



AMP hydrolysis reduction in blood plasma of breast cancer elderly patients after different treatments

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Abstract

Adenine nucleotides are important signaling molecules that mediate biological functions in many conditions, including cancer. The enzymes CD39 and CD73 produce adenosine in the extracellular milieu that has a very important role in tumor development. This study aimed to evaluate nucleotide hydrolysis in the plasma blood of breast cancer elderly patients. In this prospective cohort study, we investigated the ectonucleotidases activity in breast cancer elderly patients, at the moment of diagnosis and after treatment. Control group consisted of elderly women without cancer diagnostic. The nucleotide hydrolysis assay was performed by the malachite green method and used ATP, ADP, or AMP as substrates. Paired t test or Wilcoxon rank-sum test was used. Our data showed that breast cancer patients presented high levels of ATP and AMP hydrolyses when compared to control group at the moment of diagnosis. When analyzing the differences between the samples at the time of diagnostic and 6 months after treatment, we observed a significant reduction on CD73 activity after all treatments used: surgery, chemotherapy, radiotherapy, or hormone therapy. The results with ACP, a specific CD73 inhibitor, showed that the AMP hydrolysis was inhibited in all conditions evaluated. We observed a diminished ADPase activity in the patients without metastasis when compared to metastatic breast cancer patients. The results showed that AMP hydrolysis was reduced in the blood plasma of breast cancer elderly patients after different treatments. This study strengthens the potential role of CD73 enzyme as a biomarker for breast cancer treatment response.

Keywords Breast cancer · Elderly patients · Plasma blood · Ectonucleotidases · CD39 · CD73

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Introduction

Breast cancer (BC) is the most common type of cancer among women all over the world. Low- and middle-income countries have diagnosis in later stages of the disease, increasing treatment related morbidity, quality of life impairment, and reducing overall survival [1, 2]. Various cases have familiar predisposition, related to BRCA 1 and 2 gene mutation [2, 3]. The disease has three different predominant pathological status: hormonal receptors expression (estrogen and progesterone), overexpression of Human Epidermal growth factor Receptor 2 (HER2), and the triple-negative type (TNBC – triple-negative breast cancer) that does not express neither HR nor HER2 [4–6].

Older patients are more likely to present high estrogen receptor (ER) and progesterone receptor (PR) expression breast cancer, with or without HER2 expression [5]. The tumor size and nodal involvement increase with age and

can be explained by the late diagnosis in this population. However, the increase of nodal involvement is related to smaller tumors, suggesting small but aggressive tumors in older women [5, 7, 8]. Furthermore, the 5- to 10-year relative survival of patients older than 70 years is shorter than younger patients. This may be due to insufficient treatment, socioeconomic differences, and unequal access to healthcare in poorer countries [2, 7, 9]. Besides, elderly patients are initially excluded from clinical trials because of the age restrictions [2, 10]. The underestimation of life expectation can result in insufficient treatments, being considered a major risk factor to cancer recurrence and death [10–12].

Nucleotides (ATP, ADP, UTP, and UDP) and nucleosides (adenosine) are characterized as extracellular messengers [13–15]. They are present in normal conditions, and their exacerbated release is caused by different stressor agents, such as plasmatic membrane damages, platelet aggregation, viral or bacterial infections, and mechanical stress [13, 15]. ATP acts as an alert sign, activating the immune cells to fight microorganisms, start tissue repair responses, and to find and engulf apoptotic cells [14, 16]. Once on the extracellular milieu, ATP and ADP are quickly hydrolyzed to AMP by NTPDase1/CD39, and subsequently to adenosine (ADO) by ecto-5'-nucleotidase/CD73 or alkaline phosphatase (ALP) [13, 14, 16, 17]. The CD39 is anchored to plasmatic membrane by two transmembrane domains essential to its catalytic activity and substrate specificity [18]. This enzyme is a lymphocytic maturation marker and has fundamental role on immune system by purinergic signaling modulation [16, 19]. Evidence showed that NTPDase1 catalytically active can be released as plasma membrane fragments from expressing cells. In addition, it can be found incorporated into exosomes released by various tumor types [19].

