstruation due to loss in ovarian follicular activity, occurring with 12 consecutive months of amenorrhea (WORLD HEALTH ORGANIZATION, 1996). The production of hormones by ovaries initiates after puberty and stops after menopause. The main hormone produced in the ovaries is estrogen, and the mammary gland is highly sensitive to this hormone, which causes maturation and differentiation of the gland when bound to its receptor (NI et al., 2021).

The pathway to produce estrogen initiates with a cholesterol molecule that is converted to progestogens and androstenedione, which is then converted to estrone (E1) via aromatase (P450arom) and to estradiol (E2) via 17β-hydroxysteroid dehydrogenase (17β-HSD). In the same pathway, testosterone

can also be converted into E2 via 17β-HSD – where E2 is the active form of estrogen (Figure 2).

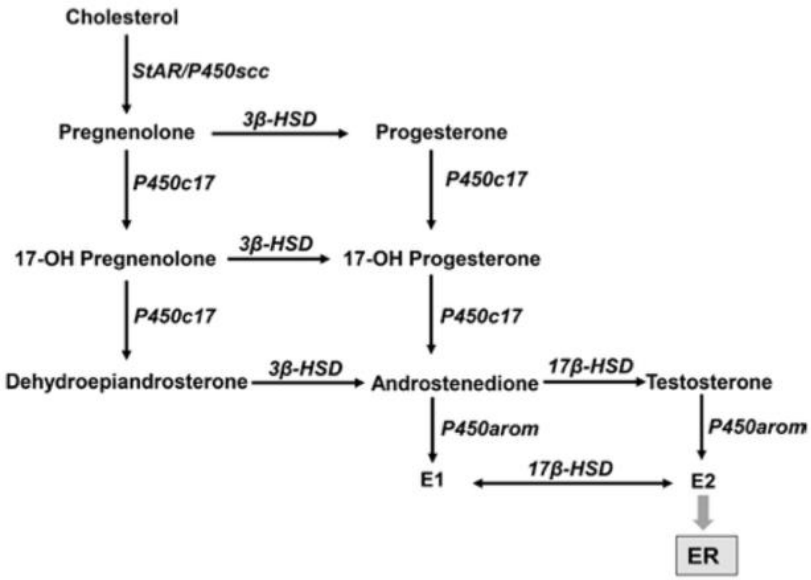

Figure 2. Estrogen biosynthetic pathway.
STAR: steroidogenic acute regulatory protein; P450scc: cholesterol side-chain cleavage enzyme; P450c17: steroid 17α- hydroxylase/17,20 lyase; P450arom: aromatase; E1: estrone; E2: estradiol; 3β-HSD: 3β-hydroxysteroid dehydrogenase; 17β-HSD: 17β- hydroxysteroid dehydrogenase; ER: estrogen receptor.
Adapted from Zhao et al. (2016).

In premenopausal women, estrogen is produced mainly in the ovary, in a cyclical form regulated by feedback. After menopause, estrogen is produced via the aromatase enzyme, which is located mostly in adipose tissue (FOLKERD; DOWSETT, 2013), and the increase in serum estradiol concentration has been related to a two-fold increased risk of developing BC in postmenopausal women (BROWN; HANKINSON, 2015). The increase risk was not observed in a study by Dorgan et al. (2010) with premenopausal women, this can be due to alteration in estrogen levels regarding menstrual cycle, however testosterone was strongly associate with BC risk (in the highest quartile OR: 3.3 (95%CI 1.5 – 7.5; p = 0.006). Increasing levels of sex hormone-binding globulin (SHBG) – a

glycoprotein that binds androgens and estrogens – decreases BC risk, indicating the relevant role of this hormone is breast carcinogenesis. Postmenopausal women with obesity have an increase in the availability of adipose tissue for estrogen production, which raises estrogen levels and decreases in SHBG, increasing the bioavailability of circulating estradiol (FOLKERD; DOWSETT, 2013). In a study by Zhang et al. (2013), postmenopausal women were at 50 to 110% higher risk of developing BC when presenting with higher circulating levels of estradiol, testosterone, and low levels of SHBG. Some modifiable factors proposed to reduce the risk of BC in postmenopausal women are controlling sex hormone, maintaining a eutrophic BMI and increasing daily physical activity, especially when the outcome is related to decreased total adiposity (MCTIERNAN, 2008; MCTIERNAN et al., 2006).

To confirm the importance of the modifiable risk factors, a study conducted with identical twins found that the contribution of hereditary factors was 27% (95%CI: 4–41%) in BC (LICHTENSTEIN et al., 2000). Implementing the lifestyle interventions proposed to prevent cases of cancer in the guidelines can decrease about 31% of BC cases (CATSBURG; MILLER; ROHAN, 2014).

Three main pillars need to be explored to understand and modulate the modifiable risk factors and BC. The first is the metabolic and hormonal influence, stimulated mainly by overnutrition, which increases growth factors, such as insulin and Insulin Like Growth Factor 1 (IGF-1), high supply of ATP, lipid membrane synthesis, dysbiosis, and excess adipose tissue. The second is a change in immune function and inflammation that can be related to excess adipose tissue and to the tumor itself. Hypertrophy of the adipose tissue causes a chronic inflammatory environment that predisposes tumorigenesis.

Additionally, a diet rich in saturated fatty acids (SFA) can activate toll-like receptors (TLR) increasing transcription of nuclear kappa-light-chain-enhancer of activated B cells (NF-κB), sustaining the proinflammatory environment. The last factor is the regulation of oxidative stress-induced DNA damage. Accumulation of reactive oxygen species (ROS) is a physiological process that can be exacerbated during overnutrition and inefficient clearance of excess free radicals that can be associated with pro-tumorigenic impact. An increase of acetaldehyde from alcohol can also increase ROS. In a malignant phenotype cell, ROS can increase the accumulation of somatic mutation and epithelial-mesenchymal transition (EMT), which is important for metastatic progression (TAN; NAYLOR, 2022).

Although important, many risk factors still present controversial results in the literature. This could probably be explained by the important differences regarding the metabolic pathways in premenopausal/postmenopausal women and the physiology of the different types of BC.

Breast cancer: Physiopathology and Staging

The female breast is a glandular organ that can also be referred as mammary gland, which is made of mainly adipose tissue, connective tissue, and breast tissue (that contains lobules – where human milk is made – and connected to ducts that lead out to the nipple) (Figure 3).

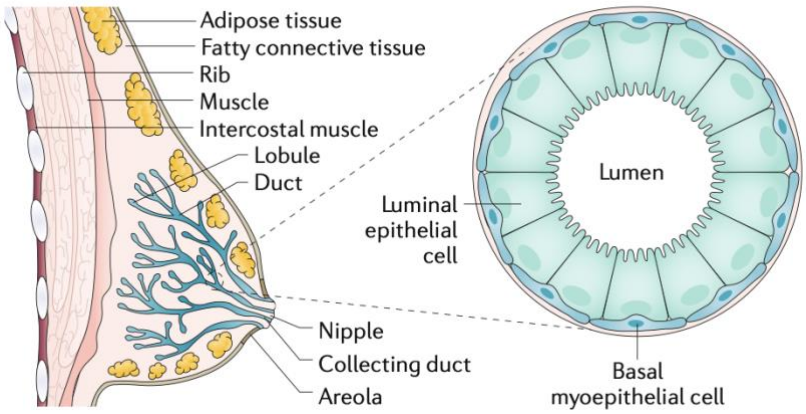

Figure 3. Female breast components.
Adapted from Harbeck et al. (2019).

BC can be defined as an abnormal growth in breast cells with malignant characteristics, where the most common type is ductal carcinoma followed by lobular carcinoma. BC is also characterized by the extension of the spreading tumor – the in situ type (45%) is the earliest form of BC that is contained in its primary structure, and the invasive type (55%) has spread to surrounding normal tissue. This classification helps to define treatment and estimate clinic prognosis; however, it does not consider important molecular and genetic aspects of the tumor (GANNON; COTTER; QUINN, 2013; MAKKI, 2015; MALHOTRA et al., 2010; RAKHA et al., 2010).

To expand the histopathologic characterization that precedes the choice of treatment, the American Joint Commission on Cancer (AJCC) in the eighth edition (AMIN, M.B., EDGE, S., GREENE, F., BYRD, D.R., BROOKLAND, R.K., WASHINGTON, M.K., GERSHENWALD, J.E., COMPTON, C.C., HESS, K.R., SULLIVAN, D.C., JESSUP, J.M., BRIERLEY, J.D., GASPAR, L.E., SCHILSKY, R.L., BALCH, C.M., WINCHESTER, D.P., ASARE, E.A., MADERA, 2017) recommends the use of tumoral staging as an essential and complementary

strategy to precisely characterization of the tumor. TNM staging – T: tumor size; N: spread of cancer in lymph nodes; M: metastasis – allows identification of tumors from staging 0 (in situ) through stage IV (any size, any amount of compromised lymph nodes, and the presence of metastasis). Due to advances in the translation field and diversity of diseases in the BC scope, the staging manual since the 2018 edition also recommends utilizing biomarkers. The recommended biomarkers are hormonal receptors (estrogen receptor – ER – and progesterone receptor – PR), HER2 (human epidermal growth factor receptor 2), and a proliferative biomarker Ki67 protein. This classification allows creation of molecular subtypes that assist in better understanding BC, defining treatment and prognosis (Table 2).

Table 2. Breast cancer molecular subtypes.

Molecular subtypes	*ER status*	*PR status*	*HER2 status*	*Ki67%*	*Target therapy*	*Prognosis*
TNBC	Neg	Neg	Neg	High	No	Poor
HER2	Neg	Neg	Pos	High	Yes	Intermediate
Luminal B HER2+ like	Pos (low)	Pos (low)	Pos	High	Yes	Intermediate
Luminal B HER2- like	Pos (low)	Pos (low)	Neg	High	No	Intermediate
Luminal A	Pos (high)	Pos (high)	Neg	Low	No	Good

Pos = positive; Neg = Negative; TNBC = triple negative breast cancer; ER = estrogen receptor; PR = progesterone receptor; HER2: human epidermal growth factor receptor 2
Adapted from Harbeck et al. (2019).

In general about 10% of BC cases are related to genetic predisposition or family history, which can also vary according to country and ethnicity (LOIBL et al., 2021). The most common germline mutation in BC is in *breast cancer 1* (*BRCA 1*) and *breast cancer 2* (*BRCA 2*). Both factors are responsible for coding tumor suppressing proteins, and its mutation causes alterations in cell replication

checkpoints (SUN et al., 2017). These mutations are rare, mainly expressed in young women (< 45 years), and have a more aggressive phenotype (TAO et al., 2015). Still is suggested that in women with this type of mutation lifestyle recommendations to maintain a healthy weight, be physically active and, controlling metabolic parameters is an opportunity to decrease risk (LAMMERT; GRILL; KIECHLE, 2018).

Oxidative stress and inflammation

Oxidative stress is an imbalance in the ratio between oxidants and antioxidants, in which the rate of production and removal of free radicals is altered (HECHT et al., 2016). The excessive formation of reactive oxygen species (ROS) is an important step for carcinogenesis and assists to sustain the proliferation (LIBERTI; LOCASALE, 2016). The increase in ROS can augments genomic instability via DNA damage that can drive carcinogenesis due to accumulating errors in DNA and influence transcription factors that can impair DNA repair and sustain proliferation of the cell (BHARDWAJ; BROWN, 2021; OKOH; DEORAJ; ROY, 2011), which is considered a key player in the development of BC (KUNDAKTEPE et al., 2021). An increase in ROS can also increase NF-κB, leading to the expression of proinflammatory mediators that increase BC risk (TOUVIER et al., 2013), alter tumor cell biology, facilitate tumorigenesis, sustain proliferation, and promote drug resistance and metastasis (BHARDWAJ; BROWN, 2021; KOLB; ZHANG, 2020; OKOH; DEORAJ; ROY, 2011). An increase in inflammatory biomarkers, due to activation of toll-like receptor 4 (TLR4) and NF-κB/TNF-α pathway, creates an inflammatory environment in the

breast tissue that contributes to apoptosis and consequent increase in crown-like structure (CLS) and proinflammatory cytokine signaling (NAIMO et al., 2020).

Pierce et al. (2009) found that women with BC that presented higher serum inflammatory biomarkers had reduced overall survival regardless of age, tumor stage, race, and BMI. However, Dai et al. (2009) described that women with BMI $\geq$ 29.0 kg/m^2 presented a higher level of isoprostanes and was associated with an increased risk of developing BC (OR= 10.27; CI95%= 2.41 – 43.8; p=0.003). Similar to these findings, Shaik and Rupasee (2011) found that women with BC had increased serum 8-OH-2'-deoxyguanosine (8-OH-dG) compared to its matching controls ($p < 0.004$), and in their BC tissue, nine-times higher concentration of 8-OH-dG was found comparted to normal tissue (MUSARRAT; WANI, 1996).

Inflammation is also associated with BC development, can be related with many cellular changes, and has relevant consequences on tumor progression and immunosuppression of the tumor microenvironment, especially chronic inflammation (DANFORTH, 2021). The etiology of this inflammation can be related to obesity and increased adiposity, ROS, stress, smoking, alcohol consumption, or the tumor itself, which can contribute to its development, progression, and recurrence (DESHMUKH et al., 2019). In a controversial way, inflammation can have an anticancer effect. A high density of tumor-infiltrating T cells can be a predictor of patient survival, but an abundance of regulatory T cells is associated with poor prognosis. Cytokines are also responsible for playing an ambiguous role in BC. Interleukin 10 (IL10) is a cytokine that can inhibit the production of inflammatory cytokine, monocyte chemoattractant protein 1 (MCP1), and NF-κB (HAMIDULLAH; CHANGKIJA; KONWAR, 2012) and has

been shown to reduce TNF-α stimulation (MARTÍNEZ-CHAĆON et al., 2018) and stimulate cytotoxic immune cells (NK and $CD8^+$ T cell) (DORSEY et al., 2002). Low levels of IL10 in the tumor and microenvironment were associated with an increased risk of recurrence and metastasis (LI et al., 2014); however, some reports also discuss the pro-tumorigenic effect of IL10 by decreasing immunosurveillance of the microenvironment due to its decrease in cytokine (HAMIDULLAH; CHANGKIJA; KONWAR, 2012). A study by Matkowski et al. (2009) found no difference in the expression of IL10 in BC tumors and normal tissue. To corroborate the ambiguous role of inflammation, a cohort studied by Oshi et al. (2020) demonstrated that women with higher inflammation scores were associated with a more aggressive BC, but for TNBC high inflammation was associated with increased survival probably due to enrichment of immune pathways that favors infiltration of anticancer immune cells such as interferon-gamma (IFN-γ) and $CD8^+$ T cells.

Insulin/Insulin-like Growth Factor-1 (IGF1) axis

In recent years the insulin-IGF1 axis have been considered relevant for the risk of developing BC. This axis includes insulin, IGF-1, insulin receptor (IR), IGF1 receptor (IGF-1R), and Insulin Growth Factor Binding Proteins (IGFBP), a family of six proteins, with IGFBP-3 being the most important.

IGF-1 is a peptide mainly produced in the liver due to growth hormone (GH) and insulin stimulus that activates expression and release of IGF-1, which can have paracrine and autocrine action, considered a mitogenic and anti-apoptosis molecule in health and malignant cells (CHRISTOPOULOS; MSAOUEL; KOUTSILIERIS, 2015; CLEMMONS, 2012; DEHKHODA et al.,

2018). Chong et al. (2006) and Voskuil et al. (2004) found increased synthesis of IGF-1 in healthy tissue adjacent to tumor tissue in the breast, confirming the essential role of the tumor microenvironment for cell proliferation. According to Hoy et al. (2017), IGF-1 can also be secreted by the adipose tissue of the breast. Insulin is produced and secreted via pancreatic β cells mainly through post prandial glucose stimulus, and it has an important role for glucose capitation in insulin-dependent tissue (POLOZ; STAMBOLIC, 2015). IGF-1 is related with proliferative pathways that favor cell growth, and chronic hyperinsulinemia can be associated with increased cancer risk, including BC (CLAYTON et al., 2011).

To stabilize the IGF-1 molecule and increase its half-life, binding proteins are necessary that create an active molecule creating a ternary structure with IGF, binding protein, and acid-labile subunit (ALS) (FANG; HWA; ROSENFELD, 2004). Subclass 3 of IGFBP is the isoform with a higher affinity for IGF-1 (CLEMMONS, 2012; FANG; HWA; ROSENFELD, 2004). Most IGF-1 found in circulation is bound to IGFBP-3, which increases affinity for the receptor (FANG; HWA; ROSENFELD, 2004). Another important component of the IGF-1 axis is the receptor, a transmembrane protein with tyrosine kinase activity that when bound to IGF-1, or with lower affinity, bound to insulin, it can stimulate mitogenic pathways, favoring the growth and proliferation of cells (DE MEYTS et al., 2004). The expression and synthesis of IGF-1R is being considered a relevant step in breast carcinogenesis, fundamental to metastasis. Furthermore, the expression of the *IGF-1R* gene has been associated with alterations in tumor suppressor genes, such as p53, WT1, and PTEN. When mutated, this protein can promote an overexpression of IGF-1R (BELARDI et al., 2013). The insulin receptor is also a transmembrane protein with tyrosine kinase activity that comes from a common

ancestral gene, and they share a degree of homology (BARBIERI et al., 2003). The receptors can have hybrid forms. Ligands of these receptors are insulin, IGF-1, and IGF-2 – the main difference is the affinity, but all receptors can bind these receptors (BOWERS et al., 2015; CLAYTON et al., 2011; LERO; SHAW, 2021).

The activation of these receptors leads to phosphorylation of insulin receptor substrate (IRS) and Shc protein with subsequent activation of phosphoinositide 3 kinase (PI3K)-Akt and MAPK pathways, respectively. Activation of Akt promotes cell survival, including inhibition of apoptosis and induction of pro-survival genes. The parallel pathway is RAS-RAF-MAPK, which stimulates cell proliferation (Figure 4) (JUNG; SUH, 2015; LERO; SHAW, 2021). Normally these pathways are regulated in normal breast tissue and play a functional role in the development and mature adult gland, but metabolic alterations such as obesity and high adiposity can alter this regulation (BELARDI et al., 2013; LERO; SHAW, 2021).

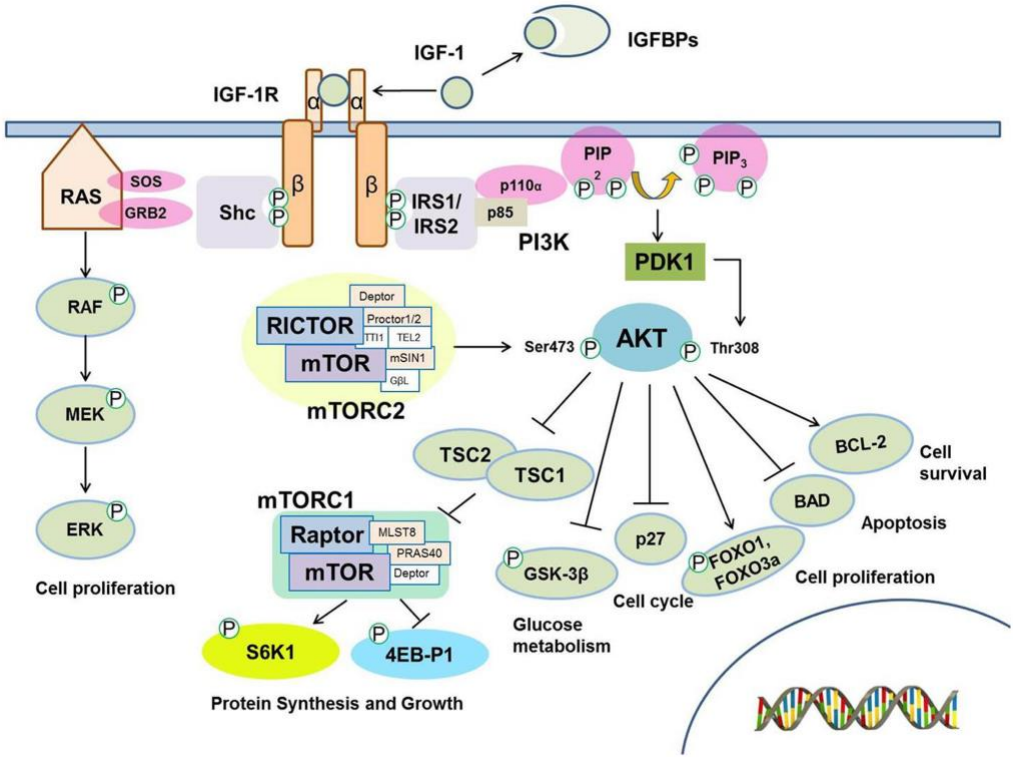

Figure 4. Insulin-like growth factor 1 (IGF-1) signaling pathway.
Adapted from Jung et al. (2015)

In an important meta-analysis that elevated concentrations of serum, IGF-1 increased the risk of BC, especially in ER-positive and women with BMI between 25.0 to 27.0 kg/m^2 (THE ENDOGENOUS HORMONES AND BREAST CANCER COLLABORATIVE GROUP, 2010). Murphy et al. (2020) in a recent study with 400,000 women also observed the relevance of IGF-1 in increasing overall BC risk suggesting a probable cause. The risk was sustained when observed in women with ER-positive, but not ER-negative (OR = 1.06, 95%CI = 1.01 – 1.11; p = 0.03 and OR = 1.02, 95%CI = 0.96 – 1.08; p = 0.58, respectively). A previous study by Goodwin et al. (2009) identified a strong correlation between insulin resistance and BMI (r = 0.61; p < 0.001) in women recently diagnosed with BC. Tin Tin et al. (2021) found that both pre- or postmenopausal women had a higher risk of developing BC according to IGF-1 status, and women with hormone-sensitive BC with higher levels of IGF-1 had a worse survival (HARTOG et al., 2013). Although the evidence is robust regarding IGF-1 in hormone-sensitive BC, preclinical studies demonstrate the important role of IGF-1 and its receptor in growth pathways of many TNBC cell lines (DAVISON et al., 2011) and an increase in IGF-1 in this population was observed being also considered a predictive factor for other types of BC (BAHHNASSY et al., 2015), which corroborates with the importance of understanding if this biomarker could be considered an independent risk factor.

Lipids

Lipoproteins, which are heavily modulated by dietary factors (VINCENT et al., 2019) are associated with cellular metabolism, differentiation, progression, and metastasis of BC. The triad of cholesterol, proinflammatory, and cancer progression is relevant to understand breast carcinogenesis (PANDRANGI et al., 2022). Cholesterol can be synthesized by cancer cells, or it can be taken up from lipoproteins. It is an essential constituent of maintaining membrane integrity, and a major component of cell membrane microdomains called lipid rafts, in which high-cholesterol lipid rafts are associated with tumor progression (LAISUPASIN et al., 2013). A study by Ha et al. (2009) found, for each 1 mmol/L increase in total cholesterol (TC) of women with eutrophic BMI, there is a 13% increased risk of developing BC. This was also observed in previous studies, especially in women with hypercholesterolemia and obesity (GARCIA-ESTEVEZ; MORENO-BUENO, 2019; NI; LIU; GAO, 2015; TOUVIER et al., 2015).

The mechanism that underlies the effect of cholesterol in BC progression is multifactorial, comprising metabolic alterations. Besides dietary components, cancer cells have increased expression of sterol regulatory element-binding protein (SREBP)-regulated genes, which enhances cholesterol synthesis and uptake, in addition to concomitant decreased expression of liver X receptors (LXR), leading to intracellular cholesterol accumulation (GOMARASCHI, 2019). Cholesterol is a precursor of several metabolites, and the role of 27-hydroxycholesterol (27HC), its main product, has gained attention. This metabolite promotes proliferation of breast cancer cell lines, suggesting that it can act as an ER agonist, leading to inhibition of LXR by this receptor, thus

augmenting cancer progression (GARCIA-ESTEVEZ; MORENO-BUENO, 2019; GOMARASCHI, 2019; NELSON; CHANG; MCDONNELL, 2014).

A decrease risk of BC was observed when compared to women with higher high-density lipoprotein cholesterol (HDL-c) values (POUCHIEU et al., 2014; TOUVIER et al., 2015). However, in a study by Nowak and Ärnlov (2018), the risk of ER-positive BC was raised in HDL-c. The effect of menopause status also is unclear regarding HDL-c. In a study by Kucharska-Newton et al. (2008), premenopausal women with high serum HDL-c had decreased BC risk. However, in a meta-analysis conducted by Ni et al. (2015), this was observed in postmenopausal and not premenopausal women.

An increase in low-density lipoprotein cholesterol (LDL-c) and very-low-density lipoprotein cholesterol (VLDL-c), the risk of developing BC increased compared to healthy controls (LAISUPASIN et al., 2013) and could be associated with tumor progression, angiogenesis, and metastasis (LU et al., 2017). Expression of LDL receptor (LDLR) in BC cells is higher than non-tumorigenic cell lines (LU et al., 2017; OHKAWA; OHISHI; YAGI, 1979). In mouse models of hyperlipidemia, tumors from BC cells with high LDLR expression grew incrementally larger with increasing serum LDL concentrations. The increased expression of LDLR led to the inhibition of caspase-3 cleavage and cell survival. Moreover, the upregulation of LDLR caused by adiponectin deficiency in mouse models was followed by greater mammary tumorigenesis (STRANZL et al., 1997) and higher cholesterol uptake, leading to conversion to 27HC by cytochrome P450 oxidase CYP27A1.

Furthermore, lipid peroxidation is associated with breast carcinogenesis due to DNA damage and decrease in DNA repair capacity (WISEMAN;

HALLIWELL, 1996). Women with BC presented more oxidized LDL (oxLDL) than healthy individuals and an increased risk of developing BC (DELIMARIS et al., 2007). The receptor responsible for internalizing oxLDL (OxLDL lecithin-like receptor 1 – OLR1) is overexpressed in human BC and correlates with tumor stage and grade (PUCCI et al., 2019); furthermore, the inhibition of this receptor suppressed invasion and migration of BC (KHAIDAKOV et al., 2011). A study by Wang et al. (2017) found that ORL1 expression could be regulated by TNFα/NF-κB pathway and worsen in a situation where this pathway is increased, such as chronic inflammation and obesity.

Breast cancer and Nutrition

Obesity

Overweight and obesity are defined as a BMI value ≥ 25.0 kg/m^2 and ≥ 30.0 kg/m^2, respectively. A recent report by Ferreira et al. (2021) observed an increase in obesity in Brazil, with the latest number presenting 25.9% of the population, and among these, more than half were women. Obesity is a complex and multifactorial disease in which increased energy intake leads to pathologic enlargement of fat cells and could cause hyperglycemia and hyperinsulinemia that alter metabolic pathways causing chronic diseases, such as Type 2 diabetes mellitus (T2DM), cardiovascular disease (CV), dyslipidemia, nonalcoholic fatty liver diseases (NAFLD), and certain types of cancer, including BC (BROWN, 2021; LIN; LI, 2021). For every 1 kg/m^2 increase in BMI, there is a 3% increase in the risk of developing BC (BERGSTROM et al., 2001). In addition, obesity is related to increase in serum estrogen, IGF-1, insulin resistance, aromatase

upregulation, and inhibition of hepatic synthesis of SHBG, increasing the availability of estrogen and stimulating the growth of ER-positive BC.

The bidirectional relation between overweight/obesity and BC is linked with an increased risk of BC and more aggressive tumor. Excess energetic substrate favors biomarkers that are expressed in serum and tumor (within the tumor and its microenvironment). This is related to the process of tumor growth and metastasis and are associated with local and systemic inflammation, increased inflammatory markers, oxidative stress, alteration in serum adipokines (mainly leptin and adiponectin), insulin, and IGF-1, due to regulation of many pathways (BLAIR et al., 2019) (Figure 5).

The expansion of adipose tissue generates hypoxia and cell death. This releases chemokines, such as MCP1, which recruits macrophages that will secrete inflammatory mediators (BROWN, 2021), and an increase in non-esterified fatty acids (NEFA), which increases substrate for BC growth and can lead to activation of TLR and afterward NF-κB, promoting inflammation (LEE et al., 2001). NEFA also induces mitochondrial and cell cycle deregulation, events commons in the lipotoxicity state, whereas in BC these fatty acids can be utilized by cancer cells as energy source, stimulating cell proliferation and tumor growth (BLÜCHER; STADLER, 2017).

The unbalanced adipokines associated with excess adipose tissue is related with higher levels of leptin and lower levels of adiponectin. Wu et al. (2009) found that women with BC had higher levels of serum leptin and the increase could be a risk factor for BC, especially in postmenopausal women (PAN et al., 2018). The rise in serum leptin signaling in breast tissue is associated with increase in ROS production and inflammation. It also impairs regulation of

molecules that control cell cycle (such as cyclin D1 and p53) and adhesion (such as E-cadherin - a molecule responsible for maintaining cell adhesion), which could increase metastasis (MANTZOROS et al., 2004). On the other hand, a meta-analysis by Yu et al. (2019) with 27 case-control studies concluded that adiponectin is inversely associated with BC risk. This could be due to activation of monophosphate-activated protein kinase (AMPK), upregulating p21 and promoting cell cycle arrest (FOGARTY; HARDIE, 2010).

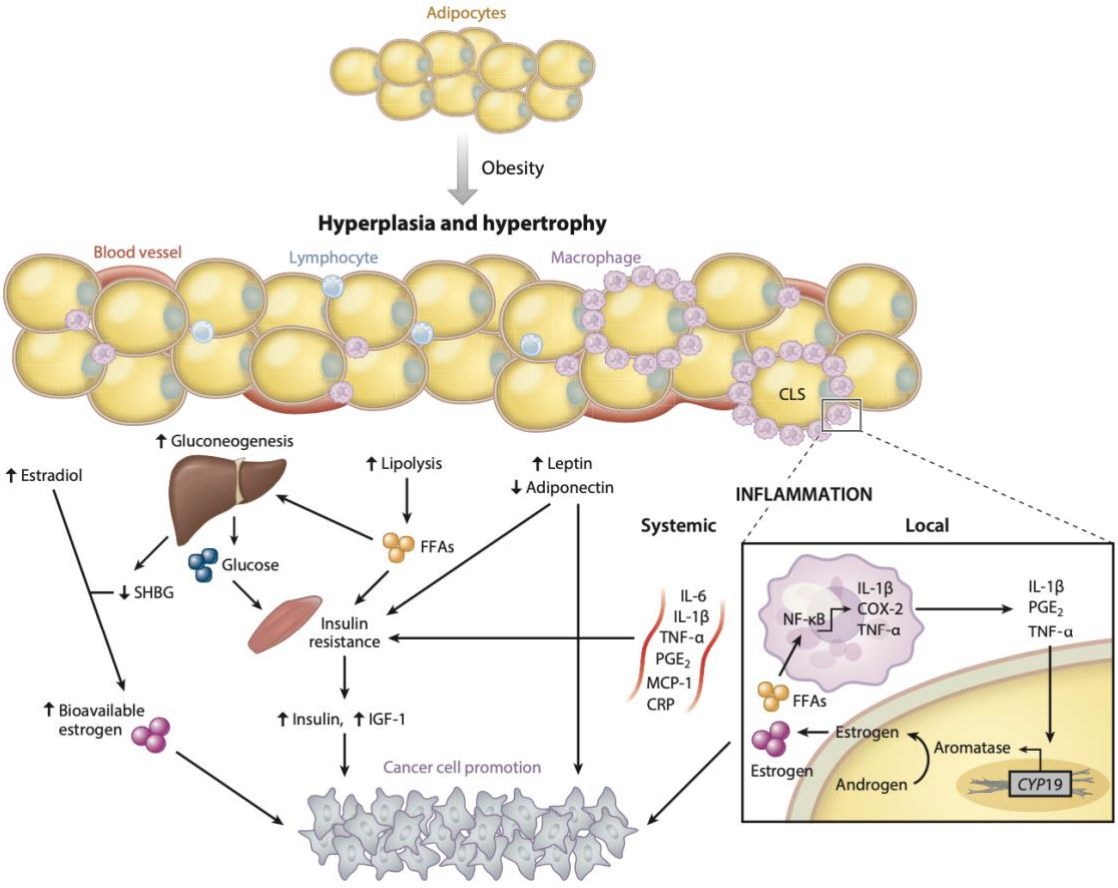

Figure 5. Local and systemic consequences of increased adiposity for carcinogenesis
Adapted from Iyengar et al. (2015)

A meta-analysis with 82 prospective studies observed a positive association between mortality from BC and BMI – regardless of the time of the BMI diagnosis (CHAN et al., 2014). Rosner et al. (2017) indicated that women

that lost more than 5.0 kg since age 18 were inversely associated with a risk of developing BC, whereas weight gain was associated with an increase in risk.

Although BMI is an important risk biomarker for BC, the relevant role of body composition is clear, especially adiposity. A study by Caan et al. (2018) on patients with non-metastatic BC found that an increase in adiposity and the presence of sarcopenia were associated with an increase in mortality.

Overweight and obesity corroborate with an increase in IGF-1 (FRYSTYK et al., 1995). In a murine model provided by Lautenbach et al. (2009), the expression of IGF-1 and leptin increased when the mice were induced to obesity by diet.

The role of obesity in BC risk is very discussed in relation to menopause status. Some studies found that in a premenopausal setting, obesity could be a protective factor against BC, whereas in a postmenopausal state is a clear risk factor (BERGSTROM et al., 2001; HARRIS et al., 2011; NEUHOUSER et al., 2015; RENEHAN et al., 2008). A recent report by García-Estévez et al. (2021) elucidated some of the possible mechanisms involved; many interactions remain unknown. Despite this observation, no formal recommendations are made for the implementation of weight gain strategies in premenopausal women due to risk in metabolic and cardiovascular risk.

Fatty acids

The consumption of some nutrients and bioactive compounds can modulate some risk factors for cancer. Fatty acids have recently gained attention because they play multiple roles, such as building blocks for many lipid species, an essential energy source to sustain proliferation and growth and gene regulators. Another relevant role of fatty acids is membrane structure via

phospholipids, which are a determinant of membrane fluidity, signaling, and organization of molecules that are anchored in the membrane (FERRERI et al., 2020; KOUNDOUROS; POULOGIANNIS, 2020).

The consumption of polyunsaturated fatty acids (PUFAS), especially omega-3 and omega-6 fatty acids, could be associated with the development of BC. The omega-3 series include α-linolenic (ALA - C18:3), eicosapentaenoic (EPA - C20:5), and docosahexaenoic (DHA - C22:6). The main source of ALA food is from vegetable sources, such as flaxseed, and for EPA and DHA, it is cold water fish, such as salmon, tuna, and cod.

For the biosynthesis of omega-3 and -6, two enzymes are needed – desaturase and elongase – which they compete for. Linoleic acid from the omega-6 series is converted to arachidonic acid (AA), and with EPA, they are substrates for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. In AA, prostaglandin 2 and leukotrienes 4 are produced, which are involved in inflammation, vasocontraction, and platelet aggregation. On the other hand, prostaglandin 3 and thromboxane and leukotrienes 5 are generated in EPA pathway and are associated with anti-inflammatory pathways, vasodilatation, and anti-platelet aggregation.

Omega-3 fatty acids exert a positive effect on suppressing inflammatory process, apoptotic stimulus, inhibition of metastasis, and tumor proliferation, modulating peroxisome proliferator-activated receptor (PPAR-α) expression, and reducing NF-kB and interleukin 6 (IL6) – a proinflammatory cytokine – and total levels of epidermal growth factor receptor (EGFR) (ZANOAGA et al., 2018). In line with these studies, supplementation of 1g/day of omega-3 fatty acids can

trigger positive effects in decreased expression of Ki67 and vascular endothelial growth factor **(**VEGF) in BC patients (DARWITO et al., 2019).

Observational studies found a positive association of the low omega-3:omega-6 ratio intake and increased BC risk (DYDJOW-BENDEK; ZAGOŹDŹON, 2020). In a case-cohort study with healthy and BC women, dietary omega-3 fatty acids were inversely associated with BC (BASSETT et al., 2016). Furthermore, EPA and/or DHA supplementation, independent of the cancer treatment, improved progression-free survival, overall survival, and quality of life of cancer patients (NEWELL et al., 2021). A meta-analysis by Zheng et al. (2013) with 26 studies observed that an increase in 0.1 g/day of PUFA could reduce the risk by 5% of developing BC. This was corroborated by Nindrea et al. (2019) who also found a positive association between omega-3 consumption and a lower risk of developing BC.

Among the 14 meta-analyses examined in an overall review, only three studies obtained a statistically significant association between omega-3 fatty acid intake and BC risk (LEE et al., 2020). Furthermore, a recent review based in 47 randomized controlled trials suggested that increased omega-3 fatty acids have little or no effect on the risk of BC diagnosis and deaths from any cancer (HANSON et al., 2020), therefore the evidence still lacking to comprehend the role of this fatty acid in BC. In a study by VanderSluis et al. (2017) it was highlighted the differential roles of EPA and DHA in different BC cell lines and that they could be considered separately regarding prevention. The main findings were that DHA have a tendency to have an anticancer activity in TNBC due to an increase in uptake of this fatty acid by membrane lipid rafts. For ER-positive cell line EPA had a higher uptake in lipid rafts. Also, the incorporation of both fatty

acids was not predicted by its ratio and EPA was preferentially incorporated in BC cell lines.

