UNIVERSITAT ROVIRA i VIRGILI

UNIVERSITAT DE BARCELONA

Final Project for the master's degree in Nutrition and Metabolism

Exploring the circulatory metabolic alterations in breast cancer patients

Project carried out in Unitat de Recerca Biomèdica

ANDREA JIMÉNEZ FRANCO

Directed by

Prof. Jorge Joven
Elisabet Rodríguez Tomàs

Tarragona, June 2022

INDEX

Acknowledgements

Primero que todo, me gustaría agradecerle al Prof. Jorge Joven por permitirme realizar las prácticas en la Unitat de Recerca Biomèdica donde he podido adquirir una gran variedad de conocimientos que me han permitido formarme como investigadora. A la Dra. Meritxell Arenas por facilitarme la recopilación de muestras para el estudio y aportarme los conocimientos oncológicos necesarios para el desarrollo del proyecto.

Gracias Elisabet por introducirme en el mundo del cáncer y enseñarme todo lo que se, por guiarme dentro del laboratorio y hacerme ver que en los momentos de frustración dentro del laboratorio con buena compañía se supera mejor. Gracias por enseñarme a ser perfeccionista y a no quedarme con la primera opción, sino que luchar hasta que quede perfecto. Helena y Gerard, gracias por siempre darme soporte, por apoyarme y por enseñarme todos vuestros conocimientos y más. Pero sobre todo, os quiero agradecer a los tres por haberme dado esta oportunidad, por haber confiado en mí, y por permitirme estar donde estoy.

Me gustaría agradecerle al Sr. Cabré, él ha sido la verdadera razón por la que estoy donde estoy, él fue el primero en confiar en mí y en dar la cara por mí, me falta vida para agradecerte lo que has hecho por mí.

Quiero agradecerle a mi familia, a mis padres y a mi hermano, por ser los pilares fundamentales en mi trayectoria, por darme la mano en las caídas y por ayudarme a dar el empujón que a veces me faltaba. Agradecerle a Jordi por la paciencia durante estos meses, por apoyarme en todo y por ser comprensivo en los momentos de más estrés. Finalmente, me gustaría agradecerles a mis yayos, sin vosotros esto tampoco hubiese sido posible, siempre habéis estado ahí cuando os he necesitado, sin importaros la hora ni el lugar, gracias por los quilómetros que habéis hecho por mí y las horas de espera en Barcelona.

Simplemente gracias a todos.

Abbreviations

AHR: Hydrocarbon receptor

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

AUC: Area under the curve

BC: Breast cancer

CA 15.3: Carcinoma antigen 15.3

CCL2: Chemokine C-C motif ligand 2

CD8: Cluster of differentiation 8

CEA: Carcinoembryonic antigen

Cl: Chlorine

CRP: C-reactive protein

CTL: Cytotoxic T lymphocytes

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ER: Estrogen receptor

HDL: High-density lipoproteins

HER2: Human epidermal growth factor receptor 2

IL-10: Interleukin 10

IL-10R: Interleukin-10 receptor

IL-6: Interleukin 6

Ki67%: Cell proliferation index

LDA: Linear discriminant analysis

LDH: Lactate dehydrogenase

LDL: Low-density lipoproteins

NK: Natural killer

PON1: Paraoxonase-1

PR: Progesterone receptor

PXR: Pregnane X receptor

ROC: Receiver operating characteristics (ROC) curves

ROS: Reactive oxygen species

TG: Triglycerides

TGF-β: Transforming growth factor beta

VLDL: Very-low-density lipoproteins

Abstract

Background and aims: Breast cancer (BC) is one of the most diagnosed cancers and the main cause of cancer-related death among the women population. The interplay between metabolism, oxidative stress, and inflammatory processes appears to play an essential role in the development and progression of BC, according to growing research.

In the present study, we aimed 1) to investigate the circulatory metabolic and immunity alterations, as well as the levels of proteins involved in oxidative stress in BC patients, and 2) to identify potential biomarkers associated with the pathophysiological characteristics of the patients.

Methods: 85 women with BC who had not yet received any oncological treatment were recruited from Hospital Universitari Sant Joan in Reus. As a control group, we analysed samples from 50 healthy women without any carcinogenic evidence. We performed targeted metabolomics, ELISA, and colorimetric analyses to quantify circulatory metabolites involved in different metabolic pathways as well as proteins related to oxidative stress (PON1) and inflammatory system (CCL2, IL-10 and TGF-β).

Results: Using multivariant analysis, we identified several circulatory alterations in BC patients including the increased levels of glucose, VLDL-cholesterol, anti-inflammatory cytokines (IL-10 and TGF-β), and a decreased PON1 activity. Post-menopausal state, others pathological anatomy, tumour histological grade III, and TN molecular subtype were associated with an increase of tumour biomarkers (CEA and CA15.3), alterations in oxidative stress (PON1 decrease) and inflammatory system.

Conclusion: BC patients showed several alterations in the levels of proteins related with oxidative stress and immune response, as well as in metabolites involved in carbohydrates pathways. PON1 activity and hypoxanthine were the best parameters to discriminate between BC patients and controls. Moreover, we could observe worse prognostic in patients with postmenopausal state, others pathological anatomy, histological grade III and TN subtype.

Key words: oxidative stress, inflammation, energy metabolism and immune response

1. Introduction

Breast cancer (BC) is one of the most diagnosed cancers and the leading cause of cancer-related death among the female population.

In 2020, 34.088 new cases of BC were diagnosed in Spain, and it is estimated that 1 in 8 women will suffer it. The incidence curve grows in parallel as women age, and due to the increase in longevity and the high risk of developing BC over the years, is important to continue research on it [1].

BC is known as a heterogeneous disease. This heterogeneity usually occurs between similar tumours, yielding a subtype (intertumoral heterogeneity), or within tumours of the same subtype (intratumoral heterogeneity). Among intratumoral heterogeneity features, the capacity to adapt to new microenvironment conditions has been related to drug resistance and poor clinical outcomes [2–5].

Focusing on tumour molecular profiles, specific subtypes (luminal A, luminal B, HER2 positive, and triple negative) are defined according to the tumour morphology and the expression or not of certain biomarkers such as estrogen receptors (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and based on the cell proliferation index (Ki67%) [2,5,6]. Each molecular subtype shows specific features being triple negative the worst in terms of BC survival.

Growing evidence suggests that an interplay between energy metabolism, oxidative stress, and inflammation processes plays a key role in the development and progression of BC. Cancer cells are characterized by an accelerated energy metabolism to acquire necessary nutrients from a frequently nutrient-poor environment for their growth, proliferation, and viability [7,8]. This metabolic reprogramming induces a mitochondrial dysfunction which in turn generates an increase of reactive oxygen species (ROS) and then, the onset of oxidative stress [9]. High levels of ROS contribute to tumour cell proliferation, cell migration, increased tumour cell proangiogenic factors, and activation of signalling pathways [10]. Moreover, alterations in mitochondrial DNA led to chemoresistance and/or invasive phenotype in cancer cells [9,11]. All these alterations contribute to overexpress inflammation pathway, which triggers increased tumour progression, metastasis, and resistance to therapy. This

deregulation is favours by the immune system, which is unable to regulate this process correctly and produces prolonged inflammation (chronic inflammation) [12,13].

Regarding to BC treatment different multimodal strategies have been applied to treat this disease which includes local treatments like surgery and radiation therapy, and systemic treatments like chemotherapy and immunotherapy. It has been demonstrated that the combination of treatments reduces the risk of recurrence according to tumour subtypes [14,15]. However, 30% of patients eventually relapse into the disease and develop distant metastasis in the subsequent years [16]. Because of this, it is important find specific signatures of the tumours based on clinicopathological characteristics of the patients.

1.1 Hypothesis and objectives

The identification of baseline biomarkers related to metabolism, oxidative stress and immune response could be relevant for the evolution of BC.
To address this hypothesis, we propose the following objectives:

- To investigate changes in energy metabolism, oxidative stress, and immune system in patients with BC comparing it with a control group.
- To identify new BC biomarkers associated with clinicopathological characteristics of the patient.

2. Materials and methods

2.1 Participants of the study

A total of 85 women with BC were included in the study between September 2020 to October 2021 in Hospital Universitari Sant Joan in Reus. As a control group, we analysed samples from 50 healthy women without any carcinogenic evidence.

To develop this investigation, it was considered some inclusion and exclusion criteria. Patients with an invasive BC and ≥ 18 years old were included while those patients with a previously oncological background, presence of Paget's nipple disease, presence of vascular collage disease, systemic lupus

erythematosus and/or scleroderma, pregnancy, or lactation at the time of inclusion, presence of psychiatric disorders or other conditions were excluded. All participants signed a written informed consent according to the Helsinki declaration. The study was approved by Ethics Committee of the Hospital Universitari Sant Joan in Reus.

2.2 Biological sample collection

Two blood samples (10ml each) were collected at the time of diagnosis before the patient received any treatment, one of them in a tube with EDTA. Samples were processed to obtain aliquots of serum and plasma via centrifugation (2.500xg, 15min, 4ºC) and stored in -80ºC until being analysis. The samples from the control group were processed in the same way.

All samples were identified with the number regarding to the anonymization process.

2.3 Biological samples analyses

2.3.1 ELISA and colorimetric analyses

Circulating levels of PON1 concentration and inflammatory proteins (CCL2, IL-10 and TGF-β) were analysed by ELISA kits from Elabsciencie company. To carry out these determinations were used serum for PON1 and plasma samples for the other proteins.

Serum PON1 arylesterase activity was measured as the rate of hydrolysis of phenylacetate at 270nm, inf 9mM Tris-HCl buffer, pH 8.0, supplemented with 0.9 mM $CaCl_2$.

2.3.2 Targeted metabolomics

Targeted metabolomics was employed to determine plasma concentration of 74 metabolites involved in different metabolic pathways. The metabolic categories analysed were carbohydrate, amino acid, lipid, metabolism of cofactors and vitamins, nucleotide, xenobiotic biodegradation, and energy metabolism.

The equipment used was constituted of a 7890A gas chromatograph coupled to 7200-quadruple time-of-flight mass spectrometer with an electron impact source. In addition, the system was equipped with a 7693 autosampler module and a

J&W Scientific HP-5MS column (30ms 0.25 mm, 0.25µm) (Agilent Technologies, Santa Clara, CA, USA). The calibration curve was obtained by means of standard concentrations as a function of the peak area.