Ecto-5'-nucleotidase/CD73 is a glycosyl phosphatidylinositol (GPI)-anchored enzyme expressed on the surface of subsets of human lymphocytes that hydrolyze the extracellular AMP into ADO, regulating extra and intracellular environment [20]. Its expression is related to cellular adhesion and proliferation, and it also works in cellular migration and invasion [21]. Soluble nucleotidases may represent an important effector system to local nucleotide inactivation that are intensely raised, especially on inflammation and injury sites [22].

It is known that ATP concentration on extracellular tumor milieu reaches molar ranges, which is different from normal tissues [23]. Thus, tumor microenvironment becomes an active ATP release/generation site and its conversion to ADO, producing growth factors and immunomodulators. In vivo studies showed that solid tumor extracellular milieu have high concentration of ADO, generated by conjunct action of CD39 and CD73 ectonucleotidases [23, 24]. In fact, CD73 has been reported to promote breast cancer occurrence and development through immune evasion,

angiogenesis and lymphangiogenesis [20, 22]. High CD73 expression may be related to bad prognosis and high doxorubicin resistance, a commonly anthracycline used on its treatment [21]. Also, CD39 expression, with consequent ATP hydrolysis and adenosine generation, may compromise antitumor immune responses, including the ones mediated by natural killer cells [18].

Considering that previous studies have described CD39 and CD73 expression and activity on different kinds of tumor samples [25–28] added to the high necessity of studies with elderly patients, we aimed to investigate the hydrolysis of these enzymes on bloodstream of elderly breast cancer patients before and after treatment.

Methods

Chemicals

Adenosine-5'triphosphate (ATP), Adenosine-5'diphosphate (ADP), Adenosine-5' monophosphate (AMP), α - β -methylene-ADP (APCP), Malaquite Green, Coomassie Blue, and Tris-HCl were purchased by Sigma-Aldrich.

Subjects

The prospective cohort study included thirty-three women above sixty (60) years old with breast cancer diagnosis. These patients were diagnosed and started their treatment at São Lucas da PUCRS Hospital's Oncology Center and formalized the participation in the study through Adherence to Informed Consent Standards (AICS) before blood collection. Inclusion criteria were as follows: patients age \geq 60 years old; diagnosed with breast cancer; and who will start or start treatment. Exclusion criteria were as follows: previous chemotherapy treatment on the past ten years and patients who have some type of cognitive impairment (Mini Mental State Examination). The control group consisted of fifteen elderly women with age above sixty years old. Previous diagnostic of cancer was used as exclusion criteria to control group.

Blood collection

Blood samples (4 mL) were collected before initiating cancer treatment and after six months. The blood was collected in appropriated plastic tubes with heparin. For plasma isolation, samples were centrifuged at 4000 rpm for 12 min. After centrifugation, the supernatant was stored at -80 °C until biochemistry analysis.

Protein analysis

The quantification of protein levels in all plasma samples was performed by Coomassie Blue method, as described by Bradford et al. [29], using bovine serum albumin as standard.

Nucleotide hydrolysis assay

The incubation protocol was performed as described by Moritz et al. [30]. For NTPDases activity determinations in blood plasma, samples were pre-incubated with Tris-HCl buffer 112.75 mM (final concentration) pH 8.0 for 10 min at 37 °C and, to start reactions, substrates (ATP, ADP and AMP) were added to samples in a final concentration of 3 mM. At 50 min after incubation, 5% (final concentration) trichloroacetic acid (TCA) was added to stop reaction, and samples were subsequently chilled on ice. The samples were centrifuged at 14,000 rpm for 12 min and, according to Chan et al. [31], the amount of inorganic phosphate (Pi) released was analyzed by the Malachite Green colorimetric method with minor modifications. Controls were performed to correct the nonenzymatic substrate hydrolysis. All samples were processed in triplicate. Enzyme activities were expressed as μmol of Pi released per minute per milligram of protein ($\mu\text{mol}/\text{min}/\text{mg}$).

Statistical analysis

Quantitative variables were described by mean, range, and standard deviation, while categorical variables were described by absolute and relative frequencies. For mean comparison, paired t test or Wilcoxon rank-sum test, the alternative nonparametric test used when conditions for t test are not met, was used. Statistical analysis was also performed using ANOVA followed by Tukey post hoc. The significance level for claim statistical difference between groups was set at 0.05. All analyses were performed using the SAS statistical software (version 9.4; SAS Institute, Inc. Cary, NC). The graphics were produced using GraphPad Prism 5.01, San Diego, CA, USA.