In humans is discussed the difficult in absorbing and incorporating EPA and DHA in cell membrane. Factors such as empty stomach, consumption of concomitant fat foods and fasting can impair the absorption (MAKI et al., 2018). The discrepancies found in studies can be partially explained by methods used to investigate the bioavailability of omega-3, such as food register, food frequency questionnaire, and 24 hours recall (NASKA; LAGIOU; LAGIOU, 2017). These problems have been partially solved by analysis of plasma and cell content of omega-3 fatty acids amply described in the literature (PICÓ et al., 2019). Therefore observing the fatty acid that are incorporated in cell membrane assist in the understanding of the mechanism regarding its role in BC and considering an individual role for each of them could assist in our future recommendations in BC prevention and treatment scenario.

OBJECTIVE

To assess the potential predictor of the nutritional status and metabolic biomarkers in women with BC and their impact in intermediate clinical outcome and survival.

MATERIAL AND METHODS

The present study is included in the cohort “Obesity and breast cancer: Assessment of risk factors associated with excess weight and adipose tissue” approved by the Research Ethics Committees of the General Hospital of Fortaleza (Nº 050507/10) (Appendix 2) and the School of Public Health, University of São Paulo (Nº 2162) (Appendix 2).

Study design and Sample size

The first design of the study is a study with BC patients of the Mastology Clinic of the General Hospital of Fortaleza (Ceará, Brazil) and matching Controls from the Gynecologic and Obstetrics Clinic collected between May 2011 to August 2012. The studied samples were collected in a probabilistic and consecutive way and were paired according with age, menpause status, BMI, smoking and alcohol consumption.

From the case group (women diagnosed with BC) there was a follow up until April 2019, characterizing a cohort study. In the different manuscripts presented in this thesis we used different cross-sectional time points that are highlighted in each methodology.

Sample size – Manuscript 1

The sample size calculation was considered using the formula for cohort studies by Charan et al. (2013) based on the study by Ha et al. (2009) that observed the association between total cholesterol and BC. Considering an α of 5% bilateral and a statistical power of 90% (β of 10%), the minimum sample size after calculation was 46 individuals.

Sample size – Manuscript 2

The sample size calculation considered the proportion of cases, and controls were paired based on the study by Akinyemiju et al. (2021) that observed the diagnosis of Luminal A BC according to BMI. Using the formula for case-control studies proposed by Charan et al. (2013) and considering an α of 5% bilateral and a statistical power of 90% (β of 10%), the minimum sample size was 71 individuals per group.

Sample size – Manuscript 3

The sample size calculation considering the proportion of cases and controls were paired based on the study by Salinas-Martínez et al. (2014), which observed the diagnosis of diabetes through the value of fasting glucose and glycated hemoglobin. Using the formula for case-control studies by Charan et al. (2013) and considering an α of 5% bilateral and a statistical power of 90% (β of 10%), the minimum sample size after calculation was 90 individuals per group.

Data collection

Patients with suspected breast malignant lesions assisted at the Mastology Clinic of the General Hospital of Fortaleza (Ceará, Brazil) were invited to participate of this study. The study was explained, and the participants voluntary expressed their interest to participate by signing the informed consent form. Only after the confirmation of BC by anatomopathological the data collection began. It was included 114 women and 15 were then diagnosed with metastatic disease and 2 with ductal carcinoma in situ (DCIS).

The Control group consisted in 100 women of the same institution selected from the Gynecologic and Obstetrics Clinic. These women were invited to participate in the study during the regular annual visit to the women's medical

care and after explaining the study and participants voluntary expressed their interest to participate in the study by signing the informed consent form the data was collected (Figure 6).

Inclusion criteria

The BC group contained women with a recent diagnosis of mammary neoplasia, confirmed by clinical and anatomopathological diagnosis, clinical stage (CS) I to III, without metastatic disease or other associated neoplasia, previous cancer treatment, Karnosfsky index above 70%, and without neoadjuvant or adjuvant treatment except surgery to remove the tumor.

The Control group were women without mammary or other neoplasia diagnoses, assessed by medical physical examination and medical records.

Non-inclusion criteria

Individuals with uncontrolled non-communicable chronic disease, with current nutrition counseling or pharmacology therapy for weight loss, with neurological or psychiatry issues were excluded from the study.

Sociodemographic profile and clinical history

The sociodemographic profile was assessed with a standard questionnaire (Appendix 2). Data regarding age, marital status, race, years of education, and per capita family income were obtained.

Clinical variables considering risk factors were obtained from medical records and direct interviews by trained researchers. The variables were menopausal status (12 continued months of amenorrhea), nulliparity, hormone replacement therapy (HRT), reproductive history, breastfeeding, smoking status,

alcohol intake (>15 g of alcohol/day), use of nutritional supplements, and family history of breast cancer.

Information was collected about the individuals in the BC group from the medical records data about their tumor. According to the American Joint Commission on Cancer (AJCC) eighth edition recommendations, BC was classified according to the TNM system – T describes the size of the tumor; N describes the spread of cancer into any nearby lymph node; M describes metastasis. We also collected from their medical chart the status of biomarkers a after biopsy (ER status; PR status; HER2 status; percentage of Ki67) and were able to determine the main molecular subtypes of BC: Luminal A (ER+, PR+, HER2-, and Ki67 low), Luminal B HER2- (ER+, PR+, HER2-, and Ki67 high), Luminal B HER2+ (ER+, PR+, HER2+, and Ki67 high), HER2 (ER-, PR-, HER2+, and Ki67 high), and TNBC (ER-, PR-, HER2-, and Ki67 high).

Anthropometry

A trained researcher collected data about current weight, height, BMI, waist circumference (WC), and body composition. To measure weight, a digital scale was used (Plenna, São Paulo, Brazil) with a maximum capacity of 150 kilograms (kg) and 100 grams (g) precision. The height was measured with a stadiometer (TBW, São Paulo, Brazil) with a maximum height of 2.1 meters (m) and 1.0-millimeter (mm) precision. BMI calculation was based on the formula: current weight (kg) / height^2 (m). WC was measured with an inelastic tape at the umbilical scar. Body composition was used to estimate muscle mass, fat mass, and phase angle by tetrapolar electrical impedance (Biodynamics®, model 450 – TWB, São Paulo, Brazil).

Blood sample

A blood sample (20 mL) was collected after 12-hour fasting by a trained professional (nursing technician or assistant) in a reserved room that provided the required biological safety. Blood was collected in a vacutainer tuber containing ethylenediaminetetraacetic acid (EDTA) (1 mg/mL) with an anticoagulant and antioxidant, kept in ice, and protected from light until plasma separation (1,500g, 10 min, 4 °C). It was added to the plasma protease inhibitors aprotinin (2 μg/mL), benzamidine (2 mM), phenylmethylsulfonyl fluoride (PMSF) (1 mM), and butylated hydroxytoluene (BHT) (20 mM). After that, plasma and red cells were aliquoted and stored at -80 °C until analysis. The sample was transported from Fortaleza (CE) to São Paulo (SP) following the International Air Transport Association (IATA) rules, category B (non-infectious biological material).

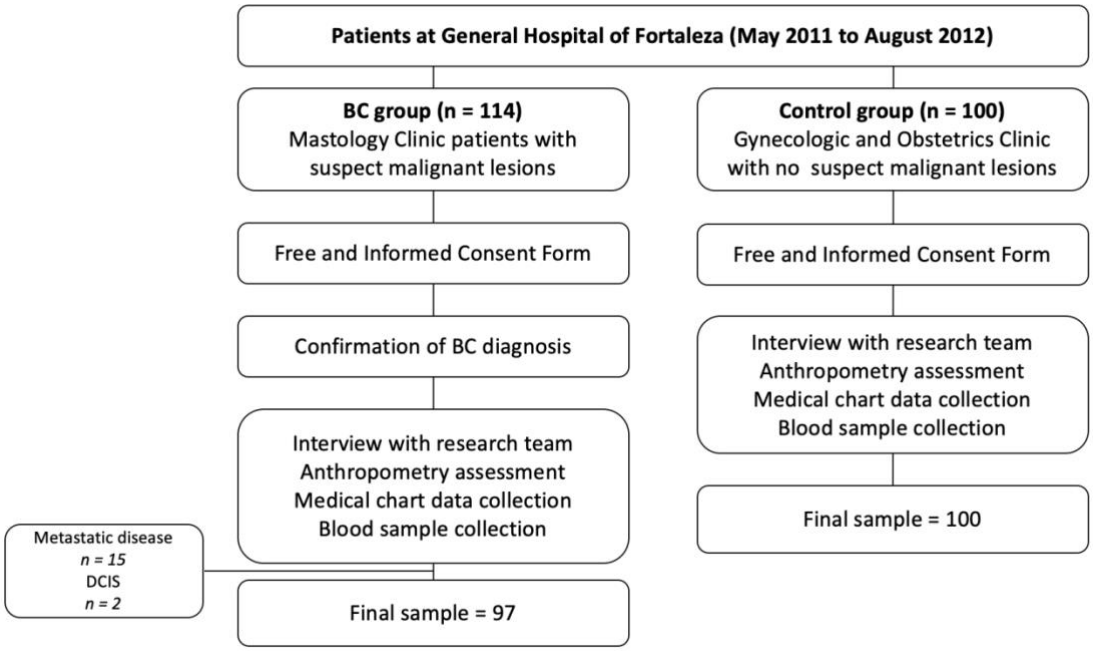

Figure 6. Data collection flowchart

DCIS = ductal carcinoma in situ

Follow up

Eight years after the baseline, a follow-up was collected from the BC group, including chart complementary information from the medical, anthropometric data, and blood sample. In addition, relapse or new primary neoplasia and mortality information was collected on the day of the last appointment. To determine the cause and date of mortality, a phone call was made to the families for conformation. There was no follow-up in the Control group.

Biochemical assays

Insulin Growth Factor 1 (IGF-1)

To determine serum plasma IGF-1, an Enzyme-Linked Immunosorbent Assay (ELISA) IGF-1 (human) ELISA Kit® (Enzo Life Sciences Farmingdale, NY, USA) based on a competitive and calorimetric immunoassay was used. The positive color reactivity of the sample was detected at a 450 nm wavelength and the results are expressed in ng/mL according to the protocol established by the manufacturer.

*Insulin Growth Factor Binding Protein 3 (*IGFBP-3*)*

To determine the plasma IGFBP-3, a commercial ELISA kit IGFBP3 Simple Step ELISA Kit® (Abcam, Cambridge, UK) based on a competitive and calorimetric immunoassay was used. The reactivity of the sample was detected at a 450 nm wavelength, and the results were in µg/mL according to the protocol established by the manufacturer.

Glucose

Glucose was measured using an enzymatic and colorimetric commercial

plasma Glucose PAP Liquiform® kit (Labtest, Minas Gerais, Brazil). The results were expressed in mg/dL according to the protocol established by the manufacturer.

Insulin

To determine plasma insulin, an ELISA Insulin Human ELISA Kit® (Life Technologies, Grand Island, NY, USA) used was based on a competitive and calorimetric immunoassay. The reactivity of the sample was detected in 450 nm wavelength, and the results were in µUI/mL according to the protocol established by the manufacturer.

Glycated hemoglobin (HbA1c)

Glycated hemoglobin was assessed using red cells and an immunoturbidimetry commercial HbA1c Turbiquest® kit (Labtest, Minas Gerais, Brazil). Results were expressed in percentages according to the protocol established by the manufacturer.

Leptin

To determine plasma leptin, an ELISA Leptin Human ELISA Kit® (Enzo Life Sciences, Farmingdale, NY, USA) used was based on a competitive and calorimetric immunoassay. The positive reactivity of the sample was monitored at a 450 nm wavelength, and the results were in ng/mL according to the protocol established by the manufacturer.

Adiponectin

To determine plasma adiponectin, an ELISA Adiponectin Human ELISA Kit® (Adipogen, San Diego, CA, USA) used was based on a competitive and calorimetric immunoassay. The reactivity of the sample was monitored at a 450

nm wavelength, and the results were in µg/mL according to the protocol established by the manufacturer.

Antioxidants vitamins (retinol, α-tocopherol, β-carotene)

The concentration of liposoluble antioxidants were determined using the High-performance liquid chromatography (HPLC) and the protocol described by Arnaud et al. (1991). Briefly, 200 µL of plasma was added 200 µL of ethanol and vortexed for 5 seconds. Then was added 500 µL of hexane and vortexed for 2 minutes. Following the solution was centrifuged at 3,000 rpm for 5 min at 4 °C. The supernatant was transferred to test tubes and concentrated in TurboVap® for 15 min. After that, the sample was resuspended with 200 µL of mobile phase (70% acetonitrile; 20% of methanol; 10% of dichloromethane in a final solution of 100 mL). The solution was briefly homogenized with vortex and filtered in 0.45 µm sterile membrane. After the filtering process, 50 µL was injected in HPLC. The peak reading was made in the following order: retinol, α-tocopherol, β-carotene. After the reading the results were calculated with the ratio of peak area and standards. The results were presented in µmol/L according to the protocol.

*Thiobarbituric acid reactive substance (*TBARS*)*

The plasma lipid peroxidation was performed according to the method proposed by Ohkawa et. al (1979). Briefly, in 50 µL of plasma, 1 mL of TBARS solution composed of thiobarbituric acid (0.046 M), trichloroacetic acid (0.92 M), and hydrochloric acid (0.25 M) was added. After that, the samples were incubated at 100 °C for 30 min. The solution was then centrifuged at 8,000 g for 15 min at 4 °C. The color intensity in the supernatant (200 µL) was monitored at a wavelength of 535 nm, and the results are in µmol/mL.

Non-esterified fatty acids (NEFA)

The concentration of the NEFA was determined by a colorimetric assay using the commercial Free Fatty Acid Quantification Kit® (Wako Chemicals USA Inc., Richmond, VA, USA). The analysis was performed in the semi-automatic system Cobas-Mira®, and the results are expressed in mg/dL.

DNA Oxidative damage

The oxidative damage was assessed through the detection and quantification of 8-OH-dG in the plasma using a commercial DNA Damage ELISA kit® (Enzo Life Sciences Farmingdale, NY, USA). Results are expressed in ng/mL.

Plasma LDL(-) detection

To determine LDL(-), a ELISA system was developed according to the standard protocol proposed by Faulin et al. (2012). Plates (Costar®, model 3690, Corning, NY, USA) were sensitized with monoclonal anti-LDL(-) antibody (MAb-1A3) (0.5 µg/mL, 50 µL/well), diluted in carbonate-bicarbonate buffer (0.25 M, pH 9.6), and incubated overnight (16 h at 4 °C). The plates were then washed three times with phosphate saline buffer (PBS)-Tween 0.05%. Free sites were blocked with skimmed milk, diluted to 2% in PBS-Tween 0.01%, and incubated for 2 h at 37 °C.

After that incubation period, the plates are washed three times with (PBS)-Tween 0.05%, 50 µL/well of plasma diluted in 1:1000 in skimmed milk, in 1% in PBS-Tween 0.01% was prepared and added, followed by an incubation time of 1 h 30 at 37 °C. After that, 50 µL/well of anti-LDL(-) monoclonal antibody (Ac-2C7b) (10 µg/mL), 1% diluted with PBS-Tween, 0.01% skimmed milk was added. The plates were again incubated for 1 h at 37 °C and washed three times with PBS-Tween 0.05%.

The next addition was streptavidin-peroxidase (1:400000), diluted in PBS-Tween 0.01% skimmed milk, and then 50 µL/well added. The plates were incubated for 1 h at 37 °C and washed four times with PBS-Tween 0.05%.

Then 50 µL/well of orthophenylenediamnine (OPD) diluted in citrate-phosphate buffer (0.1 M, pH 4.2) and H_2O_2 (30%) (250/12/10 µL/µL/µL) was added. After 5 min incubation, the color reaction was blocked with 50 µL/well sulfuric acid (H_2SO_4) (2 M), and the absorbance was read at a wavelength of 450 nm, the results being expressed in U/L.

Detection of auto-antibody anti-LDL(-) in plasma

The concentration of auto-antibody anti-LDL(-) was determined by an ELISA developed according to the method proposed by Damasceno et al. (2006), where LDL(-), isolated by Fast protein liquid chromatography (FPLC), was diluted in carbonate-biphosphate buffer (0.25 M, pH 9.6) until final dilution of 1 µL/mL and put in plates (Costar®, model 3690, Corning, NY, USA) and incubated overnight at 4 °C for sensibilization. The free spaces were blocked with skimmed milk diluted to 5% in phosphate saline 0.01 mol/L (PBS – pH 7.4) and incubated for 2 h at 37 °C. After that time, the plates were washed four times with PBS-Tween 0.05%.

The samples were diluted at 1:500 in PBS and pipetted at 50 µL/well in the sensitized plate with LDL(-), following an incubation time of 2 h at 37 °C. The plates were washed, and 50 µL/well of human anti-IgG with peroxidase diluted in PBS (1:5000) was added. Then the sample was incubated for 90 min at 37 °C, and washed one more time.

To assess the reactivity, 3,3',5,5'-tetrametilbenzine (TMB), citrate-phosphate buffer (0.1 M, pH 4.2), and H_2O_2 (30%) (250/12/10/50 µL/mL/µL) was

added. Plates were incubated for 15 min at 37 °C under light protection. The reaction was blocked with 50 µL/well of sulfuric acid (H_2SO_4) (2 M), and the absorbance was read at a wavelength of 450 nm.

Lipoprotein profile

In the BC group, lipoprotein analysis was done. Total cholesterol (TC), triacylglycerols (TG), and HDL-c were performed with commercial kits (Labtest, Minas Gerais, Brazil). The cholesterol content in LDL-c was determined with the formula by Friedewald et al. (1972), LDL-c = TG – HDL-c – TG/5 and VLDL-c = TG/5 (applied only in individuals with TG < 400 mg/dL) and non-HDL-c = TC – HDL-c.

Apolipoproteins A-I and B (Apo A-I and Apo B) were assessed with the commercial Autokit Apo A1 and Autokit Apo B (RANDOX Laboratories Ltd., Dublin, Ireland).

Fatty acid profile of erythrocyte membrane

To determine the fatty acid profile of the erythrocyte membrane, the method proposed by Masood et al. (2005) was adapted.

A 300 µL hemoconcentration with erythrocytes was lysed with iced PBS followed by three cycles of washing and centrifugation (1,000 g, 15 min, 4 °C), and then placed for 5 min in an ultrasonic processor. Next, methanol (1.75 mL), 50 µL of tridecanoic acid (C13:0) as the internal standard, and acetyl chloride (100 µL) was added. The tubes were closed and vortexed for 30 seconds, following an autoclaving for 1 h at 100 °C. Then the tubes were cooled to room temperature, and to extract the fatty acids, 1.5 mL of hexane was added.

The samples were homogenized for 1 min and centrifugated for 2 min at 4 °C and 1,500 g. After, 800 µL of supernatant was transferred, the process was

repeated by adding 750 µL of hexane.

Both supernatants collected were mixed and concentrated in CentriVap® for 20 min at 40 °C. After that, the sample was resuspended with 150 µL of hexane, put in the ultrasonic processor for 5 min, filtered in 0.22 µm membrane, transferred to an insert vial, protected from light, and analyzed using gas chromatography (Shimadzu CG-2010) with a DB-FFAP capillary column (15.0 m x 0.100 mm x 0.10 µM 0 J and W Scientific, Agilent Technologies). Hydrogen was used with a 0.27 mL/min flux, a flow rate of 35 cm/s, and a pressure of 187.8 kPa. Fluxes for synthetic air, N_2, and H_2 were 300, 30, and 30 mL/min, respectively. The temperature of the injector was 250 °C and the detector was 260 °C. The programming of the initial temperature of the column were 100 °C with retention of 0.5 min, ramp of 25 °C/min at 195 °C, 3 °C/min at 205 °C, 8 °C at 230 °C, retention of 4 min, 50 °C/min at 245 °C retaining 0.5 min. The Split ratio used in the injector was 1:100 and the total time of the race was 15.56 min.

The intern pattern used was a mixture of 37 fatty acid methyl esters (FAME 37, code 47885, Sigma ChemicalCo.). The injection volume was 2 µL in an automatic injector AOC 20i. Fatty acids were identified by comparing the retention time of the intern pattern and the samples. The results are expressed as a total percentage of fatty acids integrated into the sample. The present study assessed the following fatty acids: myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1 n9), elaidic (C18:1 n9-trans), linoleic (C18:2 n3), linolenic (C18:3 n3), cis-8,11,18 eicosatrienoic (C20:3 n6), arachidonic (C20:4 n6), eicosapentaenoic (C20:5 n3), benehic (C22:0), and docosahexaenoic (C22:6 n3).

Inflammation biomarkers

To perform the cytokines assay, 200 µL of plasma was used employing a commercial kit (Human Magnetic Panel Bead Milliplex® MAP – HCY T0 MAG – Merck, Millipore). The selected biomarkers were IL6, IL1β, TNF-α, MCP-1, and IL10. To detect the intensity of fluorescence that each microsphere emits, streptavidin phycoerythrin was used. The Luminex 200™ with xMAP® technology and acquisition of xPONENT® was used to detect the fluorescence. To integrate the data analysis, MILLIPLEX® Analyst 5.1 software was used.

Statistical analysis

Considering the presentation format of the thesis, each manuscript focuses on a specific topic of the methodology applied. In summary, quantitative variables were described and presented as frequency and percentage. The quantitative variables were calculated as mean and standard deviation (SD). Statistical difference was assessed using a *chi*-square test and the results are expressed in tables and graphics. For the quantitative variables, the distributions were considered according to the Kolmogorov-Smirnov test with normality considered at $p > 0.050$. To assess the difference, Student's t-test or a Mann-Whitney test were used, and for correlation analysis Pearson's or Spearman correlations were used.

Univariate logistic regression models were used to assess associations. For multivariable logistic regressions, a stepwise forward approach was employed to estimate the coefficients of regression (β), SE, Wald, Odds Ratio (OR), and 95% confidence interval (95%CI), and no variables were used that presented collinearity ($p < 0.025$). Survival was analyzed by Kaplan-Meier curve

with log-rank. All statistical tests were performed using Statistical Package for Social Sciences® (SPSS), version 21.0. Statistical significance was set at $p < 0.050$.

RESULTS AND DISCUSSION

The results and discussion section will be presented in three complete manuscripts, following the guideline from which journal were submitted, except the last manuscript (number three), where selection of journal and submission will occur after approval of this committee.

MANUSCRIPT 1 – Unbalanced lipid and oxidative stress in premenopausal women with breast cancer: impact on clinical staging and survival

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Unbalanced lipid and oxidative stress in premenopausal women with breast cancer: impact on clinical staging and survival

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Abstract

Aims: Breast cancer (BC) is the most common cause of cancer and leading cause of death. We aim to investigate how lipid profile, oxidative stress and inflammation in the moment of diagnosis can influence tumor size and survival outcome. **Main methods:** This is a cohort between May 2011 and April 2019 with 114 women with recent diagnosis of BC. Data were obtained by medical records, interview and blood samples collected to analyze serum lipoproteins, glucose, glycated hemoglobin and adipokines, thiobarbituric acid reactive substances (TBARS), non-esterified fatty acids (NEFA) and DNA oxidative damage. **Key findings:** Premenopausal women with CS II-III had worse serum lipid profile (higher LDL-c, non-HDL-c and Apo B and lower HDL-c). Also, LDL-c and non-HDL-c were associated with increased odds of having larger tumor size (OR = 1.028; CI95%= 1.001 – 1.057 and OR = 1.032; CI95% = 1.004 – 1.061) and HDL-c was associated with a decrease in odds of having larger tumor size (OR = 0.930; CI95% = 0.868 – 0.996) adjusted by BMI and menopause status. Premenopausal women with an increased level of TBARS and NEFA at diagnosis had a lower survival probability (p=0.019 and p=0.020, respectively). **Significance:** Altered lipid and oxidative stress metabolism in the moment of diagnosis can be associated with increased clinical staging in premenopausal women, increase in tumor size and worse survival outcome. Control of plasma cholesterol and its associated risk factors could be positive to prevent BC and enhance survival.

Keywords: Breast cancer, lipid profile, glucose metabolism, oxidative stress, inflammation, survival

INTRODUCTION

Breast cancer (BC) is the most common cause of cancer worldwide and leading cause of cancer death among women[1]. The global burden of cancer is rapidly increasing and a better understanding of factors that contribute to breast carcinogenesis and survival is necessary to implement prevention strategies[2].

BC multifactorial aspects are determined by both modifiable and non-modifiable risk factors. Age of menarche and menopause, parity, age of first child, previous occurrence of cancer and lifestyle are well-known risk factors that account for the majority of cases[3].More recently, obesity has been considered a relevant risk factor in BC as it impacts negatively the inflammatory state, and increases oxidative stress, unbalanced glucose and lipid metabolism[4]. Additionally, obesity is associated with advanced clinical staging and poor survival of women with BC[9 - 10].

Excess adipose tissue stimulates insulin resistance and increase of the non-esterified free fatty acid (NEFA) and glycerol through lipolysis as observed in obese cancer patients [7] and it can contribute to risk and development of BC[8, 9]. At peripheral tissues, the NEFA induces mitochondrial and cell cycle deregulation, events commons in the lipotoxicity state, whereas in BC these fatty acids can be utilized by cancer cells as energy source, stimulating cell proliferation and tumor growth[10]. Hypercholesterolemia is also an obesity comorbidity, and clinical studies have been linking cholesterol metabolism with BC risk[11–13] but not with survival [14].Higher levels of low-density lipoprotein cholesterol (LDL-c) and very-low-density lipoprotein cholesterol (VLDL-c) were found in BC women compared to the control group[15]. Furthermore, a high total cholesterol had a 31% increase in the risk of developing BC [16], while high-density lipoprotein cholesterol (HDL-c) was inversely associated with BC risk[11].

The mechanism that underlies the effect of cholesterol in BC progression is likely multifactorial comprising metabolic alterations, effect of cholesterol in cancer cell and the effect of its metabolite (27-hydroxycholesterol - 27-HC) in the tumor and microenvironment [17]. Cholesterol can be synthetized by cancer cells or it can be taken up from lipoproteins. It is an essential constituent of membrane, maintaining integrity and a major component of microdomains called lipid rafts, in which high-cholesterol lipid rafts are associated with tumor progression [18]. Also, it is shown that 27-HC promotes proliferation of BC cell lines, suggesting that it can act as an estrogen receptor agonist augmenting cancer progression[17–19].

Circulating cholesterol is also related to lipid peroxidation that can be a substrate to oxidative stress and contribute to carcinogenesis via tissue damage and DNA

alteration[20] and increase disease aggressiveness [21].It was found out that BC patients have an impaired control of oxidative/antioxidant ratio that favors oxidative stress and carcinogenesis [22] but its role in survival is still unclear[23, 24].

Therefore, the primary endpoint of the study is to associate blood lipids profile at the moment of BC diagnosis with clinical tumor staging, and tumor size in pre and postmenopausal women. Here, we hypothesize that the alterations in lipid profile promote disruption in lipid metabolism and oxidative stress, impacting negatively on women survival.

MATERIAL AND METHODS

Study design and participants

This is a prospective cohort study that included women referred to the Mastology Clinic of the General Hospital of Fortaleza (Fortaleza, Ceará, Brazil) between May 2011 and April 2019. Women who were > 18 years and had recent diagnosis of BC according to anatomopathological analysis, clinical staging between I to III, without metastasis or other previous neoplasia and without neo-adjuvant therapies were selected. Women were considered postmenopausal if they self-reported cessation of menses in the previous year[25].Patients with uncontrolled chronic noncommunicable diseases, use of weight reduction medications, or psychiatric or neurological disorders were excluded. The clinical staging (CS) was performed by a physician using the American Joint Committee on Cancer (AJCC) Staging Manual (8th Edition)[26]. This study was approved by the Research Ethics Committees of the General Hospital of Fortaleza (nº 050507/10) and the School of Public Health, University of São Paulo (nº 2162). All participants provided an informed written consent to participate. The study was performed in accordance with the Declaration of Helsinki.

Data collection

Sociodemographic data and risk factors for cancer (smoking, alcohol intake, breastfeeding) were obtained from the medical records and direct interview using a standard form. Anthropometric assessment was performed by a trained researcher. Body weight (kg) was measured using a digital scale (Plenna®, São Paulo, Brazil) and height (m) was measured using a portable stadiometer (TBW®, São Paulo, Brazil). The Body Mass Index (BMI) (kg/m²) was calculated according to the WHO recommendation. Waist circumference (WC) was measured by an inelastic tape. Body composition was determined with tetrapolar electrical bioimpedance (Biodynamics®, model 450 – TWB,

São Paulo, Brazil). Survival data were collected in medical chart and following up by phone.

Blood samples (20mL) were collected after a 12-hour fasting in vacutainer tubes containing EDTA (1 mg/mL). Blood was centrifuged for plasma separation (1500g, 10 minutes, 4oC) and protease inhibitors (10 µg/mL aprotinin, 10 µM benzamidine and 5 µM phenylmethylsulphonyl fluoride - PMSF) and 100 µM butylated hydroxytoluene - BHT antioxidant were added to the plasma. After that, samples were aliquoted and stored at -80 ºC until analysis.

Blood lipids

The concentration of total cholesterol (TC), HDL-c and triacylglycerols (TG) were determined using a colorimetric assay using the kits Cholesterol Liquiform®, Cholesterol HDL® and Triglycerides Liquiform®, respectively (Labtest, Minas Gerais, Brazil). The content of cholesterol in LDL-c was calculated using the formula proposed by Friedewald (1972)[27]: LDL-c = (TC-HDL-c) – (TG/5).

The apolipoprotein A-I (Apo A-I) and B (Apo B) were measured using an immune-turbidimetric assay with the commercial kit Autokit APO A-I® and Autokit APO B®, respectively (Wako Chemicals USA Inc., Richmond, VA, EUA).

Glucose, glycated hemoglobin and adipokines

Plasma glucose and glycated hemoglobin were analyzed by colorimetric and enzymatic kits(Glicose PAP Liquiform® and HbA1c Turbiquest®, respectively; Labtest, Minas Gerais, Brazil). Plasma leptin and adiponectin were analyzed using a human enzyme-linked immunosorbent assay (ELISA) commercial kit (Leptin Human ELISA Kit® - Enzo Life Sciences Farmingdle, NY, USA and Adiponectin Human ELISA Kit® - Adipogen, San Diego, CA, USA).

Thiobarbituric acid reactive substances (TBARS)

The lipid peroxidation in plasma was performed according to the method proposed by Ohkawa et al. (1979)[28]. Briefly, in 50 µL of plasma and 1mL of TBARS solution composed of for thiobarbituric acid (0.046 M), trichloroacetic acid (0.92 M) and hydrochloric acid (0.25 M) were mixed. After that, samples were incubated at 100ºC for 30 minutes. The solution was then centrifuged at 8,000g for 15 minutes at 4ºC. The color intensity in the supernatant (200 µL) was monitored at 535 nm.

Non-esterified fatty acids (NEFA)

The NEFA concentration was determined by a colorimetric assay using the commercial Free Fatty Acid Quantification Kit® (Wako Chemicals USA Inc., Richmond, VA, EUA). The analysis was performed in duplicate in the semi-automatic system Cobas-Mira®.

DNA oxidative damage

The oxidative damage was assessed through the detection and quantification of 8-OH-2'-deoxyguanosine (8-OH-dG) in the plasma using an ELISA commercial kit (DNA Damage ELISA kit® (Enzo Life Sciences Farmingdle, NY, USA).

Statistical Analysis

For analysis, the patients were divided in premenopausal and postmenopausal women, while CS was stratified in I and II-III groups. The normality of the variables was verified by the Kolmogorov-Smirnov test ($p > 0.05$). Descriptive data were expressed as absolute values and frequency or mean followed by standard deviation (SD). Categorical data were compared using the chi-square test. Comparisons between quantitative variables were performed using Student's *t* test or the Mann-Whitney test, according to normality. For the multivariable logistic regression models no variable used presented collinearity. The model was used to estimate the associations between tumor size (≤ 2.0 cm and > 2.0 cm) and lipid parameters. The models were adjusted for smoking, menopausal status and BMI using the stepwise forward approach to estimate the coefficients of regression (β), standard error (SE), Wald, Odds Ratio (OR) and 95% confidence interval (95% CI). Survival was analyzed by Kaplan-Meier curve with log-rank and for that, the cutoff point (percentile 50 - p50) was applied for all variables. Statistical analyses were performed with the software Statistical Package for Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Among the 114 women enrolled in this study, 82 met the inclusion criteria, 15 had confirmation or suspicion of metastasis, 2 had *in situ* tumors and 15 had no clinical staging data. As expected, the age of postmenopausal women was higher than premenopausal women (58.5± 9.3 y vs. 42.2±6.3 y; $p < 0.001$). The frequency of smokers was also higher in this group (53.8% vs. 27.9%; $p = 0.017$). Participants were overweight (BMI ≥ 25.0 and < 30 kg/m^2), and abdominal fat indicated visceral obesity (WC ≥ 88 cm), without statistically significant differences between groups. Lean and fat mass, alcohol intake, breastfeeding, and phase angle were similar among pre and postmenopausal women (**Table 1**). This profile was maintained after stratification by clinical staging groups (i.e., CS I and CS II-III of pre and postmenopausal women). In fact, 30.2% of premenopausal women were obese, while 76.7% and 46.5% had high WC and fat mass (data not shown).

Tumor size of pre and postmenopausal women did not statistically differ, but among premenopausal women, those within the CS II-III group had larger tumor size

than those within the CS I group (3.2 ± 1.8 cm vs. 1.6 ± 0.6 cm; p = 0.016). The BC subtypes (i.e., ER+, PR+, HER2+, and triple negative) did not differ among groups and subgroups (**Table 2**).

Postmenopausal women had higher plasma glucose (97.8 ± 13.2 mg/dL vs. 89.6 ± mg/dL; p = 0.016) and HbA1c (6.1 ± % vs. 5.6 ± %; p = 0.015) than those within the premenopausal group, however, in both groups women did not have a diabetes mellitus diagnosis. Among premenopausal women, patients within the CS II-III group had higher levels of LDL-c (129.9 ± 31.0 mg/dL vs. 105.0 ± 17.5 mg/dL; p=0.014), non-HDL-c (151.6 ± 34.9 mg/dL vs. 124.9 ± 19.2 mg/dL; p=0.017) and Apo B (105.1 ± 19.9 mg/dL vs. 89.6 ± 15.7 mg/dL; p=0.022),as well as lower plasma adiponectin (7.7 ± 4.2μg/mL vs. 12.1 ± 7.7μg/mL; p=0.023) and HDL-c (31.8 ± 7.8 mg/dL vs. 37.7 ± 9.0 mg/dL; p=0.044)when compared to those within the CS I group (**Table 3**).

Additionally, plasma LDL-c and non-HDL-c were associated with increased odds of having larger tumor size, whilst HDL-c showed opposite association. After adjustment for smoking, menopausal status, and BMI, the increase of one unit of LDL-c and non-HDL-c was associated with a 2.8% (OR = 1.028; CI95%= 1.001 – 1.057) and 3.2% (OR = 1.032; CI95% = 1.004 – 1.061) chance increase of having tumors larger than 2 cm, respectively. Inversely, the increase of one unit of HDL-c level was associated with a 7% chance reduction of having tumors larger than 2 cm (OR = 0.930; CI95% = 0.868 – 0.996). Regarding the odds for LDL-c and non-HDL-c, we did not observe significant changes after multiple adjustments, suggesting the independent effect of high cholesterol level on tumor size (**Table 4**).

The average following up time was of 53.7 (16.5) months for this sample. Lipid profile and oxidative stress did not show significant differences between survival probability in all the sample and in postmenopausal women with BC (**Supplementary Figure 1 and 3**). Similarly, premenopausal women did not show, either, difference among glucose, glycated hemoglobin, leptin, adiponectin, lipid profile and 8-OHdG (**Supplementary Figure 2**), but increased values of oxidative stress (TBARS; high > 6.1μmol/mL and low ≤ 6.1μmol/mL; p=0.019) and inflammatory marker (NEFA; high > 0.54 mmol/L and low ≤ 0.54 mmol/L, p=0.020) in premenopausal women was associated with a lower survival probability (**Figure 1**).

DISCUSSION

The current study showed that premenopausal women presenting advanced disease stage (CS II and III) had worse plasma cholesterol profile compared to those

with better clinical staging (I), characterized by higher levels of Apo B, LDL-c and non-HDL-c, as well as lower HDL-c regardless of weight, adiposity and receptor status. Furthermore, it was found that one-unit increase in LDL-c and non-HDL-c was associated with approximately a 3% risk increase of larger tumor size, whereas HDL-c was associated with a 7% risk reduction of smaller size BC, independently of menopause status, weight and smoking.

Dyslipidemia has been linked to BC and in some cell lines, in which LDL-c and VLDL-c particles promote tumor progression through increased cell proliferation, cell migration/invasion and angiogenesis[29]. In severely hypercholesterolemic mice lacking apolipoprotein E (ApoE-/-) fed with high fat-high carbohydrate - HFHC diet exhibited increased mammary tumors progression and metastasis compared to wild types[30]. Moreover, expression of LDL receptor (LDLR) in BC cells is higher than non-tumorigenic cell lines[31, 32]. It was reported that in mice models of hyperlipidemia, tumors from BC cells with high LDLR expression grew incrementally larger with increasing serum LDL concentrations. The increased expression of LDLR led to the inhibition of caspase-3 cleavage and cell survival[33]. Moreover, the upregulation of LDLR caused by adiponectin deficiency in mice models was followed by greater mammary tumorigenesis [34] and higher cholesterol uptake, leading to conversion to 27-HC by cytochrome P450 oxidase CYP27A1. This metabolite is associated with high blood cholesterol and it was found to promote BC cell growth and metastasis in cell lines[35], but postmenopausal women showed a lower risk of BC [36]. Therefore, our results confirm the role of cholesterol in multiple steps of carcinogenesis and expand the experimental previous studies showing that premenopausal women have increased risk to larger size BC tumor.