2.4 Statistical analysis

2.4.1 Standard statistics

For compare two independent groups were used Mann-Whitney U test and to evaluate descriptive variables was used Chi-squared test.

Significant differences were considered when p-value < 0.05. Frequencies were expressed as median (interquartile range) for quantitative variables and frequency (percentage) to qualitative variables.

Graph representations were performed by GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA) and statistical calculations were determinate by Statistical Package for Social Sciences (SPSS 22.0, Chicago, IL, USA), Python 3.10.0 (SW Gemini Dr., Beaverton, USA) and MetaboAnalyst 5.0 (www.Metaboanalyst.ca).

2.4.2 Graphic representations

LDA (Linear Discriminant Analysis) was used to find a linear combination of variables that characterizes the population of the study, and it was generated with Python. As of Heatmap we could observed the significant variables that induce differentiation between groups, and it was performed with Python too.

To identify the most important components we evaluated variable importance in projection (VIP) score, an estimate of the importance variables in the projection used in the partial least squares' discriminant analysis (PLS-DA) model as a quantitative estimate of the discriminatory power of each individual characteristic

For biomarker analysis, we performed receiver operating characteristics (ROC) curves by combining significative variables. ROC curves are a graph showing the output of a classification model at all thresholds of classification. This curve plots for two parameters: true positive rate and false positive rate. On the other hand, Volcano plot was performed for determine differential metabolites in target metabolomics. It is a type of scatter plot that shows statistical significance (p-value) in front of magnitude of change (fold change). It provides a quick visual identification of genes with large fold changes that are also statistically significant.

MetaboAnalyst was used to perform VIP score, ROC and Volcano plot. Finally, Boxplot were generated with GraphPad Prism to evaluate the distribution and the asymmetric grade of the significative variables.

3. Results

3.1 Clinical characteristics of the BC patients

The main clinical characteristics are shown in **Table 1**. Most patients developed cancer in a postmenopausal state. The population showed a low percentage of comorbidities, while the percentage of family oncologic antecedents was high. In general, the patients did not show positive nodes or metastasis at the time of diagnosis. The most common pathological tumour diagnosed was ductal carcinoma, and there was a predominance of histological grade II and luminal B molecular subtype.

Table 1. Clinical characteristics of breast cancer patients

	Breast cancer (n=85)
Clinical characteristics	
Diagnostic age (years)	57 (48-66)
Alcohol habit (>20g/day)	12 (13.5)
Smoking	20 (23.5)
Hypertension	23 (23.8)
Diabetes Mellitus	13 (14.6)
Dyslipidaemia	21 (24.7)
Chronic obstructive pulmonary disease	10 (11.2)
Ischemic heart disease	10 (11.2)
Family oncologic antecedents	56 (62.9)
Intake of oral contraceptives	28 (32.9)
Motherhood	71 (79.7)
Menopause state	
Premenopausal	25 (29.4)
Perimenopausal	6 (6.9)
Postmenopausal	54 (63.5)
Cancer characteristics	
Affected breast	
Left	43 (50.6)
Right	37 (43.5)
Both	5 (5.7)

Tumour size (TNM system)	
T0	-
T1	28 (32.9)
T2	38 (48.7)
T3	9 (10.5)
T4	1 (1.3)
Nodes (TNM system)	
N0	48 (56.5)
N1	27 (31.8)
N2	3 (3.7)
N3	1 (1.2)
Metastases (TNM system)	
M0	80 (94.1)
M1	2 (2.4)
Pathological anatomy of the tumour	
Ductal carcinoma	65 (76.5)
Lobular carcinoma	10 (11.8)
Others	8 (9.4)
Histological grade	
I	14 (16.9)
II	53 (62.4)
III	14 (16.5)
Positive Strogen receptor	67 (78.8)
Positive Progesterone receptors	54 (63.5)
Positive HER2 in tumour biopsy	14 (15.9)
Ki67 antigen in tumour biopsy	
Less than 15%	16 (18.8)
15-50%	54 (64.5)
More than 50%	11 (13.0)
Tumour molecular classification	
Luminal A	17 (20.0)
Luminal B	41 (48.2)
HER2 positive	13 (14.9)
Triple negative	12 (13.8)

Values are provided as frequency (percentage) or median (interquartile range).

3.2 Circulatory alterations in BC patients

Linear discriminant analyses revealed global differences between BC patients and controls according to biochemical profile and proteins associate to oxidative stress and inflammation (**Figure 1A**). Glucose, lipid-related variables (total cholesterol, VLDL and TG) and inflammatory biomarkers (TGF-β and IL-10) were significantly increased in BC patients, while HDL-cholesterol, transaminase

(AST) and inflammatory biomarkers (PON1 activity and CCL2) were significantly decreased compared to the controls (**Figure 1B and Supplementary table 1**). To identify the most predictive variables we employed VIP score, ROC, and matrix confusion analyses. We observed that PON1 activity, VLDL, IL-10, and TG concentrations were the most important parameters to discriminate BC patients and controls (AUC = 0.902) (**Figure 1C and 1D**).

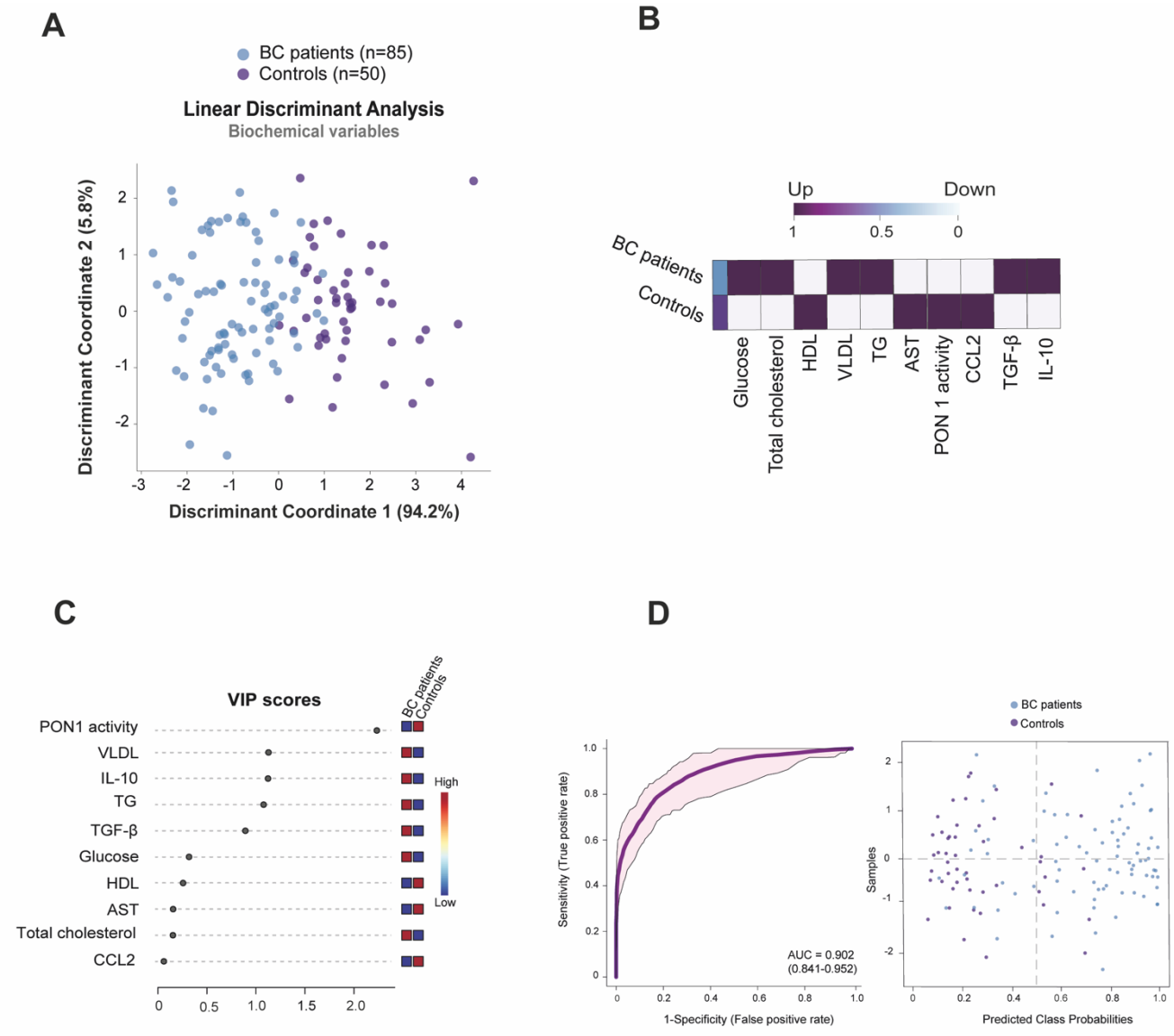


Figure 1. PON1 activity, IL-10, and lipid-related variable are predictors of BC.
(A) Linear Discriminant Analysis (LDA), (B) Heatmap of significative variables, (C) Variable Importance in Projection (VIP) score of statistically significant variables, (D) Receiver Operating characteristic (ROC) Curve and matrix confusion of PON1 activity, VLDL, IL-10 and TG.
HDL: high-density lipoproteins; VLDL: very-low-density lipoproteins; TG: triglycerides; AST: aspartate aminotransferase; CCL2: chemokine C-C motif ligand 2; TGF-β: transforming growth factor beta; IL-10: interleukin 10; PON1: paraoxonase-1. The significance was determined by the Mann-Whitney U-test, $p < 0.05$

Regarding metabolic profiles LDA showed clear differences between BC patients and controls (**Figure 2A**). Several significant metabolic alterations were found in BC. Most of these alterations were found in metabolites related to carbohydrates and amino acid metabolism (**Figure 2B and Supplementary table 2**). According to the VIP score, ROC, and matrix confusion analyses hypoxanthine, 3-phosphoglyceric acid, xylonic acid and maltose were the most predictive metabolites in BC (AUC = 0.998) (**Figure 2C and 2D**).