Results

In this study, we analyzed 32 elderly patients with breast cancer diagnosis. The mean age of these patients was 67.8 (61–82) years, and the subtypes of breast cancer identified were as follows: 5 with Luminal A (15.6%), 12 with Luminal B (37.5%), 11 with HER2 positive (34.4%), and 4 TNBC (12.5%). The evaluation of the grade showed that the majority (51.8%) were grade 2 (moderately differentiated). According to TNM Staging System, 23 patients

(71.9%) presented stages I to II, and nine patients (28.1%) had stages III and IV, which represent worse prognosis. The associated comorbidities presented in breast cancer patients were cardiovascular disease (57.6%), endocrine disease (39.3%), psychiatric conditions (42.8%), gastrointestinal disease (10.7%), bone and joint disease (25%), and respiratory tract disease (17.9%). Twenty patients were conducted to surgery (62.5%), while 26 received chemotherapy (81.3%), 16 received hormone therapy (50%), and 19 received radiotherapy (59.4%) (Table 1).

Here, we evaluated the nucleotide hydrolysis in blood plasma of breast cancer patients comparing to elderly women without cancer (Fig. 1). The results demonstrated that, at

Table 1 Clinical and pathological data of breast cancer patients

Characteristics	N	%
Age (years)		
Mean	67	
Range	61–82	
Tumor type		
Luminal A	5	15.6
Luminal B	12	37.5
HER2	11	34.4
Triple negative	4	12.5
Grade		
1 (well differentiated)	7	21.9
2 (moderately differentiated)	16	50.0
3 (poorly differentiated)	9	28.1
Clinical stage		
I and II	23	71.9
III and IV	9	28.1
Associated comorbidities		
Cardiovascular disease	19	57.6
Endocrine disease	11	39.3
Psychiatric disease	12	42.8
Gastrointestinal disease	3	10.7
Bone and articular disease	7	25
Respiratory tract disease	5	17.9
Breast cancer treatment		
Surgery		
Yes	20	62.5
No	12	37.5
Chemotherapy		
Yes	26	81.3
No	6	18.7
Radiotherapy		
Yes	19	59.4
No	13	40.6
Hormone therapy		
Yes	16	50.0
No	16	50.0