Results regarding HDL-c and BC risk are conflicting. An inverse association with BC risk was found in premenopausal women [37, 38], whereas high HDL-c level induced by genetic modulation was associated to increased BC risk [39, 40], while HDL-c was found to have a protective effect in breast carcinogenesis in postmenopausal women [12]. Here, we found a protective effect of HDL-c, even though our sample had low values of HDL-c at the time of diagnosis (HDL-c premenopausal = 33.6 ± 8.4 mg/dL and postmenopausal = 34.9 ± 10.1 mg/dL; p = 0.540), results similar to Laisupasin et al. (2013)[15].

Even with some conflicting results, HDL-c is still important due to its role in modulating intracellular cholesterol and its antioxidant proprieties, inhibiting peroxidation of cholesterol in LDL particles and reducing lipid peroxidation products from circulation. It is known that women with BC have higher levels of markers of lipid peroxidation in comparison to control women [41], inflammation [42] and an impaired level of antioxidant

capacity[43] – corroborating with an environment of oxidative stress due to unbalance in serum lipoproteins which can increase carcinogenesis by damaging DNA, and contribute to membrane destabilization[44]. It was found in women with BC an increase in TBARS – a classical marker of advanced lipid peroxidation. Similar profile was described in BC tissue compared to adjacent normal tissue, suggesting a growth advantage in this environment[45]. In our study we found out that premenopausal women with advanced disease stage (CS II and III) had higher plasma LDL-c, non-HDL-c, and Apo B, as well as lower plasma HDL-c and adiponectin concentrations. Together, these markers contributed positively to higher levels of oxidative stress (TBARS) and NEFA, an important biomarker to insulin resistance and inflammation, with consequent negative impact on lower survival ($p = 0.019$ and 0.020, respectively).

As demonstrated in previous studies, NEFA levels are higher in women with obesity and it can be related to a greater BC risk since it can be taken up by these cells and activate proliferation pathways such as mTOR and regulate cancer cell metabolism[8] and progression[46].

Comparing pre and postmenopausal groups, participants in the latter group had higher plasma glucose, HbA1c and lower adiponectin, which supports that menopause is associated with glucose homeostasis disruption and increased type 2 diabetes risk[47, 48].

In the present study, there was no significant difference in BMI and WC among pre and postmenopausal women, but both groups showed BMI mean indicative of overweight. Previous studies found that high BMI in premenopausal women might be a protector factor due to low estrogen levels and high testosterone[49].However, this association needs caution as the increase in total cholesterol, LDL-c and lower HDL-c and adiponectin level observed in our study could contribute to a more proliferative environment in this population, as previously proposed[29, 50].Overweight and obesity, translated as an excess of adipose tissue, can contribute to an increase in oxidative stress[51, 52].This oxidative stress is related to a higher risk of carcinogenesis by promoting signaling pathways for cell proliferation in BC[53, 54].Our study highlighted the relevant effect of overweight and adiposity on modulating lipid pathways, regardless of menopausal status and BC subtype, but with positive impact on tumor size.

It is suggested that lowering blood cholesterol drugs such as statins-inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) and other lipid-lowering drugs, may suppress or delay BC proliferation and metastasis. The lower levels of LDL-c can diminish cholesterol availability for tumor cell membrane as well as proinflammatory cytokines[55]. Recently, this hypothesis was tested using simvastatin. This drug

promoted decreasing tumor recurrence in patients with BC stage I-III[56], and statins reduced mortality[57] in 20,559 BC patients. On the other hand, it is important to consider statins toxic effects, especially muscle pain and its interaction with chemotherapy used on BC treatment[55]. However, the present study did not have information about medication used and omega-3 intake. Together, the lack of this information may be a potential limitation. In parallel to the medication use, lifestyle changes are recommended to lower blood cholesterol. Additionally, a recent meta-analysis showed that alterations in lifestyle, involving dietary and physical activity can improve HDL-c and LDL-c levels[58].Here, we speculate that nutrients such as omega-3 and their metabolites can improve lipid homeostasis by reduction of triglycerides[59], improvement of HDL-c functionality[60] and decreasing small dense LDL[61] potentially impacting BC cell proliferation.

This is one of the few studies that bring associations of cholesterol metabolism at the time of diagnosis and clinical staging in BC, allowing further comprehension that cholesterol can be considered a risk factor for the disease. Another strength is the use of plenty of biomarkers in association to tumor characteristics in addition to menopausal status. Therefore, it was possible to identify more susceptible profiles of women in risk to lower survival outcome.

CONCLUSION

In conclusion, the present study showed that premenopausal women diagnosed with BC advanced disease had worse plasma cholesterol profile and this negative profile increased the risk of having larger tumors and oxidative stress, thus leading to a poor survival. Cholesterol-lowering strategies may be useful to modulate BC progression. Therefore, control of plasma cholesterol and its associated risk factors, such as oxidative stress and obesity, by medications or lifestyle changes, could be positive for BC prevention, treatment and survival. Further studies are needed regarding the acute and long-term impact of improving lipid profiles on pre and postmenopausal women diagnosed with BC by randomized clinical trials using lipid-lowering therapy and lifestyle changes.

Supplementary materials:

Figure S1. Kaplan-Meier curve of survival probability in women with breast cancer according to metabolic profile, lipid profile and oxidative stress oxidative stress parameters.

Figure S2. Kaplan-Meier curve of survival probability in premenopausal women with breast cancer according to metabolic profile, lipid profile and oxidative stress oxidative stress parameters.

Figure S3. Kaplan-Meier curve of survival probability in women with breast cancer according to metabolic profile, lipid profile and oxidative stress oxidative stress parameters.

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Informed Consent Statement: All participants provided an informed written consent to participate. The study was performed in accordance with the Declaration of Helsinki.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, [NRTD], upon reasonable request.

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Table 1. Characteristics of pre and postmenopausal women according to the clinical staging (CS).

Variables	Premenopausal group				Postmenopausal group				
	Total (n=43)	**CS I (n=12)**	**CS II - III (n=31)**	***p* value***	**Total (n=39)**	**CS I (n= 16)**	**CS II - III (n=23)**	***p* value***	***p* value****
Age (years)	42.6 (5.5)	43.7 (4.3)	42.1 (6.0)	0.387	59.1 (9.9)	62.2 (11.1)	56.9 (8.4)	0.096	**<0.001**
Smoker (n, %)	12 (27.9)	5 (41.7)	7 (22.6)	0.265	21 (53.8)	7 (43.8)	14 (66.7)	0.342	**0.017**
Alcohol intake (n, %)	22 (51.2)	7 (58.3)	15 (48.4)	0.736	17 (43.6)	6 (37.5)	11 (47.8)	0.743	0.493
Breastfeeding (n, %)	29 (67.4)	8 (66.7)	21 (67.7)	> 0.999	26 (66.7)	9 (56.2)	17 (73.9)	0.493	0.226
Weight (kg)	68.9 (10.2)	69.5 (11.4)	68.6 (9.9)	0.800	66.9 (11.1)	68.1 (9.3)	66.1 (12.3)	0.586	0.409
BMI (kg/m^2)	28.0 (3.8)	28.4 (4.4)	27.8 (3.5)	0.634	28.2 (5.2)	27.5 (3.1)	28.8 (6.2)	0.457	0.785
WC (cm)	95.6 (9.4)	97.5 (11.9)	94.9 (8.5)	0.446	97.9 (10.8)	97.8 (7.5)	98.0 (12.5)	0.958	0.317
Lean mass (%)	65.4 (4.5)	66.3 (4.5)	65.0 (4.5)	0.429	63.7 (4.7)	67.7 (3.1)	63.1 (5.6)	0.287	0.112
Fat mass (%)	34.6 (4.5)	33.7 (4.6)	34.9 (4.5)	0.433	36.3 (4.7)	35.3 (3.1)	36.9 (5.6)	0.287	0.110
Phase angle (^{o})	6.5 (1.0)	6.4 (0.6)	6.5 (1.0)	0.551	6.3 (0.9)	6.4 (1.1)	6.2 (0.8)	0.544	0.256

BMI: Body Mass Intake; WC: Waist circumference. Comparisons between quantitative variables were performed using Student's *t* test or the Mann-Whitney test. according to normality and cut off point ($p<0.05$).*p* value*: Difference between CS I and CS II-III; *p* value**: Difference between premenopausal and postmenopausal groups.

Table 2. Tumor characteristics of pre and postmenopausal women according to the clinical staging (CS).

Variables	Premenopausal group				Postmenopausal group				
	Total (n = 43)	**CS I (n=12)**	**CS II - III (n=31)**	***p* value***	**Total (n = 39)**	**CS I (n= 16)**	**CS II - III (n=23)**	***p* value***	***p* value****
Tumor size (cm)	2.7 (1.7)	1.6 (0.6)	3.2 (1.8)	**0.016**	2.3 (2.0)	1.5 (0.4)	2.9 (2.5)	0.073	0.380
ER+ (n, %)	28 (65.1)	8 (66.6)	20 (64.5)	0.894	25 (64.1)	11 (68.7)	14 (60.9)	0.614	0.924
PR+ (n, %)	27 (79.4)	8 (80.0)	19 (79.2)	> 0.956	25 (83.3)	11 (84.6)	14 (82.3)	0.869	0.688
HER2+ (n, %)	4 (12.1)	2 (20.0)	2 (8.7)	0.361	3 (11.1)	1 (9.1)	2 (12.5)	0.782	0.903
Triple negative (n, %)	4 (12.1)	1 (11.1)	3 (12.5)	0.815	2 (7.4)	0 (0.0)	2 (13.3)	0.238	0.828

ER: Estrogen receptor; PR: Progesterone receptor; HER2: Human Epidermal Growth Factor Receptor type 2.Comparisons between quantitative variables were performed using Student's *t* test or the Mann-Whitney test according to normality and cut off point ($p<0.05$).*p* value*: Difference between CS I and CS II-III; *p* value**: Difference between premenopausal and postmenopausal groups.

Table 3. Biochemical parameters of pre and postmenopause women according to the clinical staging (CS)

Variables	Premenopausal group				Postmenopausal group				
	Total (n=43)	**CS I (n=12)**	**CS II - III (n=31)**	***p* value***	**Total (n=39)**	**CS I (n= 16)**	**CS II - III (n=23)**	***p* value***	***p* value****
Glucose (mg/dL)	89.6 (16.8)	91.2 (22.2)	89.0 (14.5)	0.705	97.8 (13.2)	98.0 (16.2)	97.7 (11.0)	0.955	**0.016**
HbA1c (%)	5.6 (0.6)	5.6 (0.5)	5.6 (0.6)	> 0.999	6.1 (0.9)	6.3 (0.8)	6.0 (0.9)	0.374	**0.015**
Total cholesterol (mg/dL)	177.2 (31.1)	162.5 (19.1)	183.4 (33.3)	0.050	179.9 (40.0)	190.3 (43.8)	172.0 (36.0)	0.174	0.734
LDL-c (mg/dL)	122.4 (29.8)	105.0 (17.5)	129.9 (31.0)	**0.014**	119.8 (38.0)	127.7 (43.6)	113.8 (33.0)	0.276	0.738
HDL-c (mg/dL)	33.6 (8.4)	37.7 (9.0)	31.8 (7.8)	**0.044**	34.9 (10.1)	36.2 (10.6)	33.9 (9.8)	0.489	0.540
Triacylglycerols (mg/dL)	100.3 (75.2-113.7)	87.5 (74.8-101.0)	107.0 (76.8-124.2)	0.475	114.5 (84.5-127.0)	114.5 (84.8-133.3)	114.5 (83.3-132.3)	0.859	> 0.999
Non-HDL-c (mg/dL)	143.6 (33.2)	124.9 (19.2)	151.6 (34.9)	**0.017**	144.8 (38.4)	154.1 (42.8)	137.7 (34.0)	0.203	0.882
Apo A-I (mg/dL)	115.3 (15.3)	121.0 (13.1)	112.9 (15.7)	0.123	116.5 (27.8)	114.7 (32.7)	117.7 (23.2)	0.750	0.827
Apo B (mg/dL)	100.5 (19.9)	89.6 (15.7)	105.1 (19.9)	**0.022**	105.1 (31.5)	114.5 (32.7)	98.3 (29.6)	0.132	0.457
NEFA (mmol/L)	0.6 (0.3)	0.6 (0.3)	0.6 (0.2)	0.419	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	0.664	0.119
TBARS (µmol/mL)	6.2 (1.8)	5.9 (1.0)	6.3 (2.0)	0.429	5.7 (1.1)	5.5 (1.2)	5.8 (1.0)	0.386	0.132
8-OH-dG (ng/mL)	17.3 (6.1)	16.3 (5.7)	17.7 (6.3)	0.522	18.3 (4.5)	18.1 (3.7)	18.5 (5.1)	0.794	0.457
Leptin (ng/mL)	29.9 (15.9)	25.4 (15.2)	32.0 (16.0)	0.258	31.7 (21.8)	25.7 (23.0)	35.9 (20.6)	0.219	0.707
Adiponectin (µg/mL)	9.0 (5.8)	12.1 (7.7)	7.7 (4.2)	**0.023**	8.8 (5.4)	9.3 (5.5)	8.4 (5.4)	0.637	0.853

HbA1c: Glycated hemoglobin; LDL-c: Low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol; Non-HDL-c: Non-High-density lipoprotein cholesterol; Apo A-I: Apolipoprotein A-I; Apo B: Apolipoprotein B; NEFA: Non-esterified fatty acids; TBARS: Thiobarbituric acid reactive substances; 8-OH-dG: 8-OH-2'-deoxyguanosine.Comparisons between quantitative variables were performed using Student's *t* test or the Mann-Whitney test. according to normality and cut off point ($p<0.05$).*p* value*: Difference between CS I and CS II-III; *p* value**: Difference between premenopausal and postmenopausal groups.

Table 4. Logistic regression models according to tumor size.

Variables	Model 1					Model 2				
	β	**SE**	**Wald**	**OR**	**95% CI**	**β**	**SE**	**Wald**	**AOR**	**95% CI**
Total cholesterol (mg/dL)	0.019	0.010	3.189	1.019	0.998-1.040	0.020	0.012	3.000	1.020	0.997-1.044
LDL-c (mg/dL)	0.028	0.013	4.884	1.029	**1.003-1.055**	0.028	0.014	4.018	1.028	**1.001-1.057**
HDL-c (mg/dL)	-0.640	0.033	3.835	0.938	0.880-1.000	-0.730	0.035	4.359	0.930	**0.868-0.996**
Triglycerides (mg/dL)	0.003	0.005	-0.327	1.003	0.993-1.012	0.006	0.005	1.289	1.006	0.996-1.017
Non-HDL-c (mg/dL)	0.029	0.012	5.420	1.029	**1.005-1.055**	0.032	0.014	5.149	1.032	**1.004-1.061**

LDL-c: Low-density lipoprotein cholesterol; HDL-c: High-density lipoprotein cholesterol; Non-HDL-c: Non-High-density lipoprotein cholesterol; OR: Odds ratio; AOR: Adjusted odds ratio. Model 1 - OR: without adjustments. Model 2 - AOR: adjusted by menopause, smoking and body mass index.

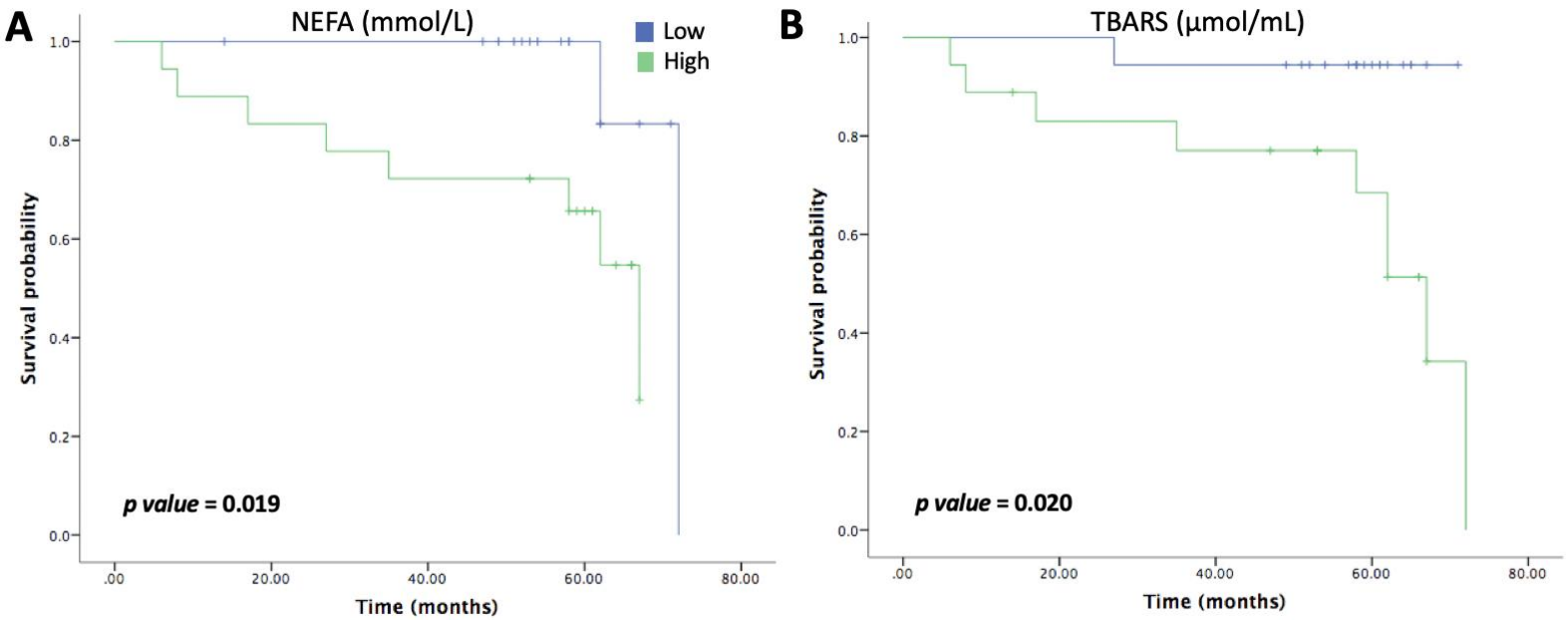

Figure 1. Kaplan-Meier curve of survival probability in premenopause women with breast cancer according to oxidative stress parameters. NEFA: Non-esterified fatty acids; TBARS: Thiobarbituric acid reactive substances. Analysis use median value of variables and cut off point ($p<0.05$).

Supplementary materials

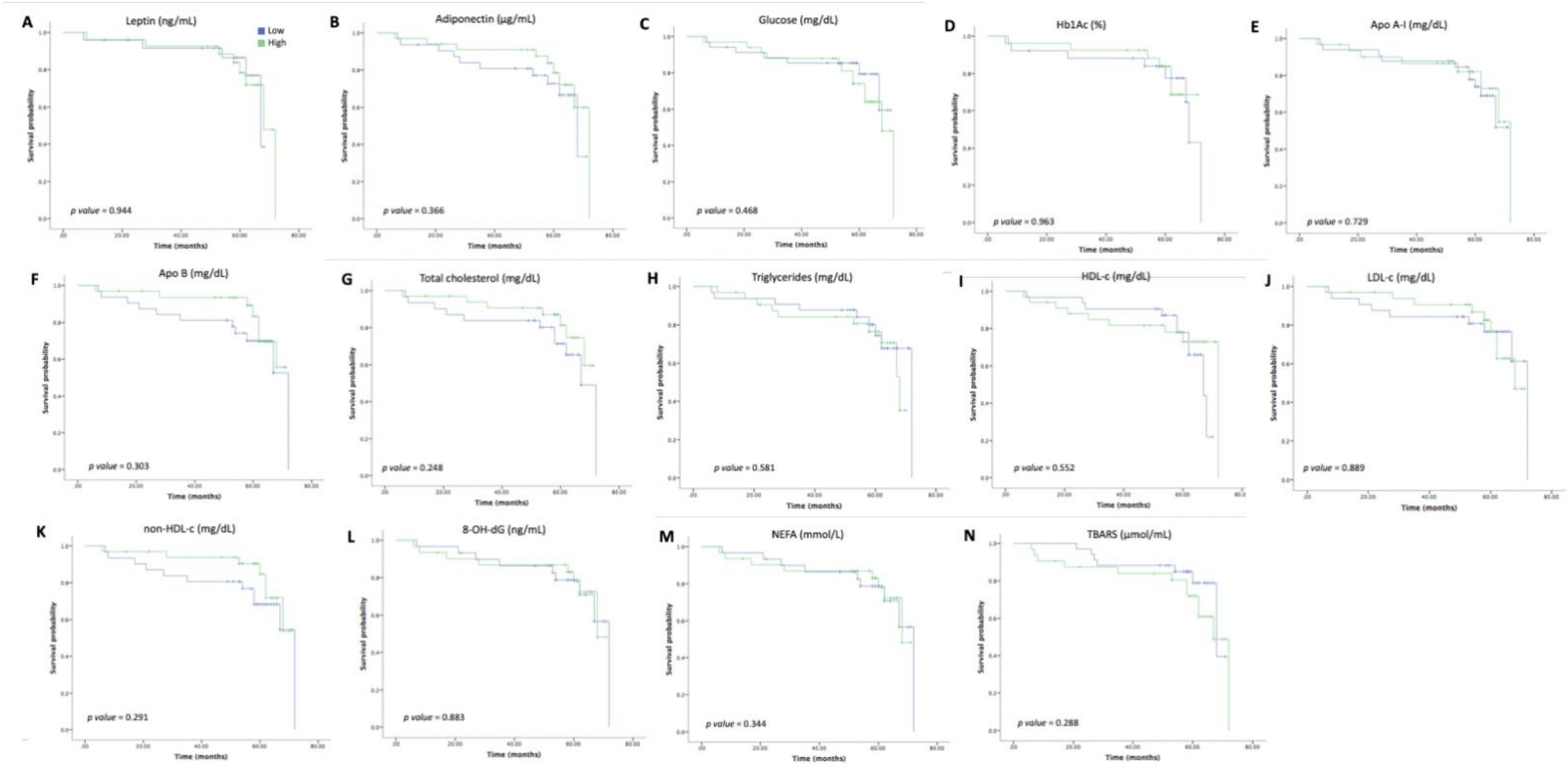

Figure S1. Kaplan-Meier curve of survival probability in women with breast cancer according to metabolic profile, lipid profile and oxidative stress parameters.

HbA1c: Glycated hemoglobin; Apo A-I: Apolipoprotein A-I; Apo B: Apolipoprotein B; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; Non-HDL-c: Non-High-density lipoprotein cholesterol; 8-OH-dG: 8-OH-2'-deoxyguanosine; NEFA: Non-esterified fatty acids; TBARS: Thiobarbituric acid reactive substances. Analysis use median value of variables and cut off point ($p<0.05$)

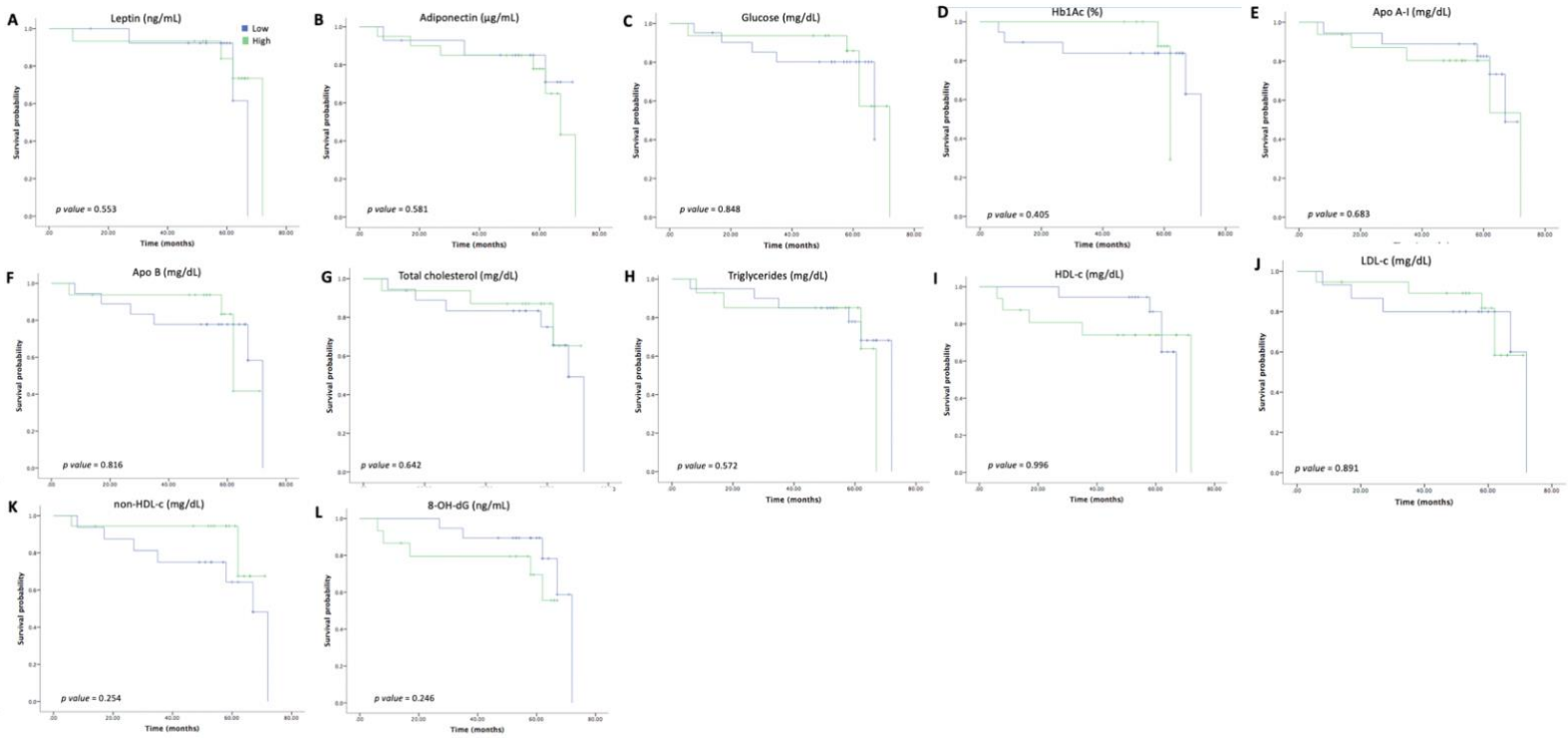

Figure S-2. Kaplan-Meier curve of survival probability in premenopausal women with breast cancer according to metabolic profile, lipid profile and oxidative stress parameters.

HbA1c: Glycated hemoglobin; Apo A-I: Apolipoprotein A-I; Apo B: Apolipoprotein B; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; Non-HDL-c: Non-High-density lipoprotein cholesterol; 8-OH-dG: 8-OH-2'-deoxyguanosine. Analysis use median value of variables and cut off point ($p<0.05$).

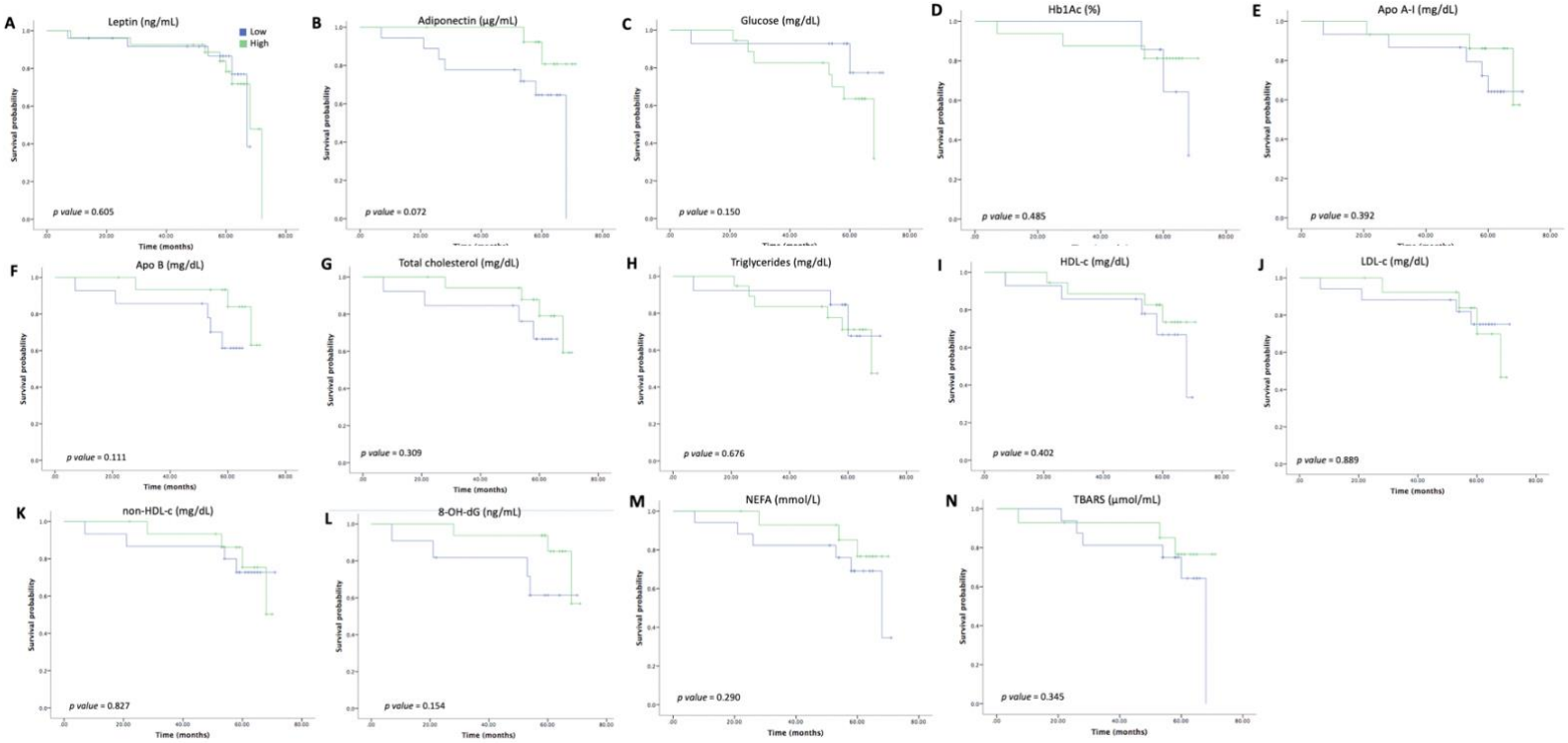

Figure S3. Kaplan-Meier curve of survival probability in postmenopausal women with breast cancer according to metabolic profile, lipid profile and oxidative stress oxidative stress parameters.

HbA1c: Glycated hemoglobin; Apo A-I: Apolipoprotein A-I; Apo B: Apolipoprotein B; HDL-c: High-density lipoprotein cholesterol; LDL-c: Low-density lipoprotein cholesterol; Non-HDL-c: Non-High-density lipoprotein cholesterol; 8-OH-dG: 8-OH-2'-deoxyguanosine; NEFA: Non-esterified fatty acids; TBARS: Thiobarbituric acid reactive substances. Analysis use median value of variables and cut off point ($p<0.05$).

MANUSCRIPT 2 – Oxidative stress and inflammation increase risk to Luminal A breast cancer independent of menopause and obesity: a case-control study

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Pivotal role of oxidative stress and inflammation in increased risk of luminal A breast cancer independent of menopause and obesity: a case-control study

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ABSTRACT

Purpose: Breast cancer (BC) is the main cause of cancer in women. BC can be stratified into diverse molecular subtypes, and Luminal A has the highest incidence. Estrogen is a risk factor associated with inflammation, oxidative stress, adipokines, insulin, and insulin-like growth factor 1 (IGF-1). We aim to understand the interplay between these factors and the risk of developing this type of BC. **Methods:** The study included 47 women with Luminal A BC and 100 matching controls. Blood samples were collected to analyze IGF-1, IGF-binding protein 3, insulin, glucose, glycated hemoglobin, leptin, adiponectin, thiobarbituric acid reactive substances (TBARS), non-esterified fatty acids, DNA oxidative damage and inflammatory biomarkers. **Results:** Individuals were similar regarding menopause status and both groups were overweight/obese. Women with Luminal A BC had higher levels of TBARS, glucose and IGF-1. Pro-inflammatory markers IL-1**β** and IL6 were higher in the BC group whereas anti-inflammatory marker (IL10) was lower in these patients. High levels of TBARS, glucose and insulin were associated with increase odds of having Luminal A BC (AOR = 3.291; CI95% 1.621 – 6.642; AOR = 6.106; CI95% 2.773 – 13.444; AOR = 3.057; CI95% 1.235 – 7.569, respectively). Increase in IL10 has proven to be a protective factor for Luminal A BC (AOR = 0.263; CI95% 0.102 – 0.683). **Conclusion:** Women diagnosed with Luminal A BC had impaired inflammatory biomarkers, oxidative stress, adiponectin, and alterations in the insulin/IGF axis. An increased risk was observed in women with higher levels of glucose, insulin, and TBARS independently of menopause and BMI.

Keywords: breast cancer, luminal A, risk factor, oxidative stress, inflammation

INTRODUCTION

The increased prevalence of breast cancer (BC) worldwide has led it to being the main cause of cancer and mortality in women[1], therefore it is urgent to understand the risk factors that can contribute to the increased BC incidence. Due to its heterogeneous characteristics, BC can be stratified into diverse categories including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and nuclear protein Ki67 (a proliferation marker) which can determine treatment response, therefore, classifying BC in molecular subtypes is relevant to improve prognosis[2].Nowadays, there are four main types of BC molecular classification: Luminal A, Luminal B, HER2-positive, and Triple negative[3]. Of all types, the luminal A BC (ER+, PR+, HER2- and Ki67 low) presents the highest incidence[4].

Estrogen is a risk factor for BC due to its binding to ER that activates transcription factors in the nucleus, stimulating the development and growth of tumor cells and it has an impact on the tumor microenvironment[5, 6]. ER also interacts with growth cell receptors, enhancing proliferation and survival of the tumor, and women with Luminal A BC have a high prevalence of *PIK3CA* mutation[6]. This mutation increases PIK3C/Akt/mTOR signaling pathway, responsible for cell metabolism, growth, proliferation, apoptosis, and angiogenesis in tumor cells. The main natural ligands of this pathway are insulin-like growth factor (IGF), insulin, and estrogen[7].

In parallel, obesity is a clear risk factor for developing BC and the increase of adipose tissue is linked to a positive stimulus for estrogen circulation[8]. In a previous report, it was found that women with obesity and Luminal A BC had a higher mortality[5]. Obesity is also linked with more tumor aggressiveness associated with the local and systemic, chronic, and low-grade inflammatory process, stimulating inflammatory markers, oxidative stress, unbalance in serum adipokines such as leptin and adiponectin, in addition to the increased levels of insulin and IGF-1[5].

Moreover, estrogen can contribute to the production of reactive oxygen species (ROS) in a mitochondrial-dependent way[9, 10] as confirmed by increased generation of oxidative markers in ER+ BC[11]. High levels of ROS induce increase genomic instability that can drive carcinogenesis and influence transcription factors that can sustain the proliferation of the cell[12]. An increase in ROS can also favor phosphorylation of NF-κB, especially in an obese environment, leading to the expression of pro-inflammatory mediators that increase BC risk[13] and alter tumor cell biology besides facilitating tumorigenesis and metastasis[12], however, the association with Luminal A BC is still unclear.

This study aims to understand the association of the metabolic biomarkers related to glucose metabolism, oxidative stress and inflammation on the risk of women Luminal A BC compared to its matching controls.

MATERIAL AND METHODS

Study design and participants

This is a prospective case-control study that included women referred to the Mastology Clinic of the General Hospital of Fortaleza (Fortaleza, Ceará, Brazil) and matching controls with no report of previous BC or other neoplasia who attended the Gynecologic and Obstetrics Clinic from May 2011 to August 2012 for annual health care. Women who were ≥18 years old and had a recent diagnosis of BC according to anatomopathological analyses, and were classified as Luminal A BC (ER+, PR+, HER2- and Ki67 low) according to immunohistochemical analyses and without previous neo-adjuvant therapies, were selected. Post-menopausal status was considered if women self-reported cessation of menses at least in the previous consecutive 12 months[14]. Patients with uncontrolled chronic noncommunicable diseases, use of weight reduction medications, or psychiatric or neurological disorders were excluded. This study was approved by the Research Ethics Committees of the General Hospital of Fortaleza (nº 050507/10) and the School of Public Health, University of São Paulo (nº 2162). All women provided informed written consent before starting study. The study was performed following the Declaration of Helsinki.