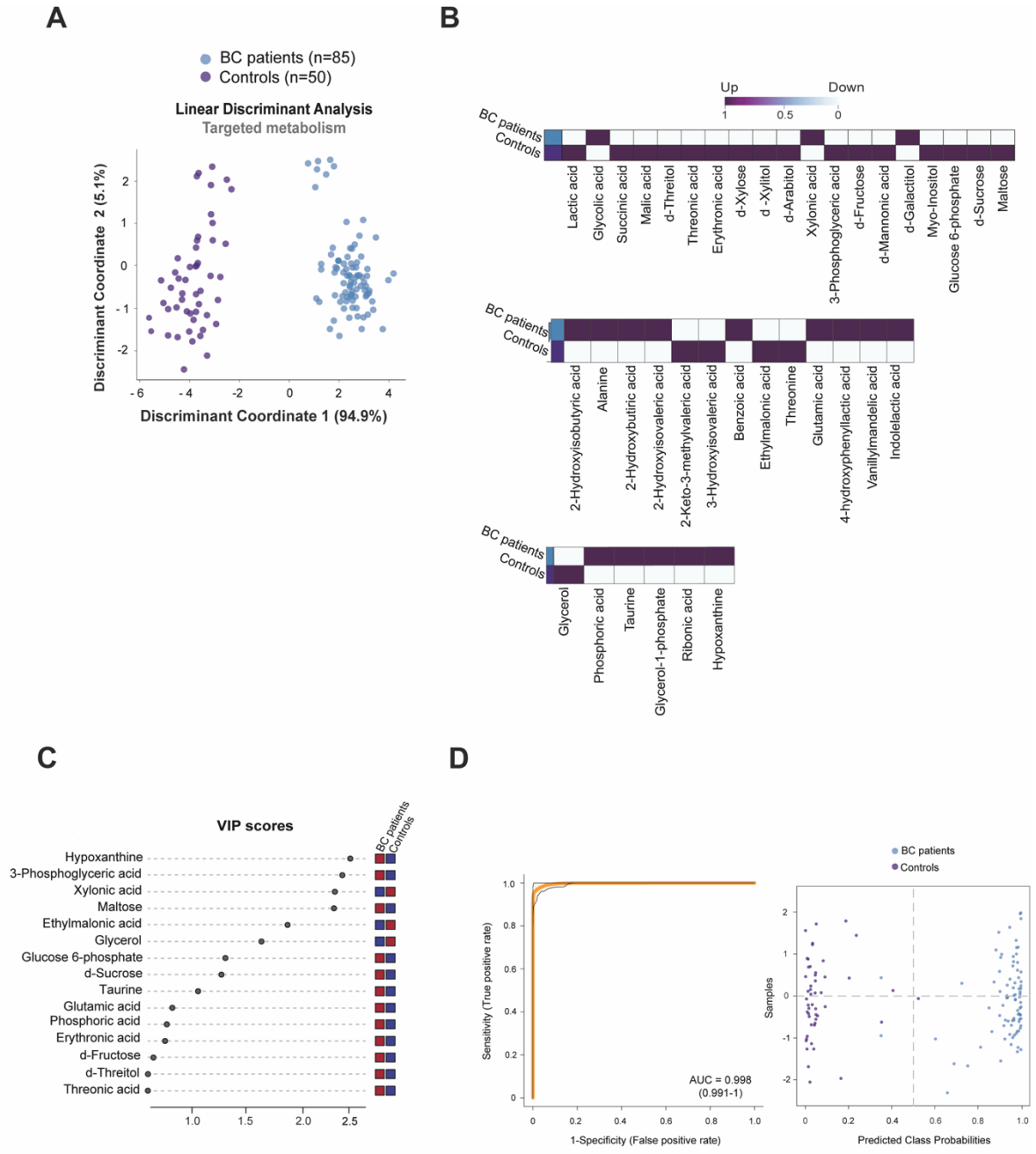


Figure 2. Hypoxanthine, 3-phosphoglyceric acid, xylonic acid and maltose metabolites are predictors of BC.
(A) Linear Discriminant Analysis (LDA), (B) Heatmap of significative variables, (C) Variable Importance in projection (VIP) score of statically significant variables, (D) Receiver Operating characteristic (ROC) Curve and matrix confusion of hypoxanthine, 2-phosphoglyceric acid, xylonic acid and maltose.
The significance was determined by the Mann-Whitney U-test, $p < 0.05$

3.2 Circulatory alterations associated with clinicopathological characteristics of BC patients

Among clinical characteristics of the BC patients, postmenopausal state was associated with a significant increase of tumour biomarkers (CEA and CA15.3), glucose, Na, urea, LDH-cholesterol, hemoglobin, inflammatory biomarkers (CRP and IL-6), transaminases (AST and ALT) and alkaline phosphatase (**Figure 3A**). Regarding circulatory metabolites we compared both menopausal status (pre- vs postmenopausal), and we observed alterations in carbohydrates, amino acid, and

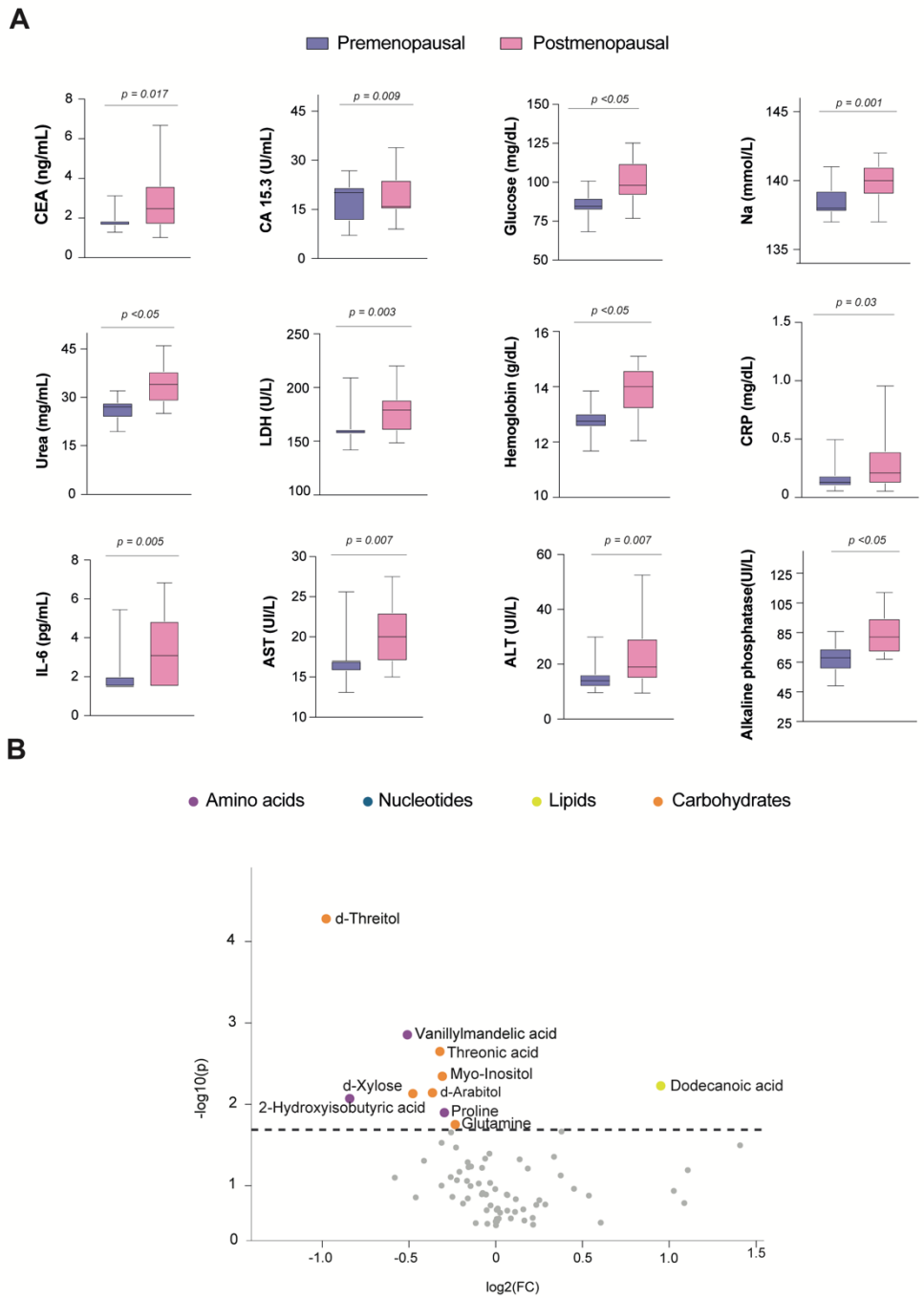


Figure 3. Menopausal state was associated with biochemical and metabolic alterations
(A) Boxplot for biochemical variables, and (B) mean log2 for energy metabolism were depicted. Significative metabolites were coloured depending on the class. CEA: carcinoembryonic antigen; CA 15.3: cancer antigen 15.3; LDH: low-density lipoproteins; CRP: C-reactive protein; IL-6: interleukin 6; AST: aspartate aminotransferase; ALT: alanine aminotransferase. The significance was determined by the Mann-Whitney U-test, p< 0.05

lipids metabolism. Notably, all significant metabolites were increased in postmenopausal state. To determine which metabolites altered could be useful as biomarkers of BC, we performed ROC and matrix confusion with the combination of d-threitol, dodecanoic acid and vanillylmandelic acid. The AUC was 0.816 (**Supplementary figure 1A**).

