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ORIGINAL ARTICLE

Hydrolysis of ATP, ADP, and AMP is increased in blood plasma of prostate cancer patients

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Abstract

Prostate cancer is among the major malignancies that affect men around the world. Adenine nucleotides are important signaling molecules that mediate innumerous biological functions in pathophysiological conditions, including cancer. These molecules are degraded by several ectoenzymes named ectonucleotidases that produce adenosine in the extracellular medium. Some of these ecto-enzymes can be found in soluble in the blood stream. Thus, the present study aimed to evaluate the hydrolysis of adenine nucleotides (ATP, ADP, and AMP) in the plasma blood of patients with prostate cancer. Peripheral blood samples were collected, and questionnaires were filled based on the clinical data of the medical records. The nucleotide hydrolysis was performed by Malachite Green method using ATP, ADP, and AMP as substrates. Plasma from prostate cancer patients presented an elevated hydrolysis of all nucleotides evaluated when compared to healthy individuals. NTPDase inhibitor (ARL67156) and the alkaline phosphatase inhibitor (levamisole) did not alter ATP hydrolysis. However, AMP hydrolysis was reduced by the CD73 inhibitor, APCP, and by levamisole, suggesting the action of a soluble form of CD73 and alkaline phosphatase. On microvesicles, it was observed that there was a low expression and activity of CD39 and almost absent of CD73. The correlation of ATP, ADP, and AMP hydrolysis with clinic pathological data demonstrated that patients who received radiotherapy showed a higher AMP hydrolysis than those who did not, and patients with lower clinical stage (CS-IIA) presented an elevated ATP hydrolysis when compared to those with more advanced clinical stages (CS-IIB and CS-III). Patients of all clinical stages presented an elevated AMPase activity. Therefore, we can suggest that the nucleotide hydrolysis might be attributed to soluble ecto-enzymes present in the plasma, which, in a coordinate manner, produce adenosine in the blood stream, favoring prostate cancer progression.

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Keywords Prostate cancer · ATP · ADP · AMP · Hydrolysis · Plasma blood

Introduction

Prostate cancer (PC) is one of the most common malignancies that affect men. In 2012, there were registered 1.1 million of cases [1, 2] and PC is considered one of the main causes of death among men in the world [3]. In 2014, there were about 3 million men with prostate cancer living in the United States [4]. In Brazil, it is estimated that in the biennium 2018–2019, 600 thousand new cancer cases per year will be diagnosed. From these, approximately 68 thousand will be new cases of PC [5]. It is known that between 65 and 75% of PC patients evolved to bone metastasis, and early diagnosis and treatment can prevent such events, tending to prolong survival rate [6]. Prostatic cancer main risk factor is related to longevity, being rare in men younger than 50 years and common among men over 65, concentrating approximately 65% of the diagnosis in this age group [3].

Nowadays, the tracking methods used to determine tumor degree in PC include rectal touch (RT) and evaluation of prostatic serum antigen (PSA) levels, which are considered as biomarkers used for early detection and follow-up of the disease [7, 8]. In addition, digital rectal examination and core needle biopsy determine the tumor grade that is named as Gleason score [9, 10]. After the diagnosis, the tumor needs to be staged in accordance with the 8th edition of the American Joint Committee on Cancer (AJCC—8th) and grouped as its clinical stage (CS) [2, 11]. The main treatment used to early stages of PC recommends radical prostatectomy or transurethral resection followed by radiotherapy plus androgenic suppression. The prognosis is good, with 80% until 5 years of survival [12, 13].

The tumor microenvironment (TME) presents great relevance to control cancer development and suppression of the tumor immune response [14]. Among the uncountable molecules that are in TME, we can highlight the nucleotide ATP (adenosine triphosphate) and the nucleoside adenosine (ADO), which are present in the hypoxic environment of solid tumors [15]. Both are extracellular molecules that promote important functions related to tumor progression. ATP is involved in the initial tumor establishment to recruit immune cells to the tumor host. It is released to the extracellular medium by cell death and induces secretion of IL-1β and IL-18 by the dendritic cells. These cytokines promote the activation of NK cells, T-cells, and macrophages, generating an inflammatory environment. On the other hand, ADO, which is a product of ATP hydrolysis, suppresses the antitumor immune system and subsequently protects the tumor mass favoring its progression [16].