Data collection

Sociodemographic data and risk factors for BC (menopausal status, smoker, alcohol consumption, hormone replacement therapy - HRT, nulliparity, breastfeeding) were obtained from the medical records and direct interviews using a standard form. The anthropometric assessment was performed by a trained researcher. Body weight (kg) was measured using a digital scale (Plenna®, São Paulo, Brazil) and height (m) was measured using a portable stadiometer (TBW®, São Paulo, Brazil). The Body Mass Index (BMI) (kg/m²) was calculated according to the WHO recommendation. Waist circumference (WC) was measured by an inelastic tape. Body composition was determined with tetrapolar electrical bioimpedance (Biodynamics®, model 450 – TWB, São Paulo, Brazil). Blood samples (20mL) were collected after 12-hour fasting in vacutainer tubes containing EDTA (1 mg/mL). Blood was centrifuged for plasma separation (1,500g, 10 minutes, 4ºC) and protease inhibitors (10 µg/mL aprotinin, 10 µM benzamidine, and 5 µM phenylmethylsulphonyl fluoride - PMSF) and 100 µM butylated

hydroxytoluene - BHT antioxidant were added to the plasma. After that, samples were aliquoted and stored at -80 ºC until analysis.

Glucose, glycated hemoglobin, insulin, IGF-1 system, and adipokines

Plasma glucose and glycated hemoglobin were analyzed by colorimetric and enzymatic kits (Glucose PAP Liquiform® and HbA1c Turbiquest®, respectively; Labtest, Minas Gerais, Brazil). The concentration of insulin, IGF-1, and IGFBP-3 were assessed using a human enzyme-linked immunosorbent assay (ELISA) commercial kit (*Insulin ELISA Kit*® - Enzo Life Sciences Farmingdale, *IGF-1 human ELISA Kit*® - Enzo Life Sciences Farmingdale, NY, USA, and *IGFBP3 Simple Step ELISA Kit*® - Abcam, Cambridge, UK). Plasma leptin and adiponectin were also analyzed using a commercial ELISA kit (Leptin Human ELISA Kit® - Enzo Life Sciences Farmingdale, NY, USA, and Adiponectin Human ELISA Kit® - Adipogen, San Diego, CA, USA).

Thiobarbituric acid reactive substances (TBARS)

The plasma lipid peroxidation was performed according to the method proposed by Ohkawa et al. (1979)[15]. Briefly, 50 µL of plasma and 1mL of TBARS solution composed of thiobarbituric acid (0.046 M), trichloroacetic acid (0.92 M) and hydrochloric acid (0.25 M) were mixed. After that, samples were incubated at 100ºC for 30 minutes. The solution was then centrifuged at 8,000g for 15 minutes at 4ºC. The color intensity in the supernatant (200 µL) was monitored at 535 nm in duplicate analysis.

DNA oxidative damage

The oxidative damage was assessed through the detection and quantification of 8-OH-2'-deoxyguanosine (8-OH-dG) in the plasma using an ELISA commercial kit (DNA Damage ELISA kit®, Enzo Life Sciences Farmingdale, NY, USA).

Inflammatory markers

The inflammatory markers were measured using the commercial Human Magnetic Panel Bead Milliplex® MAP kit (HCY T0 MAG-, Merck Millipore®). The selected biomarkers were: IL6, IL1β, TNF-α, MCP-1 and IL10. To detect the intensity of fluorescence each microsphere emits, streptavidin phycoerythrin was used. Luminex 200™ with xMAP® technology and acquisition of xPONENT® was used to detect the fluorescence. To integrate the data analysis MILLIPLEX® Analyst 5.1 software was used.

Statistical Analysis

The Kolmogorov-Smirnov test verified the variables' normality ($p > 0.05$). Descriptive data were expressed as absolute values and frequency or mean followed by the standard deviation (SD). Comparisons between quantitative variables were performed using Student's *t*-test or the Mann-Whitney test, according to normality. For the multivariable logistic regression models, no variable tested presented collinearity.

The model used to estimate the risk of women Luminal A BC according to metabolic parameters adopted as cutoff points was the percentile 50 (p50). The models were adjusted for menopausal status and BMI using the stepwise forward approach to estimate the coefficients of regression (β), standard error (SE), Wald, Odds Ratio (OR), and 95% confidence interval (95%CI). Statistical analyses were performed with the software Statistical Package for Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

RESULTS

One hundred forty-seven women were enrolled in the study (100 controls and 47 Luminal A BC). The mean age in the control group was 48.2 ± 12.9 years and in the Luminal A BC group was 50.2 ± 12.5 years with no difference ($p = 0.275$). Individuals were also similar regarding menopausal status and other BC risk factors (HRT, nulliparity, breastfeeding, smoking and alcohol). Women in both groups were overweight according to BMI (control group= 27.7 ± 4.3 kg/m^2*vs.* BC= 27.7 ± 4.2 kg/m^2; $p = 0.446$) and had similar lean mass and fat mass, but women in Luminal A BC group had higher WC compared to control group (WC= 91.2 ± 10.1 cm*vs.*WC= 95.7 ± 10.2 cm; $p = 0.020$) (**Table 1**).

Regarding oxidative stress parameters, women with Luminal A BC had higher levels of TBARS in comparison to their matching controls ($p < 0.001$). Also, women with Luminal A BC presented an increased level of glucose (control group= 83.0 ± 14.9 mg/dL*vs.* Luminal A BC= 94.1 ± 16.1 mg/dL; $p < 0.001$) and IGF-1 (control group= 178.8 ± 121.5 ng/dL*vs.* Luminal A BC= 267.5 ± 203.1 ng/dL; $p = 0.017$). Similarly, pro-inflammatory markers IL-1**β** and IL6 were higher in Luminal A BC group (control group= 2.0 ± 4.5pg/dL*vs.* BC= 5.0 ± 2.6pg/dL; $p < 0.001$ and control group=1.8 ± 1.3pg/dL*vs.* Luminal A BC=3.3 ± 7.7pg/dL; $p < 0.001$, respectively), but anti-inflammatory marker (IL10) was lower in these patients (control group= 5.5 ± 9.7mg/dL*vs.* Luminal A BC=4.5 ± 9.9 mg/dL; $p < 0.001$) (**Table 2**).

We have also found out that TBARS was associated with increased odds to have Luminal A BC(OR = 3.133; CI95%= 1.581 – 6.210), even after adjustment for menopausal status (AOR = 3.292; CI95% = 1.643 – 6.594), menopause and BMI (AOR = 3.291; CI95% = 1.621 – 6.642). Similarly, a significant association with increased risk of Luminal A BC was found with glucose (> 88.0 mg/dL) and insulin (> 5.69 µUI/mL) in the unadjusted model and after adjustment for menopausal status and BMI (AOR = 6.106; CI95%= 2.773 – 13.444 and AOR= 3.057; CI95%= 1.235 – 7.569, respectively).

On the other hand, adiponectin has proven to be protective on Luminal A BC in the unadjusted model (OR = 0.469; CI95% = 0.238 – 0.925) and after adjustment for menopause (OR = 0.479; CI95% = 0.242 – 0.947), however, when BMI was added this association was lost (OR = 0.510; CI95% = 0.254 – 1.025). Lastly, higher levels of IL10 were related to reduced risk for Luminal A BC after simultaneous adjustment for menopausal status and BMI (AOR = 0.263; CI95% = 0.102 – 0.683).

DISCUSSION

In this study, we demonstrated that an increase in glucose, insulin, and advanced oxidative products (TBARS) promoted a significant increase in risk for women with Luminal A BC, while IL10 was associated with a protection factor, both independently of menopausal status and BMI. Additionally, these women, when compared to their matching controls showed higher serum levels of oxidative and inflammatory biomarkers in spite of the similar menopausal status and BMI.

Many previous studies described the negative impact of excess weight on the incidence, recurrence, and survival of BC women[16–18]. Here, by applying a more conservative cut-off point for BMI (> 24.9 kg/m^2), considered adequate for healthy women, we were able to identify associations with unbalanced oxidative stress, glucose, and inflammatory markers. This profile suggests that women Luminal A BC can be more susceptible to the negative role of excess weight. Additionally, in both groups, women were classified as overweight, but women in the Luminal A BC presented a higher level of adiposity due to higher WC (91.2 ± 10.1 cm *vs.* 95.7 ± 10.2 cm; p = 0.020). This profile signals the relevance of monitoring adiposity over isolated weight or BMI.

BC lies in a complex microenvironment of cells, in which fibroblasts, adipose tissue, inflammatory and immune cells, and endothelial cells act in a complex network that produces several activated molecules (hormones, growth factors, cytokines). During obesity, high levels of plasma free fatty acids can contribute to an increase in inflammatory biomarkers due to its role on the activation of toll-like receptor 4 (TLR4) and NF-κB/TNF-α pathway, creating an inflammatory environment that contributes to apoptosis and consequent increase in crown-like structure (CLS) and pro-inflammatory cytokine signaling[19]. Together, these alterations induce as well as maintain tumorigenesis, sustain proliferation, promoting drug resistance, and metastasis[20, 21].

BC is associated with a high level of serum leptin[22] and its receptor is shown to increase in BC cells, which can be related to distant metastasis and opposite to the peripheral leptin resistance[23]. In the study by Catalano et al.[24] MCF-7 cell lines leptin induced ER functional activation. On the other hand, adiponectin can stimulate the

AMPK pathway and have an anti-BC effect by regulating the PI3K/Akt pathway and decrease its proliferative effect on the cell[25].Similarly to our findings, Korner et al.[26] observed a decrease in the risk of developing BC in women with high adiponectin levels, and corroborating this finding a recent meta-analysis concluded that adiponectin is inversely associated with BC risk[27].In fact, women in our study showed similar prevalence of overweight, contributing to no significant differences in leptin and adiponectin.

Furthermore, in our study, we demonstrated that women with Luminal A BC presented an increase in inflammatory cytokines (IL1β and IL6) and a decrease in IL10. High inflammatory biomarkers are associated with reduced overall survival of BC women[28]. Oh et al.[29] also described hat IL1β induces IL6 production in MCF-7 cells through NF-κB and PI3K-dependent way, increasing the tumor aggressiveness. . Higher levels of IL6 were observed in women with ER+ compared with ER- BC[30]. On the other hand, IL10, a cytokine that can inhibit the production of inflammatory cytokines, MCP1, and NF-κB[31], has shown to reduce TNF-α stimulation and decrease regulation of aromatase expression in adipose tissue of breast stroma[32]. In a Murine model, IL10 induced a protective and anti-tumor immune response mediated by stimulation of cytotoxic immune cells (NK and $CD8^+$ T cell)[33] , and low levels of IL10 in the tumor and microenvironment were associated with an increased risk of recurrence and metastasis[34]. Some reports also discuss the pro-tumorigenic effect of IL10 by decreasing microenvironment immunosurveillance due to its decrease in cytokine[31], which was demonstrated in an *in vivo* study of knockout mice that had an increase in tumor rejection[35] and in a study that associated increased IL10 with BC in women[36], whereas a study by Matkowski et al.[37] found no difference in the IL10 expression in BC tumors and normal tissue. Our findings, however, corroborate with a protective role of IL10 in women diagnosed with Luminal A BC.

In previous studies, estrogen has been linked to inducing breast carcinogenesis due to an increase in ROS by direct and independent cell damage[38, 39]. The increment of ER-estrogen complex in cell surface can stimulate DNA damage by ROS and create replication stress accumulating errors that overwhelm the capacity of DNA repair, stimulating growth by increasing cellular respiration and oxidative phosphorylation in a mitochondrial-dependent way[9, 21].In fact, comparing ER- patients with women with ER+ BC, the former had higher ROS[30]. In a MCF-7 cell model, estrogen was responsible for increasing oxidative stress and DNA damage as well as decreasing the antioxidant capacity[40]. Also, the oxidative stress found in this type of cell was associated with a

more aggressive cancer[41]. Supporting the mechanism discussed by Okoh et al.[12] , our study demonstrates that women with Luminal A BC had a higher level of TBARS increasing three times the risk in this type of BC.

The excess of circulating estrogen can increase growth hormones such as IGF-1 due to a cross-talk of these biomarkers and ER[42–44], impacting negatively the BC risk, especially ER+ subtype[45]. Metformin, a glucose-lowering medication, inhibited the growth of estradiol-induced cells by activating the AMPK pathway[46].In line with our results, Haseen et al.[47] found out that BC women presented higher serum glucose, whereas Medina et al.[48] described that ZR-75 human BC cells (ER+, PR+) in a glucose-rich environment had an increase in GLUT expression, favoring conditions for cell proliferation. Hyperglycemia is considered a significant risk factor for BC[49], in women without diabetes[50] inclusive. Additionally, previous studies described the proliferative role of insulin and its negative effect on Luminal A BC, especially in postmenopausal women[51, 52].

This study presents an important stratification of a BC subtype regarding the possible metabolic alterations that this cancer subtype presents as to its matching controls as well as menopause status and BMI, allowing us to comprehend mechanisms that were described previously in *in vitro* and *in vivo* models. A relevant limitation of the study is the sample size and the absence of other BC subtypes. Further studies are needed to fully understand the role of these main metabolic alterations in the serum and its relationship with microenvironment and tumor cells in different BC subtypes. This issue helps us to better understand the disease development and prognosis.

In conclusion, the present study demonstrated that women diagnosed with Luminal A BC had increased serum levels of serum glucose, IGF-1, TBARS, IL1β, IL6, and a decrease in IL10 when compared to their matching controls. Regarding the risk of developing Luminal A BC, we observed that independently of menopause and BMI higher levels of glucose, insulin, and TBARS represented an increase in the risk of developing this type of BC, and higher levels of IL10 were associated with a decreased risk, possibly due to its particular subtype. Finally, we speculate that the serum estrogen and ER expression in the cell surface create an environment to breast carcinogenesis by stimulus of oxidative and inflammatory pathways. Although, here, this process occurred regardless of menopause status and BMI, it is plausible that in presence of obesity, Luminal A BC has a worsening prognosis.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author, [NRTD], upon reasonable request.

Ethics approval: This study was carried out in line with the principles of the Declaration of Helsinki. Approval was granted by the Research Ethics Committees of the General Hospital of Fortaleza (Nº. 050507/10) and the School of Public Health, University of São Paulo (Nº. 2162).

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Table 1. Characteristics of control and women with Luminal A breast cancer groups.

Variables	Control	Luminal A Breast Cancer	*p-value*
	n = 100	**n = 47**	
Age, years	48.2 (12.9)	50.2 (12.5)	0.275
Menopause, yes	58 (58.0)	23 (48.9)	0.197
HRT, yes	6 (6.0)	7 (14.9)	0.075
Nullyparity, yes	21 (21.0)	11 (23.4)	0.448
Breastfeeding, yes	63 (80.7)	32 (88.9)	0.211
Smoker, yes	35 (35.0)	17 (36.2)	0.516
Alcohol, yes	45 (45.0)	19 (40.4)	0.367
Weight, kg	65.7 (11.1)	66.9 (10.2)	0.524
BMI, kg/m^2	27.7 (4.3)	27.7 (4.2)	0.446
WC, cm	91.2 (10.1)	95.7 (10.2)	**0.020**
Lean mass, %	65.5 (4.7)	65.2 (4.1)	0.756
Fat mass, %	34.5 (4.7)	34.7 (4.1)	0.748

HRT: Hormone replacement therapy; BMI: Body Mass Intake; WC: Waist circumference. Comparisons between quantitative variables were performed using Student's t test or the Mann-Whitney test. For qualitative variables we used χ^2-test according to normality and cut off point ($p<0.05$).

Table 2. Inflammation, oxidative stress and other metabolic parameters for control and women with breast cancer.

Variables	Control	Breast Cancer Luminal A	*p-value*
	n = 100	**n = 47**	
TBARS, µmol/mL	4.7 (1.1)	5.9 (1.7)	**< 0.001**
8-OHdG, ng/mL	20.2 (28.2)	17.3 (5.2)	0.334
Leptin, ng/mL	29.9 (17.5)	32.0 (19.4)	0.568
Adiponectin, µg/mL	11.7 (6.6)	10.2 (6.3)	0.204
Glucose, mg/dL	83.0 (14.9)	94.1 (16.1)	**< 0.001**
HbA1c, %	5.8 (0.8)	5.7 (0.7)	0.920
IGF-1, ng/mL	178.8 (121.5)	267.5 (203.1)	**0.017**
IGFBP-3, µg/mL	0.3 (0.5)	0.6 (0.7)	0.114
Insulin, µUI/mL	6.1 (1.7)	6.6 (2.9)	0.284
MCP1, pg/mL	295.83	315.7 (117.6)	0.437
TNF-**α**, pg/mL	29.3 (18.2)	73.0 (192.7)	0.887
IL10, pg/mL	5.5 (9.7)	4.5 (9.9)	**<0.001**
IL1**β**, pg/mL	2.0 (4.5)	5.0 (2.6)	**<0.001**
IL6, pg/mL	1.8 (1.3)	3.3 (7.7)	**<0.001**

TBARS: Thiobarbituricacidreactivesubstances; 8-OH-dG: 8-OH-2'-deoxyguanosine; HbA1c: Glycatedhemoglobin; IGF-1: InsulinGrowth Factor 1; IGFBP-3: Insulingrowthfactorbindingprotein 3; MCP1: Monocytechemoattractant protein-1; TNF-**α**: Tumor necrosisfactor**α**; IL-10: Interleukin 10; IL-1**β**:Interleukin 1**β**; IL-6: Interleukin 10. Comparisons between quantitative variables were performed using Student's t test or the Mann-Whitney test, according to normality and cut off point ($p<0.05$).

Table 3. Multivariable logistic regression according to Luminal A breast cancer.

Variables	Model 1	Model 2	Model 3
	OR (CI 95%)	**AOR (CI 95%)**	**AOR (CI 95%)**
TBARS, µmol/mL			
⩽ 4.96	1	1	1
> 4.96	**3.133**	**3.292**	**3.291**
	(1.581 - 6.210)	**(1.643 - 6.594)**	**(1.621 - 6.642)**
Adiponectin, µg/mL			
⩽ 9.67	1	1	1
> 9.67	**0.469**	**0.479**	0.510
	(0.238 - 0.925)	**(0.242 - 0.947)**	(0.254 - 1.025)
Glucose, mg/dL			
⩽ 88.03	1	1	1
> 88.03	**5.188**	**6.511**	**6.106**
	(2.520 - 10.682)	**(2.979 - 14.233)**	**(2.773 - 13.444)**
Insulin, µUI/mL			
⩽ 5.69	1	1	1
> 5.69	**2.917**	**3.022**	**3.057**
	(1.227 - 6.934)	**(1.244 - 7.338)**	**(1.235 - 7.569)**
IL-10, pg/mL			
⩽ 2.30	1	1	1
> 2.30	**0.275**	**0.281**	**0.263**
	(0.108 - 0.700)	**(0.110 - 0.718)**	**(0.102 - 0.683)**

TBARS: Thiobarbituric acid reactive substances. OR: Odds ratio; AOR: Adjusted odds ratio. Model 1 - OR: without adjustments. Model 2 - AOR: adjusted by menopause. Model 3 - AOR adjusted by menopause and body mass index.

MANUSCRIPT 3 – Insulin Growth Factor 1 (IGF-1) as an independent predictor for breast cancer in women: a case-control study

This article will be submitted after approval of this committee.

Insulin-like Growth Factor 1 (IGF-1) as an independent risk factor for breast cancer in women: a case-control study

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ABSTRACT

Introduction: Breast cancer (BC) is the most common type of cancer worldwide. Obesity as a complex disease promotes metabolic dysfunction in insulin and insulin-like growth factor 1 (IGF-1), but their isolated and synergistic roles in BC are not fully understood. The aim of this study was to investigate alterations of the insulin/IGF-1 axis in women with BC and the role of overweight/obesity. **Methods:** The study included 114 women with suspicious breast lesions and 100 matching controls between May 2011 and August 2012. Data were obtained from medical records, interviews, and anthropometric measures. Blood samples were collected to determine IGF-1, IGF-binding protein 3 (IGFBP-3), insulin, glucose, glycated hemoglobin (HbA1c), leptin and adiponectin. Categorical data were compared using the chi-square test and differences in quantitative variables were analyzed using Student's *t*-test or the Mann-Whitney test, according to normality. Univariate logistic regression models were developed to estimate associations between women with and without BC, and multivariate logistic regressions were performed using a stepwise forward approach. **Results:** Women with BC had lower serum adiponectin (7.0 ± 5.2 *vs.* 11.7 ± 6.6 µg/mL; *p-value*: 0.001), and higher glucose (94.1 ± 16.3 *vs.* 83.0 ± 14.9 mg/dL; *p-value*: < 0.001), IGF-1 (242.5 ± 160.2 *vs.* 178.8 ± 121.5 ng/mL; *p-value*: 0.002), IGFBP-3 (0.7 ± 0.7 *vs.* 0.3 ± 0.5 µg/mL; *p-value*: 0.025) and insulin (7.4 ± 4.0 *vs.* 6.1 ± 1.7 µUI/mL; *p-value*: 0.026) relative to matching controls. Women with BC classified as overweight/obese and with higher WC had significantly higher IGF-1 and IGFBP-3. Also, higher glucose, insulin and IGF-1 were associated with an increased risk of developing BC, regardless of WC and menopause status. **Conclusion:** Women diagnosed with BC had an increased insulin/IGF-1 axis and overweight/obese subjects had sustained high IGF-1 and IGFBP-3. The increased risk of developing BC associated with higher glucose, insulin and IGF-1 persisted even after adjustment. These factors should therefore be considered when assessing risk of BC in women, especially in overweight and obese individuals.

Keywords: breast cancer, insulin-like growth factor, insulin, obesity, risk factor

INTRODUCTION

Breast cancer (BC) is the most common type of cancer worldwide, with an estimated 2.3 million new cases annually, and constitutes the leading cause of death in women[1]. As a multifactorial disease, only 5-10% of patients have a genetic predisposition for developing BC[2]. The disease has numerous known risk factors, such as age, menopausal status, breastfeeding, use of hormonal replacement therapy (HRT) and lifestyle[3] , all of which are considered important variables contributing to the risk of developing BC[4]. Other variables are also being studied, with obesity and metabolic dysfunctions, such as type 2 diabetes mellitus (T2DM), now also recognized as important risk factors[5]. Women with grade I and grade II-III obesity exhibited a 56% and 82% higher risk of developing BC, respectively, compared to normal weight women[6]. In light of this new evidence, it is important to seek novel biomarkers potentially associated with obesity to help predict the risk of BC and prevent future cases.

Serum levels of growth factor molecules, such as insulin and insulin-like growth factor 1 (IGF-1), tend to be elevated in overweight and obese women. Insulin and IGF-1 represent an important metabolic axis and are known to play an important role in mammary tissue and BC[5] given their involvement in malignant transformation of the tissue and maintenance of its malignant phenotype – characteristics that can lead to a poorer prognosis for patients with BC[8]. On the other hand, a congenital deficiency of IGF-1 has been shown to decrease the risk of developing cancer[9,10]. A non-linear relationship between BMI, waist circumference (WC) and IGF-1 has been observed in healthy women[7].

When binding to its receptor, IGF-1 and insulin promote metabolic signaling stimulating the phosphatidylinositol-3-kinase (PI3K)/AKT pathway and activating the RAS/RAF/MAPK/ERK cascade, predominantly promoting breast tumor progression by sustaining proliferation, survival and migration[11]. However, the stimulus of the mitogenic pathway, regulating cell growth, differentiation and apoptosis function of the IGF-1 molecule, depends on the fine balance of IGF-binding protein (IGFBP), responsible for maintaining molecule stability and modulation of its receptor (IGF-1R)[11]. Also, IGF-1 can be produced in breast tissue and have an autocrine or paracrine role[12]. Regarding the multiple roles of IGF-1 in BC, Chong et al. (2006)[13] and Voskuil et al. (2004)[14] found increased synthesis of IGF-1 in healthy tissue adjacent to tumor tissue in the breast, confirming the essential role of the tumor microenvironment for cell proliferation.

Obesity promotes disruption in the insulin/IGF-1 axis and a collaborative study has confirmed this connection, showing that even women who were only slightly

overweight (body mass index – BMI – of 25.0-27.0 kg/m^2) had a higher level of circulating IGF-1 and increased risk for BC[15]. Alterations in the balance of adipokines, such as leptin and adiponectin, can partially explain the disruption in IGF-1 metabolism. Mauro et al. (2015)[16] demonstrated that a low concentration of adiponectin can increase phosphorylation in IGF-1R, especially in estrogen-receptor positive (ER+) BC, sustaining tumorigenesis. In a recent study by Houghton et al.(2021)[17], central adiposity, as measured by waist circumference (WC), was a risk factor for BC in pre and post-menopausal women, reinforcing the potential relationship of obesity and growth factors in increasing BC risk.

Therefore, the primary objective of the present study was to investigate an altered insulin/IGF-1 axis in women with BC and the role of overweight/obesity. Also, the hypothesis that an imbalanced axis is a risk factor for developing BC was explored.

METHODS

Study population

The study included women with suspicious breast lesions who were referred to the Mastology Clinic of the General Hospital of Fortaleza (Ceará, Brazil) and matching controls from the Gynecologic and Obstetrics Clinic treated between May 2011 and August 2012. Women aged ≥ 18 years with recent diagnosis of BC according to anatomopathological analysis, clinical staging (CS) of I to III, without metastasis, other previous neoplasms or neo-adjuvant therapies. The CS was performed by a physician using the AJCC (8th Edition)[18] criteria. A BC diagnosis was established for 114 cases after further examination. A total of 15 patients had confirmed or suspicion of metastasis, while 2 had in situ tumors, and were subsequently excluded. There were 100 matching controls with no history of previous BC or other neoplasms. Patients with uncontrolled chronic non-communicable diseases, in use of weight reduction medications, or with psychiatric or neurological disorders were excluded. This study was approved by the Research Ethics Committees of the General Hospital of Fortaleza (nº 050507/10) and the School of Public Health of the University of São Paulo (nº 2162). All participants signed an informed written consent form. The study was performed in accordance with the Declaration of Helsinki.

Data Collection

Sociodemographic (age, race and education) data, risk factors (menopausal status, smoker, alcohol consumption, hormone replacement therapy - HRT, nulliparity, breastfeeding) and tumor characteristics (subtype, clinical staging, tumor size, lymph nodes and receptor status) were obtained from medical charts and by direct interview

using a standard form. Menopause was defined as cessation of 12 consecutive months of amenorrhea due to natural loss of follicular activity19. Anthropometric assessment was performed by a trained researcher. Body weight (kg) was measured using digital scales (Plenna®, São Paulo, Brazil) and height (m) was measured using a portable stadiometer (TBW®, São Paulo, Brazil). Body Mass Index (BMI) (kg/m²) was calculated according to WHO recommendations. Waist circumference (WC) was measured using an inelastic tape.

Biomarker Assessment

Blood samples (20 mL) were collected after a 12-hour fast in vacutainer tubes containing EDTA (1 mg/mL). Blood was centrifuged for plasma separation (1,500 g, 10 min, 4ºC) and protease inhibitors were added to the plasma (aprotinin 2 µg/mL; benzamidine 2mM; phenylmethylsulfonyl fluoride 1mM; and butylated hydroxytoluene 20mM). Samples were then aliquoted and stored at -80ºC until further analysis.

Concentrations of IGF-1, insulin-like growth factor-binding protein 3 (IGFBP-3), insulin, leptin and adiponectin biomarkers were assessed using a human enzyme-linked immunosorbent assay (ELISA) commercial kit (*IGF-1 human ELISA Kit*® - Enzo Life Sciences Farmingdle, NY, USA; *IGFBP3 Simple Step ELISA Kit*® - Abcam, Cambridge, UK; *Insulin ELISA Kit*® - Enzo Life Sciences Farmingdle, NY, USA; *Leptin Human ELISA Kit*® - Enzo Life Sciences Farmingdle, NY, USA; *Adiponectin Human ELISA Kit*® - Adipogen, San Diego, CA, USA, respectively). Glucose was assessed using an enzymatic colorimetric commercial kit (*Glicose PAP Liquiform*® - Labtest, Minas Gerais, Brazil) and glycated hemoglobin (HbA1c) was assessed using an immunoturbidimetric assay (*HbA1c Turbiquest*® - Labtest, Minas Gerais, Brazil). All analyses were performed using an automated Cobas system®.

Statistical Analysis

The normality of the variables was checked using the Kolmogorov-Smirnov test ($p > 0.05$). Descriptive data were expressed as frequency or mean followed by standard deviation (SD). Categorical data were compared using a chi-square test. Comparison of quantitative variables was performed using Student's *t*-test or the Mann-Whitney test, according to normality. Univariate logistic regression models were developed to estimate associations between women with and without BC. Multivariable logistic regressions were performed using a stepwise forward approach to estimate coefficients of regression (β), SE, Wald, Odds Ratio (OR) and 95% confidence interval (CI) with BC status as the dependent variable. In the first model, WC (≥ 88.0 vs < 88.0 cm) was a control factor, whereas in the second model both WC (≤ 88.0 *vs* > 88.0 cm) and menopause (yes *vs* no) were used as control factors. All statistical analyses were performed using the

software Statistical Package for Social Sciences, version 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

RESULTS

The mean age of the BC group was 50.2 (11.3) years and control group was 48.1 (13.0) years, without statistical difference (*p-value* = 0.226) *(*data not shown). There was no difference between groups regarding the sociodemographic variables. Both the BC and control groups had an equal distribution for menopausal status, smoking, HRT, nulliparity and breastfeeding. Also, no difference in BMI status was found between groups, but more than half of the total sample was classified as overweight (25.0 - 29.9 kg/m^2) or obese (≥ 30.0 kg/m^2). Women in the Control group had lower WC than women in the BC group, indicating higher adiposity (91.2 ± 10.1 cm *vs.* 96.5 ± 10.1 cm; *p-value* = 0.001) in the latter. **(Table 1)**.

Regarding BC group characteristics, cases were predominantly ductal subtype and CS II and most had tumor size classified as T1 and no positive disease in lymph nodes. Regarding receptors, 66.6% of cases were positive for estrogen, 81.2% for progesterone and 11.7% for HER2 **(Table 2)**.

The biochemical parameters for both groups are presented in **Table 3.** There were no significant group differences in leptin or HbA1c, but adiponectin was higher in the Control group relative to the BC group (11.7 ± 6.9 µg/mL *vs.* 7.0 ± 5.2 µg/mL; *p-value* = 0.001). Glucose and insulin were higher in the BC group (p-value <0.001 and = 0.026, respectively). The biomarker IGF-1 was significantly higher in the BC group (242.5 ± 160.2 ng/mL *vs.* 178.8 ± 121.5 ng/mL; *p-value* = 0.002), while IGFBP-3 was lower in the Control group (0.7 ± 0.7 µg/mL *vs.* 0.3 ± 0.5 µg/mL; *p-value* 0.025) **(Figure 1)**.

The difference in IGF-1 and IGFBP-3 levels between groups, according to BMI status and WC, is shown In **Figures 2 and 3**, respectively. Women with BC classified as overweight/obese and with higher WC (> 88.0 cm) had higher values for both markers compared to matching controls.

Based on the HbA1c parameter, 9 (11.7%) (out of the 77 women with BC had DM versus 9 (14.3%) out of 63 matching controls (diagnostic criteria HbA1c ≥ 6.5%). According to serum glucose, 1 (1.0%) out of the 97 women with BC had T2DM versus 1 (1.0%) out of 100 controls after applying the cutoff point (serum glucose ≥ 126 mg/dL) (AMERICAN DIABETES ASSOCIATION, 2019) (data not shown).

Further elucidating possible risk factors, higher levels of WC (OR= 3.57; 95% CI= 1.24 – 10.28), glucose (OR= 2.25; 95% CI= 1.16 – 4.35), insulin (OR= 2.83; 95% CI=

1.34 – 5.98) and IGF-1 (OR= 1.52; 95% CI= 1.13 – 2.01) were positively associated with increased risk of BC in the women assessed (**Figure 4**).

In order to better understand the mechanisms linking glucose, insulin and IGF-1 with BC, multivariate regression models were developed **(Table 4)**. The first model shows women with higher insulin and IGF-1 had an increased risk for BC, independently of WC. In the second model, the negative impact of higher serum glucose, insulin and IGF-1 on BC risk was significant and sustained, independently of both WC and menopausal status.

DISCUSSION

The present study showed that the women with BC had a higher serum concentration of molecules in the insulin/IGF-1 axis, increasing risk for BC, regardless of WC and menopause status. In contrast with the study by Monson et al. (2020)[21], the present study detected higher IGF-1 in the BC group compared to the control group (242.5 vs. 178.8 ng/mL; *p-value* 0.002). The strength of the association of IGF-1 with BC risk proved similar to that reported in a pooled analysis of 17 prospective studies (OR= 1.52; 95% CI= 1.13 – 2.01 and OR= 1.28; 95% CI= 1.14 – 1.44, respectively)[15].

The level of plasma IGF-1 can lie in the 150-400 ng/mL range, for a family of six main IGFBP, with the most relevant being IGFBP-3, now recognized as an important factor involved in BC pathogenesis. IGFBP have a higher affinity to IGF receptors and can sequestrate IGF and block its interaction with the receptor[22]. Some authors have also described the IGFBP-3 as a regulator in IGF function, since it can work as a reservoir that unbinds in the microenvironment[23]. According to Probst-Hensch et al. (2010)[24], IGFBP-3 expression was positively associated with high BMI. Among the groups assessed in the present study, an over 2-fold increase in IGFBP-3 concentration (*p-value*, 0.025) was observed, regardless of BMI. This result may correlate with an increased risk of developing BC[21,25]. The underlying mechanism involved remains unclear, but a murine model demonstrated that IGFBP-3 promoted BC growth and progression[26] and that IGFBP-3 in an obesity model promoted by a high-fat diet can increase mammary tumor growth, stimulate expansion of adipose tissue, and impair immune function, promoting a carcinogenic effect[27].

In the context of obesity, dysregulation in adipokines is also observed. In the present study, adiponectin level was found to be lower in the BC group compared with the control group (*p-value* < 0.001). Adiponectin is lowered in obesity and some studies suggest that low adiponectin levels are associated with increased risk for several types of cancers, including BC, and also that low adiponectin can increase the aggressiveness

of BC[28,29]. Furthermore, a crosstalk between the adiponectin receptor and IGF-1R has been investigated, in which low levels of adiponectin can increase phosphorylation of IGF-1R in an ER-positive dependent manner. Other studies have found that this increase in phosphorylation occurs regardless of ER status, representing an important factor for signaling in IGF-1 and for increasing carcinogenesis[16,30]. Although no differences in leptin values were detected among groups, Saxena et al. (2008)[31] found an important crosstalk between IGF-1 and leptin in BC cells, where both sustain an increased phosphorylation signaling in their respective receptors and an increase in migration via activation of epidermal growth factor receptor (EGFR).

Obesity, as evidenced by high BMI status, can correlate with an increase in adipose tissue, and consequently with higher WC[32]. Chang et al. confirmed a correlation of high visceral adiposity with increasing inflammation that can promote an environment that enhances cancer development[33]. In obesity, insulin resistance can occur, leading to hyperinsulinemia. This elevated level of insulin is a risk for developing BC, as seen in the present study, as well as in other investigations. The mechanism is associated with increased progression pathways, higher estradiol availability, and stimulus for angiogenesis[34]. Also, hyperinsulinemia promotes the production of hepatic IGF-1 and a decrease in IGFBP, increasing bioavailability for free IGF-1 to bind with its receptor. Increased adipose tissue and decreased adiponectin also stimulate the IGF-1 pathway, causing an increase in signaling of tumorigenic pathways.

Tin Tin et al. (2021)[35] found that both pre or post-menopausal women had a higher risk of developing BC according to IGF-1 status. This result contrasts with other studies which have observed an important role of mammary estradiol stimulating IGF-1[36]. In the current study, in the presence of high glucose, insulin and IGF-1 serum levels, menopause status was also found to be an important risk factor.

This study has several strengths, including the homogenous control group regarding age, menopausal status and BMI, parameters that are potential confounders in the insulin/IGF-1 axis. However, the number of participants in the present study was relatively small, and so future investigations involving larger samples are warranted. Also, biomarker levels remained within the normal range for both groups, and a single measure for biomarkers can introduce errors. For future studies, understanding the gene polymorphism in the IGF-1 gene is important to ascertain the relevance of this marker in BC risk and its role in different BC subtypes. Also, new therapeutic targets are being studied to include the inhibition of the IGF-1 pathway in BC treatment, especially regarding the inhibition or downregulation of IGF-1R in the tumor and its microenvironment.

CONCLUSION

In summary, the study results show that women diagnosed with BC had an increased insulin/IGF-1 axis and that subjects classified as overweight or obese and with greater WC exhibited sustained higher IGF-1 and IGFBP-3 relative to matching controls. Also, high WC, serum glucose, insulin and IGF-1 were associated with an increased risk of developing BC, where this risk persisted even after adjusting for WC and menopause status. These factors should therefore consider when assessing risk of BC in women, especially in overweight and obese individuals.

List of abbreviations:

BC = Breast Cancer; BMI = Body Mass Index; CI: Confidence Intervals; ER = Estrogen Receptor; HbA1c = Glycated Hemoglobin; HER2 = Human Epidermal Growth Factor Receptor 2; HRT = Hormone Replacement Therapy; IGF-1 = Insulin-like Growth Factor 1; IGF-1R = Insulin-like Growth Factor 1 Receptor; IGFBP-3 = Insulin-like Growth Factor-Binding Protein 3; OR = Odds Ratio; PR = Progesterone Receptor; T2DM = Type 2 Diabetes.

DECLARATIONS

Ethical approval and informed consent

This study was approved by the Research Ethics Committees of the General Hospital of Fortaleza (nº050507/10) and the School of Public Health, University of São Paulo (nº 2162). All participants signed a written informed consent form. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

I.R.N. contributed to the statistical analysis, critical review, and writing of the manuscript. N.R.T.D. and A.C.S.D.B. contributed to the study design, critical review and writing of the manuscript. S.M.M.LV contributed to the study design and data collection.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Characteristics of breast cancer and control groups.