The pathological anatomy of the tumour was also associated with certain circulating alterations. As we could see in **Figure 4A**, tumour biomarker (CEA), leukocytes and inflammatory biomarker (TFG-β) were increased in others anatomy, which is the least frequent pathological anatomy. It should be noted that we consider other pathological anatomy to be that which cannot be classified as lobular or ductal, so would be tubular, spinal, mucinous, colloid… Furthermore,

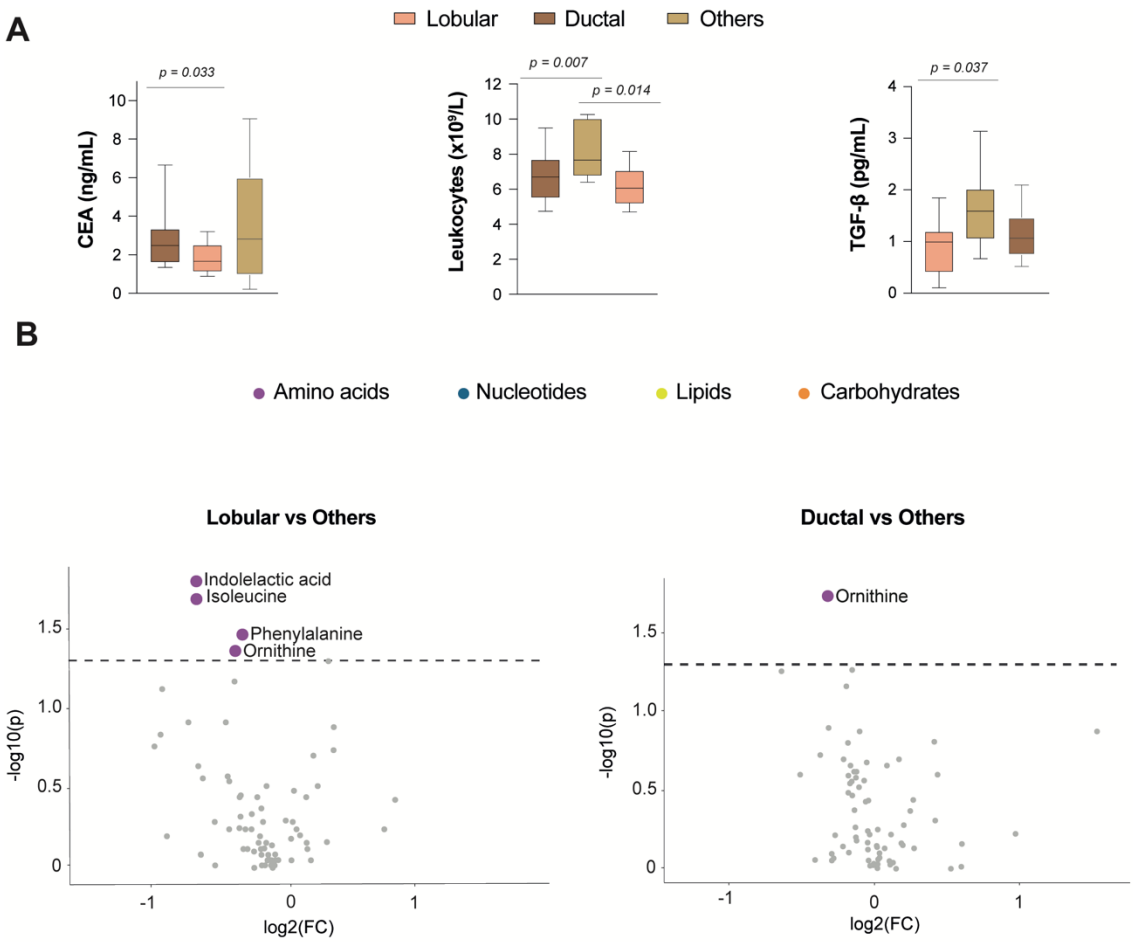


Figure 4. The others pathological anatomy was associated with alterations in biochemical and energy metabolism.
(A) Boxplot for biochemical variables, and (B) mean log2 for energy metabolism were depicted. Significative metabolites were coloured depending on the class. CEA: carcinoembryonic antigen; TGF-β: transforming growth factor beta
The significance was determined by the Mann-Whitney U-test, $p < 0.05$

significant differences in amino acid metabolism were found when we made the comparisons lobular vs others and ductal vs others pathological anatomy. It is to highlight that in both comparatives, the significant metabolites that we could observe in **Figure 4B**, were increased in others pathological anatomy.

On the other hand, we analysed the histological grade of the BC patients. CD8 was higher in grade III than in grade I or II (**Figure 5A**). Moreover, when we compared grade I vs grade II, amino acid metabolism was altered, having increased indole-3-propianoic acid in grade I, while in the comparison between grade II vs grade III, carbohydrates and nucleotides metabolism were altered showing increased levels of pyruvic acid and uracil in grade II (**Figure 5B**).

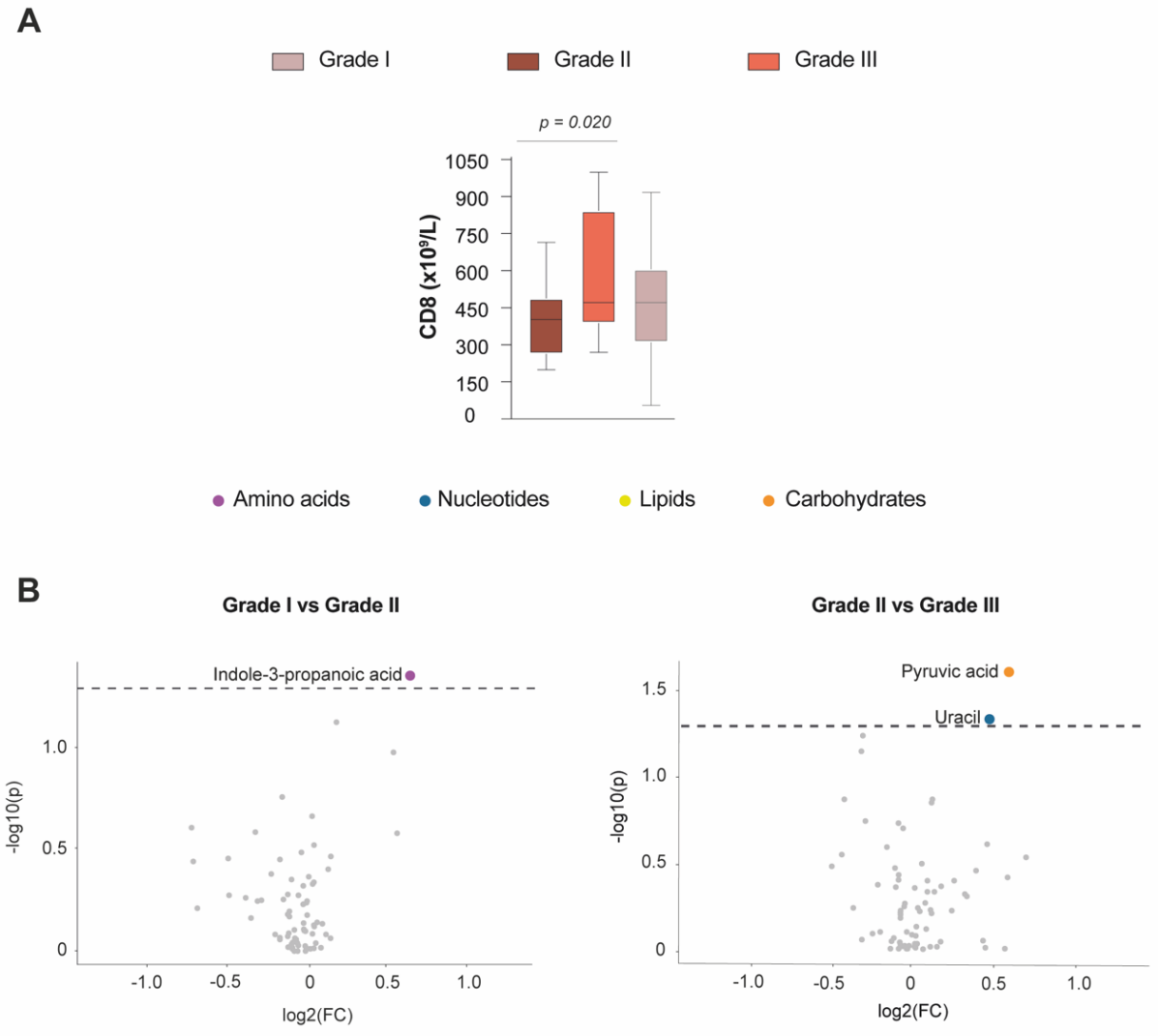


Figure 5. Histological grade was associated with biochemical and metabolism disorder.
(A) Boxplot for biochemical variables, and (B) mean log2 for energy metabolism were depicted. Significative metabolites were coloured depending on the class. CD8: cluster of differentiation 8
The significance was determined by the Mann-Whitney U-test, $p < 0.05$

Finally, in **Figure 6** we can observe the tumour molecular subtype classification. We observed that those patients with a TN molecular subtype showed tumour biomarker (CA 15.3), LDH-cholesterol and inflammation biomarker (IL-6) increased, while glucose, chlorine (Cl), TG and VLDL were increased in HER2.