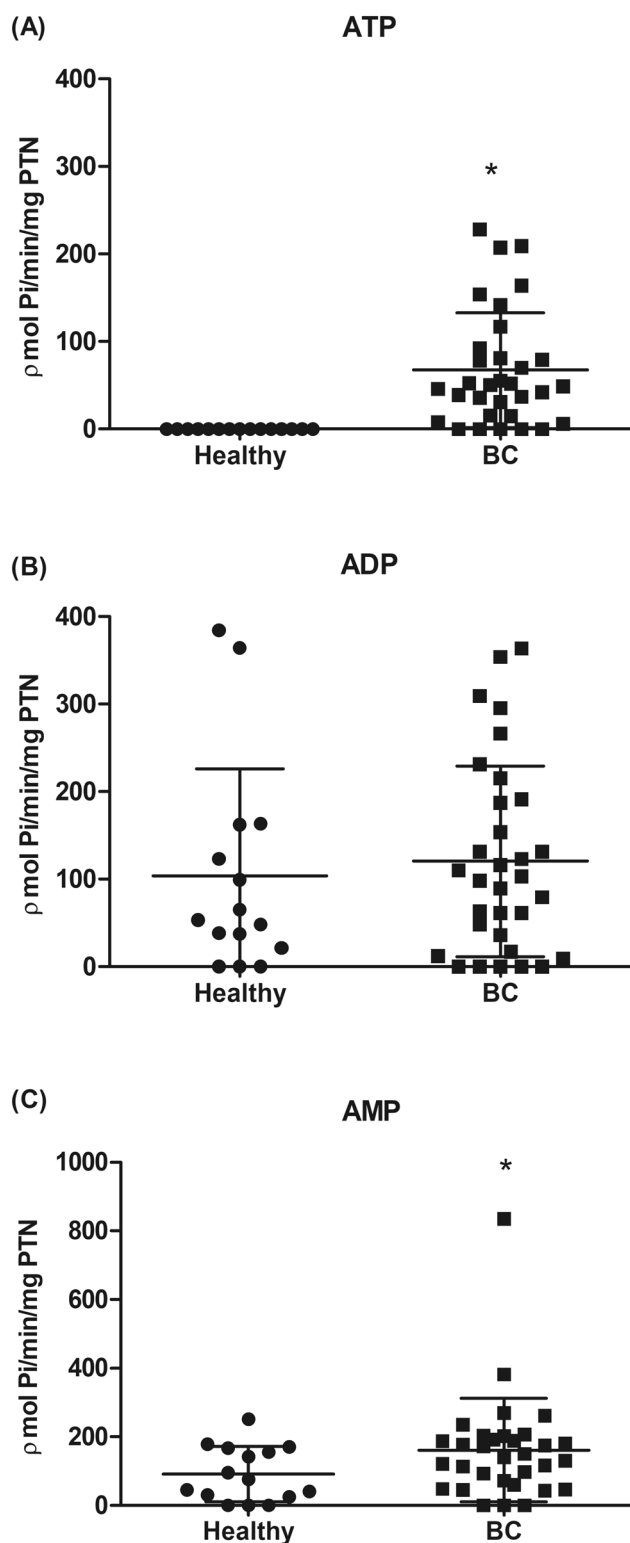


Fig. 1 Nucleotide hydrolysis—Comparison between BC and Healthy patients. ATP, ADP, and AMP hydrolyses were evaluated in blood plasma as described in Materials and Methods section, and final values were described as $\mu\text{mol/min/mg protein}$. The experiments were performed in triplicate. Data are expressed as mean \pm SD and analyzed by t test with independent samples on SPSS statistical program, and the results were considered significant when p value > 0.05

the moment of diagnosis, breast cancer patients presented high levels of ATP hydrolysis ($67.46 \pm 65.54 \mu\text{mol/min/mg PTN}$) in comparison to control group ($0.058 \pm 0.085 \mu\text{mol/min/mg PTN}$). Both groups hydrolyzed in a similar way the ADP (BC: $120.4 \pm 73.95 \mu\text{mol/min/mg PTN}$ and control: $103.8 \pm 122.1 \mu\text{mol/min/mg PTN}$). In addition, BC patients also hydrolyzed AMP at higher levels ($161.0 \pm 150.8 \mu\text{mol/min/mg PTN}$) than control women evaluated ($91.8 \pm 125.28 \mu\text{mol/min/mg PTN}$) (Fig. 1).

Interestingly, when we compared the nucleotide hydrolysis in BC patients' blood plasma before starting the treatment (at the moment of diagnosis) with the samples collected after 6 months, it was observed a significant reduction of AMPase activity after treatment (157.15 ± 72.67 and $76.23 \pm 81.85 \mu\text{mol/min/mg PTN}$, respectively). The ATP and ADP hydrolysis profile did not present significant differences (ATPase: 48.31 ± 56.65 to 65.92 ± 104.32 and ADPase: 91.23 ± 82.69 to $120.85 \pm 122.48 \mu\text{mol/min/mg PTN}$, respectively) (Fig. 2).

It has been already described that Ecto-5' nucleotidase/CD73 (CD73) is the main enzyme capable to hydrolyze AMP in the extracellular medium [21]. In order to evaluate if this enzyme was present in the experimental conditions tested here, we evaluated the AMP hydrolysis in blood plasma samples at the presence of the APCP, a specific CD73 inhibitor (Fig. 3). The results showed that the AMP hydrolysis was inhibited in all groups (control, BC patients at moment of diagnosis, and BC patients after six months of treatment). At the sequence, we evaluated the correlation between breast cancer patients' nucleotide hydrolysis at diagnosis and after six months, considering each treatment. The analysis showed a significant reduction of AMPase activity in all treatment groups studied: surgery, chemotherapy, radiotherapy, and hormone therapy (Table 2).