Two main enzymes are responsible in the control of the extracellular ATP and ADO levels—NTPDase1/CD39 (CD39) and ecto-5′-NT/CD73 (CD73), respectively. Both are extracellular enzymes linked to the cell membrane where CD39 promotes ATP and ADP hydrolysis, while CD73 hydrolyzes AMP and, in an organized way, produces ADO [15–17]. It was described that these ectoenzymes might be released in a soluble form associated to exosomes. These microvesicles (MVs) were found in supernatant of cancer cell culture and in human blood samples [18–20]. It is very well established that CD39 and CD73 promote the tumor mass progression in different kinds of cancer [17] and that they act by modulating the immune system to ensure tumor progression [16]. Previous studies demonstrated a high CD73 activity in blood serum of patients with head and neck cancer [21] and with intracranial neoplasias [22]. Clayton and collaborators have shown that the supernatant of different kinds of tumor cells secreted exosomes with CD39 and CD73 activity, which modulated the isolated T-cells [19]. In addition, samples of platelet-rich plasma of breast cancer patients present ATP, ADP, and AMP hydrolysis, which was attributed to CD39 and CD73 enzymes [23]. Other studies show the involvement of ATP and ADO, as well as CD39 and CD73, in PC cell lines [17]; however, a few studies demonstrate the action of these enzymes in patients' blood samples.

Currently, there is no single recommendation to early PC detection. As mentioned previously, the incidence of PC is elevated and the late diagnosis increases the relapse, prolongs the time of illness, and reduces patient's quality of life. Therefore, the search for new biomarkers and tools that would facilitate faster diagnosis, reducing the time of treatment, is very important. In addition, knowing that CD39 and CD73 can regulate in a positive manner the tumor progression, mainly through the modulation of the immune system, this study aims to evaluate ATP, ADP, and AMP hydrolysis, which are substrates of these enzymes in blood plasma samples of PC patients. Additionally, we intended to correlate these values with clinic pathological data, in order to find new biomarkers for PC detection.

Methods

Chemicals

Adenosine-5′triphosphate (ATP), adenosine-5′diphosphate (ADP), adenosine-5′monophosphate (AMP), α-β-methylene-ADP (APCP; inhibitor of CD73), ARL67156 (inhibitor of NTPDases), levamisole (inhibitor of alkaline phosphatase), Malachite Green, Coomassie Blue, and Tris–HCl were purchased from Sigma Aldrich.

Subjects

Twenty-nine (29) patients with prostate cancer, monitored at Centro de Oncologia e Hematologia de Cruz Alta (COHCA), participated in this transversal study. Seventeen (17) healthy volunteers, without altered liver function or the presence of chronic or acute disease, were included as controls in the study. All of them formalized their participation in the study through the adherence to the informed consent standards before blood collection. The PC diagnosis was determined by a pathologist through pathological analysis, and the patients were grouped by the clinical stage (CS), graduation in the Gleason score (GS), PSA, and specific treatments that were submitted. All clinical and pathological data of the patients studied are summarized in Table 1. The plasma blood collections were performed in patients at prostate cancer remission state after their respective treatments, with the exception of 11 patients who were receiving hormone therapy.

Data collection

Healthy and PC patients submitted for peripheral blood collected, in which blood (4 mL) was stored in plastic tubes with heparin. For blood plasma isolation, the samples were centrifuged at 4000 rpm for 12 min. At the sequence, the supernatant was stored at − 80 °C until enzymatic analysis.

Protein analysis

The quantification of protein levels in healthy and patients' plasma samples was performed by Coomassie Blue method, as described by Bradford et al. using bovine serum albumin as standard [24].