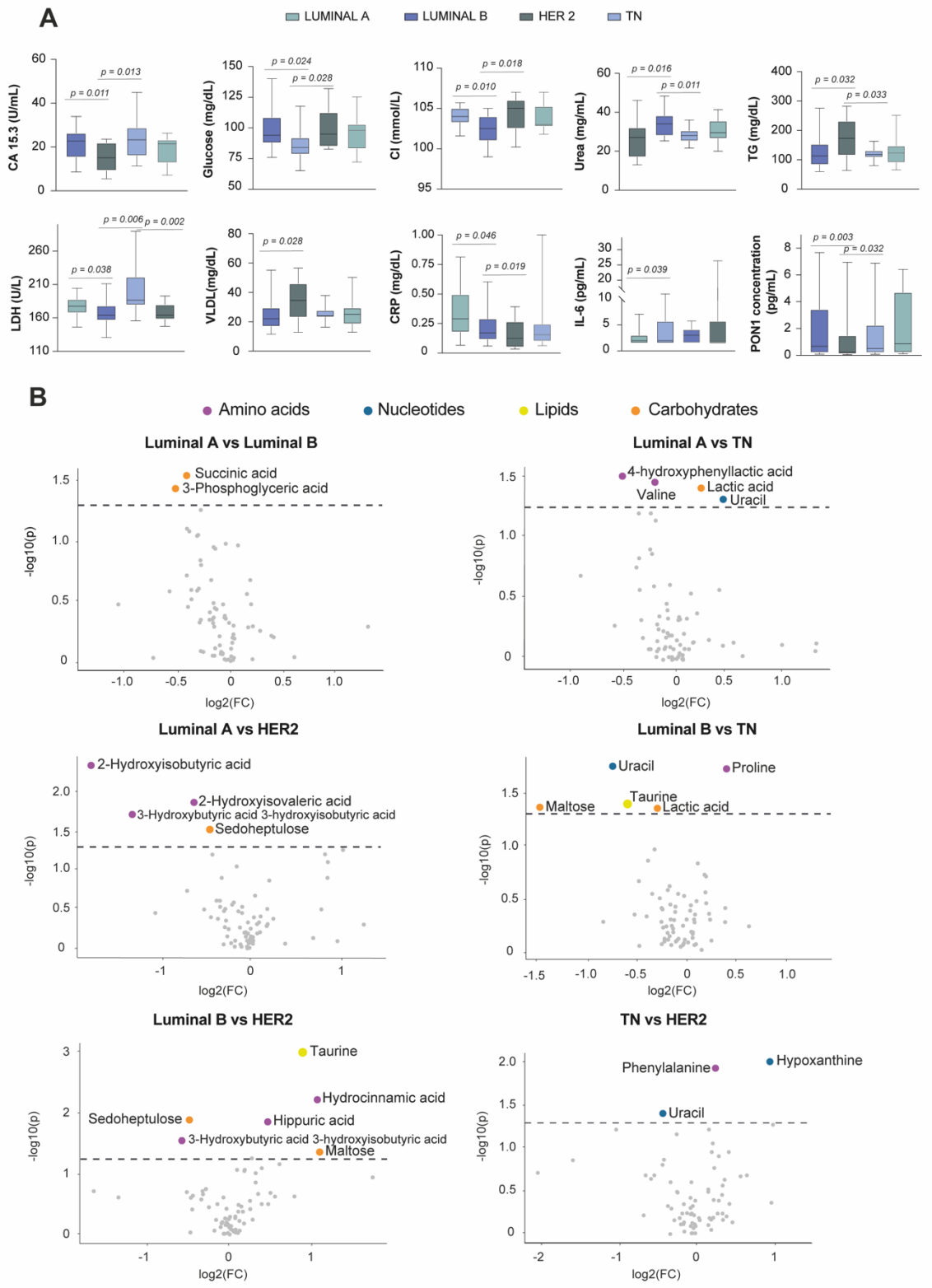


Figure 6. Molecular subtype was associated with biochemical and metabolism disruption.
(A) Boxplot for biochemical variables, and (B) mean log2 for energy metabolism were depicted. Significative metabolites were coloured depending on the class. CA 15.3: 15.3: cancer antigen; Cl: chlorine; TG: triglycerides; LDH: low-density lipoproteins; VLDL: very-low-density lipoproteins; CRP: C-reactive protein; IL-6: interleukin 6; PON1: paraoxonase-1
The significance was determined by the Mann-Whitney U-test, $p < 0.05$

On the other hand, urea was higher in luminal B and, CRP and PON1 concentration were enhanced in luminal A (**Figure 6A**).
Considering the volcano plots, depending on the comparison, different metabolic pathways were altered (**Figure 6B**). We focus on the comparison between TN and HER2 because patients with these subtypes have worse prognosis. In this comparison we found significant differences in nucleotide and amino acid metabolism, with high concentrations of hypoxanthine and phenylalanine for TN subtype, while uracil presented a reduced concentration compared to HER2. To determine which variables altered could be useful as biomarkers of BC, we performed ROC and matrix confusion with the combination of CA 15.3, TG, PON1 concentration, glucose, LDH, hypoxanthine, phenylalanine, and uracil. The AUC was 0.906 (**Supplementary figure 1B**).

4. Discussion

Currently, the search for cancer biomarkers provides tools for achieving personalised medicine. In this sense, is so important to search tumour biomarkers for cancer diagnosis and to relate them with the patient's prognosis and response to treatment.

In recent years, emphasis has been placed on the importance of studying the relationship between oxidative stress, inflammation, and metabolism in cancer, due to the key role they play in tumour development and progression.
The present study shows the specific circulatory alterations presented in BC patients as well as the relationship between these alterations and the clinicopathological characteristics of the patients.

Different significant alterations were observed in BC patients compared to controls, highlighting higher levels of glucose in the group of BC patients. Several studies suggest that the presence of high glucose levels, increase the incidence of cancer because of key role of glucose on cell proliferation [17,18].

In other matters, alterations in plasma lipoproteins, cholesterol metabolism and oxidative stress have been observed in cancer patients in recent years [19,20].

In our study, total cholesterol was increased in BC patients. There are studies like Kitahara et al. [21,22] that found a positive association between an increase in total cholesterol and the risk of BC.

On the other hand, as is already known, HDL promotes reverse cholesterol transport, and has antioxidant and anti-inflammatory properties based on its interaction with PON1 [20]. In our study, patients with BC had low HDL levels compared to controls, these results are also present in Mazzuferi et al. [19]. Camps et al. [23] have found an inverse correlation between HDL and BC indicating that low HDL concentrations increase the risk of BC.

As it has mention above, HDL function is associated with PON1 enzyme. PON1 is an antioxidant enzyme, that could be found in the membrane of most cells or bound to HDL. PON family protects cells from mitochondrial dysfunction and alterations in metabolism, although this has not been clearly seen in PON1. On the other hand, this enzyme degrades the products of lipid peroxidation, which is one of the causes of oxidative stress in the organism. An increase in oxidative stress promotes the development of cancer because cellular damage can be caused via several pathways. Deregulation in the activity of PON1 has been observed in BC patients, and in the studies reported by Mazzuferi et al., Mustafa et al. and Arenas et al. [19,20,24]. In our study, PON1 activity was decreased in BC patients compared to controls, as also reported in Camps et al. and Arenas et al. [23,24]. These two studies indicates that the reduction in PON1 activity occurs in the presence of inflammation.

Inflammation has also an important role in the development and progression of the tumour. Because of this, in our study we have determined the levels of some pro- and anti-inflammatory biomarkers. We identified high concentration of TGF-β in BC patients. It has been correlated with tumour stimulation as it promotes collagen production in the area, as well as being associated with better tumour progression, as is discussed in Acerbi et al. and Drabsch et al. [25,26]. However, in our study we could not observe a correlation between tumour aggressiveness and BC cell subtype, as has been shown in Acerbi et al. [25]. Probably, we could not see it because of the heterogeneity between patients, the number of patients recruited in our study, or because they determined TGF-β in tissue samples, and we determined it in circulation.

On the other hand, we observed high levels of IL-10 in BC patients. IL-10 is an anti-inflammatory pleotropic cytokine that protect us from exaggerated responses to pathogens and microbiota. This cytokine has an immunosuppressive function, but very high levels of this cytokine facilitate the immune escape of the tumour [27]. It is important mention that its role in tumorigenesis is still controversial. At early stages of this disease, IL-10 stimulate NK and CTL mediated killing of cancer cells. However, in advanced tumour stages, cancer cells re-write themselves to express IL-10R, so in this case IL-10 acts as a cancer promoter [28]. One reason of why we found elevated levels of IL-10 in BC patients would be because we determined it at baseline levels, possibly at the beginning of the pathology, so this increase in concentration could indicate that IL-10 acts as a tumour suppressor and begins to recruit immune cells.

In the case of CCL2, it is a pro-inflammatory cytokine and chemokine. We found low levels of this protein in BC patients. In Steiner et al. [29] reported that the expression of CCL2 in BC correspond with increased tumour progression and worse patient's prognosis. They found high levels in BC patients, while we found low levels, these discrepancies could be for the heterogeneity of the tumour or for the tumour status of our patients. By contrast, Li et al. [30] indicated that patients with low CCL2 levels have longer progression-free survival than patients with higher CCL2 concentration.

Omics science provides a large number of molecular measurements in tissues, cells or fluids. These techniques (proteomics, lipidomics, metabolomics, transcriptomics, and genomics) can be applied to a biological system with the interest to obtain the maximum information about it and the underlying system. On the other hand, omics studies can associate molecular measurements with clinical outcomes of interest, such as cancer. Focussing on metabolomics, which is an accurate method that allows us to study the metabolome, a set of small metabolites that make up a biological sample. It should be noted that metabolome is dynamic and presents variations in the same individual and between individuals of the same species due to the number of factors that influence, such as cancer, environmental factors, hormonal status, among others [31]. Metabolomics has been used in recent years as a technique to identify new diagnostic and prognostic biomarkers for different types of tumours, including BC.

In our study, from 74 metabolites involved in 7 categories, we found different altered metabolites, but we highlighted hypoxanthine, 2-phosphoglyceric acid, xylonic acid and maltose which were the metabolites that in the results section we pointed out as the most predictive metabolites for BC. As possible biomarkers, hypoxanthine and xylonic acid were found to be increased in BC, while 3-phosphoglyceric acid and maltose were found to be decreased. Based on the VIP score, hypoxanthine would be the best candidate. As reported by Park et al. [32], hypoxanthine is a metabolite involved in the purine degradation pathway. An alteration in the purine metabolic pathway increases ATP synthesis, thus favours tumour cell growth. Therefore, elevated blood levels of hypoxanthine are indicative of an overexpression of the purine pathway, and this may indicate the presence of cancer [32].

Due to the wide heterogeneity of BC, it is essential to stratify and evaluate the clinicopathological characteristics of patients to assess with more accuracy this disease. Based on our results, menopausal status was associated with the circulatory alterations in patients with BC. It has been observed that younger patients (premenopausal stage) present more aggressive tumours, while older patients (postmenopausal stage) who generally presented less aggressive tumours [33,34]. Moreover, we have observed a significant increase of tumour markers (CEA and CA 15.3) in postmenopausal state. Similar results were found in Zhao et al. [35], where they indicate that CEA and CA15.3 could be taken as markers in the presence of BC for the menopausal stage. Regarding to metabolism, we observed alterations in amino acid metabolism, which can be explained by the tumour's dependence on this metabolism for progression and proliferation [36]. Moreover, most of the metabolites altered were presented in carbohydrate metabolism. Variations in this metabolism have also been reported in Dias et al. [37]. It is important to note that the altered metabolites from amino acid and carbohydrates metabolism were elevated for the post-menopausal state.

In relation to tumor histology, BC can appear in different structures of the breast, like ducts, lobules, mucins... On this basis, the most frequent histology is ductal BC, followed by lobular BC. Ductal and lobular neoplasms are characterized by invasion of proximal tissue and the ability to metastasize distant organs.

Moreover, ductal neoplasms are defined by lymph node invasion. Others anatomy is the group of tumours that could not be classified in the previous tumour histology [38]. Due to the high prevalence of metastases depending on the tumour histology, the analysis of circulatory parameters could improve the patient's prognosis, as the stage of tumour development would be known. From the parameters obtained at the circulatory level, significant differences have been observed at the level of the others anatomy compared to the ductal and lobular anatomies. In addition, an increase of CEA biomarkers and TGF-β were observed in the others anatomy, they are associated with the progression of the pathology [39,40]. In relation to the altered metabolic pathways, higher amino acid levels were found in patients with others tumour anatomies. Zhang et al. [7] indicate that the increase in amino acid synthesis is due to the elevated tumour cell proliferation.