Variables	Breast cancer	Control	*p-value*
Age, years			
≤ 48	51.5	52.0	0.946
> 48	48.5	48.0	
Race			
White	26.3	16.0	0.076
Non-white	73.7	84.0	
Education level, years			
0 - 8	43.4	34.0	0.370
9 - 11	22.2	28.0	
≥ 12	34.3	38.0	
Menopausal status, %			
Pre, Peri	50.5	42.0	0.229
Post	49.5	58.0	
Smoker, %			
Never	59.6	65.0	0.702
Currently	8.1	8.0	
Former	32.3	27.0	
Alcohol consumption, %			
Never	54.5	55.0	0.976
Currently	19.2	20.0	
Former	26.3	25.0	
HRT, %			
Yes	9.1	6.0	0.409
No	90.9	94.0	
Nulliparity, %			
Yes	19.2	21.0	0.750
No	80.8	79.0	
Breastfeeding, %			
Yes	82.5	63.0	0.853
No	17.5	37.0	
BMI, kg/m^2			
≤ 24.9	23.2	33.0	0.220
25.0 - 29.9	44.4	42.0	
≥ 30.0	32.4	25.0	
WC, cm	96.5 (10.1)	91.2 (10.1)	**0.001**

HRT: Hormonal Replacement Therapy; BMI: Body Mass Index; WC: Waist Circumference

Table 2. Tumor characteristics of breast cancer group.

Variables		n	%
Subtype			
	Lobular	15	17.0
	Ductal	73	83.0
Clinical staging			
	I	28	34.1
	II	33	40.2
	III	21	25.7
Tumor size (T)			
	T1	33	42.8
	T2	21	27.3
	T3	9	11.7
	T4	14	18.2
Lymph nodes (N)			
	N0	49	63.6
	N1	27	35.1
	Nx	1	1.3
ER			
	Positive	53	64.6
	Negative	29	35.4
PR			
	Positive	52	81.2
	Negative	12	18.8
HER2			
	Positive	7	11.7
	Negative	53	88.3

ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor 2

Table 3. Biochemical parameters of breast cancer and control groups.

Variables	Breast cancer	Control	*p-value*
Leptin (ng/mL)	30.0 (17.7) 2.5 - 75.8	29.9 (17.5) 2.2 - 78.2	0.967
Adiponectin (µg/mL)	7.0 (5.2) (1.4 - 27.2)	11.7 (6.6) (2.0 - 36.5)	**0.001**
Glucose (dL/mL)	94.1 (16.3) 35.5 - 129.7	83.0 (14.9) 53.4 - 116.5	**<0.001**
HbA1c (%)	6.0 (1.1) 4.7 - 10.9	5.8 (0.8) 4.5 - 8.5	0.243
IGF-1 (ng/mL)	242.5 (160.2) 75.8 - 801.5	178.8 (121.5) 33.6 - 735.8	**0.002**
IGFBP-3 (µg/mL)	0.7 (0.7) 0.02 - 2.8	0.3 (0.5) 0.02 - 2.6	**0.025**
Insulin (µUI/mL)	7.4 (4.0) 2.6 - 25.5	6.1 (1.7) 4.6 - 14.5	**0.026**

HbA1c: Glycated hemoglobin; IGF-1: Insulin-like Growth Factor 1; IGFBP-3: Insulin-like Growth Factor-Binding Protein 3

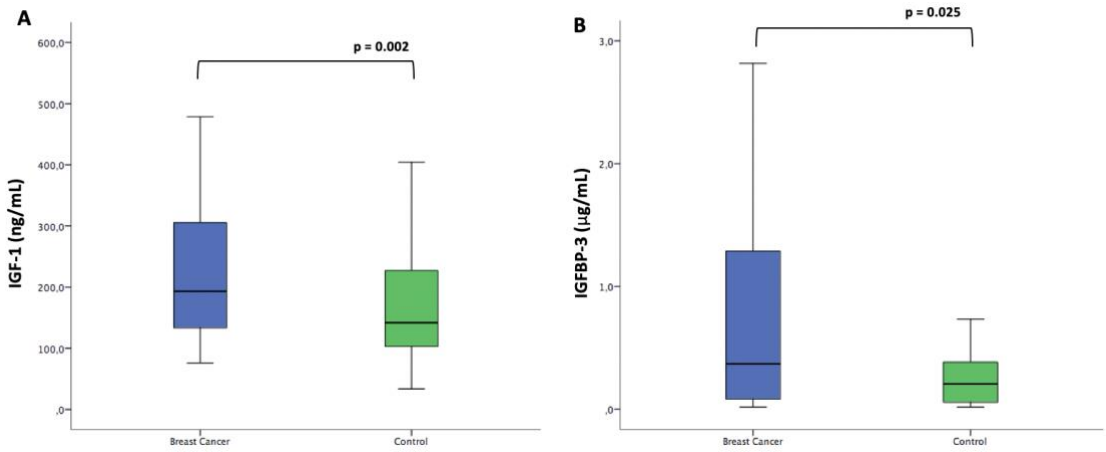

Figure 1. Box plot of IGF-1 and IGFBP-3 of breast cancer and control groups.

IGF-: Insulin-like Growth Factor 1; IGFBP-3: Insulin-like Growth Factor-Binding Protein 3

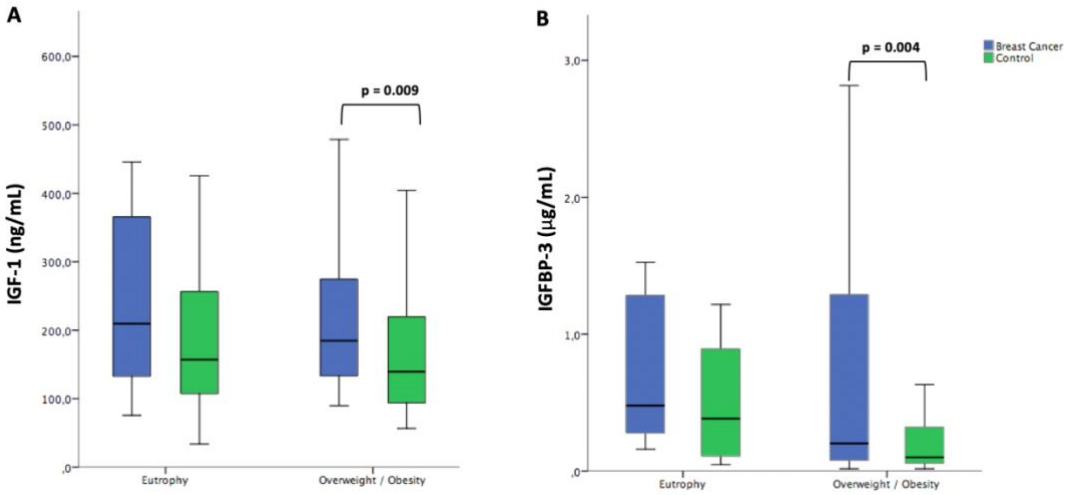

Figure 2. Box plot of IGF-1 and IGFBP-3 of breast cancer and control groups according to BMI.

IGF-1 = Insulin-like Growth Factor 1; IGFBP-3 = Insulin-like Growth Factor-Binding Protein 3

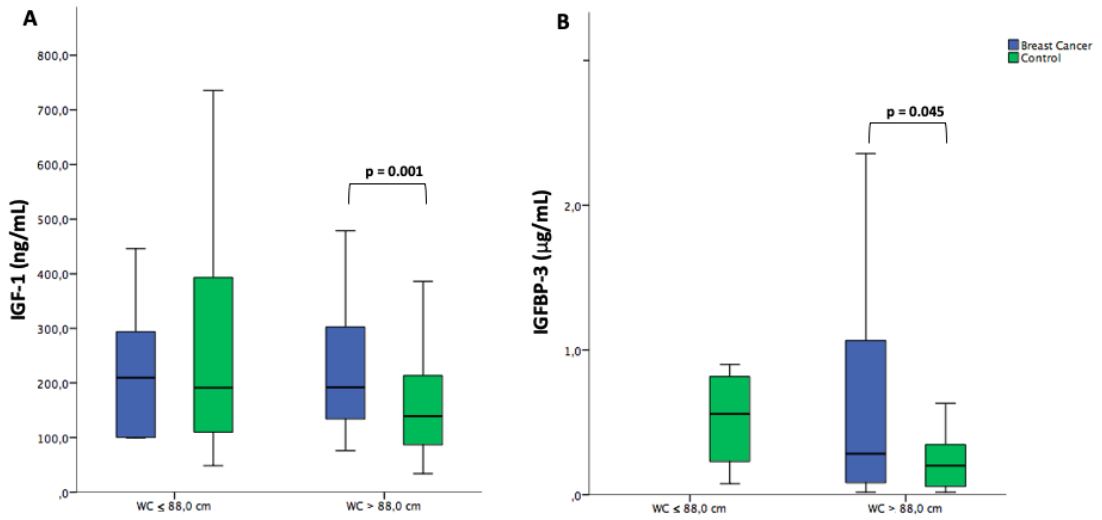

Figure 3. Box plot of IGF-1 and IGFBP-3 of breast cancer and control groups according to WC.

IGF-1 = Insulin-like Growth Factor 1; IGFBP-3 = Insulin-like Growth Factor-Binding Protein 3; WC: Waist Circumference

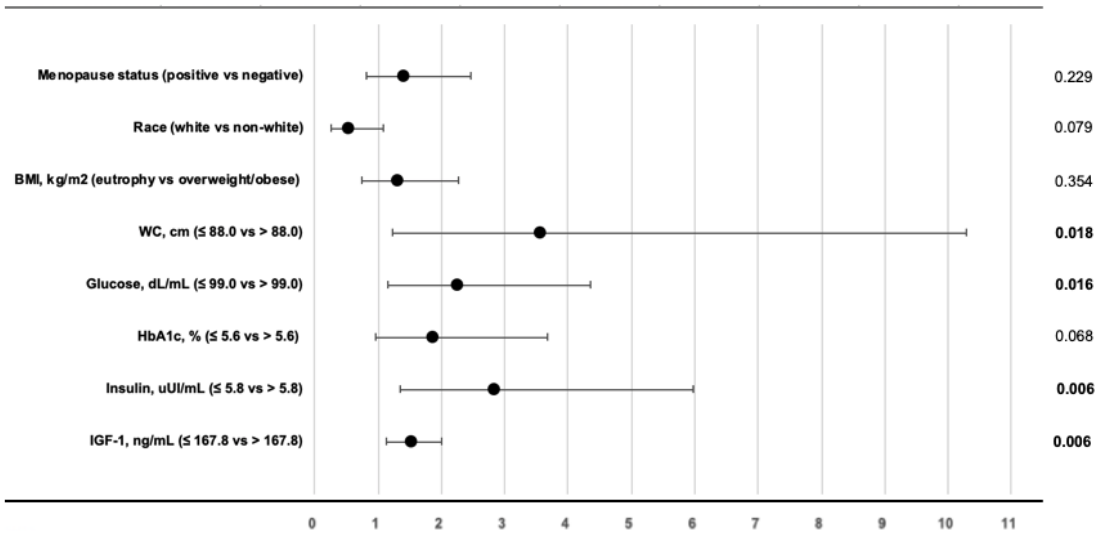

Figure 4. Univariate logistic regression of breast cancer and control groups.

BMI: Body Mass Index; HbA1c = Glycated hemoglobin; WC: Waist Circumference; IGF-1 = Insulin-like Growth Factor 1

Table 4. Multivariate logistic regression of breast cancer group.

Variables	OR	95% CI	*p-value*
Model 1			
Glucose, dL/mL (≤ 99.0 *vs* > 99.0)	1.97	0.99 - 3.98	0.055
Insulin, µUI/mL (≤ 5.8 *vs* > 5.8)	2.54	1.15 - 5.62	**0.021**
IGF-1, ng/mL (≤ 167.8 *vs* > 167.8)	1.53	1.11- 2.10	**0.009**
Model 2			
Glucose, dL/mL (≤ 99.0 *vs* > 99.0)	2.29	1.12 - 4.70	**0.024**
Insulin, µUI/mL (≤ 5.8 *vs* > 5.8)	2.58	1.15 - 5.78	**0.021**
IGF-1, ng/mL (≤ 167.8 *vs* > 167.8)	1.48	1.07 - 2.04	**0.017**

Model 1: Waist circumference (≤ 88.0 *vs* > 88.0 cm); Model 2: Waist circumference (≤ 88.0 *vs* > 88.0 cm) + Menopause (yes *vs* no)
IGF-1 = Insulin-like Growth Factor 1

FINAL CONSIDERATIONS

Due to the increasing incidence of BC worldwide identification and comprehension of new risk factors are relevant to the prevention of new cases and the establishment of more specific treatment, impacting positively on disease-free survival, recurrence, and mortality. In our study, we found that women with BC had alterations in metabolic biomarkers – especially in the IGF-1/insulin axis, lipoproteins, oxidative stress, and inflammation.

For women with BC when compared to their control we found increase serum levels of the IGF-1/insulin axis, especially in women classified as overweight/obese and with higher central adiposity. This axis was considered an independent risk factor for women. Also, we were able to detect these risk factors according to relevant stratification of these women – we observed that premenopausal women had a worse lipoprotein profile at the moment of diagnosis and oxidative stress biomarkers could worse survival outcome. Contrary to our first hypothesis in which obesity could be a worsening factor; our results confirm that this issue is not applied to all types of cancer. In the most incident of BC type – Luminal A – that have a relevant growth response for estrogen, obesity did not alter risk, but the metabolic alterations such as oxidative stress, inflammation, and insulin did.

So, we hope that these results improve our view of nutrition factors in BC women, highlighting the relevance to identify and monitoring the individual profile of tumors that respond better to a specific intervention. We have an important opportunity to create public health policies to have better control of metabolic alterations and corroborate the role of nutrition during all steps of the treatment.

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Appendix 1: Complementary production

Article: DHA in Red Blood Cell Membrane is Associated with Lower Tumor Size in Women with Breast Cancer

This article was submitted at Nutrition and Cancer (Impact Factor 2.816 in 2021 and B1 in Public Health and Nutrition).

Fwd: Submission received for Nutrition and Cancer (Submission ID: 225822586)

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DHA in red blood cell membrane modulates tumor size but has no impact on survival of women with breast cancer

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Ethical approval and participation consent

The study was submitted before and approved by the Research Ethics Committees of the HGF (nº 050507/10) and FSP / USP (nº 2162). All procedures were performed only after participants had signed the informed consent form for the study. Figure 1 describes the flowchart of the study protocol. The study was performed in accordance with the Declaration of Helsinki.

Data availability

The data are available from authors upon request.

Disclosure statement

The authors report there are no competing interests to declare

Funding information

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Abstract

Breast cancer (BC) remains the leading cause of mortality in women worldwide. Omega-3 fatty acids have been proposed as a relevant nutrient due to their role in BC. However, the effect of n-3 on inflammatory and antioxidant markers, tumor size and its impacts on survival has not yet been investigated. This is a case-control study of women with newly diagnosed BC. For both BC and control groups, clinical and demographic characteristics were obtained and blood samples were collected to determine plasma inflammatory and antioxidant (TBARS, LDL(-), antibodies anti-LDL(-) and 8-OHdG) levels, and fatty acids in red blood cell membranes. A total of 87 BC cases and 100 controls were enrolled. TBARS and anti-LDL(-) antibody levels were higher in BC women. IL-1β and IL-6 levels were higher in the BC group, while IL-10 was lower in BC women. These differences were associated with tumor size, where BC women with high tumor size had reduced DHA in red blood cell membranes, but within this group, women with higher DHA content had reduced odds of high tumor size. These results confirm the association of DHA and inflammatory, oxidant pathways and tumor size, and that n-3 fatty acids had no impact on BC women survival.

Keywords: breast cancer, docosahexaenoic acid, tumor size, polyunsaturated fatty acids

INTRODUCTION

Breast cancer (BC) is the most common form of cancer in women worldwide[1]. In Brazil, there is an estimated risk of 61.6 new cases for every 100,000 women[2]. Traditionally, the main risk factors include age, early menarche, menopause, tobacco use, alcohol intake, sedentary lifestyle and high BMI[3–5], where approximately 5-10% of BC cases are of hereditary origin[6]. However, about 35% of cancers in women can be attributed to modifiable factors, such as diet[7]. A healthy dietary pattern, including a balance of vegetables, fruit, whole foods, together with a low intake of saturated fatty acids, red and processed meat, has been associated with a lower risk of developing breast cancer, highlighting the relevance of an adequate consumption of nutrients and bioactive compounds[8].

The essential long-chain fatty acids polyunsaturated omega 3 (n-3), α-linolenic acid (ALA, 18:3), and its bioactive lipids eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (DPA, 22:5) and docosahexaenoic acid (DHA, 22:6) have been inversely associated with different types of cancer[9–12]. Preclinical studies have demonstrated the modulatory role of n-3 in inflammatory and oxidative processes and its effect on cell proliferation[13, 14], DNA damage[15], and antioxidant status[16, 17] in BC. Additionally, the antitumor effect of n-3 involves apoptosis, pyroptosis and inhibition of tumor growth. Based on these actions, and the limited human bioconversion of ALA to EPA or DHA (0.13-0.05%)[18, 19], many clinical trials have been performed, although results are conflicting. Besides the BC diagnosis, some interpersonal features can, at least partially, explain these disparate results, such as an empty stomach, fatty food during n-3 intake, fasting, and consequent bioavailability of n-3[20, 21]. Moreover, there has been some discussion over the type of n-3 consumed (vegetable/animal sources, purified/fish oil fatty acids, mono/di/triglycerides, phospholipids, and ethyl esters)[20, 22, 23]. With the aim of removing these potential biases, both plasma and cell content of n-3 has emerged as a good strategy for studying the benefits of these fatty acids in biological system[13, 14]. Furthermore, the balance of n-3 and omega-6 (n-6) can guide the modulatory role of these fatty acids in BC development.

Cell proliferation in BC is modulated by inflammatory and oxidative stress processes. Excessive oxidative damage to DNA may favor disruption of homeostasis and negative feedback in the control of the tumorigenic and inflammatory environment[15]. Furthermore, elevated oxidative substances, concomitant with low antioxidant status, has been associated with the development of BC and with more invasive forms[16, 17].

Against this background, the primary aim of this study was to investigate the association of n-3 incorporated in red blood cell membrane with tumor size in women with BC. Additionally, the modulatory role of fatty acids regarding oxidative and inflammatory biomarkers, and their impact on survival of women with BC, was investigated.

MATERIAL AND METHODS

Data collection

An observational, analytical, case-control study including women with newly diagnosed BC, selected based on a convenience sample in a non-probabilistic, consecutive fashion from the Mastology outpatient clinic of the General Hospital of Fortaleza – HGF was conducted (Ceará, Brazil). The BC group comprised 114 women with primary recent clinical and anatomopathological diagnosis of BC, with TNM classification from T1 to T3, and clinical staging from 0 to IIIc, without previous neoplasia, antineoplastic treatment or metastasis. Patients with uncontrolled chronic noncommunicable diseases, use of weight reduction medications, or psychiatry/neurological medication were excluded. For the Control group, 100 healthy women were matched to cases for age and menopausal status – women were considered postmenopausal if they self-reported cessation of menses in the previous year[24]. Using the clinical staging proposed by the AJCC (eighth edition[25), women in the BC group were stratified according to clinical staging (CS) into two sub-groups: Low group - women with low BC staging (0 to II) and High group - high BC staging (IIa to IIIc). For both groups, women were evaluated by direct interview with a structure questionnaire and consulting medical record. After applying the selection criteria, 15 patients were excluded due to metastatic disease and 12 had incomplete information in biochemical analysis, giving a total of 87 women in the BC group. The study was submitted before and approved by the Research Ethics Committees of the HGF (nº 050507/10) and FSP / USP (nº 2162). All procedures were performed only after participants had signed the informed consent form for the study. Figure 1 describes the flowchart of the study protocol.

Clinical evaluation and follow-up

A structured questionnaire was applied by direct interview collecting demographic (ethnicity and age) and risk factors (hormone replacement therapy - HRT, nulliparity, breastfeeding, family history of BC, smoking), anthropometric (weight, height and waist circumference) and body composition (resistance, reactance, phase angle, lean and fat

mass) data, as described elsewhere[26]. Risk factors for breast cancer (menopause, smoking, hormone replacement therapy, breastfeeding, nulliparity, and family history of BC) were also investigated. Menopausal status was defined when the woman self-reported ≥12 months without menses or surgery procedures for bilateral oophorectomy, hysterectomy or both.

Women were monitored for BC recurrence and total and BC mortality for 72 months after the diagnosis.

Inflammatory and oxidative markers

Blood samples (20 mL) were collected after 12-hour fasting in ethylenediaminetetraacetic acid (EDTA) tubes. Both plasma and red blood cells were separated by centrifuging (3,000 rpm, 15 min, 4°C) and samples were then aliquoted and stored at -80°C until analysis. Approximately 25 µL of plasma was used for the cytokine (IL-10, IL-6, IL-1β, TNF-α and MCP-1) assay employing the commercial Human Magnetic Panel Bead Milliplex® MAP kit (HCY T0 MAG-, Merck Millipore®). Leptin and adiponectin levels were assayed by a competitive and colorimetric Enzyme-Linked Immunosorbent Assay (ELISA) kit (ELISA Leptin Human ELISA Kit® Enzo Life Sciences® and ELISA Adiponectin Human ELISA Kit® Adipogen, respectively).

Thiobarbituric acid reactive substance (TBARS) were obtained according to the method described by Ohkawa et al. (1979)[27], with modification. In 50 µL of plasma, 1 mL of TBARS solution composed of thiobarbituric acid (0.046 M), trichloroacetic acid (0.92 M), and hydrochloric acid (0.25 M) was added then samples were incubated at 100 °C for 30 min. The solution was centrifuged at 8,000 g for 15 min at 4 °C. The color intensity in the supernatant was monitored at a wavelength of 535 nm.

Electronegative low-density lipoprotein (LDL-) and its antibodies were performed as validated by our group[28, 29]. Oxidative DNA damage was assayed by the 8-hydroxy-2'-deoxyguanosine (8-OHdG) biomarker using the competitive ELISA kit (DNA Damage ELISA kit® Enzo Life Sciences®).

Fatty acids in Red Blood Cells

After removing plasma, 300 µL of red blood cells (RBC) were washed with iced phosphate saline buffer (PBS; pH 7.4; 1:10 v/v) and centrifuged (3,000 rpm, 15 min, 4°C) until complete cell lysis of membrane. From pellets, fatty acids were extracted with a mix composed of 1.75mL hexane and 100 µL acetyl chloride, followed by vortex (30 sec) and heating (90°C, 60 min). In this step, 50 µL of internal standard (tridecanoic acid, C13:0) was added. Subsequently, 1.5 mL hexane was added, and tubes were then centrifuged (1,500 rpm, 2 min, 4°C). This step was repeated twice to optimize fatty acid extraction. The fatty acids in RBC were identified in a gas chromatograph (Shimadzu, CG-2010)

and capillary column DB-FFAP (15 m x 0.100 mm x 0.10 µm; Agilent Technologies) using continuous hydrogen flux (0.27 mL/min) and pressure (187.8 kPa). All peaks were integrated automatically by comparison with external standard (FAME 37, code 47885, Sigma Chemical Co.). Analyses were performed in duplicate and results expressed in percentage of area for each fatty acid in relation to total fatty acids. For all analysis, the recovery of internal standard exceeded 85%.

Histopathology and Immunohistochemistry of tumor

After surgery, data on tumor size (cm) and subtype (lobular or ductal) were obtained by histopathological analysis on the medical chart. These data, in conjunction with positive lymph nodes and metastasis, were used to determine TNM classification. Expression of progesterone receptor (PR+), estrogen receptor (ER+) and human epidermal growth factor receptor 2 (HER2+) was then determined by immunohistochemical analysis.

Statistical analysis

The $\chi 2$ test was used for qualitative variables. In order to define the most suitable analysis, the distribution of the variables was previously tested using the Kolmogorov-Smirnov test ($p>0.05$). For variables with a normal distribution, results were expressed as mean and standard deviation, and Pearson's correlation and Student's *t*-tests were used. The Mann-Whitney test and Spearman correlation were applied for non-parametric variables.

For logistic regression analyses, tumor size, oxidative stress, and cytokines were considered as dependent variables, while n-6 and n-3 fatty acids, and their sums and ratios, were adopted as independent variables. Given there are no reference values for these variables, a cut-off point based on the 50^{th} percentile (p50) was applied. Only the n-3 index, EPA and DHA fulfilled all assumptions for the regression models. Based on these conditions, models adjusted for age, smoking and menopausal status were tested. Survival curves were tested for these fatty acids using p50 and p75 cut-off points and multiple adjustments (menopausal status, phase angle, BMI and WC). The Statistical Package for the Social Sciences - SPSS, 21.0 (SPSS Incorporation) was used for statistical analysis. GraphPad Prism version 6.0 was used for figures. The significance level was set at $p <0.05$ for all tests.

RESULTS

Participant characteristics

Patients in the BC group had a mean age of 50.6±11.3 (min=22.2; max=88.0) years, whereas mean age in the Control group was 48.2±12.9 (min=21.2; max=77.4)

years. There were no significant differences between groups regarding ethnicity, menopausal status, hormone replacement therapy (HRT), nulliparity, breastfeeding, family history of BC or smoking (Table 1). However, smokers in the BC group (20.4±14.8 years) had smoked for more years than smokers in the Control group (13.5±11.2 years; p=0.031).

Both groups had similar weight (BC group=67.9±10.8kg vs. Control group=65.7±11.1kg; p=0.058). According to BMI, both groups were overweight, but the BC group had a higher waist circumference (WC) than the Control group (96.2±10.7cm vs. 91.2±10.1cm; p=0.002).

Regarding tumor profile, there were no differences between the Low CS and High CS groups according to tumor subtype, or expression of the estrogen receptor (ER), progesterone receptor (PR), or tyrosine-protein kinase erbB-2 receptor (HER-2). As expected, there was a significant difference in tumor size between the CS groups (Low CS=1.6±0.6cm versus High CS group=3.2±2.2cm; p<0.001), directly correlating with differences observed in clinical staging (p=0.023) (Table 2). The rate of BC recurrence during the 72-month follow-up time was 12 cases, of which 66% died. Overall, 33% of deaths occurred within the first 2 years after diagnosis.

Oxidative stress and adipokines

The oxidative stress biomarkers according to BC diagnosis and CS are shown in Figure 2. Levels of 8-OHdG were similar for both the Low CS and High CS groups (17.8±5.1 vs. 17.9±5.9 ng/dL; p=0.997; respectively) and also for the BC and Control groups (p=0.145) (Figure 2A). However, TBARS in the BC group was significantly higher than in the Control group (p=0.001). This profile was confirmed by significant differences in both the High CS group and Low CS group, and the Control group, for TBARS and anti-LDL(-) antibody levels (p<0.001; for all) (Figure 2B-D). Interestingly, only the Low CS group had higher LDL(-) values than the Control group (p=0.002) (Figure 2C). Leptin level was similar for both groups, but adiponectin in the BC group differed to that of the Control group (p=0.001) where this profile was correlated with the High CS group (p=0.005) (Figure 2E-F).

Inflammatory cytokines

As expected, women with BC had lower levels of IL-10 (5.5±1.2 pg/mL vs 2.8±1.1); Figure 3A), while IL-6 (1.6±0.1 pg/mL vs 0.9±0.2; Figure 3B), IL-1β (2.8±2.2 pg/mL vs 1.3±0.1 pg/mL; Figure 3C) and MCP-1 (295.8±11.4 vs 357.8±28.4; Figure 3E) were higher relative to the Control group. TNF-α was similar for both groups (Figure 3D). Except for MCP-1 and TNF-α, other cytokines were significantly higher in the High CS group than in the group with lower tumor size.

Analysis of fatty acids in red blood cells

Regarding the oxidative and inflammatory status in the BC group, an analysis of the fatty acid content in red blood cell membranes was performed (Table 3). Except for DHA, which was significantly higher among women in the Low CS group (4.0±1.3%) than in the High CS group (3.4±1.3%; p=0.020), all others fatty acids were similar for both these groups.

Correlations, Logistic regression models and Survival analysis

All correlations tested between n-3 fatty acids and tumor size, inflammatory and oxidative biomarkers are presented in Table 1 (Supplementary material 1). No significant relationships were observed between anti-inflammatory cytokines or oxidative parameters and tumor growth. However, when testing n-3 and inflammatory and oxidative markers, DHA was found to correlate with increase in IL-10 (r=0.343; p=0.023), DPA and leptin (r=-0.234; p=0.043), n-3 sum and 8-OHdG (r=0.241; p= 0.030) and adiponectin (r=0.231; p=0.024). Adiponectin also correlated with n-6/n-3 ratio (r=0.296; p=0.004).

Furthermore, the logistic regression model adjusted for age, smoking and menopausal status showed that high DHA level was associated with lower tumor size (OR=0.54, 95% CI=0.31-0.86) (Figure 4).

Lastly, n-3 and its ratio were not associated with mortality, irrespective of the cutoff points and adjustment tested (Figure 5).

DISCUSSION

In the present study, high DHA in red blood cell membranes was associated with low tumor size through modulation of inflammatory and oxidative biomarkers. Previously, Rahrovani et al. (2017)[29] and Hirko et al. (2017)[30] found that individuals with carcinoma had up to 3% less n-3 acid in red blood cell membranes than healthy individuals. Additionally, the BC women had a high proportion of saturated and trans-fatty acids.

Many individual and life-style characteristics can modulate BC risk in women. The women enrolled in both groups of the present study were overweight or obese, but the individuals in the BC group had a higher WC. Moreover, these women had a lower level of adiponectin, where this reduction was correlated with high tumor size. This profile was recently confirmed in a meta-analysis[31] of 27 case-control studies which observed an inverse association between adiponectin and BC, and found that low levels of adiponectin were associated with large tumors (> 2 cm) and higher histological grade,

suggesting a more aggressive phenotype[32]. Although leptin was similar between groups, its imbalance with adiponectin is common in obesity. The increased adiposity observed in BC women represented a positive stimulus for oxidative stress and an inflammatory environment able to induce and maintain cell proliferation as proposed previously[33].

In spite of the low IL-10, BC women had an inflammatory status compared with healthy women. In the literature, analyses of IL-10 alone have led to controversial conclusions. According to Hamidullah et al., (2012)[34] , women with BC have high IL-10 levels and its role in the carcinogenesis process is directly dependent on the compartment investigated. In fact, increased IL-10 in the microenvironment was associated with tumor development, while plasma IL-10 content was associated with a better prognosis[35]. Within the microenvironment, intense anti-inflammatory interleukins are believed to act as a protective mechanism to the tumor, acting to modulate the local inflammatory process in tumor cells. Contrary to this assumption, the present study results confirm the systemic inflammatory response in BC women and its relationship with tumor size.

In addition, with regard to the intensity of the inflammatory response, this study showed that TBARS were more elevated in BC women than in controls, and their level was associated with tumor size. TBARS is a classical oxidative biomarker for lipid peroxidation. Similar to the present results, Goswami et al. (2010)[13] identified high plasma TBARS content in BC women, while elevated lipid peroxidation evaluated by TBARS and reduced antioxidant capacity were described by Suddek (2014)[36] in response to tamoxifen treatment of hormone-dependent BC.

For the last 20 years, our group has tested LDL(-) as a biomarker for oxidative stress due to pro-oxidative and antioxidant balance. Regarding the high TBARS levels detected in BC women and its relationship with tumor size, this biomarker was detected to provide an estimate of oxidative stress in these patients. In fact, LDL(-) was higher in BC women with higher tumor size, reinforcing the TBARS levels observed in these women. Abplanalp et al., (1999)[37] was one of the first groups to propose that oxidized LDL and lipid peroxidation could negatively impact BC women with hormone-dependent tumors. In a later study, Panis et al. (2012)[38] found higher concentrations of TBARS in women with higher BC staging, concomitantly with a reduction in catalase activity and high concentrations of lipid peroxidation and nitric oxide. Anti-LDL(-) antibodies indicate an immune system response against oxidized LDL products and serves as an indirect parameter for estimating oxidative stress in a biological environment. The BC women studied had higher anti-LDL(-) levels than controls, although it was not possible to

observe the impact of tumor staging on both LDL(-) and their antibodies. Singh et al. (2020)[39] reported that oxidized LDL induced significant reactive oxygen species (ROS) production in BRCA2-silenced endothelial cells and exacerbated DNA damage. In the present study, both the total sample and subgroups of BC women stratified according to tumor size showed no significant differences in DNA damage, as evaluated by 8-OHdG, although oxidative stress in the BC women was confirmed by other biomarkers and correlation with n-3 sum ($r=0.241$; $p=0.03$).

Given the exacerbated oxidative stress and inflammation in BC, n-3 has been investigated for its relevant role in inflammation resolution[40]. However, more recently, many studies have reported a specific type and dose-protective response to n-3. EPA and DHA, but not linolenic acid, are able to disrupt lipid rafts and microdomains in cell membranes, impacting signaling proteins and consequent cell proliferation[41]. Furthermore, nuclear factor kappa B (NF-κB) signaling is reduced in the presence of EPA and DHA, favoring apoptosis, metalloprotease, cytokines production and adhesion molecules[42]. In the current investigation, an inverse association of DHA with reduced tumor size (but not for EPA) was identified, where this protective role was confirmed by increased risk of larger tumors in women with lower DHA content in red blood cell membrane. In line with these results, Pizato et al., (2018)[43] found that DHA induced pyroptosis-programmed cell death in breast cancer cells, suggesting potential benefits of DHA intake and supplementation, improving prevention and treatment of BC women. Although the present study did not investigate inflammation and oxidation directly in BC tumors, previous studies such as that by Aslan et al., (2020)[44] show additional mechanisms related to the effect of DHA in the inhibition of genes and miRNA pro-angiogenic in BC cells. The specific-type response to n-3 in BC was recently investigated by Brown et al. (2019)[45], who observed that different effects of DHA and EPA can be partially explained by their conversion into endocannabinoid derivates (docosahexaenoyl ethanolamide -DHEA - and eicosapentaenoyl ethanolamide - EPEA, respectively). Both these derivatives demonstrated greater anti-cancer roles than DHA and EPA in two BC cell types, although DHEA proved more effective. Therefore, the present study results further the state of the art, showing that BC women can benefit from DHA intake through its modulation of inflammation and oxidation in BC women, in addition to several other mechanisms previously observed in cell studies.

Although these results support a relationship of DHA with tumor size through modulation of inflammation and oxidation, no changes in survival as a function of DHA or other n-3 investigated were evident. In 2020, the review of Donovan et al. (2020)[46] revealed that adjuvant treatment of BC using DHA improved clinical outcomes for women

with triple-negative BC, in part due to downregulation of Bcl-2 and phosphorylated Akt and increased levels of cleaved caspase-3 and BAX. In contrast with the positive effect of DHA observed in cell and animal studies, an analysis of a sample from the Nurse´s Health Study (n=2729) failed to identify an association of diet quality indices and total and BC mortality (Kim et al. (2011)[47]. In a later study, Makarem et al. (2013)[48] evaluated the impact of fatty acids pre- and post-BC diagnosis. Similarly, to the present results, the authors found no association of n-3 with recurrence or mortality, probably due the low number of studies reviewed.

The limitations of the present study include the type of study design. Although results are promising, the observational nature of the study only allowed associations to be explored. Nevertheless, the sample size and direct measure of fatty acids yielded improved results compared with the use of traditional food registers, avoiding systematic and randomized bias of information. The inclusion of a control group represents another strength of the study because cut-off points have not been defined for some biomarkers.

In summary, we conclude that BC women with larger tumors had more intense inflammatory and oxidative response and reduced DHA levels in red blood cell membranes. Furthermore, women with high DHA content showed a reduced risk of larger tumors, irrespective of other traditional risk factors. However, results suggest there were no changes in survival of BC women. Therefore, these results represent an exciting opportunity to optimize strategies of improving DHA for the prevention and treatment of BC through diet and/or supplementation. Future randomized clinically controlled trials are warranted to confirm these findings.

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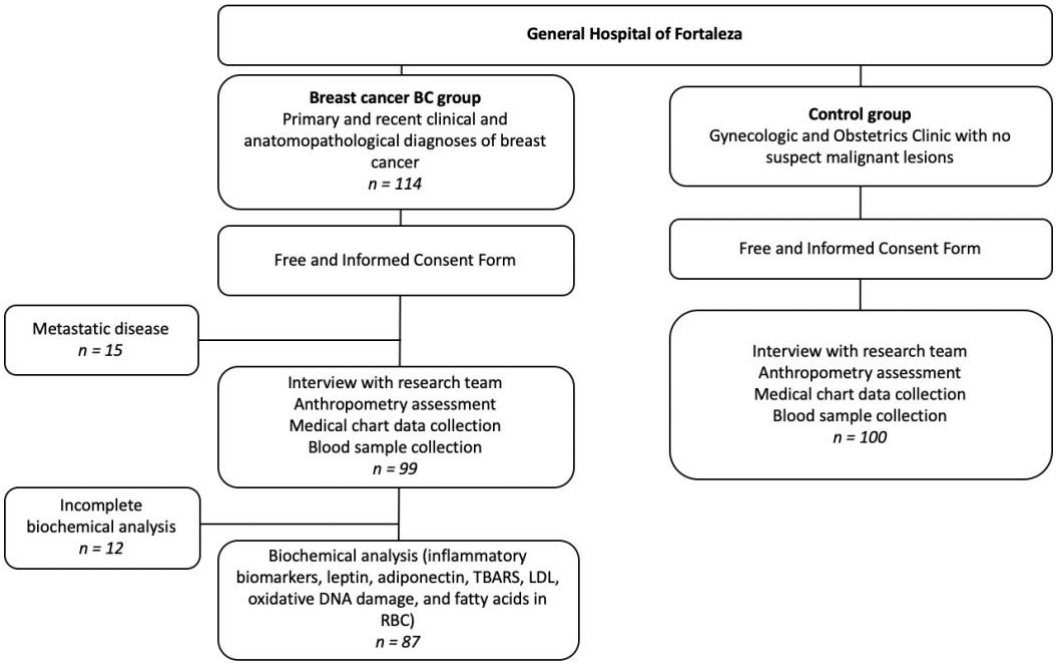


Figure 1. Flowchart of study protocol.