In addition, we compared breast cancer patients' nucleotide hydrolysis in relation to Ki67 proliferation marker status. High Ki67 levels ($> 20\%$) correspond to high proliferation rates, and low Ki67 levels ($\leq 20\%$) correspond to low proliferation. The results showed that 47% of the patients presented low Ki67 score and 52.2% had a high proliferation rate without differences in ATPase, ADPase, or AMPase activity among them. The group presenting low Ki67 status had less AMPase activity when compared to high Ki67 (Table 3). Besides that, we divided the patients in two groups based on Clinical Stage (CS) defined by TNM classification, which defines tumor size, lymph nodes affected, and presence of metastasis. The first group consisted of patients arranged in I and II stages (71.9%), and the second of patients in III and IV stages (28.1%). There were no significant differences in nucleotide hydrolysis in the groups, ATP, ADP, or AMP hydrolysis according to CS nor in different grades (1 to 3). Finally, we evaluated the relation between metastasis with the nucleotide hydrolysis. Interestingly, our

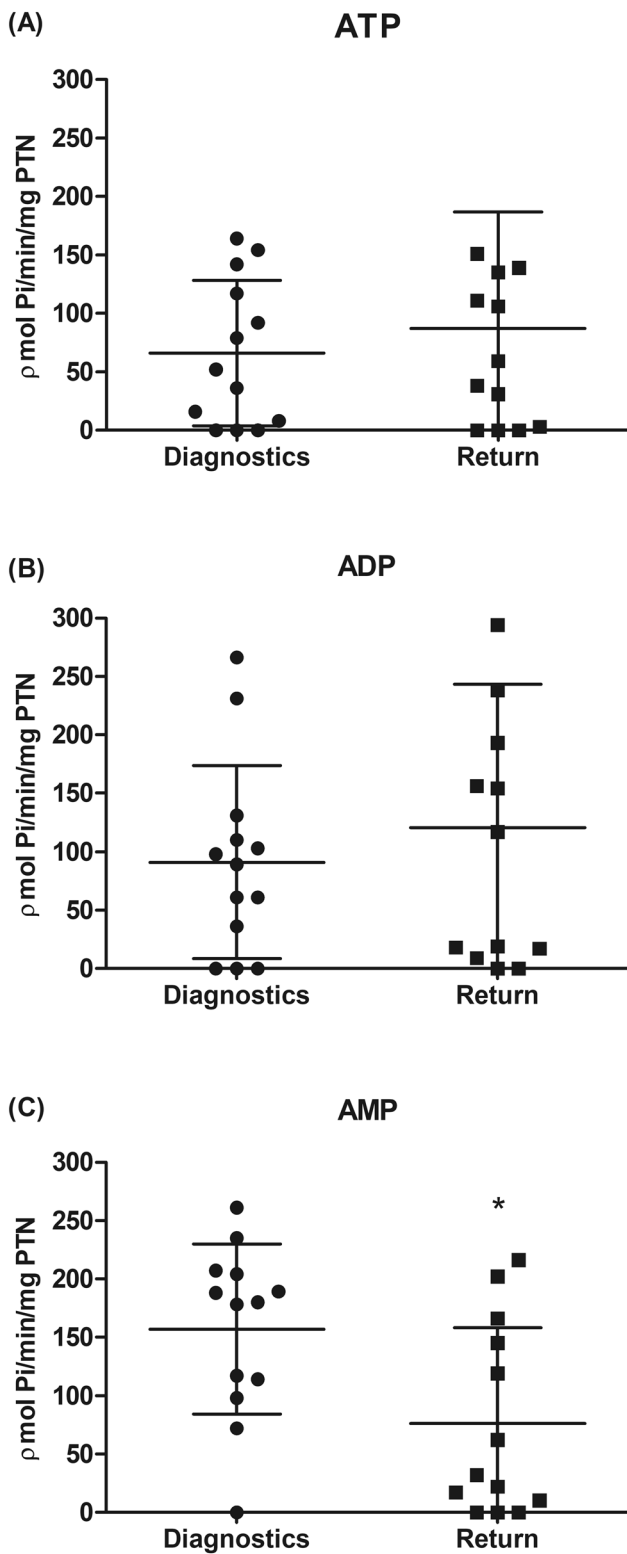


Fig. 2 Nucleotide hydrolysis—Comparison between diagnosis and after 6 months of treatment BC patients. The analysis was performed as described in Materials and Methods section, and final values were described as $\mu\text{mol}/\text{min}/\text{mg}$ protein. The experiments were performed in triplicate. Data are expressed as mean \pm SD and analyzed by paired t test on SPSS program, and the results were considered significant when p value > 0.05