The histological grade is a measure of the degree differentiation of the tumour tissue. It is a semi-quantitative evaluation of morphological characteristics [41]. It has been described that this grade is important for determine patient's prognosis and is a predictive tool for BC survival. Grade III has the highest frequency of metastasis and has been related with a low survival percentage. The study of biomarkers related to histological grade would allow us to focus the patient´s treatment, as well as to know their life expectancy [42].

Based in our results, those patients with a histological grade III showed an increase in CD8 levels compared to grade I and II. These results have also been observed in the study presented by Kristi et al. [43]. This study determines the CD8 concentration of both ER status and histological grade. They were able to determine that high CD8 levels are present in grade III and ER-negative tumours. By contrast, in our study we have not been able to correlate ER and grade III.

On the other hand, we have found significative differences in the metabolism of the amino acid (indole-3-propianoic acid (IPA)) between grade I and II. IPA concentration was higher in grade I than in grade II. From various studies such as Sári et al. [30], it has been observed that *in vitro* IPA could reduce the levels of infiltration into adjacent tissues. Through this study, it has been observed that IPA contains cytostatic activity on the aryl hydrocarbon receptor (AHR) and the pregnane X receptor (PXR). The high expression of these two receptors in tumour

cells, favours better survival in BC patients due to an inversion relationship between the expression of these receptors and cell proliferation, and low tumour stage and grade [44]. Therefore, according to the results obtained in our study, grade I has been related with a greater survival and less cell proliferation than grade II. Regarding the comparisons between grade II vs grade III, we could determine several carbohydrates (pyruvic acid) and nucleotide (uracil) metabolism alterations. It is to highlight that both metabolites were increased in grade II. Alterations in carbohydrate and nucleotide metabolism favour the proliferation and progression of the tumour [45,46].

Moreover, molecular subtype of the tumour plays a crucial role on patient's prognosis and response to treatment. As we mention before, TN subtype has been associated with poor BC patient survival. In our study, we have identified and increase of tumour marker (CA 15.3), LDH and IL-6 levels in patients with TN subtype. These parameters indicate elevate levels of inflammation and tumorigenesis, favouring resistance to therapy, as has been observed in Vagia et al. [47]. It should be noted that for the luminal A subtype an increase of CRP and PON1 concentrations were observed. These findings have been associated with acute inflammation [48,49]. Regarding metabolomic analyses, we observed significant alterations in metabolites involved in pathway related to amino acid, nucleotides, lipids, and carbohydrates metabolism. Focusing on the comparison between TN and HER2, which would be the two subtypes with worst prognosis, we observed huge alterations in amino acid (phenylalanine) and nucleotides metabolism (uracil and hypoxanthine). A high concentration of phenylalanine and hypoxanthine and a lower concentration of uracil were found in TN subtype. Taking into account the previous mentions on hypoxanthine, it is consistent to find elevated levels in the TN subtype as this metabolite favours cell progression and we have mentioned that patients with TNBC have a worse prognosis.

Finally, the possible limitations of this study could be related to the low number of patients. Studies with a larger number of patients are necessary to validate these preliminary results.

5. Conclusions

Significant alterations in metabolism, oxidative stress and immune response were observed in BC patients. PON1 activity, hypoxanthine, 2-phosphoglyceric acid, xylonic acid and maltose could be possible biomarkers of BC. Postmenopausal state, some histological features of the tumour (others pathological anatomy and histological tumour grade III) and triple negative molecular subtype were associated with high levels of tumour biomarkers, inflammatory components, carbohydrates, lipids, and amino acid metabolism.

Finally, indicate that possible studies could be carried out based on the evidence found in this study.

Bibliography

1. Data explorer | ECIS [Internet]. [cited 2021 Dec 1]. Available from: https://ecis.jrc.ec.europa.eu/explorer.php?$00$1All$2All$42$329$60,85$52008,2008$77$CEstByCountry$X0_83$X0_19AE27$X0_20No$CEstBySexByCountry$X1_83$X1_19AE27$X1_11$CEstByIndiByCountry$X2_83$X2_19AE27$X2_20No$CEstRelative$X3_83$X3_9AE27$X3_19AE27$CEstByCountryTable$X4_19-AE27
2. Januškevičienė I, Petrikaitė V. Heterogeneity of breast cancer: The importance of interaction between different tumor cell populations. Life Sciences. 2019;239:117009.
3. Lüönd F, Tiede S, Christofori G. Breast cancer as an example of tumour heterogeneity and tumour cell plasticity during malignant progression. British Journal of Cancer. 2021;125(2):164–75.
4. Sousa B, Ribeiro AS, Paredes J. Heterogeneity and plasticity of breast cancer stem cells. Advances in Experimental Medicine and Biology. 2019;1139:83–103.
5. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: How special are they? Molecular Oncology. 2010;4(3):192–208.
6. Li J, Chen Z, Su K, Zeng J. Clinicopathological classification and traditional prognostic indicators of breast cancer. International Journal of Clinical and Experimental Pathology. 2015;8(7):8500–5.
7. Zhang D, Xu X, Ye Q. Metabolism and immunity in breast cancer. Frontiers of Medicine. 2021;15(2):178–207.
8. Islam RA, Hossain S, Chowdhury EH. Potential Therapeutic Targets in Energy Metabolism Pathways of Breast Cancer. Current Cancer Drug Targets. 2017;17(8):707–21.
9. Hsu CC, Tseng LM, Lee HC. Role of mitochondrial dysfunction in cancer progression. Experimental Biology and Medicine. 2016;241(12):1281–95.
10. Lee JD, Cai Q, Shu XO, Nechuta SJ. The Role of Biomarkers of Oxidative Stress in Breast Cancer Risk and Prognosis: A Systematic Review of the Epidemiologic Literature. Journal of Women's Health. Mary Ann Liebert Inc.; 2017;2:467–82.
11. Księzakowska-Łakoma K, Zyła M, Wilczyński JR. Mitochondrial dysfunction in cancer. Przeglad Menopauzalny. 2014;18(2):136–44.
12. Deshmukh SK, Srivastava SK, Poosarla T, Dyess DL, Holliday NP, Singh AP, et al. Inflammation, immunosuppressive microenvironment and breast cancer: opportunities for cancer prevention and therapy. Annals of Translational Medicine. 2019;7(20):593–593.
13. Benjamin Chun-Kit Tong. 乳鼠心肌提取 HHS Public Access. Physiol Behav. 2017;176(5):139–48.
14. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. American Family Physician. 2010;81(11):1339–46.

15. Castaneda SA, Strasser J. Updates in the Treatment of Breast Cancer with Radiotherapy. Surgical Oncology Clinics of North America. 2017;26(3):371–82.
16. El cáncer de mama en España - GEICAM - Investigación en Cáncer de Mama. [cited 2022 May 12]. Available from: https://www.geicam.org/sala-de-prensa/el-cancer-de-mama-en-espana
17. Sun S, Sun Y, Rong X, Bai L. High glucose promotes breast cancer proliferation and metastasis by impairing angiotensinogen expression. Bioscience Reports. 2019;39(6):1–9.
18. Brown KA. Metabolic pathways in obesity-related breast cancer. Nature Reviews Endocrinology. 2021;17(6):350–63.
19. Mazzuferi G, Bacchetti T, Islam MO, Ferretti G. High density lipoproteins and oxidative stress in breast cancer. Lipids in Health and Disease. 2021;20(1):1–13.
20. Kaya MO, Sinan S, Güler ÖÖ, Arslan O. Is there a relation between genetic susceptibility with cancer? A study about paraoxanase (PON1) enzyme activity in breast cancer cases. Journal of Enzyme Inhibition and Medicinal Chemistry. 2016;31(6):1349–55.
21. Kitahara CM, de González AB, Freedman ND, Huxley R, Mok Y, Jee SH, et al. Total cholesterol and cancer risk in a large prospective study in Korea. Journal of Clinical Oncology. 2011;29(12):1592–8.
22. Cedó L, Reddy ST, Mato E, Blanco-Vaca F, Escolà-Gil JC. HDL and LDL: Potential new players in breast cancer development. Journal of Clinical Medicine. 2019;8(6):1–21.
23. Camps J, Castañé H, Rodríguez-Tomàs E, Baiges-Gaya G, Hernández-Aguilera A, Arenas M, et al. On the role of paraoxonase-1 and chemokine ligand 2 (C-C motif) in metabolic alterations linked to inflammation and disease. a 2021 update. Biomolecules. 2021;11(7):1–26.
24. Arenas M, Rodríguez E, Sahebkar A, Sabater S, Rizo D, Pallisé O, et al. Paraoxonase-1 activity in patients with cancer: A systematic review and meta-analysis. Critical Reviews in Oncology/Hematology. 2018;127(2):6–14.
25. Acerbi I, Cassereau L, Dean I, Shi Q, Au A, Park C, et al. Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration. Integrative Biology. 2015;7(10):1120–34.
26. Drabsch Y, ten Dijke P. TGF-β signaling in breast cancer cell invasion and bone metastasis. Journal of Mammary Gland Biology and Neoplasia. 2011;16(2):97–108.
27. Saraiva M, Saraiva M, Vieira P, Vieira P, Vieira P, O'Garra A, et al. Biology and therapeutic potential of interleukin-10. Journal of Experimental Medicine. 2020;217(1):1–19.
28. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. Cancer Letters. 2015;367(2):103–7.
29. Steiner JL, Angela Murphy E. Importance of chemokine (CC-motif) ligand 2 in breast cancer. International Journal of Biological Markers. 2012;27(3):179–85.