Table 1. Demographic characteristics, anthropometry, and risk factors of women according to BC diagnosis.

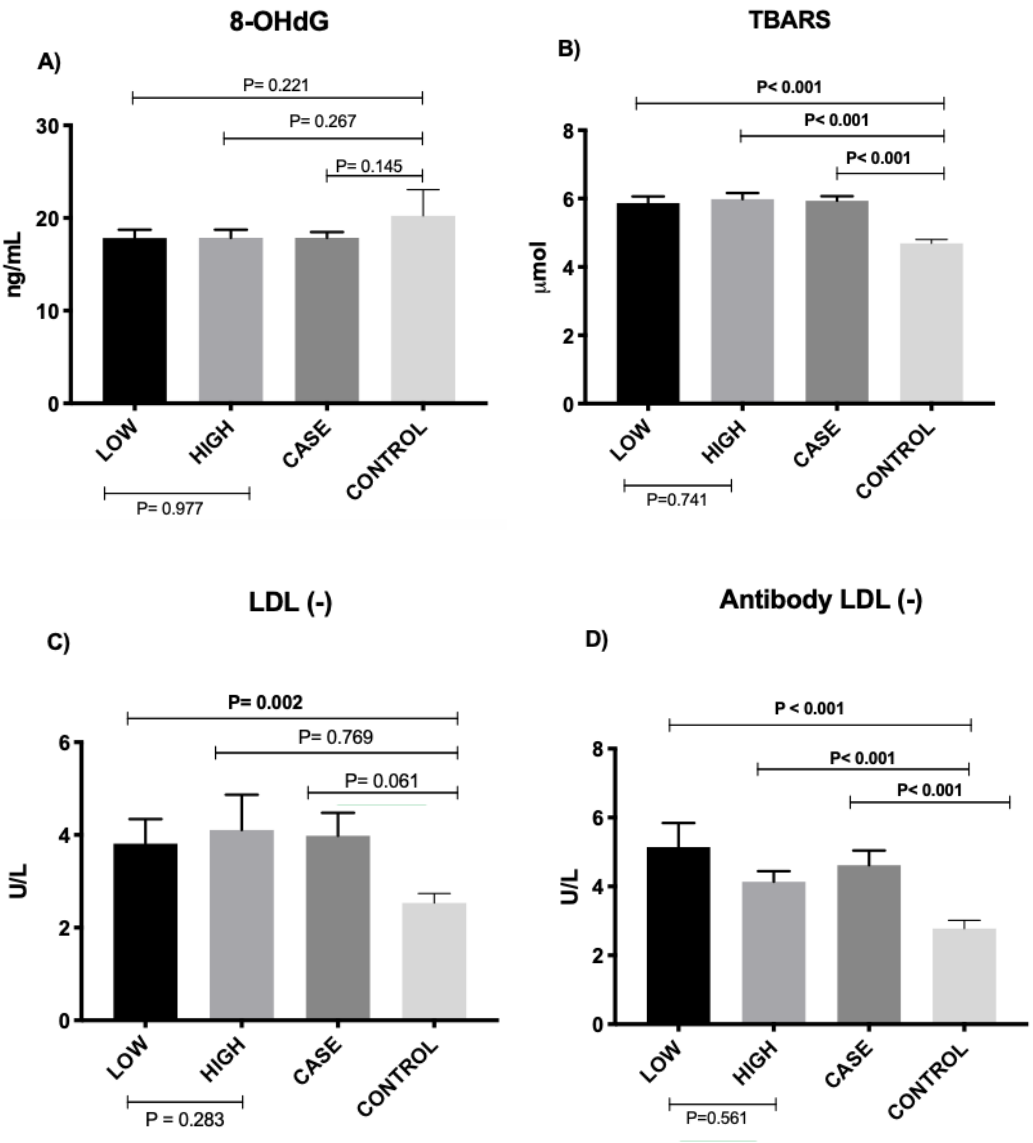
Variables	Case (n=87)	Control (n=100)	*p-value*
Age, years	50.6 (11.3)	48.2 (12.9)	0.168
Ethnicity (%)			
White	19 (21.8)	16 (16.0)	
Non-white	48 (55.2)	74 (74.0)	0.057
Other	20 (23.0)	10 (10.0)	
Menopause, yes (%)	43 (49.4)	58 (58.0)	0.303
HRT, yes (%)	8 (9.2)	6 (6.0)	0.421
Nulliparity, yes (%)	15 (17.2)	21 (21.0)	0.579
Breastfeeding, yes (%)	60 (69.0)	63 (63.0)	0.660
Family history of BC, yes (%)	63 (72.4)	66 (66.0)	0.428
Smoking, yes (%)	37 (42.5)	35 (35.0)	0.297
Smoking, years	20.4 (14.8)	13.5 (11.2)	**0.031**
Weight, kg	67.9 (10.8)	65.7 (11.1)	0.058
BMI, kg/m^2	28.1 (4.7)	27.7 (4.3)	0.156
WC, cm	96.2 (10.7)	91.2 (10.1)	**0.002**
Resistance (R)*	566.9 (96,4)	586.3 (78.4)	0.096
Reactance (Ω)*	63.5 (10.1)	64.6 (10.0)	0.361
Phase angle (°)*	6.4 (1.0)	6.3 (0.7)	0.984
Fat mass (%)*	35.1 (4.8)	34.5 (4.7)	0.424
Lean mass (%)*	64.9 (4.8)	65.5 (4.7)	0.432

Variables expressed as absolute value (n) and percentage (%) or mean and standard deviation. Differences between groups were assessed using χ2 test for categorical variables, Student's *t*-test for continuous parametric variables and *Mann-Whitney test for continuous non-parametric variables. Significance level adopted for all tests was $p<0.05$. Smoker = current or former smoker. HRT = Hormone replacement therapy; BMI = Body mass index; WC = Waist circumference.

Table 2. Tumor profile in women with BC according to clinical staging.

Variables	Case (n=87)	Low CS (n=36)	High CS (n=51)	*p-value*
Tumor subtype, n (%)				
Lobular	9 (10.3)	6 (16.7)	3 (5.9)	0.156
Ductal	69 (79.3)	28 (77.8)	41 (80.4)	
Tumor size, cm*	2.6 (1.9)	1.6 (0.6)	3.2 (2.2)	**< 0.001**
Clinical staging, n (%)				
0	4 (4.6)	4 (11.1)		
I	1 (1.1)	1 (2.8)		
I a	28 (32.2)	28 (77.8)		
II	3 (3.4)	3 (8.3)		
II a	19 (21.8)		19 (37.3)	**0.023**
IIb	11 (12.6)		11 (21.6)	
III	2 (2.3)		2 (3.9)	
IIIa	3 (3.4)		3 (5.9)	
III b	12 (13.8)		12 (23.5)	
IIIc	4 (4.6)		4 (7.8)	
Tumor receptors, n (%)				
ER+	53 (64.6)	8 (66.6)	20 (64.5)	0.894
PR+	52 (81.2)	8 (80.0)	19 (79.2)	0.956
HER+	7 (11.7)	2 (20.0)	2 (8.7)	0.361

Variables expressed as *mean and standard deviation and absolute (n) value and percentage (%). Differences between groups were assessed using $\chi 2$ test for categorical variables and Student's *t*-test for continuous parametric variables. Significance level for $p < 0.05$. Tumor stage according to AJCC (eighth edition) PR+: positive progesterone receptor, ER+: positive estrogen receptor, HER2+: positive human epidermal growth factor receptor 2.


8-OHdG
A)
P= 0.221
P= 0.267
P= 0.145
ng/mL
30
20
10
0
LOW
HIGH
CASE
CONTROL
P= 0.977
TBARS
B)
P< 0.001
P< 0.001
P< 0.001
µmol
8
6
4
2
0
LOW
HIGH
CASE
CONTROL
P=0.741
LDL (-)
C)
P= 0.002
P= 0.769
P= 0.061
U/L
6
4
2
0
LOW
HIGH
CASE
CONTROL
P = 0.283
Antibody LDL (-)
D)
P < 0.001
P< 0.001
P< 0.001
U/L
8
6
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2
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LOW
HIGH
CASE
CONTROL
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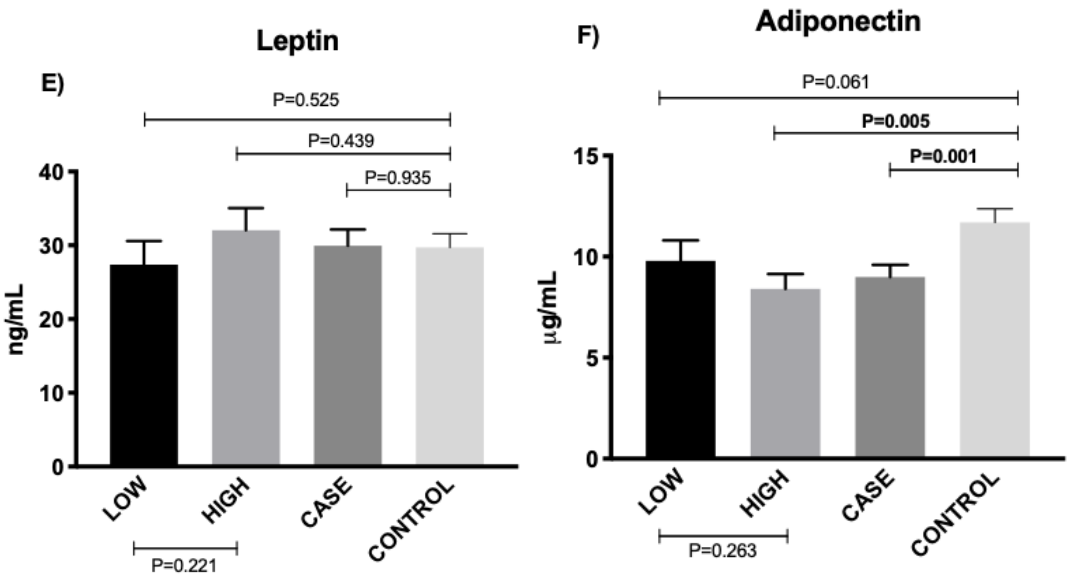

Figure 2. Plasma oxidative stress markers and adipokines according to BC diagnosis and clinical stage.

Values expressed as average and standard error of the mean. Difference between groups determined by Mann-Whitney test. Significance value p <0.05. Low: Low CS group; High: High CS group; Control: women free of BC; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; TBARS: thiobarbituric acid reactive substances; LDL(-): electronegative low-density lipoprotein.

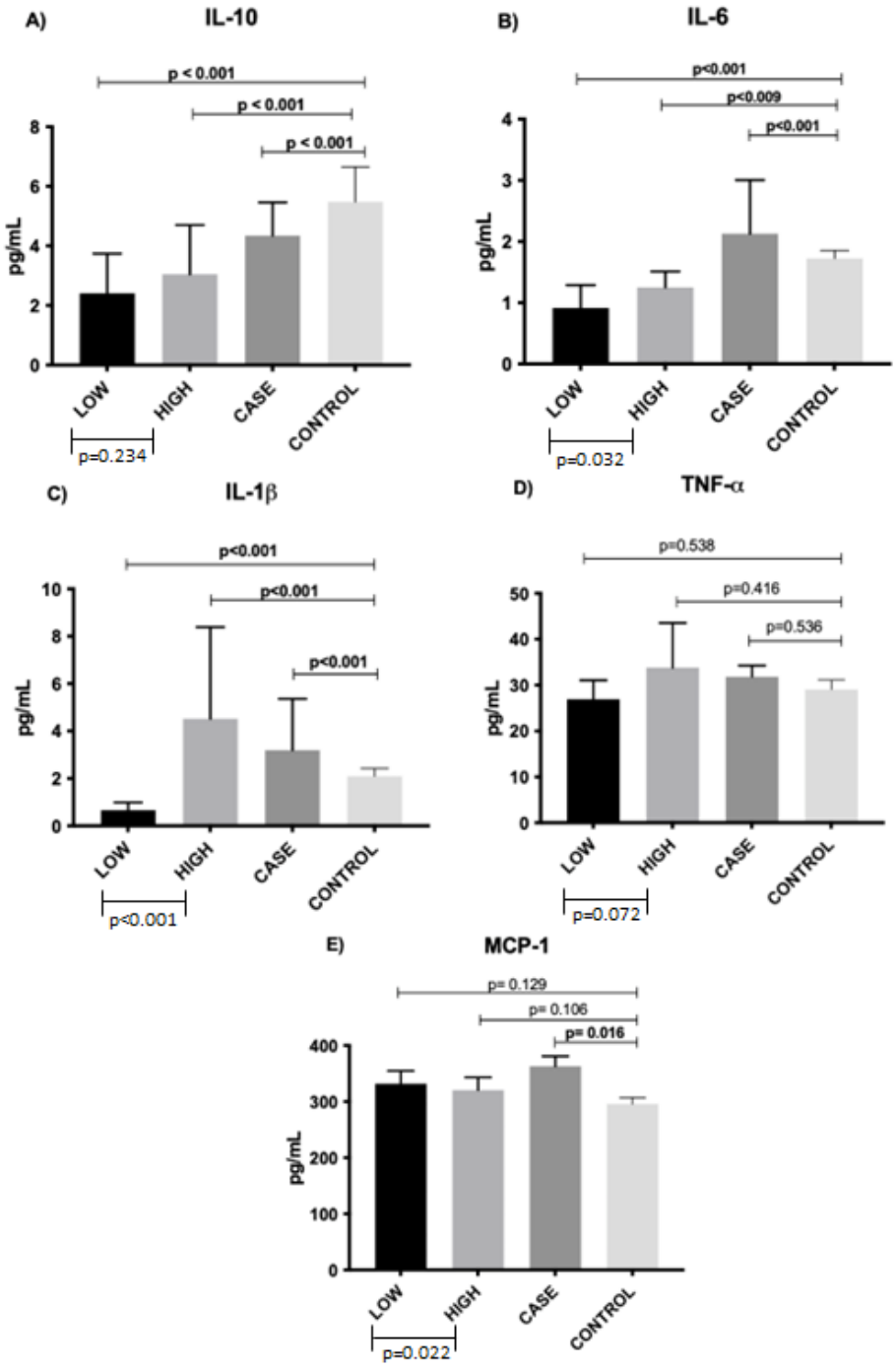

Figure 3. Plasma cytokines according to BC diagnosis and clinical stage.

Results expressed as average and standard error of the mean. Differences between groups determined by Mann-Whitney test. Significance value p <0.05. Low: Low CS group; High: High CS group; Control: women free of BC; IL: interleukin; TNF-α: tumor necrosis factor alpha; MCP-1: monocyte chemoattractant protein 1.

Table 3. Characterization of fatty acids in erythrocyte membrane of women with BC according to clinical staging.

Fatty acids (%)	Low CS (n = 36)	High CS (n = 51)	Case group (n = 87)	*p-value*
SFA	39.3 (3.3)	39.8 (3.2)	39.6 (3.2)	0.716
Palmitic acid	17.1 (4.9)	16.9 (4.9)	16.9 (4.7)	0.928
Stearic acid	22.4 (2.1)	22.3 (2.1)	22.6 (2.1)	0.508
MUFAS	16.4 (2.2)	16.8 (2.3)	16.5 (2.2)	0.137
Oleic acid	16.4 (2.2)	16.8 (2.3)	16.5 (2.3)	0.137
PUFAS	44.7 (3.9)	43.4 (3.7)	43.9 (3.8)	0.243
Linoleic acid	10.6 (1.7)	10.9 (1.4)	10.8 (1.5)	0.429
Arachidonic acid	21.2 (3.2)	20.4 (2.8)	20.8 (2.3)	0.228
Linolenic acid	2.1 (0.4)	2.2 (0.6)	2.6 (0.2)	0.634
n-6	31.7 (3.1)	31.0 (3.1)	32.6 (6.8)	0.362
EPA	0.6 (0.3)	0.8 (0.8)	0.7 (0.8)	0.580
DPA	2.4 (0.6)	2.2 (0.5)	2.3 (0.6)	0.166
DHA	4.0 (1.3)	3.4 (1.3)	3.6 (1.3)	**0.020**
n-3	7.2 (1.2)	6.9 (1.1)	7.0 (1.2)	0.322
n-3 index	4.6 (1.5)	4.2 (0.9)	4.4 (1.1)	0.214
n-6/n-3 ratio	4.4 (1.0)	4.8 (2.9)	4.7 (1.6)	0.431

Results expressed as percentage of area of all fatty acids analyzed. Differences between groups (Low CS vs High CS) determined by Student's *t*-test for parametric variables and Mann-Whitney for non-parametric variables. Significance level $p < 0.05$. SFA: sum of saturated fatty acids, MUFA: sum of monounsaturated fatty acids, PUFA: sum of polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DPA: docosapentaenoic acid, DHA: docosahexaenoic acid; n-6: sum of linoleic acid + arachidonic acid; n-3: alpha linolenic acid + EPA + DPA +DHA; n-3 index: EPA + DHA; n-6/n-3 ratio: sum of n-6 to sum of n-3 ratio.

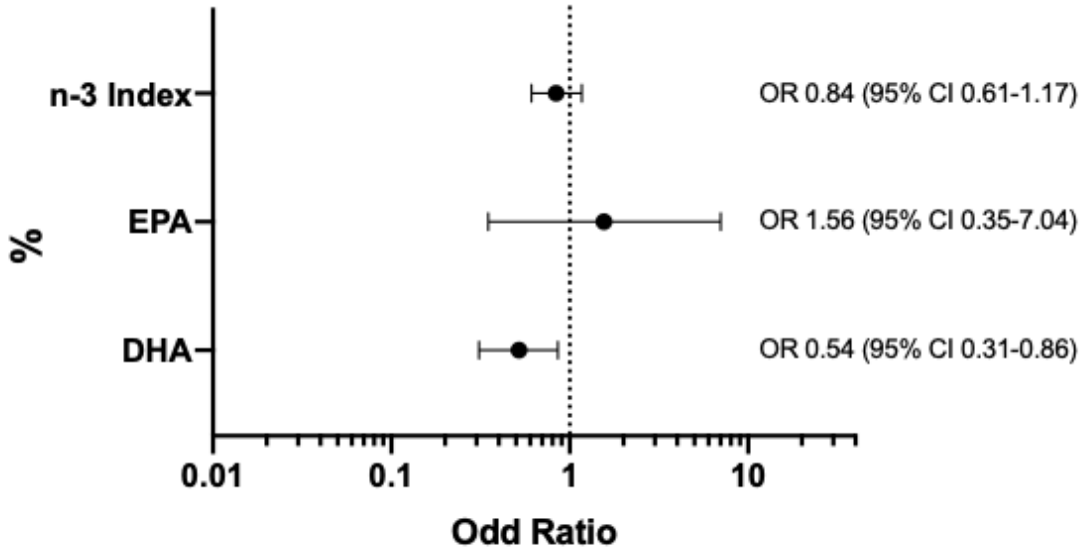

Figure 4. Logistic regression models between n-3 fatty acids and tumor size, adjusted for age, smoking and menopausal status.

Concentration of polyunsaturated fatty acids, expressed as percentage of total area comprising all fatty acids, analyzed by gas chromatography. EPA - eicosapentaenoic fatty acid; DHA - docosahexaenoic fatty acid; n-3 index - sum of EPA and DHA; OR - Odds ratio and CI - confidence interval.

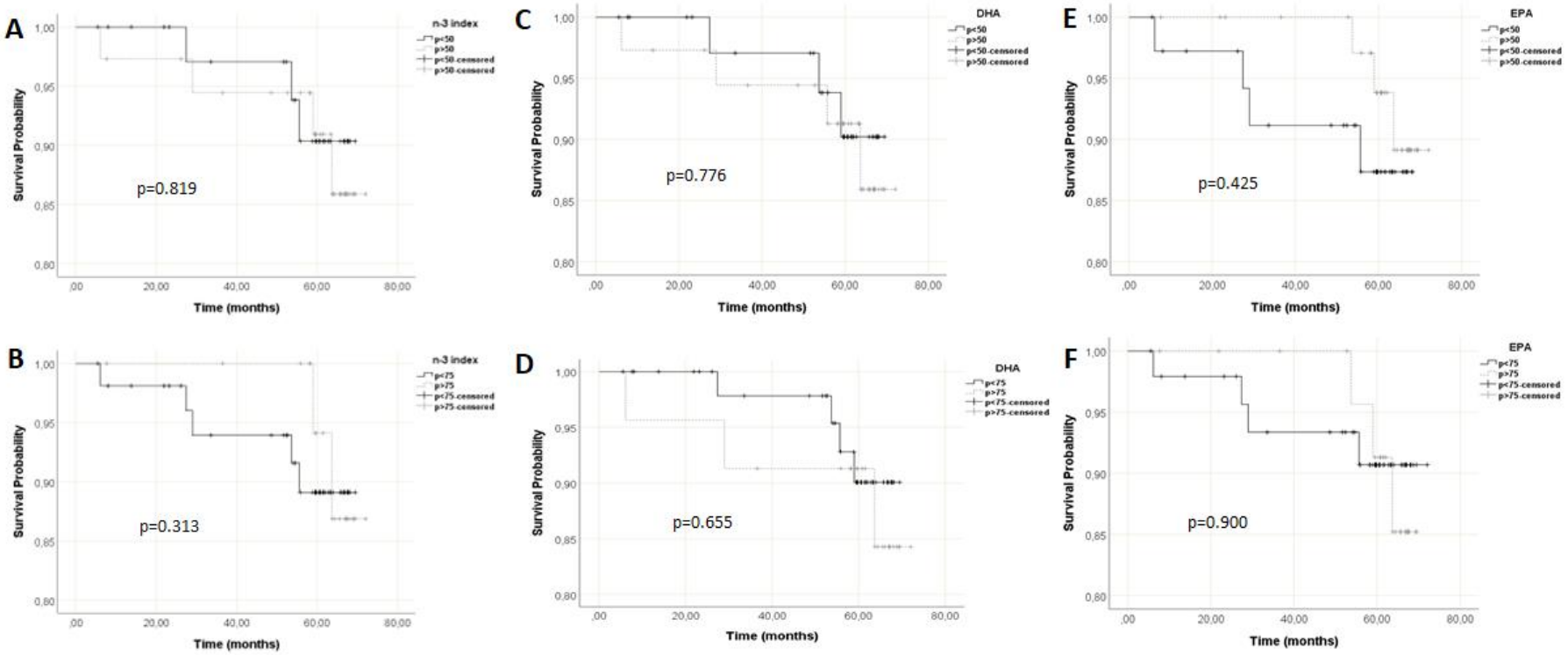

Figure 5. Survival curves of women with BC according to n-3 index, DHA and EPA during 72-month follow-up.

Supplementary material 1. Correlations between tumor and fatty acids and oxidative and inflammatory biomarkers.

	Tumor size (cm)										
	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	-0.175	0.213	0.092	-0.132	0.362	-0.118	-0.074	-0.006	-0.063	0.004	-0.058
p	0.197	0.111	0.502	0.362	0.053	0.404	0.586	0.963	0.642	0.978	0.670

	DHA (%)										
	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	0.108	-0.015	-0.117	-0.075	-0.082	0.066	-0.090	***0.343***	0.054	-0.014	-0.106
p	0.309	0.887	0.274	0.516	0.485	0.534	0.396	***0.023***	0.609	0.897	0.314

	EPA (%)										
	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	-0.058	0.006	0.062	0.067	0.018	0.068	-0.122	-0.109	-0.035	-0.083	-0.014
p	0.586	0.951	0.563	0.559	0.875	0.524	0.247	0.299	0.742	0.434	0.896

	DPA (%)										
	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	0.163	0.036	-0.154	-0.151	***-0.234***	0.198	-0.100	-0.037	-0.115	-0.043	-0.096
p	0.123	0.736	0.147	0.187	***0.043***	0.060	0.342	0.724	0.276	0.683	0.362

Linolenic acid (%)

	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	-0.008	0.146	-0.042	-0.022	-0.215	-0.163	0.103	0.020	-0.014	0.060	0.010
p	0.938	0.164	0.694	0.849	0.065	0.124	0.327	0.852	0.892	0.568	0.924

n-3 index (%)

	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	0.010	0.032	-0.032	-0.020	-0.044	0.061	-0.079	0.018	0.094	0.004	-0.065
p	0.927	0.764	0.761	0.864	0.709	0.568	0.456	0.868	0.374	0.967	0.536

n-3 sum (%)

	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	0.125	-0.030	0.014	***-0.241***	-0.091	***0.231***	0.075	0.070	-0.008	0.067	0.101
p	0.228	0.769	0.891	***0.030***	0.440	***0.024***	0.468	0.497	0.942	0.515	0.330

n6/n3 ratio

	LDL(-) (U/L)	anti-LDL(-) antibodies (mU/L)	TBARS (μmol/mL)	8-OHdG (ng/mL)	Leptin (ng/mL)	Adiponectin (μg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	IL-6 (pg/mL)	IL-1β (pg/mL)	MCP-1 (mg/mL)
r	0.101	0.082	-0.161	0.199	0.106	***0.296***	0.075	0.102	0.172	0.089	0.197
p	0.334	0.429	0.122	0.075	0.368	***0.004***	0.467	0.321	0.095	0.390	0.054

Article: Omega-3 fatty acids are associated with reduced oxidative stress and inflammation in post-menopausal women with breast cancer and ER+

This article is still in writing process.

Omega-3 fatty acids in red blood cell are associated with reduced oxidative stress and inflammation in post-menopausal women with breast cancer and ER+

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INTRODUCTION

Breast cancer (BC) is the main cause of cancer death in females worldwide and is estimated to account for nearly one-third of the 934,870 new cancer cases in 2022[1]. The incidence of BC is modified by genetic, environmental, and lifestyle factors, whereas modifiable risk factors account for about 42.0% of all cancer incidences[2]. An estimated 5.2% of new cancer cases are attributable to dietary patterns[3].

Nutrients and bioactive components in foods can modulate many cancer risk factors by different metabolic pathways[4,5]. Consumption of foods rich in polyunsaturated fatty acids (PUFAS), especially, omega-3 and omega-6 fatty acids, and their bioactive metabolites and ratios may be associated with the development of BC by competitive modulation of the different metabolic pathways[6–8]. These mechanisms are complex and not completely elucidated, however, have been described that omega-3 fatty acids exert a positive effect on suppressing inflammatory process, apoptotic stimulus, inhibition of metastasis, and tumor proliferation[9]. Additionally, omega-3 fatty acids can modulate peroxisome proliferator-activated receptor (PPAR-α) expression, reduce interleukine-6 (IL6) and factor nuclear kappa B (NF-kB) transcript mRNA, cell surface of lipid rafts, and total levels of epidermal growth factor receptor (EGFR)[10–12].

At least in part, these mechanisms can explain observational studies that verified the positive association between the low omega-3:omega-6 ratio intake and increased BC risk[13]. In a case-cohort with health and BC women, dietary omega-3 fatty acids were inversely associated with BC[14]. In previous studies, higher dietary intake of fish and omega-3 fatty acids was associated with a 16% and 34% reduction in risk of additional BC events and all-cause mortality, respectivelly[15,16]. Furthermore, eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) supplementation, independent of the cancer treatment, improved progression-free survival, overall survival, and quality of life in cancer patients[17]. Despite that, many studies were not able to confirm the connection between BC and omega-3 intake. Among the 14 meta-analyses examined in an overall review, only 3 studies showed a statistically significant association between omega-3 fatty acids intake and BC risk[18]. Also, a recent review based on 47 randomized controlled trials suggested that increased omega-3 fatty acids have little or no effect on the risk of BC diagnosis and deaths from any cancer[19]. These controversies can be partially explained by methods used to investigate the bioavailability of omega-3 such as food registers, questionnaires of food frequency, and 24 hours recall[20]. In the last decade, numerous efforts have been dedicated to solving the inherent limitations of these methods, intentional misreporting or failure to recall consumption, and limitations of the databases applied to record the type and amount of food consumed. These problems

have been partially solved by analysis of plasma and cell content of omega-3 fatty acids amply described in literature[21].

Additionally, the clinical and genetic profile of patients and specific tumor characteristics could help to identify individuals more responsive to omega-3 modulation, with the characterization of the tumor using biomarkers such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-type 2 (HER2) and proliferative factors (vascular endothelial growth factor – VEGF, Ki67) to expand our view about the specific profile of BC in addition to the traditional TNM risk estimate. Together, these characteristics can build a personal signature that allows the design of better individual protocol treatment, improving prognosis and increasing disease-free survival, reducing relapse and mortality. Omega-3 fatty acids combined with all-*trans* retinoic acid promoted synergistic inhibition of cell proliferation of three types of BC cell lines (ER-positive MCF7, HER2-positive SK-BR-3, and triple-negative MDA-MB-231), confirming the positive impact of these fatty acids in specific BC by modulation of caspases signals[22]. Also, omega-3 fatty acids can induce apoptosis in BC by inhibiting of PI3K/Akt pathway and can be an adjuvant for treatment of triple-negative breast cancer (TNBC) as proposed in a recent review[23]. In line with these studies, omega-3 fatty acids supplementation can trigger a positive effect on the decrease of the expression of Ki67 and VEGF in BC patients[24]. So, exploring the effect of omega-3 fatty acids in specific types of BC can add value to acute and chronic steps of antineoplastic treatment.

The study aims to investigate the association of omega-3 fatty acids in BC considering the expression of ER, PR, and HER2, and menopausal status. To avoid bias in omega-3 data collection from diet information, we used a validated method to determine de omega-3 fatty acids content incorporated in membrane cells of erythrocytes of women with recent BC diagnosis without previous cancer treatment.

MATERIAL AND METHODS

Study population

This cross-sectional study included women with a recent anatomopathological diagnosis of BC who attended at the General Hospital of Fortaleza (Ceará, Brazil). Were included women with BC (n=99), clinical staging 0 to III, without metastasis, and previous antineoplastic treatment. The exclusion criteria included women under nutritional counseling or using weight loss drugs, with no-controlled transmissible chronic diseases, transmissible diseases, neurological or psychiatric complications, previous diagnosis of cancer, and women under neoadjuvant treatment for BC. The study was approved by the Research Ethics Committee of the General Hospital of Fortaleza (Nº 050507/10) and

the University of São Paulo School of Public Health (Nº 2162). Free and informed consent forms were obtained from all of the participants and all procedures adopted the rules established by the Declaration of Helsinki for human research.

Data collection

Using medical records, direct interviews and structured questionnaires were collected demographic data (age and ethnicity), use of hormone replacement therapy (HRT), reproductive history, breastfeeding, smoking, alcohol intake, and family history of BC. Menopausal status was defined as 12 continued months of amenorrhea[25]. Clinical tumor staging was based on TNM system[26], and immunohistopathologic analysis was performed to detect positive estrogen receptor (ER+), progesterone receptor (PR+), human epidermal growth factor receptor type 2 (HER2+), and Ki67.

Current weight (kg) was measured using a digital scale (Plena, São Paulo, Brazil), height (cm) was measured using a stadiometer (TBW, São Paulo, Brazil), and waist circumference (WC) was assessed using an inelastic tape (cm). Body mass index was calculated according to the WHO recommendation (weight/height^2; obesity was classified as a BMI > 29.9 kg/m^2) and cardiometabolic risk associated with increased waist circumference (WC > 80 cm)[27,28]. Body composition was determined using bioelectrical impedance analysis (Biodynamics 450 model; TBW, São Paulo, Brazil), and values of percentage of fat mass (FM) and lean mass (LM) were estimated. Fat mass was classified as proposed by Lohman[29].

Biochemistry analysis

After 12 hours of fasting, blood samples (20mL) were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA; 1mg/mL) and stored on ice and shielded from light until obtention of plasma (1,500g, 10min, 4°C). Protease inhibitors were added to the plasma: aprotinin (2ug/mL), benzamidine (2mM), phenylmethylsulfonyl fluoride (1mM), and butylated hydroxytoluene (20mM). Plasma samples were aliquoted and stored at -80ºC until analyses.

Plasma interleukine-1β (IL1β), IL6, interleukine-10 (IL10), tumor necrosis factor α (TNF-α), and Monocyte chemoattractant protein-1 (MCP-1) were analyzed using the commercial Human Magnetic Panel Bead Milliplex® MAP kit (HCY T0 MAG-, Merck Millipore®). The content of plasma antioxidant (retinol, α-tocopherol e ß-carotene) were measured in High-performance liquid chromatography (HLPC) according to Fortis and Faver (1991). Oxidative DNA damage (8-OH-2'-deoxyguanosine - 8-OH-dG) was analyzed using a commercial kit (Enzo Life Sciences®), thiobarbituric acid reactive substances (TBARS) were determined by colorimetric reactions[31] and oxidized low-density lipoproteins (oxLDL) and its antibodies were determined by Enzyme-linked

immunosorbent assay (ELISA) using monoclonal antibody anti-LDL(-) developed by our group[27].

Erythrocyte fatty acids

Erythrocyte membrane fatty acids analyses were performed using a modified protocol proposed by Masood et al[32]. Phosphate-buffered saline (PBS - 5mL; 10:1, pH 7.4) was added to the hemoconcentration (300 µL). The samples were mixed (30 sec), sonicated (2 min), and centrifuged (1,000g, 30 min 4ºC). The supernatant was discarded, and the pellet was washed three times until remove hemoglobin.

The fatty acids in erythrocyte lysate samples were extracted by addition of methanol (1.75 mL), tridecanoic acid (0.1 g of internal standard, 50 µL), and acetyl chloride (100 µL) followed by sonication (5 min). After, the samples were homogenized (30 sec) and maintained in a water bath (60 min; 100ºC). Subsequently, hexane (1.5 mL) was added, and the samples were mixed in a vortex (1 min) and centrifuged (1,500g, 2 min, 4 ºC). After, the supernatant (800 µL) was removed. The process was repeated two times by adding hexane (750 µL).

The supernatant was evaporated (20 min; 40°C), and the sample was resuspended with hexane (150 µL), filtered (0.22µm membrane), and transferred to the vial for further analysis in gas chromatography. The fatty acid profile was determined on a Shimadzu gas chromatograph, CG-2010, equipped with a DB-FFAP capillary column (15 m x 0.100 mm x 0.10 µm 0 J and W Scientific, Agilent Technologies). The results expressed percentage of fatty acids present in the erythrocyte membrane.

Statistical analysis

The normality of the variables was verified with the Kolmogorov-Smirnov test ($p > 0.05$). Categorical data were described and presented as frequencies and percentages and statistical differences were analyzed using the Chi-Square test. Descriptive data were expressed as a frequency or mean followed by the standard deviation (SD). According to normality, comparing quantitative variables was performed using the Student's t-test or Mann-Whitney. For correlation analysis, we used Pearson's or Spearman's correlation, according to normality. All statistical tests were performed using Statistical Package for Social Sciences ® (SPSS), version 21.0. Statistical significance was set at $p < 0.05$.

RESULTS

The distribution of demographic characteristics and risk factors of BC according menopausal status are presented in **Table 1**. The mean age of women was 50.3 years, with the premenopausal group significantly younger than postmenopausal women (42.2

vs. 58.4 years; $p<0.05$). The postmenopausal group has significantly more former- and smokers (51.0% vs. 30.0%; $p= 0.03$) compared to the premenopausal group. Similarly, postmenopausal group was more women under HRT (2.0% vs. 16.3%; $p=0.01$). According **Table 2**, the most of women were in stage II (33.7%), had tumors up to 2.0 cm (42.9%), and without spread to nearby (63.6%), without differences between menopause status groups.

Concerning the oxidative plasma profile, postmenopausal status was associated with significant increase in plasma retinol (1.5 μmol/L vs. 1.8 μmol/L; $P<0.05$) and α-tocopherol (10.6 μmol/L vs. 11.9 μmol/L; $p=0.01$). For all others oxidative and inflammatory biomarkers, both groups were similar (**Table 3**). On the erythrocyte fatty acid analyses, higher levels of oleic fatty were associated with postmenopausal status (11.6% vs 12.9%; $p=0.01$). Also, in this group higher levels of arachidonic (11.1% vs 12.9%; $p=0.01$) and EPA (0.3% vs 0.4%; $p=0.01$) were found (**Table 4**).

Regarding that oxidative stress and inflammatory biomarkers were similar in both, pre-menopausal and post-menopausal women, despite that different antioxidants and omega-3 fatty acids, we tested the impact of receptors. Post-menopausal-(ER+) women was associated with higher levels of EPA (0.26% vs 0.35%; $p=0.04$) than pre-menopausal-(ER-) patients. In contrast, post-menopausal-(ER-) women was associated with lower DHA levels (2.66% vs 1.32%; $p=0.01$), total omega-3 (3.01% vs 1.91%; $p=0.04$) and omega-3 index (2.92% vs 1.62%; $p=0.02$), as well as, higher levels of arachidonic (10.68 vs 13.17%; $p=0.01$). The impact of fatty acids on oxidation and inflammation markers were reinforced when post-menopausal-(ER+) and (ER-) women in which higher levels of DHA (2.31% vs. 1.32%; $p=0.03$), total omega-3 (2.86% vs. 1.91%; $p=0.04$), and omega-3 index (2.68% vs. 1.62%; $p=0.02$) were found observed in (ER-) patients (**Figures 1-3**).