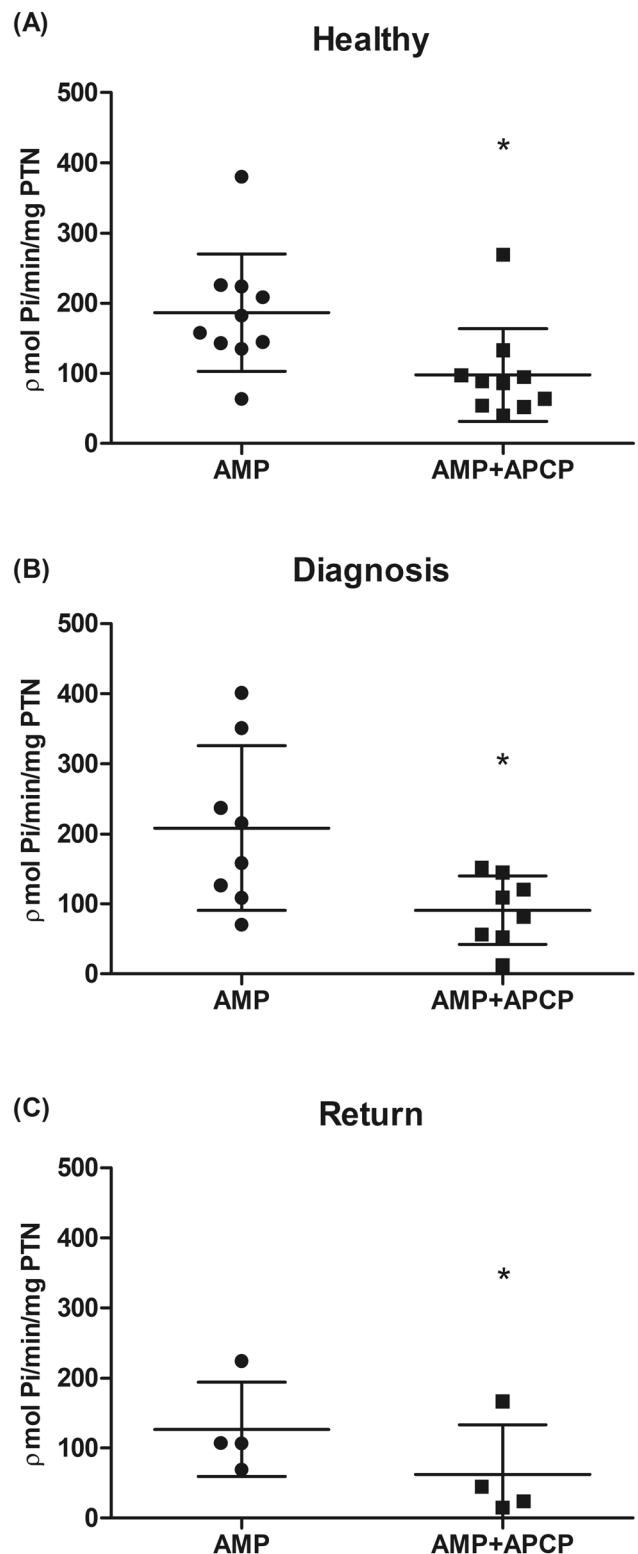


Fig. 3 Effect of APCP on AMP hydrolysis profile. The AMP hydrolysis was analyzed in three groups: healthy woman, BC patients at diagnostic and after 6 months of treatment. APCP, a specific CD73 inhibitor, was used at final concentration of $30 \mu\text{M}$. The experiments were performed in triplicate. Data are expressed as mean \pm SD and analyzed by paired t test on SPSS program, and the results were considered significant when p value > 0.05

Table 2 Evaluation of nucleotide hydrolysis according to different kinds of treatment

Treatment	Nucleotide hydrolysis (pmol Pi/min/mg PTN)		
	ATP	ADP	AMP
Chemotherapy			
Diagnosis (<i>n</i> =12)	67.32 ± 64.95	93.75 ± 85.84	153.00 ± 74.27
After treatment (<i>n</i> =12)	91.67 ± 102.77	129.42 ± 123.79	80.75 ± 83.78*
Surgery			
Diagnosis (<i>n</i> =9)	87.11 ± 63.41	80.00 ± 76.05	176.22 ± 56.16
After treatment (<i>n</i> =9)	102.56 ± 112.68	122.56 ± 124.29	80.11 ± 79.07*
Radiotherapy			
Diagnosis (<i>n</i> =10)	83.660 ± 60.81	78.10 ± 71.95	179.30 ± 53.83
After treatment (<i>n</i> =10)	95.40 ± 108.62	112.10 ± 121.76	74.30 ± 76.78*
Hormone therapy			
Diagnosis (<i>n</i> =6)	58.00 ± 46.86	94.83 ± 82.07	139.50 ± 86.03
After treatment (<i>n</i> =6)	115.33 ± 131.06	151.00 ± 116.313	41.17 ± 62.10*