30. Li D, Ji H, Niu X, Yin L, Wang Y, Gu Y, et al. Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. Cancer Science. 2020;111(1):47–58.
31. Micheel CM, Nass SJ, Omenn GS, Policy HS. Evolution of Translational Omics: Lessons Learned and the Path Forward IOM Committee on Omics-based Predictive Tests On Behalf of the IOM Committee: Institute of Medicine. 2012.
32. Park J, Shin Y, Kim TH, Kim DH, Lee A. Plasma metabolites as possible biomarkers for diagnosis of breast cancer. PLoS ONE. 2019;14(12):1–12.
33. Unlu O, Kiyak D, Caka C, Yagmur M, Yavas HG, Erdogan F, et al. Risk factors and histopathological features of breast cancer among women with different menopausal status and age at diagnosis. Journal of BUON. 2017;22(1):184–91.
34. Lao C, Elwood M, Kuper-Hommel M, Campbell I LR. Impact of menopausal status on risk of metastatic recurrence of breast cancer. Menopause. 2021;28(10):1085–92.
35. Zhao S, Mei Y, Wang Y, Zhu J, Zheng G, Ma R. Levels of CEA, CA153, CA199, CA724 and AFP in nipple discharge of breast cancer patients. International Journal of Clinical and Experimental Medicine. 2015;8(11):20837–44.
36. Cha YJ, Kim ES, Koo JS. Amino acid transporters and glutamine metabolism in breast cancer. International Journal of Molecular Sciences. 2018;19(3).
37. Dias AS, Almeida CR, Helguero LA, Duarte IF. Metabolic crosstalk in the breast cancer microenvironment. European Journal of Cancer. 2019;121:154–71.
38. Vuong D, Simpson PT, Green B, Cummings MC, Lakhani SR. Molecular classification of breast cancer. Virchows Archiv. 2014;465(1):1–14.
39. Morikawa M, Derynck R, Miyazono K. TGF-b and the TGF-b Family: Context-Dependent Roles in Cell and Tissue Physiology. Cold Spring Harbor Perspectives in Biology. 2016;1–24.
40. Hao C, Zhang G, Zhang L. Serum CEA levels in 49 different types of cancer and noncancer diseases. Progress in Molecular Biology and Translational Science. 2019;163:213–227.
41. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, Fox SB, Ichihara S, Jacquemier J, Lakhani SR, Palacios J, Richardson AL, Schnitt SJ, Schmitt FC, Tan PH, Tse GM, Badve S EI. Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Research. 2019;12(4):207.
42. Fernández M, Brenes J, Núñez C. Cáncer De Mama: Características Anatomopatológicas. Revista Medica De Costa Rica Y Centroamerica Lxx. 2013;(607):395–9.
43. Baker K, Lachapelle J, Zlobec I, Bismar TA, Terracciano L, Foulkes WD. Prognostic significance of CD8+ T lymphocytes in breast cancer depends upon both oestrogen receptor status and histological grade. Histopathology. 2011;58(7):1107–16.

44. Sári Z, Mikó E, Kovács T, Jankó L, Csonka T, Lente G, et al. Indolepropionic acid, a metabolite of the microbiome, has cytostatic properties in breast cancer by activating ahr and pxr receptors and inducing oxidative stress. Cancers (Basel). 2020;12(9):1–27.
45. Lv Y, Wang X, Li X, Xu G, Bai Y, Wu J, et al. Nucleotide de novo synthesis increases breast cancer stemness and metastasis via cGMP-PKG-MAPK signaling pathway. PLoS Biology. 2020;18(11):1–24.
46. Wu Z, Wu J, Zhao Q, Fu S, Jin J. Emerging roles of aerobic glycolysis in breast cancer. Clinical and Translational Oncology. 2020;22(5):631–46.
47. Vagia E, Mahalingam D, Cristofanilli M. The landscape of targeted therapies in TNBC. Cancers (Basel). 2020;12(4).
48. Deme D, Telekes A. Prognostic importance of plasma C-reactive protein (CRP) in oncology. Orvosi Hetilap. 2017;158(7):243–56.
49. Devarajan A, Shih D, Reddy ST. Inflammation, infection, cancer and all that...the role of paraoxonases. Advances in Experimental Medicine and Biology. 2014;824:33–41.

Supplementary material

Supplementary table 1. Biochemical characteristics of breast cancer patients and controls.

	Breast cancer (n=85)	**Control group** (n=50)	***pvalue***
Biochemical variables			
Hemoglobin, g/dL	13.5 (13.0-14.3)	13.6 (13.2-14.3)	0.330
Platelets, $x10^9$/L	258 (227-290)	241.5 (214.25-284)	0.179
Leukocytes, $x10^9$/L	6.5 (5.5-7.5)	6.3 (5.3-7.7)	0.852
Glucose, mg/dL	94 (86-105)	81.1 (75.7-92.8)	**$3.06x10^6$**
Creatinine, mg/dL	0.7 (0.6-0.8)	0.7 (0.6-0.8)	0.904
GGT, U/L	16 (13-24)	10.2 (7.29-16.05)	**$4.22x10^6$**
AST, U/L	18 (16-21)	19.9 (17.5-22.9)	0.075
ALT, U/L	15 (13-22)	14.4 (12.3-19.9)	0.228
Total cholesterol, mg/dL	206 (183-228)	185.6 (158.5-216.5)	**0.026**
VLDL, mg/dL	23 (18-31)	14.9 (11.9-20.8)	**$9.81x10^8$**
LDL, mg/dL	116 (102-140)	108.7 (86.9-122.7)	0.052
HDL, mg/dL	58 (49-67)	61.2 (52.9-77.9)	**0.046**
TG, mg/dL	115 (89.5-154)	75.2 (59.7-106.2)	**$3.24x10^7$**
CRP, mg/dL	0.16 (0.1-0.3)	1.3 (0.5-3.1)	**$1.28x10^9$**
CCL2, pg/mL	70.8 (59.1-92.6)	77.1 (73.2-83.1)	**$4.00x10^2$**
IL-10, pg/mL	4.3 (3.1-6.2)	2.7 (2.2-4.1)	**$5.10x10^7$**
TGF-β, pg/mL	1.1 (0.8-1.5)	0.8 (0.4-1.2)	**0.005**
PON1 activity, U/L	73.6 (26.1-172.7)	176.5 (138.2-209.7)	**$3.37x10^6$**
PON1 concentration, pg/mL	0.7 (0.2-2.4)	0.8 (0.3-1.9)	0.633

Values are provided as median (interquartile range). GGT: gamma-glutamiltransferasa; AST: aspartate aminotransferase; ALT: alanine aminotransferase VLDL: very-low-density lipoproteins; LDL: low-density lipoproteins; HDL: high-density lipoproteins; TG: triglycerides; CRP: C-reactive protein; CCL2: chemokine C-C motif ligand 2; IL-10: interleukin 10; TGF- β: transforming growth factor beta
p< 0.05 by the Mann-Whitney U-test.

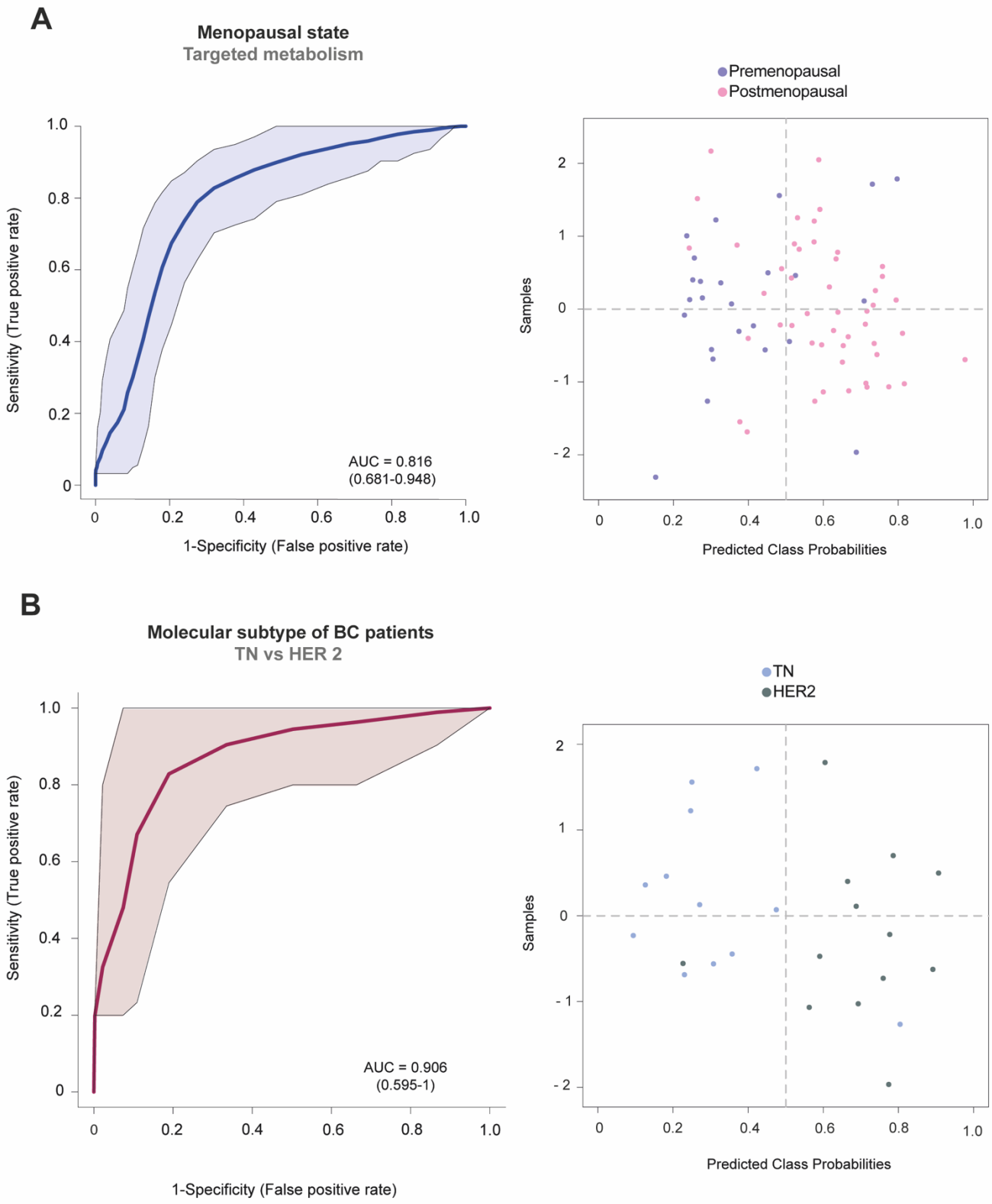
Supplementary table 2. Energy metabolism characteristics of breast cancer patients and controls.