Postmenopausal women showed that higher palmitic acid levels were moderately and significantly associated with higher IL1β levels ($r=0.49$; $p=0.03$), while higher myristic acid was associated with higher IL-10 levels ($r=0.75$; $p<0.05$). On the other hand, higher EPA levels were moderately and significantly associated with lower levels of IL1β ($r=-0.49$; $p=0.03$) and IL10 ($r=-0.54$; $p=0.02$). Similarly, higher content of LDL(-) was correlated with lower α-linolenic acid (ALA) levels ($r=-0.37$; $P=0.03$). High omega-6:omega-3 ratio was moderately and significantly associated with a higher level of the pro-inflammatory mediator MCP-1 ($r=0.49$; $p=0.03$). Regarding the premenopausal group, no significant correlations were found for all correlations (**Figure 4**).

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Table 1. Demographic characteristics and risk factors according premenopausal and postmenopausal BC women groups.

Variables	**Total (n = 99)**		**Premenopausal (n = 50)**		**Postmenopausal (n = 49)**		***p-value***
Age (years)[a]	50.3	(11.3)	42.2	(6.3)	58.4	(9.2)	**<0.05**
Ethnicity, n (%)[b]							
White	26.0	(26.3)	10.0	(20.0)	16.0	(32.7)	
Asian	12.0	(12.1)	5.0	(10.0)	7.0	(14.3)	0.35
Brown	55.0	(55.6)	31.0	(62.0)	24.0	(49.0)	
Black	6.0	(6.1)	4.0	(8.0)	2.0	(4.1)	
Smoking, n (%)[b]	40.0	(40.4)	15.0	(30.0)	25.0	(51.0)	**0.03**
Alcohol intake, n (%)[b]	45.0	(45.5)	25.0	(50.0)	20.0	(40.8)	0.36
HRT, n (%)[b]	9.0	(9.1)	1.0	(2.0)	8.0	(16.3)	**0.01**
Breastfeeding, n (%)[b]	66.0	(82.5)	33.0	(89.2)	33.0	(76.7)	0.14
Nullyparity, n (%)[b]	19.0	(19.2)	13.0	(26.0)	6.0	(12.2)	0.08
Family history of BC, n (%)[b]	69.0	(69.7)	35.0	(70.0)	34.0	(69.4)	0.95
Weight (kg)[a]	67.9	(10.8)	68.3	(10.2)	67.5	(11.5)	0.71
BMI (kg/m^2)[a]	28.0	(4.4)	27.6	(3.8)	28.3	(4.9)	0.40
WC (cm)[a]	96.5	(10.1)	94.5	(9.8)	98.5	(10.2)	0.06
Fat mass (%)[a]	35.1	(4.8)	34.2	(4.8)	36.1	(4.7)	0.05
Lean mass (%)[a]	64.9	(4.8)	65.8	(4.8)	63.9	(4.7)	0.05

*Categorical variables were shown in absolute value and percentage and continuous values were showed in mean and standard deviation. [a]The statistical analysis was performed by independent samples t-test. [b]The statistical analysis was performed with the Pearson Chi-square test. For all tests the p value accepted was <0.05. HRT = Hormone replacement therapy; BMI = Body Mass Index; WC = Waist circumference.

Table 2. Tumor characteristics among premenopausal and postmenopausal breast cancer patients.

Variables	Total		Premenopausal		Postmenopausal		*p*-value
Clinical stage							
0	2.0	(2.0)	0.0	(0.0)	2.0	(4.1)	
I	28.0	(28.6)	12.0	(24.5)	16.0	(32.7)	0.36
II	33.0	(33.7)	20.0	(40.8)	13.0	(26.5)	
III	21.0	(21.4)	11.0	(22.4)	10.0	(20.4)	
Tumor size							
T1	33.0	(42.9)	15.0	(39.5)	18.0	(46.2)	
T2	21.0	(27.3)	11.0	(28.9)	10.0	(25.6)	0.87
T3	9.0	(11.7)	4.0	(10.5)	5.0	(12.8)	
T4	14.0	(18.2)	8.0	(21.1)	6.0	(15.4)	
Lymph node							
N0	49.0	(63.6)	21.0	(55.3)	28.0	(71.8)	0.23
N1	27.0	(35.1)	16.0	(42.1)	11.0	(28.2)	
Nx	1.0	(1.3)	1.0	(2.6)	0.0	(0.0)	
Molecular subtypes							
ER+	40.0	(87.0)	21.0	(84.0)	19.0	(90.5)	0.52
PR+	37.0	(80.4)	19.0	(76.0)	18.0	(85.7)	0.41
HER2+	16.0	(39.0)	8.0	(36.4)	8.0	(42.1)	0.71

*Categorical variables were shown in absolute value and percentage. [a]The statistical analysis was performed with the Pearson Chi-square test. For all tests the p value accepted was <0.05. The statistical analysis was performed with the Pearson Chi-Square test. ER=Estrogen receptor; PR=Progesterone receptor; HER2=Human Epidermal Growth Factor Receptor 2.

Table 3. Plasmatic inflammatory and oxidative profile among premenopausal and postmenopausal breast cancer patients.

Variables	Total (n = 99)		Premenopausal (n = 50)		Postmenopausal (n = 49)		*p*-value
IL1β (pg/mL)[a]	2.9	(16.1)	5.2	(23.0)	0.6	(1.6)	0.83
IL-6 (pg/mL)[a]	2.1	(5.8)	1.4	(2.9)	2.7	(7.6)	0.93
IL-10 (pg/mL)[a]	4.3	(12.8)	6.5	(17.4)	2.2	(5.8)	0.77
TNF-α (pg/mL)[a]	32.0	(42.5)	36.8	(60.0)	27.8	(16.6)	0.68
MCP-1 (pg/mL)[b]	349.6	(190.4)	339.0	(256.6)	359.7	(95.7)	0.71
Retinol (μmol/L)[b]	1.7	(0.5)	1.5	(0.4)	1.8	(0.5)	**<0.05**
α-tocopherol (μmol/L)[b]	11.2	(2.7)	10.6	(2.3)	11.9	(3.0)	**0.01**
β-carotene (μmol/L)[b]	0.5	(0.4)	0.5	(0.3)	0.5	(0.4)	0.51
TBARS (μmol/mL)[b]	6.0	(1.6)	6.1	(1.8)	5.8	(1.3)	0.43
oxLDL (U/L)[a]	4.1	(5.4)	4.3	(5.8)	4.0	(5.0)	0.87
anti-LDL (mU/L)[a]	4.6	(3.0)	5.0	(3.3)	4.2	(2.7)	0.14
8-OHdG (ng/mL)[b]	18.2	(5.9)	17.3	(6.1)	19.1	(5.6)	0.18

[a]The statistical analysis was performed with the Mann-Whitney U Test. [b]The statistical analysis was performed with independent samples t-test. For all tests the p value accepted was <0.05. IL1β= Interleukine-1β; IL-6= Interleukine-6; IL-10= Interleukine-10; TNF-α = Tumor necrosis factor α; MCP-1= Monocyte chemoattractant protein-1; TBARS= Thiobarbituric acid reactive substances; oxLDL= Oxidized low-density lipoprotein; 8-OHdG= 8-hydroxydeoxyguanosine.

Table 4. Erythrocyte fatty acids profile among premenopausal and postmenopausal breast cancer patients.

Fatty acids (%)	Total (n = 76)		Premenopausal (n = 42)		Postmenopausal (n = 34)		*p*-value
Myristic acid[b]	6.8	(1.4)	6.8	(1.5)	6.8	(1.1)	0.97
Palmitic acid[b]	29.9	(3.2)	30.5	(3.7)	29.1	(2.3)	0.06
Stearic acid[b]	24.2	(4.4)	24.9	(4.5)	23.4	(4.1)	0.13
Oleic acid[b]	12.2	(2.4)	11.6	(2.5)	12.9	(2.1)	**0.01**
Elaidic acid[b]	1.0	(0.3)	1.0	(0.3)	1.1	(0.3)	0.43
Linoleic acid[b]	7.9	(1.8)	8.0	(1.8)	7.8	(1.7)	0.58
α-Linolenic acid[a]	0.2	(0.4)	0.2	(0.4)	0.2	(0.5)	0.43
Eicosatrienoic acid[b]	1.4	(0.5)	1.4	(0.5)	1.4	(0.5)	0.90 [b]
Arachidonic acid[b]	11.9	(3.2)	11.1	(3.4)	12.9	(2.5)	**0.01**
EPA acid[a]	0.3	(0.2)	0.3	(0.2)	0.4	(0.3)	**0.01**
Behenic acid[b]	1.8	(1.0)	1.7	(1.0)	1.9	(1.0)	0.46
DHA acid[a]	2.3	(1.7)	2.6	(1.7)	2.1	(1.8)	0.16
Total omega-3[a]	2.9	(1.8)	3.0	(1.8)	2.7	(1.8)	0.29
Omega-6:Omega-3 ratio[a]	10.6	(8.5)	9.2	(5.1)	12.5	(11.2)	0.18
Omega-3 index[b]	2.7	(1.8)	2.8	(1.7)	2.4	(1.8)	0.34

Variables expressed as mean and standard deviation. **[a]**Statistical analysis was performed with the Mann-Whitney U Test. For all tests the p value accepted was <0.05. **[b]**Statistical analysis was performed with independent samples *t-test*. EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; total ômega-3 – sum of α-Linolenic acid, EPA and DHA; omega-3 index=sum of percentage of DHA and Spearman and Pearson correlation test according normality of variables. EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; total ômega-3 – sum of α-Linolenic acid, EPA and DHA; omega-3 index=sum of percentage of DHA and EPA.

Figure 1. Erythrocyte fatty acid profile among breast cancer patients according to ER molecular subtype.

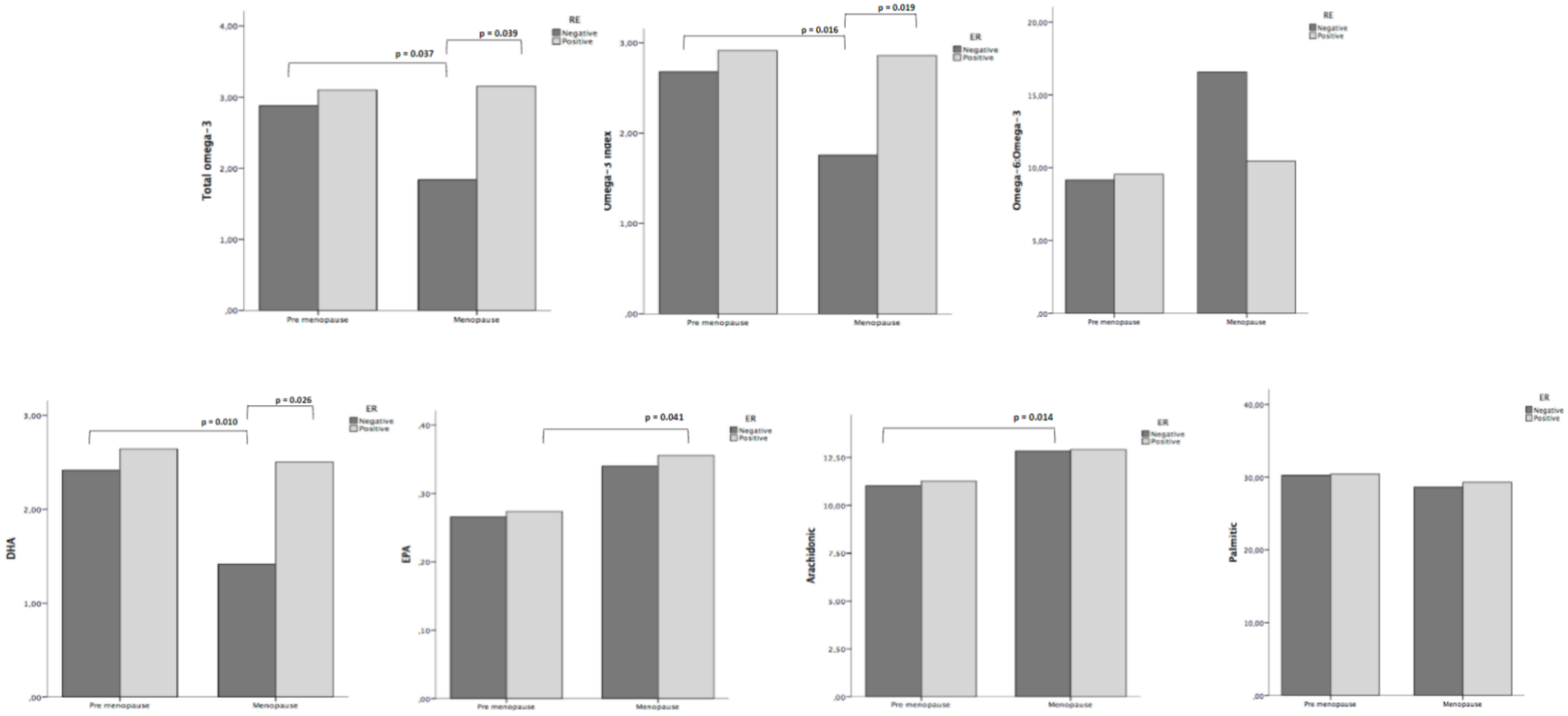

Figure 2. Erythrocyte fatty acid profile among breast cancer patients according to PR molecular subtype.

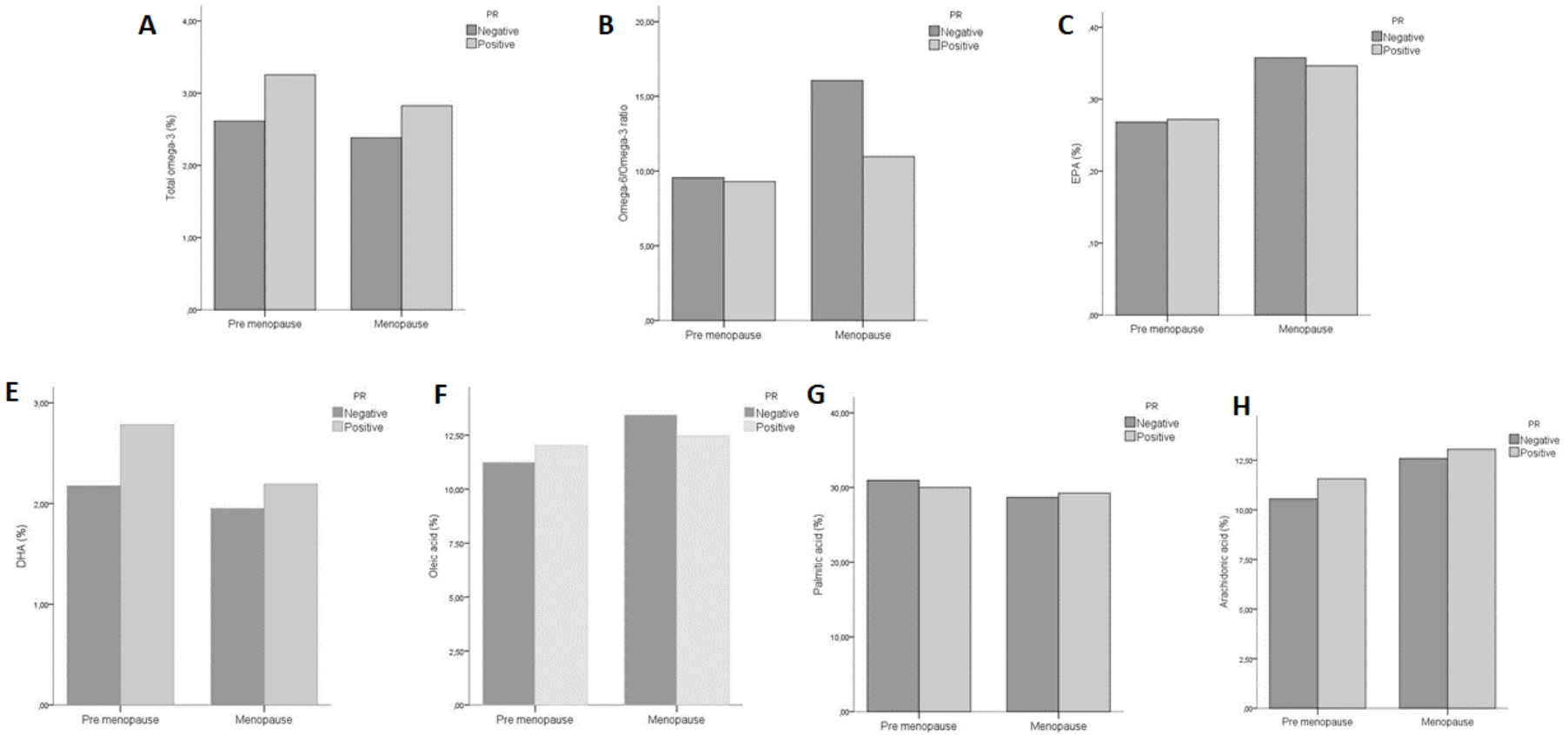

Figure 3. Erythrocyte fatty acid profile among breast cancer patients according to HER2 molecular subtype.

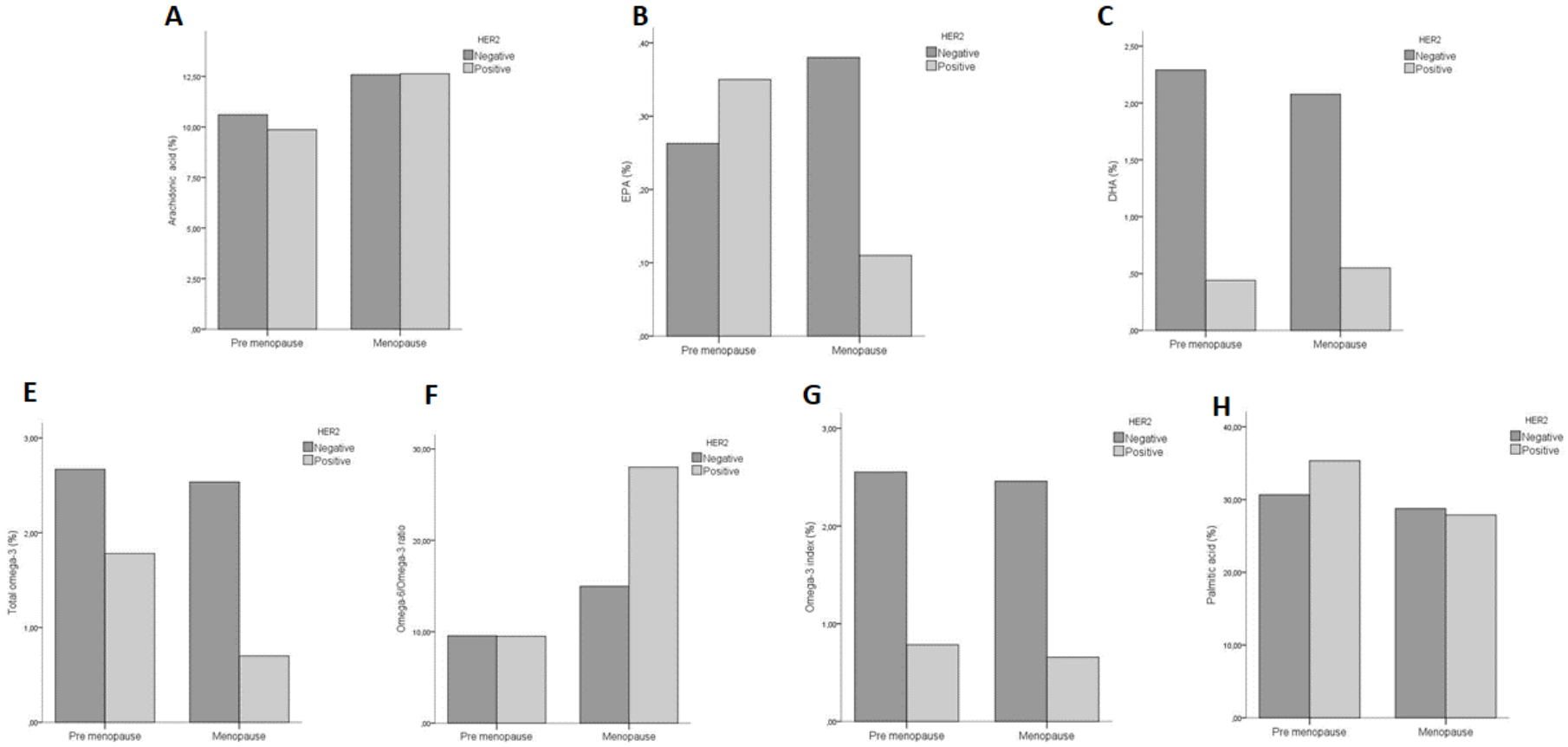

Figure 4. Heatmap of erythrocyte fatty acids, tumor size, inflammation and oxidation profile among premenopausal and postmenopausal breast cancer patients.

Article: Nutritional Counseling Protocol for Colorectal Cancer Patients after Surgery Improves Outcome

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NUTRITION AND CANCER
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Nutritional Counseling Protocol for Colorectal Cancer Patients after Surgery Improves Outcome

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ABSTRACT

Incidences of colorectal cancer (CRC) have continued to grow. Surgery is the main treatment and the only curative factor is nutritional status, which has an enormous influence on postoperative evolution. This study proposes a protocol for nutritional intervention beginning preoperatively and lasting up to three months postoperatively. Twenty patients with confirmed diagnosis of colon adenocarcinoma who underwent resection surgery were included. Anthropometric and food intake data–assessed through two 24-hour recalls, one weekday and one weekend–were collected at baseline, one month postoperative (PO), and three months PO. Anthropometric evaluation showed a decrease in the first month PO in weight, BMI, Hand grip strength, and arm circumference ($P < 0.05$), but these parameters recovered after 3 mo, PO and concomitant with the increase in protein and dietary fiber intake. In addition, collateral symptoms, such as abdominal distension, abdominal pain, and post prandial fullness, decreased between baseline and three months postoperative ($P < 0.05$). The nutritional counseling protocol for patients undergoing surgery due to CRC was positive in the recovery of nutritional status and improve of symptoms.

ARTICLE HISTORY
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Introduction

According to GLOBOCAN estimates, colorectal cancer (CRC) in 2018 was the third most common cancer (1.8 million new cases) in both men and women, and it ranked second in terms of mortality worldwide (1). The increased incidence of this cancer may be related to environmental risk factors such as high alcohol consumption and a diet with high inflammatory index and few anti-inflammatory foods such as minerals, vitamins, and dietary fiber (2).

Compared to other cancers, CRC patients have a higher prevalence of malnutrition at diagnosis, which is associated with higher mortality, morbidity, and increased hospitalization days (3).

Preoperative nutritional status significantly influences clinical prognosis. After CRC surgery, an increase of approximately 20% in the nutritional risk of these patients was observed (4). CRC patients also had a high readmission rate after 30 day postoperatively due to lack of understanding about self-care, with dehydration and intestinal obstruction the most common causes (5).

Nutritional follow-up after CRC surgery can significantly increase postoperative recovery, decreasing hospital stay and postoperative complications due to proper symptom management, improving quality of life, avoiding weight loss, and reducing hospitals expenses (5, 6).

The aim of the present study was to evaluate the impact of a nutritional intervention protocol on food intake and nutritional status of patients undergoing intestinal resection for colorectal neoplasms with three months of follow-up.

Materials and Methods

This longitudinal nutritional intervention is a time series single-arm trial with 3-month follow-up. The study was conducted between May 2013 and January 2017 at the Universitary Hospital of the University of São Paulo (HU/USP).

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Book Chapter 1

Dieta e Câncer

Tratado de Nutrição Funcional em Oncologia – 1 ed. 2022 (ISBN: 978-65-992200-4-3)

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INTRODUÇÃO

O câncer é uma doença crônica resultante da interação entre fatores ambientes e genéticos complexos, que pode demorar décadas até sua primeira manifestação clínica. Do ponto de vista epidemiológico, o câncer é a segunda principal causa de morte ao nível mundial, permanecendo como um grave problema de Saúde Pública. Apesar dos avanços em termos de diagnóstico e tratamento, que têm favorecido um bom prognóstico clínico e menor recidivo e mortalidade, a prevenção do câncer ainda permanece o ponto central dos programas de políticas públicas. Nesse contexto, a Nutrição se apresenta como um fator chave que pode agir não só diretamente nas diversas etapas da carcinogênese, mas também como importante adjuvante durante o tratamento oncológico e no controle de morbidades associadas como a obesidade, as dislipidemias e o diabetes. Insolada ou em conjunto essas morbidades agem negativamente na prevenção e tratamento do câncer. Considerando, o papel singular da dieta no contexto do câncer, instituições nacionais e internacionais têm compilado evidências cientificas que podem orientar indivíduos e profissionais de saúde a adotarem condutas dietoterápicas clinicamente seguras e eficazes. Este capítulo convida o leitor a fazer reflexões sobre as evidências científicas mais robustas, mas também aborda o atual estado da arte sobre o uso de padrões alimentares menos convencionais, mas que veem sendo estudadas no tratamento do câncer.

RECOMENDAÇÕES CLÁSSICAS SEGUNDO DIRETRIZES

A relação entre dieta/alimentação e câncer é inquestionável e no Brasil 5,1% dos casos de câncer são atribuídos à dieta[1], independente do peso, percentual semelhante ao observado nos Estado Unidos[2]. As agências internacionais de combate ao câncer, *Word Cancer Research Fund International (WCRF)[3], International Agence for Research on Cancer (IARC)[2],* e a *American Cancer Society (ACS)[4],* têm recomendações sobre dieta e prevenção do câncer e sua recidiva. No Brasil o Instituo Nacional do Câncer (INCA)[5] apresenta recomendações alimentares baseadas nessas diretrizes e orienta que os pacientes sobreviventes, após o tratamento oncológico, também sigam essas recomendações. Além disso, o INCA indica o uso do Guia Alimentar para a População Brasileira[6] como fonte confiável de informações sobre alimentação.

De um modo geral, essas diretrizes destacam que atualmente as recomendações sobre alimentação têm sido apoiadas por estudos que envolvem o padrão alimentar dos indivíduos e consideram não mais o nutriente ingerido, mas sim o alimento, pois é dessa forma que as pessoas consomem suas refeições[2,4].

CAPÍTULO 10

DIETAS E CÂNCER: DA PREVENÇÃO AO TRATAMENTO

10.1. DIETA VEGETARIANA, DIETA CETOGÊNICA, JEJUM INTERMITENTE E RESTRIÇÃO CALÓRICA

Nágila Raquel Teixeira Damasceno
Sara Maria Moreira Lima Verde
Isabelle Novelli

Introdução

O câncer é uma doença crônica resultante da interação entre fatores ambientais e genéticos complexos, que pode demorar décadas até sua primeira manifestação clínica. Do ponto de vista epidemiológico, o câncer é a segunda principal causa de morte em nível mundial, permanecendo como um grave problema de saúde pública. Apesar dos avanços em termos de diagnóstico e tratamento, que têm favorecido um melhor prognóstico clínico e menor recidiva e mortalidade, a prevenção do câncer ainda permanece o ponto central dos programas de políticas públicas.

Nesse contexto, a nutrição se apresenta como um fator-chave que pode agir não só diretamente nas diversas etapas da carcinogênese, mas também como importante adjuvante durante o tratamento oncológico e no controle de morbidades associadas como a obesidade, as dislipidemias e o diabetes. Isoladas ou em conjunto, essas morbidades [illegible] negativame na prevenção e no tratamento d[illegible]ncer.

Considerando o papel singu[illegible]la dieta no c texto do câncer, instituições na[illegible]is e interna nais têm compilado evidências c[illegible]ficas que pod orientar indivíduos e profissiona[illegible] saúde a ad rem condutas dietoterápicas cli[illegible]nente segura eficazes. Este capítulo convida [illegible]or a fazer re xões sobre as evidências científi[illegible] mais robust também aborda o atual estado da [illegible]te sobre o usc padrões alimentares menos convencionais, mas veem sendo estudados no tratamento do câncer.

Recomendações clássicas segundo diretrizes

A relação entre dieta/alimentação e câncer é questionável e, no Brasil, 5,1% dos casos de cân

Book Chapter 2

Obesidade e câncer

Unpublished

Isabelle Novelli
Bruno Dantas
Nágila Raquel Teixeira Damasceno

EIXO INFLAMAÇÃO

Inflamação sistêmica

Coerente com o aumento exponencial de sobrepeso e obesidade no Brasil e no mundo, a incidência de câncer também tem crescido, principalmente em neoplasias que sofrem influência do padrão alimentar, tais como câncer colorretal, de mama, etc. Essa associação desperta interesse para a possível relação entre o excesso de gordura corporal e o desenvolvimento dessas doenças.

Paralelamente, ao longo das últimas décadas o tecido adiposo deixou de ser considerado um simples reservatório energético para ser aceito como um tecido com alta capacidade endócrina sendo capaz de produzir diversas substâncias (adipocinas, citocinas, etc.) (DENG et al., 2016).

Diante das mudanças alimentares que ocorreram ao longo dos últimos 100 anos, com a oferta crescente de produtos industrializados, densamente energéticos, ricos em açúcares e gorduras saturadas, não se observou aumento da atividade física, resultando num balanço energético positivo com consequente acúmulo de tecido adiposo (FILHO; BATISTA, 2010). Conforme este tecido se hipertrofia o conteúdo lipídico intracelular resultante da alta ingestão energética também se acumula. Com a cronificação desse processo, ocorre a hipóxia ao nível dos adipócitos, levando a menor vascularização e favorecendo a necrose e a apoptose dessas células em um processo concomitante a infiltração de macrófagos. Isso leva a formação de estruturas em forma de coroa, classicamente denominadas *crown like struture* (CLS) (REVELO et al., 2014).

Existe um corpo de evidências que mostra a associação entre a alta adiposidade e o risco aumentado para o desenvolvimento de câncer, destacando-se aqui o câncer de mama, sobretudo na pós menopausa (NAMAZI, NAZLI et al., 2018). Embora a obesidade per si mantenha associação com o câncer, atualmente, sabe-se que aspectos qualitativos desse acúmulo de tecido adiposo podem influenciar o prognóstico clínico, pois sabe-se que, ao contrário do tecido adiposo subcutâneo, a adiposidade visceral tem intenso papel pró-inflamatório (HIMBERT et al., 2018). Embora fatores genéticos possam explicar parcialmente o acúmulo de tecido adiposo, fatores ambientais e, principalmente, a dieta exercem forte papel na no desenvolvimento da obesidade, na carcinogênese e relação entre ambas as doenças. A associação de uma dieta rica em açúcares, gorduras saturadas, pobre em fibras e o desbalanço entre ácidos graxos ômega 3 e 6 vem demonstrando associação com marcadores inflamatórios e inflamação crônica de baixa intensidade (SHIVAPPA et al., 2018). Esse perfil de dieta tem mostrado ser determinante, podendo aumentar em até 17% o risco de desenvolver neoplasias que indivíduos que não consomem esse padrão de dieta (NAMAZI, N; LARIJANI; AZADBAKHT, 2018).

No tecido adiposo de indivíduos eutróficos (Figura 1) a interleucina 4 (IL-4), produzida majoritariamente pelos eosinófilos, age como um regulador chave para manter três tipos de células: T helper 2 (Th2), macrófagos tipo M2 e células T regulatórias (Treg). Essas três linhagens celulares promovem um ambiente homeostático, ou seja, em equilíbrio metabólico no tecido adiposo. Ainda na ausência de obesidade, a adiponectina atua estimulando a produção de outras citocinas anti-inflamatórias como, interleucina 10 (IL-10), interleucina 15 (IL-15) e interleucina 33 (IL-33), assim como na manutenção das células Treg, na redução de interferon gama (IFN-γ), expressão de interleucina 12 (IL-12), e na expressão do complexo de histocompatibilidade de classe maior II (MHCII) que, em conjunto com a secreção das proteínas tipo *fizzled* 5, auxiliam na composição de um ambiente anti-inflamatório (DENG et al., 2016). Contrário ao cenário de eutrofia, na obesidade há hipertrofia crescente dos adipócitos, disfunção mitocondrial e diminuição das células anti-inflamatórias Treg e Th2, além da mudança da polarização dos macrófagos de M2 para M1, alterando seu comportamento anti-inflamatória para inflamatório. Nesse contexto, existe maior produção de leptina, que induzirá maior produção de citocinas inflamatórias interleucina 1 (IL-1), interleucina 6 (IL-6) e IL-12, assim como TNF-α pelas células do sistema imune. As células T se diferenciarão em células Th1 por ação da leptina e inibirão as células Treg. A alta concentração de leptina também leva ao aumento de IFN-γ que por sua vez induzirá polarização M1 aos macrófagos e produção de IL-6 e IL-1 pelas células Th2 e célula tipo 2 linfoide inata (ILC2) (DENG et al., 2016). Todas essas mudanças transformam os adipócitos e as células do sistema imune em células secretórias de substâncias pró-inflamatórias, desencadeando a inflamação crônica de baixa intensidade típica da obesidade e que atuará com amplo espectro em tecidos adjacentes em nível sistêmico, estimuladores de oncogêneses ou quando a lesão neoplásica já estiver presente, aumentando a agressividade e/ou proliferação das células tumorais (IYENGAR; HUDIS; DANNENBERG, 2015).

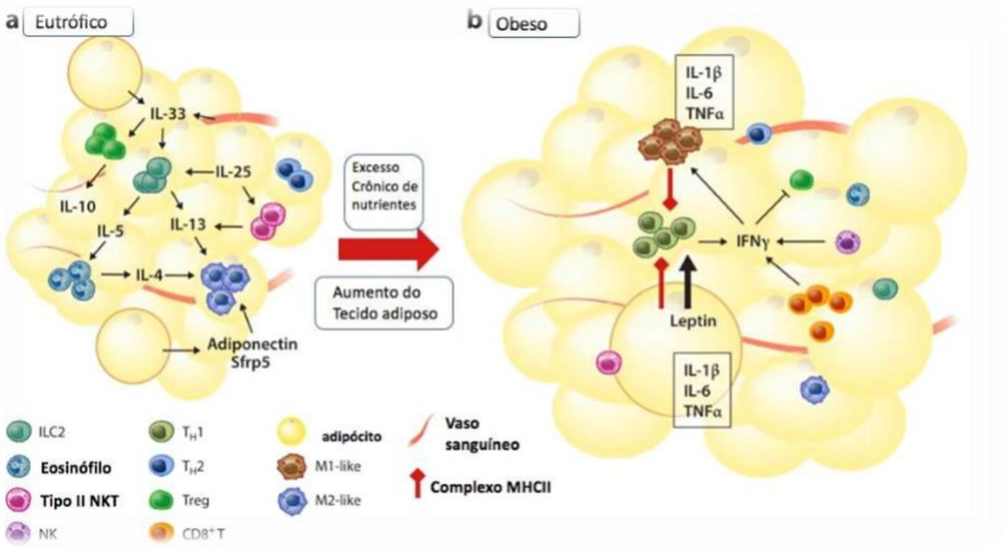

Figura 1. Regulação da inflamação pelo tecido adiposo. (A) Em indivíduos eutróficos o tecido adiposo dispõe de várias substâncias anti-inflamatórias que auxiliam a diminuir o estado inflamatório do tecido (ILC2s, Tregs, eosinófilos, NKT tipo II, células Th2 e macrófagos M2-like). (B) com o desenvolvimento da Obesidade os adipócitos expressão mais leptina e juntamente com o complexo MHCII estimulam a ativação de células Th1. IFY gama produzida por essas

células, pelas NK e as CD8+ irão inibir a produção de substâncias anti-inflamatórias e aumentaram a secreção de citocinas inflamatórias tais como Il-1, IL-6 e TNF-α. Abreviações: IFN-γ: interferon gama; IL: interleucina; ILC2: célula Tipo 2 linfoide inata; MHC-II: complexo maior de histocompatibilidade II; NK: célula *natural killer*; NKT: célula *natural killer* T; SFRP5: proteína tipo *frizzled* 5; Th1: célula T helper tipo 1; Th2: célula T helper tipo 2; TNF-α fator de necrose tumoral alfa; Treg: célula T regulatória. Figura traduzida e adaptada de DENG et al, 2016.

Alguns autores ao discutirem inflamação e câncer propuseram uma classificação de acordo com a importância da mesma nos fatores de risco para o seu desenvolvimento. Segundo eles esses fatores são independentes quando levamos em consideração dano ao DNA, tais como a radiação e produtos químicos, causas hereditárias e agentes desconhecidos que poderiam agem como indutores do câncer. Os fatores dependentes da inflamação são aqueles provocadas por infecções microbiológicas (como *H. pylori* no câncer gástrico e HPV-vírus do papiloma humano, nas neoplásicas ginecológicas), assim como a própria inflamação crônica induzida na obesidade e em doenças inflamatórias, como a doença de Crohn (Figura 2). Tanto as causas dependentes, quanto independentes resultarão em um quadro de inflamação crônica à medida que o câncer atinge estágio mais avançado (HUGO GONZALEZ et al, 2018).