*Represents a *p* value < 0.05 when comparing nucleotide hydrolysis at diagnosis and after 6 months of treatment. The values represent mean ± SD. Only the patients who had the 6 months return were analyzed

Table 3 Relation between Ki67 score, clinical stage, grade, and metastasis with nucleotide hydrolysis

	Nucleotide hydrolysis (pmol Pi/min/mg PTN)		
	ATP	ADP	AMP
Ki67**			
High (> 20%) (<i>n</i> =17)	69.19 ± 57.25	130.05 ± 102.32	199.39 ± 109.15
Low (≤ 20%) (<i>n</i> =14)	69.05 ± 77.44	109.91 ± 122.68	120.65 ± 73.59
Clinical stage			
I and II (<i>n</i> =23)	70.62 ± 64.51	115.02 ± 110.42	141.88 ± 95.42
III and IV (<i>n</i> =9)	59.41 ± 71.40	134.12 ± 109.54	209.72 ± 243.58
Grade			
1 (<i>n</i> =7)	65.93 ± 68.41	170.05 ± 134.19	171.58 ± 106.12
2 (<i>n</i> =16)	73.77 ± 71.04	119.95 ± 106.22	171.21 ± 195.35
3 (<i>n</i> =9)	57.44 ± 58.97	82.56 ± 86.02	134.48 ± 83.62
Metastatic			
Yes (<i>n</i> =10)	59.52 ± 61.52	187.61 ± 116.83	230.87 ± 221.35
No (<i>n</i> =22)	71.07 ± 68.38	89.84 ± 92.08*	129.18 ± 95.78

*Represents a *p* value < 0.05 when comparing nucleotide hydrolysis at diagnosis and after 6 months of treatment. The values represent mean ± SD

**One patient did not have Ki67 information related to medical chart, and, to this analysis, her data were not considered

results showed a diminished ADPase activity in the patients without metastasis when compared to metastatic breast cancer patients (Table 3).

Discussion

Breast cancer is the most common and the highest mortality cause of women in whole world [1, 6]. Although breast cancer in elderly patients have more favorable features than younger patients regarding hormone receptor expression profile, tumor grade, and proliferation rate, these patients

are often diagnosed in later stages and undertreated due to physicians concerns about appropriate therapy and surgical choose [5, 32]. In fact, Gal et al. [32] demonstrated that biologic features between 65–75 years and above 75 years old do not differ, but the second group receive less treatment. Also, there are few evidences concerning treatment on this population since clinical trials, usually, do not include them, making the treatment decisions based on extrapolation of studies with younger patients. Tumor biological features and treatment tolerance of elderly breast cancer patients vary, besides other mortality risks [5, 7, 33]. So, studies including and focusing elderly patients are important.

Looking closely to the tumoral scenario, purinergic system has a crucial role to cancer development and can avoid organism attempts to beat it. In fact, it is well known that ATP and ADO work in a synchronized manner to favoring the tumor growth [23]. In this study, we compared the nucleotide (ATP, ADP, and AMP) hydrolysis in blood plasma of breast cancer patients with elderly women without cancer. The data presented herein showed that elderly breast cancer patients presented high levels of ATP hydrolysis when compared to control. This could be explained since ATP hydrolysis into extracellular adenosine limits immune response [18]. Data on nucleotide hydrolysis in the blood of breast cancer patients was previously shown by Do Carmo Araújo et al. [34]. They analyzed the nucleotide hydrolysis on platelets of female patients with BC, aged 20 to 85 years, according to the length of tamoxifen use, and concluded that AMP hydrolysis was not significantly altered [34].