	Breast cancer (n=85)	**Control group** (n=50)	***pvalue***
Carbohydrate metabolism			
Fructose and mannose			
d-Fructose	5.9 (4.4-9.5)	4.8 (3.9-6.3)	**0.011**
d-Mannonic acid (RU)	0.03 (0.02-0.03)	0.02 (0.02-0.03)	**0.018**
Galactose metabolism			
d-Galactitol (RU)	0.02 (0.01-0.02)	0.02 (0.01-0.02)	**0.006**
Glycolysis			
3-Phosphoglyceric acid (RU)	0.07 (0.04-0.14)	0.02 (0.02-0.04)	**$3.47x10^{13}$**
Glucose 6-phosphate (RU)	0.009 (0.006-0.014)	0.005 (0.004-0.007)	**$1.00x10^{8}$**
Lactic acid (μM)	1425.2(1259.8-1714.5)	1303.3 (996.0-1459.8)	**0.001**
Nucleotide sugar			
d-Arabinose (RU)	0.20 (0.02-0.03)	0.018 (0.014-0.023)	0.065
d-Threitol (RU)	0.03 (0.02-0.03)	0.019 (0.016-0.021)	**$7.37x10^{8}$**
d-Xylose (RU)	0.009 (0.006-0.015)	0.007 (0.005-0.010)	**0.035**
Erythronic acid (RU)	0.03 (0.02-0.04)	0.020 (0.015-0.027)	**$9.64x10^{7}$**
Threonic acid (RU)	0.3 (0.3-0.4)	0.2 (0.2-0.3)	**$1.92x10^{5}$**
Xylonic acid (RU)	0.015 (0.012-0.020)	0.05 (0.04-0.05)	**$5.78x10^{21}$**
Pentose glucoronate interconversion			
d-Arabitol (RU)	0.011 (0.009-0.013)	0.008 (0.007-0.010)	**$1.64x10^{5}$**
d -Xylitol (RU)	0.001 (0.001-0.002)	0.001 (0.001-0.001)	**0.005**
Galacturonic acid (RU)	0.02 (0.01-0.02)	0.018 (0.015-0.022)	0.954
Myo-Inositol (μM)	26.3 (21.3-31.6)	20.3 (17.1-24.8)	**$6.37x10^{5}$**
Sucrose metabolism			
d-Sucrose (RU)	0.007 (0.003-0.012)	0.003 (0.002-0.005)	**$1.42x10^{4}$**
Maltose (RU)	0.019 (0.009-0.032)	0.006 (0.004-0.009)	**$5.49x10^{11}$**
TCA			
α-ketoglutaric acid (μM)	40.6 (29.7-53.3)	39.5 (30.4-46.6)	0.832
Citric acid (μM)	106.5 (85.8-144.1)	94.1 (76.1-110.8)	**0.014**
DL-2-Hydroxyglutaric acid (μM)	1.1 (0.9-1.4)	1.1 (0.9-1.4)	0.871
Fumaric acid (μM)	0.7 (0.6-0.9)	0.74 (0.62-0.86)	0.637
Glutamine (μM)	1289.6 (1019.2-1543.2)	1362.8 (1080.7-1745.4)	0.091
Malic acid (μM)	4.3 (3.2-5.9)	3.5 (2.8-4.3)	**0.004**
Pyruvic acid (μM)	197.8 (65.08-152.75)	108.8 (73.1-141.4)	0.717
Succinic acid (μM)	3.5 (2.7-4.8)	2.8 (2.4-3.3)	**$9.02x10^{4}$**
Glyoxylate and dicarboxylate			
Glycolic acid (μM)	3.8 (3.3-4.5)	5.0 (4.3-5.7)	**$8.94x10^{8}$**
Pentose phosphate pathway			
Sedoheptulose (RU)	0.01 (0.01-0.02)	0.02 (0.011-0.020)	0.486
Amino acid metabolism			

Alanine and aspartate			
Alanine (μM)	323.5 (287.0-389.8)	273.5 (235.7-323.9)	**$7.16x10^{5}$**
Glutamic acid (μM)	63.1 (44.7-97.8)	43.5 (36.4-52.8)	**$5.86x10^{6}$**
Arginine and proline			
2-Hydroxyisobutyric acid (RU)	1.4 (1.03-1.7)	1.1 (0.9-1.4)	**0.003**
Urea (μM)	2485.1 (1985.7-3086.2)	2426.6 (1757.4-2870.4)	0.106
Proline (μM)	112.8 (91.3-154.9)	100.5 (80.5-127.5)	0.063
4-Hydroxyproline (μM)	7.2 (5.5-10.7)	8.1 (6.2-11.2)	0.265
Ornithine (μM)	69.5 (53.1-80.8)	70.8 (52.6-91.0)	0.504
Oxoproline (RU)	24.6 (21.04-28.8)	25.7 (21.6-29.8)	0.344
Glycine and Serine			
Glyceric acid (μM)	1.3 (1.0-1.7)	1.7 (1.3-2.2)	**0.003**
Glycine (μM)	116.4 (104.7-123.1)	111.1 (102.2-120.7)	0.201
Serine (μM)	102.7 (84.2-120.8)	112.7 (93.3-121.6)	0.130
Threonine (μM)	121.2 (101.1-140.7)	133.1 (107.1-153.8)	**0.045**
Tyrosine metabolism			
Vanillylmandelic acid (RU)	0.007 (0.006-0.011)	0.006 (0.005-0.008)	**0.003**
Valine, leucine, and isoleucine			
2-Hydroxybutyric acid (μM)	37.7 (26.4-55.0)	31.3 (19.5-43.6)	**0.018**
2-Hydroxyisovaleric acid (RU)	5.2 (3.8-7.7)	4.3 (3.1-5.3)	**0.004**
2-Keto-3-methylvaleric acid (RU)	1.1 (0.89-1.31)	1.2 (0.9-1.5)	**0.037**
3-Hydroxybutyric acid_3 hydroxyisobutyric acid (RU)	8.0 (4.9-20.7)	8.6 (5.1-22.5)	0.947
3-Hydroxyisovaleric acid (μM)	0.8 (0.7-1.2)	0.9 (0.6-1.1)	**0.004**
3-methyl-2-oxobutyric acid_alphaketoisovaleric acid (RU)	0.5 (0.4-0.8)	0.6 (0.4-0.7)	0.267
Ethylmalonic acid (RU)	2.1 (1.6-2.9)	5.3 (4.4-6.3)	**$9.04x10^{19}$**
Isoleucine (μM)	63.1 (52.8-78.5)	61.5 (53.1-70.9)	0.424
Leucine (μM)	114.3 (99.8-1340.0)	114.9 (103.9-126.9)	0.687
Valine (μM)	215.0 (184.2-257.6)	206.9 (193.4-230.5)	0.356
Cysteine and methionine metabolism			
Methionine (μM)	47.3 (44.4-51.4)	48.3 (46.1-50.8)	0.275
Tryptophan metabolism			
Indole-3-propanoic acid (μM)	1.5 (0.9-2.2)	1.2 (0.9-2.0)	0.305
Indolelactic acid (RU)	0.16 (0.13-0.20)	0.14 (0.11-0.17)	**0.010**
Phenylalanine metabolism			
4-hydroxyphenyllactic acid (RU)	0.2 (0.1-0.2)	0.1 (0.1-0.2)	**0.029**
Benzoic acid (RU)	0.6 (0.5-0.7)	0.5 (0.3-0.7)	**0.022**
Hippuric acid (RU)	0.2 (0.1-0.4)	0.3 (0.1-0.4)	0.172
Hydrocinnamic acid (RU)	0.05 (0.03-0.1)	0.07 (0.03-0.10)	0.259
Phenylalanine (μM)	57.2 (50.1-65.9)	55.9 (52.8-60.1)	0.403

Lipid metabolism			
Lipids			
Dodecanoic acid (RU)	0.6 (0.4-0.8)	0.5 (0.4-0.8)	0.072
Linoleic acid (RU)	2.3 (1.5-4.0)	2.8 (1.5-4.3)	0.546
Oleic acid (RU)	17.3 (10.9-24.8)	14.6 (7.8-26.0)	0.408
Tetradecanoic acid (RU)	1.4 (1.0-2.2)	1.6 (1.1-2.1)	0.354
Glycolipid metabolism			
Ethanolamine (μM)	3.1 (2.5-3.6)	3.1 (2.7-3.7)	0.634
Glycerol (μM)	108.1 (80.9-147.8)	239.4 (187.6-312.6)	**5.42×10^{17}**
Glycerol-1-phosphate (RU)	0.2 (0.1-0.3)	0.2 (0.1-0.2)	**0.034**
Primary bile acid biosynthesis			
Taurine (RU)	0.8 (0.6-1.2)	0.5 (0.4-0.7)	**5.67×10^{8}**
Metabolism of cofactors and vitamins			
Cofactor biosynthesis			
α-tocopherol (RU)	0.2 (0.2-0.3)	0.2 (0.1-0.4)	0.660
Nucleotide metabolism			
Purine and pyrimidine			
Hypoxanthine (RU)	0.5 (0.3-1.3)	0.2 (0.1-0.3)	**5.76×10^{13}**
Ribonic acid (RU)	0.004 (0.003-0.006)	0.003 (0.002-0.004)	**0.013**
Uracil (μM)	0.01 (0.07-0.1)	0.09 (0.07-0.11)	0.196
Uric acid (μM)	186.1 (122.50-365.2)	181.2 (113.7-288.4)	0.298
Xenobiotic biodegradation			
Benzoate degradation			
4-Hydroxybenzoic acid (RU)	0.12 (0.12-0.13)	0.123 (0.120-0.126)	0.172
Energy metabolism			
Oxidative Phosphorylation			
Phosphoric acid (RU)	191.1 (163.4-234.9)	158.7 (65.1-209.4)	**0.002**

Values are provided as median (interquartile range)
$p < 0.05$ by the Mann-Whistney U-test



Supplementary figure 1. (A) Receiver Operating characteristic (ROC) Curve and complexity matrix of menopausal estate, it is a combination of d-threitol, dodecanoic acid, and vanillylmandelic. It is shown a complexity matrix too. (B) Receiver Operating characteristic (ROC) Curve and complexity matrix of the comparative TN vs. HER2 for molecular subtype, it is a combination of CA 15.3, triglycerides, PON1 concentration, glucose, LDH (low-density lipoproteins), hypoxanthine, phenylalanine, and uracil.
The significance was determined by the Mann-Whitney U-test, $p < 0.05$