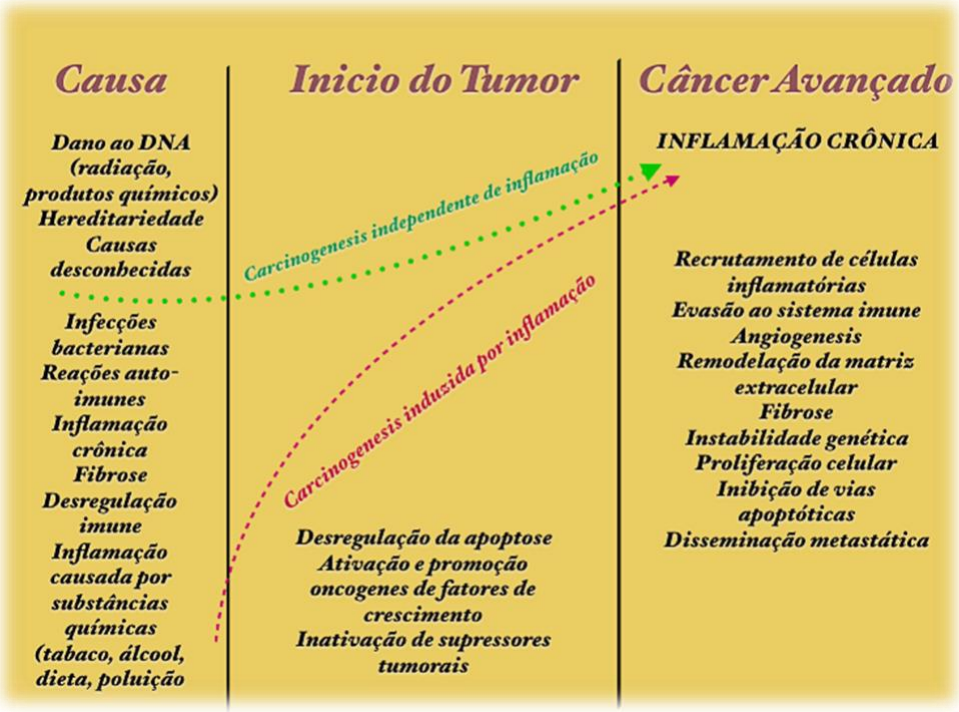

Figura 2. Diferentes causas estão associadas com a promoção da carcinogênese, as quais podem ser independentes ou dependentes de um ambiente pró-inflamatório. No evoluir da doença diversos fatores contribuem para a disseminação tumoral e um ambiente sistêmico de inflamação crônica. Adaptado de HUGO GONZALEZ et al, 2018.

Inflamação no ambiente micro tumoral

O ambiente tumoral não é composto somente de células neoplásicas, existe um conjunto dinâmico composto de células disfuncionais (células típicas do tecido que

alteram seu metabolismo em prol do tumor), de células do sistema imune e do estroma adjacente (CORRÊA et al., 2017).

Diante do quadro de obesidade há um excesso de citocinas e leptina ao nível sistêmico que perpetuam a inflamação crônica levando ao acúmulo de subprodutos do metabolismo celular como espécies reativas de nitrogênio e oxigênio que produzem danos ao DNA, do mesmo modo que ativam cronicamente o NF-κB (KAWANISHI et al., 2017). Tais estímulos constantes podem promover erros ao nível de troca das bases do DNA, diminuição de genes supressores tumorais ou em outras etapas da transcrição, tradução e até mesmo em etapas epigenéticas, que resultarão em evasão à apoptose, estímulos de crescimento e características malignas à célula afetada.

Como principais sinalizadores, as citocinas desempenham papel fundamental em todas as etapas do desenvolvimento do câncer. As citocinas IL-6, IL-1 e TNF-α aumentam no decorrer da doença e são um dos responsáveis por alguns sintomas clássicos: perda de peso, náuseas, êmese e alterações metabólicas concomitantes a anorexia progressiva (MACCIO; MADEDDU, 2012).

É bem descrito na literatura que a inflamação sistêmica possui um papel determinante nos processos de carcinogênese, manutenção e progressão tumoral. Apesar disso, algumas vias metabólicas no microambiente tumoral têm trazido novas perspectivas sobre as vias de evasão tumoral relacionadas com o sistema imune. Um exemplo dessa notável diferenciação é a repolarização dos macrófagos M1 para M2 (anti-inflamatórios) no microambiente tumoral, enquanto ao nível sistêmico esses permanecem na forma M1 (pró-inflamatórios) (CORRÊA et al., 2017; GRIVENNIKOV; GRETEN; KARIN, 2011) (Figura 3).

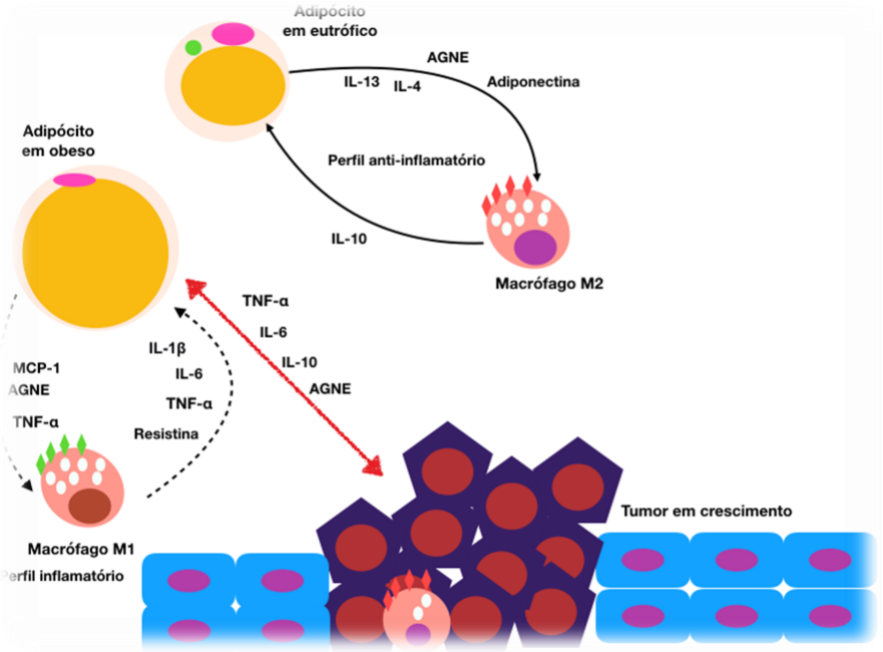

Figura 3. Interação entre o ambiente sistêmico e micro tumoral em situação de eutrofia e obesidade. Há um antagonismo entre o ambiente sistêmico e o ambiente tumoral. Na eutrofia há um equilíbrio entre o sistema imune e os adipócitos com produção constante de IL-10, adiponectina e baixa concentração de AGNE. Na obesidade há hipertrofia dos adipócitos que produzirão citocinas inflamatórias no ambiente sistêmico (IL-1β, IL-6 e TNF-α) esta inflamação por sua vez junto ao estimulo das células tumorais recrutarão células do sistema imune para o

ambiente micro tumoral, mas os macrófagos mudarão sua polarização para M2, perfil anti-inflamatório contribuindo para um ambiente propicio a evasão imune e perpetuação do crescimento neoplásico. LEGENDA: IL- interleucina, AGNE- ácido graxo não esterificado, MCP-1- proteína quimioatrativa de monócito, TNF-α- fator de necrose tumoral alfa.

EIXO INSULINA

Introdução

Nas últimas décadas houve um aumento de fatores que favorecem um balanço energético positivo no metabolismo, como maior consumo de alimentos com alta densidade calórica e diminuição da atividade física. O consumo excessivo e crônico de alimentos com alta densidade calórica provoca o desbalanço da homeostase energética a favor da reduzida mobilização de energia. Desta forma, o excesso de peso e a obesidade se tornaram uma condição crônica que gera impactos negativos na saúde (HEYMSFIEL & WADDEN, 2017).

Um dos mecanismos de manutenção da obesidade é a alteração na concentração das principais adipocinas: leptina e adiponectina. A obesidade estimula uma excessiva produção de leptina, entretanto uma falha no seu receptor leva a um estado de resistência, reduzindo o sinal de saciedade ao nível cerebral, além de induzir uma resposta anabólica ao nível sistêmico. Como resultado, a falta de estímulo pela leptina causa hiperfagia e resistência à insulina (RI). Por outro lado, a adiponectina diminui conforme o aumento do tecido adiposo. Níveis reduzidos de adiponectina estimulam a produção de citocinas inflamatórias como o fator de necrose tumoral α (TNF-α) e a interleucina-6 (IL-6) (GALLANGHER & LeROIT, 2015). A adiponectina também inibe a via de proliferação celular controlada pelo mammalian target of rapamycin (mTOR) e quando em menor quantidade essa sinalização pode ficar alterada, levando a uma maior proliferação (GUTTIÉREZ-SALMERÓN, 2016).

O excesso de tecido adiposo também representa uma fonte significativa de síntese de marcadores de inflamação no indivíduo obeso, pois o mesmo está infiltrado com macrófagos e células T que estimulam de forma latente e crônica a inflamação de baixo grau com a produção de TNF-α, IL-6 e intercelucina 1β (IL-1β) mediado principalmente pela maior expressão e síntese do fator nuclear kappa B (NFk-B) (GALLANGHER & LeROIT, 2015). Os ácidos graxos livres provenientes da dieta e, principalmente aqueles liberados da hidrólise de triglicerídeos nos adipócitos também causam um estimulo positivo às vias pró-inflamatórias moduladas pelo NFk-B através dos Toll-Like Receptors (TLR). Essa inflamação persistente na obesidade eleva de forma sistêmica as citocinas pró-inflamatórias que também estão relacionadas com a RI, pois muitas vias de sinalização da inflamação bloqueiam diretamente a ação da insulina (REILLEY & SATIEL, 2017).

O consumo elevado de carboidratos na dieta leva à longo prazo a obesidade e ao excesso de glicose no plasma. Quando esta está elevada existe uma compensação para a sua absorção à custo de uma maior produção de insulina pelas células β pancreáticas. Este sistema se manterá elevado sempre que existir um excesso desse substrato. Caso esse estímulo persista este sistema de glicose/insulina apresenta uma falha, pois a quantidade necessária de insulina para normalizar a glicose já não sensibiliza a célula da mesma forma, sendo necessária uma maior quantidade de insulina, caracterizando então a RI (GALLANGHER & LeROIT, 2015). A elevação do estímulo da insulina também causa aumento na produção de fatores de crescimento como o fator de crescimento semelhante à insulina (IGF) e ao nível celular, ativa vias

como a do mTOR e do seu substrato, S6 quinase 1 (S6K1), que estimulam vias de proliferação celular.

A persistente RI no indivíduo obeso pode evoluir para o diabetes mellitus tipo 2 (DM2), envolvendo etapas de pré diabetes, e alterações sistêmicas associadas à síndrome metabólica, que envolve fatores como baixa tolerância à glicose, dislipidemia e hipertensão (SHLOMAI et al, 2016; GALLANGHER & LeROIT, 2015).

Os mecanismos de manutenção da obesidade e, especialmente, a hiperinsulinemia, representam fatores que permeiam vias metabólicas e moléculas necessárias para o desenvolvimento do câncer estimulando proliferação, migração e invasão (SHLOMAI et al, 2016). Segundo estimativas, em 2012 5,7% de todos os novos casos de câncer no mundo foram atribuídos ao elevado IMC (> 25kg/m2) e associados ao diabetes (PEARSON-STTARD et. al, 2018). Essa incidência mostra a importância que os mecanismos de controle de peso e da obesidade e resistência à insulina possuem no desenvolvimento e prevenção de cânceres (GALLANGHER & LeROIT, 2015).

Na última década têm crescido o número de evidências sobre a relação do excesso de peso e a DM, assim como seus fatores associados – hiperglicemia e hiperinsulinemia – com o aumento no risco de desenvolvimento de vários tipos de neoplasias, incluindo o câncer de mama (BOYLE et al, 2012), próstata (SABOORI et. al, 2018), colorretal (PENG et. al, 2018), pâncreas (BOSETTI et. al, 2014) e endométrio (HERNANDEZ et. al, 2015).

Mecanismo de ação

A insulina é produzida e secretada por células β do pâncreas através principalmente do estímulo da glicose. O seu papel no metabolismo energético é fundamental para a captação de glicose pelos tecidos insulinodependentes (tecido adiposo e muscular), além de inibir a síntese hepática de glicose (gliconeogênese). A insulina também é responsável por estimular o armazenamento de gorduras pelos adipócitos e a produção de glicogênio pelo fígado e músculo. Nos adipócitos ela inibe lipólise, induz lipogênese e a absorção de ácidos graxos via estímulo de expressão e atividade da lipase lipoproteica (POLOZ & STAMBOLIC, 2015). Além destas funções, a insulina também está intimamente relacionada com estímulos proliferativos que favorecem o crescimento tumoral, atuando em vias metabólicas após a sua ligação com o seu receptor (VIGNERI et. al, 2016). No caso de uma hiperinsulinemia crônica é possível observar a maior frequência de doenças relacionadas com o aumento da proliferação celular, entre elas o câncer (VIGNERI et. al, 2016).

Na corrente sanguínea a insulina, ou com menos afinidade, o IGF, se ligam ao seu receptor (RIn) localizado na membrana celular da célula-alvo ou no tumor. O RIn possui diversas variantes, entre elas as mais conhecidas são a isoforma A e B. A via da insulina assume uma ação predominantemente metabólica quando ligado ao RIn-B, estimulando inibição da gliconeogenese e captação da glicose atavés da via PI3K/Akt. Entretanto é observado em alguns estudos que a isoforma A do receptor (RIn-A) está mais relacionada à via mitogênica da insulina, e assim, associado ao crescimento celular e às células neoplásicas através da via Ras-MEK-ERK (BELFIORI et. al, 2017), conforme foi observado em tumores de mama (HUANG et. al, 2011), endométrio (WANG et. al, 2013) e fígado (CHETTOUH et. al, 2013) (Figura 4).

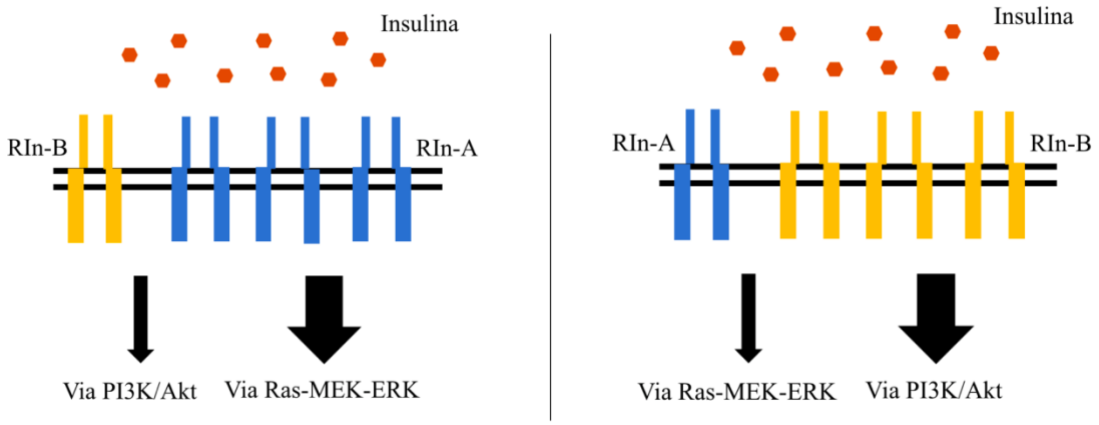

Figura 4. Isoforma do receptor de insulina e ativação de vias celulares. Seta grossa representa maior estímulo e seta fina menor estímulo. Legenda: RIn-A, receptor de insulina A; RIn-B, receptor de insulina B. Adaptado de Belfiori et al, 2017.

A via considerada como metabólica e mais ativada pelo RIn-B é a do PI3K/Akt. Apesar do seu papel importante no metabolismo energético essa via possui ação relevante na carcinogênese (Figura 2). O substrato da molécula Akt, o AS160, regula a translocação do receptor de glicose 4 (GLUT4) para a membrana celular, favorecendo o mecanismo de captação de glicose pela célula. Além disso o Akt fosforila a fosfofrutoquinase-2 (PKF-2) induzindo a glicólise. Ao estimular essa via a glicose-6-fostato – primeiro metabólito da glicólise – serve como substrato para a via das pentoses, que produz NADPH e nucleotídeos, auxiliando a replicação celular e evitando a apoptose a partir de mecanismos antioxidantes. Além disso, essa via também promove a desativação do fator de transcrição FOXO, ocorrendo a inibição de gliconeogenese e a apoptose, e do promotor de morte associado à Bcl-2 (BAD), também inibindo a apoptose celular (DOERSTILING SS et. al 2017; POLOZ & STAMBOLIC, 2015).

Outra via metabólica estimulada pela fosforilação do receptor de insulina é a Ras-MEK/ERK (Figura 5) que está intimamente relacionada com a característica mitogênica da insulina. A molécula ERK (quinase reguladora por sinal extracelular) atua como um fator de transcrição nuclear que regula genes envolvidos na proliferação celular, sobrevivência, crescimento e diferenciação da célula (POLOZ & STAMBOLIC, 2015).

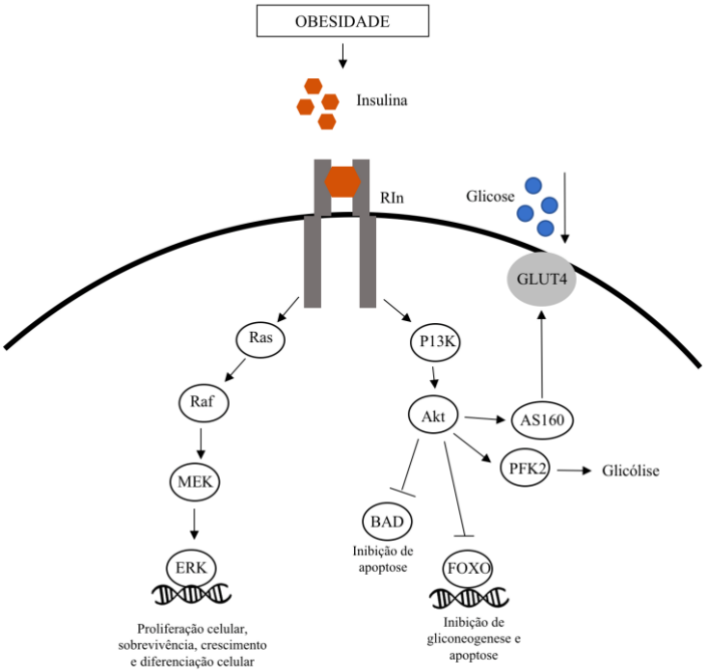

Figura 5. Via de sinalização celular da insulina. Ao se ligar no RIn a via com característica metabólica ativada pelo PI3K/Akt estimulam a inibição de gliconeogenese e inibem a apoptose, além de estimular a o GLUT4 para captação de glicose. A via com característica mitogênica, também estimulada pela insulina e seu receptor, ativa Ras/Raf/MEK/ERK que estimula a proliferação celular, sobrevivência, crescimento e diferenciação celular.
RIn, receptor de insulina; PFK, fosfofrutoquinase-2; BAD, promotor de morte associado à Bcl-2; GLUT4, transportador de glicose 4. Adaptado de POLOZ & STAMBOLIC, 2015.

O excesso de insulina circulante também estimula o sistema do IGF, que abrange sua proteína de ligação (IGFBP) e receptor (IGF-R). Os RIn possuem similaridades com o IGF-R, podendo haver estímulo na mesma via de sinalização da insulina (BELFIORI, 2017). O IGF-R tem se mostrado importante na carginogênese, visto que o controle da expressão do gene IGF-1R foi relacionado à várias proteínas supressoras tumorais, tais como p53, WT1 e PTEN. Quando mutadas essas proteínas promovem uma super expressão deste receptor, podendo haver uma sinalização elevada da via que estimula crescimento tumoral (BELARDI et al., 2013).

Já foi observada correlação entre elevadas concentrações de IGF-1 em mulheres com excesso de peso (índice de massa corporal entre 25 e 27 kg/m2) com aumento no risco de câncer de mama (THE ENDOGENOUS HORMONES AND BREAST CANCER COLLABORATIVE GROUP, 2010).

Portanto, tanto a insulina como a glicose possuem um papel importante na tumorigênese. Em estudos in vitro foi observado um maior crescimento em tumores de mama, colorretal, melanomas e leucemias quando estimuladas pela presença de insulina. Além disso, outros estudos in vitro observaram que um aumento da glicose no meio – estímulo robusto à secreção de insulina – pode amplificar as taxas de proliferação tumoral entre 7-44%.

A célula tumoral possui elevada sensibilidade à insulina, entretanto ainda não está totalmente elucidado a partir de qual concentração a insulina e a glicose passam a agir positivamente na proliferação e progressão tumoral em um sistema in vivo, apesar

de efeitos supressores terem sido observados quando houve controle do estímulo de insulina (KLEMENT & FINK, 2016).

Com o intuito de diminuir o risco e progressão do câncer padrões alimentares têm sido amplamente estudados. Baseado na diminuição da concentração de glicose e, consequentemente, de insulina circulante se propôs o uso de dietas hiperlipídicas (normocaloricas e com baixo teor de carboidratos) e/ou com restrição de calorias, onde se destacam a dieta cetogênica e dieta de Atkins. Em modelos animais esse padrão alimentar tem mostrado efetivo em controlar a progressão tumoral (LV et al, 2014), entretanto não existe na literatura evidências de um efeito positivo em humanos quando administrada de modo isolada ou adjuvante aos protocolos de tratamento oncológico convencionais, salvo em casos específicos como o glioblastoma (OLIVEIRA et al 2018).

Por outro lado, um estudo caso-controle multicêntrico com mais de 6.000 mulheres observou que aquelas que seguiam uma dieta com característica Mediterrânea (rica em vitaminas, flavonoides, azeite de oliva e baixo consumo de carnes vermelhas) obtiveram um risco 20% menor de desenvolver câncer de mama (TURATI et al, 2018) e que uma dieta rica em frutas, vegetais e com características da dieta Mediterrânea diminui o risco para câncer de endométrio (RICEERI et al, 2017). Estas evidências mostram os benefícios de se seguir recomendações relacionadas com alimentação saudável que contemplam toda a necessidade nutricional do indivíduo.

Apesar do papel negativo do excesso glicídico, induzido por uma dieta hipercalórica, sobre o peso corporal e proliferação de células tumorais, faltam evidências convincentes de eficácia e segurança das dietas hiperlipídicas ou baixa em carboidratos no contexto do paciente oncológico. Portanto, o manejo do excesso de peso e a prevenção da obesidade devem fazer parte das estratégias de prevenção, tratamento e redução do risco de recidiva em mulheres diferentes tipos de câncer, mas sobretudo naquelas com diagnóstico e com elevado risco de câncer de mama e de endométrio.

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Appendix 2

COEP FSP/USP study approval

COMITÊ DE ÉTICA EM PESQUISA - COEP
Faculdade de Saúde Pública
Universidade de São Paulo

OF.COEP/209/12

27 de novembro de 2012.

Prezadas pesquisadora e orientadora,

O Comitê de Ética em Pesquisa da Faculdade de Saúde Pública da Universidade de São Paulo, em sua **10.ª/12 Sessão Ordinária**, realizada em **23/11/2012**, analisou de acordo com a Resolução n.º 196/96 do Conselho Nacional de Saúde e suas complementares, o protocolo de pesquisa n.º **2162**, intitulado "**OBESIDADE E CÂNCER DE MAMA: AVALIAÇÃO DOS FATORES DE RISCO ASSOCIADOS AO EXCESSO DE PESO E TECIDO ADIPOSO**", do **grupo III**, sob responsabilidade da pesquisadora **Sara Maria Moreira Lima Verde** e orientação da Professora **Nágila Raquel Teixeira Damasceno,** considerando **APROVADO** a inclusão de novas análises no projeto.

Cabe lembrar que, de acordo com a Res. CNS 196/96, são deveres do(a) pesquisador(a): **1)** Comunicar de imediato qualquer alteração no projeto e aguardar manifestação deste Comitê de Ética em Pesquisa para dar continuidade à pesquisa; **2)** Manter sob sua guarda e em local seguro, pelo prazo de 5 (cinco) anos, os dados da pesquisa, contendo fichas individuais e todos os demais documentos recomendados pelo COEP, no caso eventual auditoria; **3)** Comunicar formalmente a este Comitê por ocasião do encerramento da pesquisa; **4)** Elaborar e apresentar relatórios parciais e final; **5)** Justificar perante o COEP interrupção do projeto ou a não publicação dos resultados.

Atenciosamente,

Prof. Tit. Cláudio Leone
Coordenador do Comitê de Ética em Pesquisa - FSP/USP

Ilm.ª Sr.ª
Prof.ª Dr.ª **Nágila Raquel Teixeira Damasceno**
Departamento de Nutrição
Faculdade de Saúde Pública/USP

Av. Dr. Arnaldo, 715 – Cerqueira César – CEP 01246-904 – São Paulo – SP
Contato: (55 11) 3061 7779 | coep@fsp.usp.br | www.fsp.usp.br

CEP HGF study approval

COMITÊ DE ÉTICA EM PESQUISA – CEP/HGF

Fortaleza, 06 de maio 2010.

IIlma. Sra.
Pesquisadora: **Sara Maria Moreira Lima Verde**
Projeto Intitulado: **Obesidade e câncer de mama: Avaliação dos fatores de riscos associados ao excesso de peso e tecido adiposo**
Área de conhecimento: **Ciências da Saúde / Nutrição**
Data da entrada no CEP: **13/04/10**
Protocolo do CEP: **050507/10**

O Comitê de Ética em Pesquisa do HGF em reunião ordinária no dia 05/05/10 analisou e considerou o referido projeto **APROVADO,** pois atende as recomendações da Resolução nº 196/96do CNS/MS.

Lembramos ao pesquisador o cumprimento da Resolução do CNS 196/96 na condução cientifica do seu projeto e ainda, o encaminhamento ao CEP do relatório da pesquisa bem como à devolução dos resultados à comunidade.

Atenciosamente,

Drª. Maria Veraci Oliveira Queiroz
Coordenadora do Comitê de Ética em Pesquisa – CEP/HGF

Disclosure of information form

Universidade de São Paulo
Faculdade de Saúde Pública
Av. Dr. Arnaldo, 715 – CEP 01246-904 – São Paulo – Brasil

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidada como voluntária a participar da pesquisa: **PAPEL DA ADIPOSIDADE SOBRE A INFLAMAÇÃO, OXIDAÇÃO E ADIPOCITOCINAS NA NEOPLASIA MAMÁRIA.**

O motivo que nos leva a estudar o problema da obesidade é o desenvolvimento do câncer de mama, o grande número de indivíduos com esse diagnóstico e sua relação com o excesso de peso. A pesquisa se justifica pela necessidade de avaliar quais fatores podem contribuir para o desenvolvimento dessa doença em mulheres com excesso de peso. Portanto, o objetivo desse projeto é identificar possíveis fatores presentes no excesso de peso e de gordura corporal que possam influenciar no desenvolvimento do câncer de mama. A coleta de material será da seguinte forma: será coletada uma amostra de sangue (20ml) e também será aplicado um questionário sobre seu nível socioeconômico, cultural e clinico.

Existem desconfortos e risco mínimo que envolvem enjôos em função do jejum e pequenos hematomas no local de punção, ambos relacionados a coleta de material. Os resultados obtidos através desse projeto trarão benefícios às pacientes sobreviventes de câncer de mama no momento em que mostrarem a obesidade como fator de risco e incentivarem políticas públicas de orientações sobre alimentação saudável, redução de peso e de gordura corporal.

Em caso de evidencias de risco a saúde pelos resultados encontrados as participantes serão orientadas a procurar o atendimento de um nutricionista.

Você será esclarecida sobre a pesquisa em qualquer aspecto que desejar. Você é livre para recusar-se a participar, retirar seu consentimento ou interromper a participação a qualquer momento. A sua participação é voluntária e recusar em participar não irá acarretar qualquer penalidade ou perda de benefícios.

Sua identidade será mantida em sigilo e sob a responsabilidade dos pesquisadores Os resultados dos exames serão enviados para você e permanecerão confidenciais. Seu nome ou o material que indique a sua participação não será liberado sem a sua permissão. Você não será identificada em nenhuma publicação que possa resultar deste estudo. Uma cópia deste consentimento informado será arquivada no Departamento de Nutrição da Faculdade de Saúde Pública da Universidade de São Paulo e outra será fornecida a você.

A participação no estudo não acarretará custos e você também não será beneficiada com nenhuma compensação financeira.

Eu, _____ fui informada dos objetivos da pesquisa acima de maneira clara e detalhada e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e motivar minha decisão se assim o desejar. A pesquisadora Sara Maria Moreira Lima Verde certificou-me de que todos os dados desta pesquisa serão confidenciais.

Também sei que todos os gastos estão inclusos no orçamento da pesquisa. Em caso de dúvidas poderei chamar a pesquisadora Sara Maria Moreira Lima Verde ou o(a) professor(a) orientador(a) Profa. Dra. Nagila Raquel Teixeira Damasceno no telefone (11) 3061-7865 ou os Comitês de Ética em Pesquisa (COEP) identificados abaixo:

1. COEP da Faculdade de Saúde Pública da Universidade de São Paulo, sito à Av. Dr. Arnaldo, 715, Cerqueira César – São Paulo, SP. Telefone: (11) 3061.7779.

2. COEP do Hospital Geral de Fortaleza, sito à Rua Ávila Goulart, 900, Papicu – Fortaleza, Ce. Telefone: (85).3101.7078.

Declaro que concordo em participar desse estudo. Recebi uma cópia deste termo de consentimento livre e esclarecido e fui esclarecida sobre minhas dúvidas

Nome	Assinatura do Participante	Data

Nome	Assinatura do Pesquisador	Data

Nome	Assinatura da Testemunha	Data

Data collecting questionnaire

AVALIAÇÃO SOCIO-ECONÔMICA, CULTURAL, CLÍNICA E ANTROPOMÉTRICA	**NÚMERO DO PRONTUÁRIO: _____** **DATA DA COLETA: ___ / ___ / _____**
1. B1 **AVALIAÇÃO SOCIO-ECONÔMICA**	
B1.1 **Nome**	B1.2 **Idade**
Endereço:	
Telefone: Res - Cel. - Trab. -	
B1.3 **Estado Civil** 1 ( ) Casada 2 ( ) Solteira 3 ( ) Viúva 4 ( ) Divorciada 5 ( ) Outros	B1.4 **Etnia*** 1 ( ) Branco 2 ( ) Amarelo 3 ( ) Pardo 4 ( ) Negro 5 ( ) Indígena
B1.5 **Escolariadade** 1 ( ) Ensino fundamental incompleto – 4ª série 2 ( ) Ensino fundamental completo – 8ª série 3 ( ) Ensino médio incompleto 4 ( ) Ensino médio completo – 3º ano 5 ( ) Superior incompleto 6 ( ) Superior completo 7 ( ) Outros	
B1.6 **Renda familiar *per capta:*** 1 ( ) < 1 SM 2 ( ) 2-| 6 SM 3 ( ) 7-| 10 SM 4 ( ) >10 SM	
B1.7 **Menarca:** anos B1.8 **Menopausa:** anos B1.9 **DUM:** B1.10 **TRH:** ( ) Não ( ) Sim	
B1.11 **Nuliparidade:** ( ) Não ( ) Sim B1.12 **Amamentação** ( ) Não ( ) Sim	
B1.13 **Fumo:** 1( )Não 2( )Fuma (atual) Tempo: _____ 3( ) Fumou (anterior) Tempo: _____ B1.14 **Álcool:** 1( ) Não 2( ) Consome bebida alcóolica (atual) 3( )Consumiu Quantidade:__* *>150mL para mulheres = consumo prejudicial à saúde (Sociedade Brasileira de Hipertensão, 2006)	

B1.15 **Antecedentes familiares do câncer:** 1( )Não 2( )Sim B1.16 **Localização** 1( )Mama 2( ) Outros		
B1.17 **Quem** 1( ) Mãe 2( ) Irmã 3( ) Avó 4( ) Tia		
2. B2 **AVALIAÇÃO CLÍNICA**		
Diagnóstico: Neoplasia mamária B2.1 **SUBTIPO** 1( ) Lobular 2( ) Ductal		
B2.2 **TNM:** T: ___ N: ___ M: ___ B2.3 **Estadiamento clínico (EC):** 1( )ECI 2( )EC II 3( ) EC III B2.4 **Tamanho do tumor:** ____ B2.5 **Linfonodos comprometidos:** 1.( ) N^- 2.N^+ Total: ___ B2.6 **Metástase:** 1. ( ) M^- 2. ( ) M^+ B2.7 **Grau histopatológico:** _____		
B2.8 **Informações importantes:** B2.8.1 **Faz uso de suplementos de vitaminas ou minerais? :** 1.( )Não 2.( )Sim Qual? _____ Dose diária: _____ Há quanto tempo? _____		
3. B3 **AVALIAÇÃO ANTROPOMÉTRICA**		
B3.1 Peso atual (kg):		B3.2 Peso habitual (kg):
B3.3 Altura (m):	B3.4 IMC:	B3.5 CC:
B3.6 Reactância (Xc):		B3.7 Resistência (R):
B3.8 % água:	B3.9 % gordura	B3.10 % massa magra:
B3.11 Ângulo de fase:		B3.12 TMB:

*Fonte: IBGE, senso demográfico 2002

CURRÍCULO LATTES

Isabelle Romero Novelli

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Última atualização do currículo em 28/07/2022

Doutoranda em Nutrição pela Faculdade de Saúde Publica da Universidade de São Paulo (FSP/USP). Pós graduação em Nutricional Clínica Funcional pelo Instituto VP (SP). Especialista em oncologia pelo Hospital Sírio Libanês - São Paulo, modalidade residência com período sanduíche no New York Preabyterian Hospital, Nova York (EUA). Graduada em Nutrição pela Universidade de Brasilia em 2015. Bolsista de Iniciação Científica pela Fundação Universidade de Brasília (FUB) na área de tecnologia dos alimentos com orientação da Prof. Dra. Lívia Pinelli em 2013. **(Texto informado pelo autor)**

Identificação

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Formação acadêmica/titulação

2018	Doutorado em andamento em Nutrição em Saúde Pública. Universidade de São Paulo, USP, Brasil. Título: Nutritional factors and metabolic biomarkers: Impact in Risk and Survival of Women with Breast Cancer, Orientador: Nágila Raquel Teixeira Damasceno. Coorientador: Alfredo Carlos Simões Dornellas de Barros. Palavras-chave: Câncer de mama; IGF-1.
2016 - 2018	Especialização em Residência Multiprofissional no Cuidado ao Paciente Oncológico. (Carga Horária: 5760h). Hospital Sírio-Libanês, SIRIO-LIBANÊS, Brasil. Título: Implementação da Avaliação Subjetiva Global Produzida Pelo Paciente (ASG PPP) nos Centros Oncológicos do Hospital Sírio Libanês. Orientador: Erika Yuri Hirose.
2011 - 2015	Graduação em Nutrição. Universidade de Brasília, UnB, Brasil. Título: Comportamento alimentar e sucesso cirúrgico tardio em mulheres submetidas a gastroplastia redutora em Y-de-Roux.. Orientador: Eliane Said Dutra.
2009 - 2011	Ensino Médio (2º grau). Centro Educacional Sigma, CES, Brasil.

Nágila Raquel Teixeira Damasceno

Bolsista de Produtividade em Pesquisa do CNPq - Nível 2

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Última atualização do currículo em 20/04/2022

Professora Associada do Departamento de Nutrição da Faculdade de Saúde Pública da Universidade de São Paulo (FSP-USP), responsável pelo grupo de pesquisa em Oxidações Biológicas e Metabolismo Lipídico Aplicado às Doenças Crônicas Não Transmissíveis (OxLIPID) e coordenadora do Laboratório de Bioquímica da Nutrição Aplicada às Doenças Crônicas Não Transmissíveis. Nos últimos anos tem coordenado pesquisas na área de Nutrição e Doenças Crônicas (DCV, Obesidade, câncer, Epilepsia e Doenças Neurodegenerativas), com ênfase em aspectos lipídicos, oxidativos e inflamatórios modulados por ácidos graxos e outros componentes nutricionais. É Diretora da Divisão de Nutrição e Dietética do Hospital Universitário (HU-USP), membro da Diretoria do Departamento de Nutrição da SOCESP, membro do Conselho Deliberativo do Instituto Nacional de Fluidos Complexos (INCT-FCx) e Núcleo de Pesquisas em Fluidos Complexos (NAP-FCx), além de fazer parte de comissões universitárias (Congregação, Conselho do Departamento de Nutrição, Biossegurança e Residência Multiprofissional). Coordenou o Programa de Cooperação Internacional Brasil-Suécia (CAPES-STINT, 2014-2018) e, atualmente, colabora com projeto de cooperação internacional Brasil-Alemanha (FAPESP-FAPESC-Greifswald). Mantém colaborações científicas nacionais (UFPI, UECE, UFC, UFF, FURB) e internacionais (Universidade de Harvard, Universidade de Barcelona, Universidade de Umea e Universidade de Greifswald). Tem intensa atuação ao nível de extensão universitária, tendo concedido mais de 50 entrevistas e coordenado mais de 20 projetos nos últimos 5 anos. Em parceria com a SOCESP, Centro Universitário São Camilo e INCT-FCx, é coordenadora da Plataforma EAD para capacitação de professores de ensino básico e médio sobre Educação Alimentar e Nutricional como estratégia para a prevenção de doenças cardiovasculares e morbidades associadas ? PEDUCA. **(Texto informado pelo autor)**

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