Former studies showed that adenosine has anti-inflammatory activity, helping the tumor to evade the immune system [23]. Furthermore, recent studies showed that CD39 antagonism can stabilize ATP pro-inflammatory extracellular to restore antitumor immunity [35]. Adenosine activates the P1 purinergic receptors subtypes A2A and A2B and limits the effector T cell functions [16]. Also, increased levels of serum CD73 may indicate an increased expression of CD73 within the tumor microenvironment, probably as a consequence of tissue-associated inflammation/hypoxia [36]. The assessment of cancer patient's serum CD73 levels is a promising approach that may help predict a response to immunotherapy based on adenosine-targeting agents [25, 37].

The results also showed that elderly BC patients hydrolyze more AMP than elderly women without cancer, indicating that this malignancy may cause alterations on nucleotide metabolism generating an increased amount of circulating ADO. These data are in accordance with previous results demonstrated by Morello et al. [35] and Gardani et al. [25] that showed high AMPase activity in blood samples of stage IV melanoma and prostate cancer patients, respectively [25, 35]. Both studies confirmed AMPase activity to CD73 by performing tests with inhibitors, proposing this enzyme activity as a possible plasma biomarker to the cancer types evaluated [25, 38]. Besides, another study demonstrated the expression of CD73 on breast cancer cells and tumor-infiltrating leukocytes [39], reinforcing the idea that this enzyme is responsible for the increased adenosine levels in cancer patients.

In another scenario, when we compared the AMP hydrolysis at the moment of diagnosis with samples collected after 6 months of treatment, we observed a significant reduction in all treatment groups: surgery, chemotherapy, radiotherapy, and hormone therapy. The important reduction of AMPase activity in patients after treatment could indicate CD73 as a marker to treatment

effectiveness. Our study did not show significant alterations in the activity of CD39 enzyme, once the ATP and ADP hydrolyses were similar in the BC patients before and after treatment. Corroborating to our data, Bastid et al. [32] did not find significant alterations in CD39 activity on breast cancer cell lines. Araújo et al. [40] analyzed CD39 and CD73 activity on breast cancer patients' platelets with and without treatment intervention, between 20 and 85 years old. Results showed nucleotide hydrolysis alteration on these patients' samples, indicating that these molecules metabolism changes may interfere, also, on platelet activation.

This could be associated with CD73 expression and activity and a worse prognosis, once higher AMPase activity is present on plasma patients with more aggressive tumors. These results are in accordance with Morello et al. [37], who demonstrated worse prognosis in blood samples of stage IV melanoma patients that had higher CD73 activity. Loi et al. [21] also showed high levels of CD73 expression on TNBC patients' lymphocytes with bad prognosis and anthracycline resistance. Nevertheless, the association of CD73 with long-term survival is still a matter of debate, may be due to the strong heterogeneity of breast cancers [41], and a study by Supernat et al. [42] relates high levels of CD73 to better overall survival in breast cancer stages I to III.

In this study we also observed a significative decrease in the ADP hydrolysis in patients without metastasis when comparing to metastatic patients. Other studies have demonstrated the involvement of CD39 in the metastatic process [14]. Künzli et al. [43] have shown that high levels of CD39 expression correspond to an increase of liver metastasis in a mouse model of colorectal metastases. Another study found lower concentrations of ADP and ATP in the bronchoalveolar lavage (BALF) of patients with nonsmall-cell lung cancer when compared to chronic obstructive pulmonary disease [44].

It is important to consider that nucleotide hydrolysis was carried out at the moment of diagnosis and after 6 months of treatment, which limits the observation of the disease progression. Besides, due to its prospective study design, a small number of patients was enrolled.

In conclusion, our data showed that AMP hydrolysis was reduced in blood plasma of elderly patients after six months of treatment with chemotherapy, radiotherapy, hormone therapy, or surgery. This study reinforces the potential role of CD73 enzyme as a biomarker for breast cancer treatment response.

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Author contributions F.B.M, D.R, and A.R.C participated in the research design. F.V.G, A.R.C, J.B.S, R.O.M, B.Z.M, C.A.M, and L.R conducted the experiments. PE, A.P.F.L, and A.R.C performed data analysis. F.V.G, F.B.M, A.R.C, L.R, and wrote or contributed to the writing of the manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was carried out following the Declaration of Helsinki, and all subjects signed an Informed Consent Form previously approved by the Local Ethics Committee. The study was approved by the Ethical Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (CAAE: 65653717.0.0000.5336).

Informed consent Informed consent was obtained from all individual participants included in this study.

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