Nucleotide hydrolysis evaluation

Briefly, for determination of nucleotide hydrolysis in the blood plasma, the samples were incubated with Tris–HCl buffer 112.75 mM at final concentration and pH 8.0. The incubation protocol was performed as described by Moritz et al. The samples plus Tris–HCl buffer were pre-incubated for 10 min at 37 °C, and to start the reaction, nucleotides (ATP, ADP, and AMP) were added at 3 mM as final concentration. After 50 min, the reaction was stopped with trichloroacetic acid (TCA) 5% at final concentration and subsequently chilled on ice. Next, the samples were centrifuged at 14,000 rpm for 12 min [25]. In accordance with Chan et al., the amount of inorganic phosphate (Pi) was determined by Malachite Green method with minor modifications. A control of spontaneous hydrolysis was performed to exclude the non-enzymatic nucleotide hydrolysis. To this, blood plasma was added after the reactions had been stopped with TCA. In addition, a screening of specific enzymatic inhibitors was performed where ARL67156 was used to evaluate NTPDases at final concentration of 83 μM, APCP to evaluate CD73 at 30 μM, and levamisole to evaluate alkaline phosphatase at 1 mM at final concentration. The inhibitors were added with the samples and Tris–HCl buffer during the pre-incubation time and remained during all time of incubation (50 min). All experiments were performed in triplicate. The levels of nucleotide hydrolysis were determined as nmol Pi/min/mg protein.

Table 1 Clinical and pathological data of prostate cancer patients

Characteristics	*N*	%
Age (years)		
Mean	63.3	
Range	51 to 82	
PSA		
< 10	20	71.4
10 to 20	5	17.9
> 20	3	10.7
Gleason grade		
Low grade	24	82.7
High grade	5	17.3
Histology		
Adenocarcinoma	29	100
Clinical stage		
CS-I	1	3.4
CS-IIA	8	27.6
CS-IIB	14	48.3
CS-III	6	20.7
Surgery		
Yes	28	96.6
No	1	3.4
Transurethral resection of the prostate (TURP)		
Yes	17	58.6
No	12	41.4
Radical prostatectomy		
Yes	18	62.1
No	11	37.9
Radiotherapy		
Yes	13	55.2
No	16	4.8
Hormone therapy		
Yes	24	82.8
No	5	17.2

Extracellular microvesicle isolation

The blood plasma samples were prepared for microvesicle (MV) isolation as described by Jiang et al. [20]. Briefly, the samples were centrifuged at 10,000 ×*g* for 30 min at 4 °C. At the sequence, the supernatants were again centrifuged at

10,000 ×*g* at 4 °C for 90 min. Pellets containing the microvesicles were then suspended into the PBS buffer at pH 7.4 and used for flow cytometry and nucleotide hydrolysis by HPLC analysis.

Flow cytometry

MVs were analyzed by flow cytometry as described by Suárez et al. [26]. Briefly, to evaluate the expression of CD39 and CD73 on the microvesicle membrane, isolated microvesicles were incubated with aldehyde/sulfate-latex beads (ø = 4 μm Invitrogen, Carlsbad, CA) in 1 mL of blocking buffer overnight on rotation. Bead-coupled MVs were then centrifuged at 2000 ×*g* for 20 min. The pellets were, then, washed with 1 mL of blocking buffer and centrifuged at 2000 ×*g*. Samples were suspended in blocking buffer plus primary antibodies: APC mouse antihuman CD39 1:20 (BD Biosciences, San Jose, CA, USA), PE mouse antihuman CD73 1:20 (BD Biosciences, San Jose, CA, USA), and Annexin-V/FITC 1:20 (BD Biosciences, San Jose, CA, USA), and incubated for 30 min at 4 °C. Then, the samples were washed twice with 1 mL of blocking buffer and centrifuged at 2000 ×*g* for 10 min. After the last centrifugation, the samples were suspended in PBS pH 7.4 and analyzed by flow cytometry (BD Accuri™ flow cytometer and the C6 software, BD Biosciences, San Jose, CA, USA).

HPLC

Metabolites of ATP hydrolysis were evaluated by HPLC in MVs isolated from blood plasma of prostate cancer patients. MVs (10 μg of protein) plus incubation medium (2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM Hepes buffer, pH 7.4) were pre-incubated for 10 min at 37 °C, and to start the reaction, ATP was added at 25 μM as final concentration. After 30, 60, and 90 min, the reaction was stopped by cooling on ice. All samples

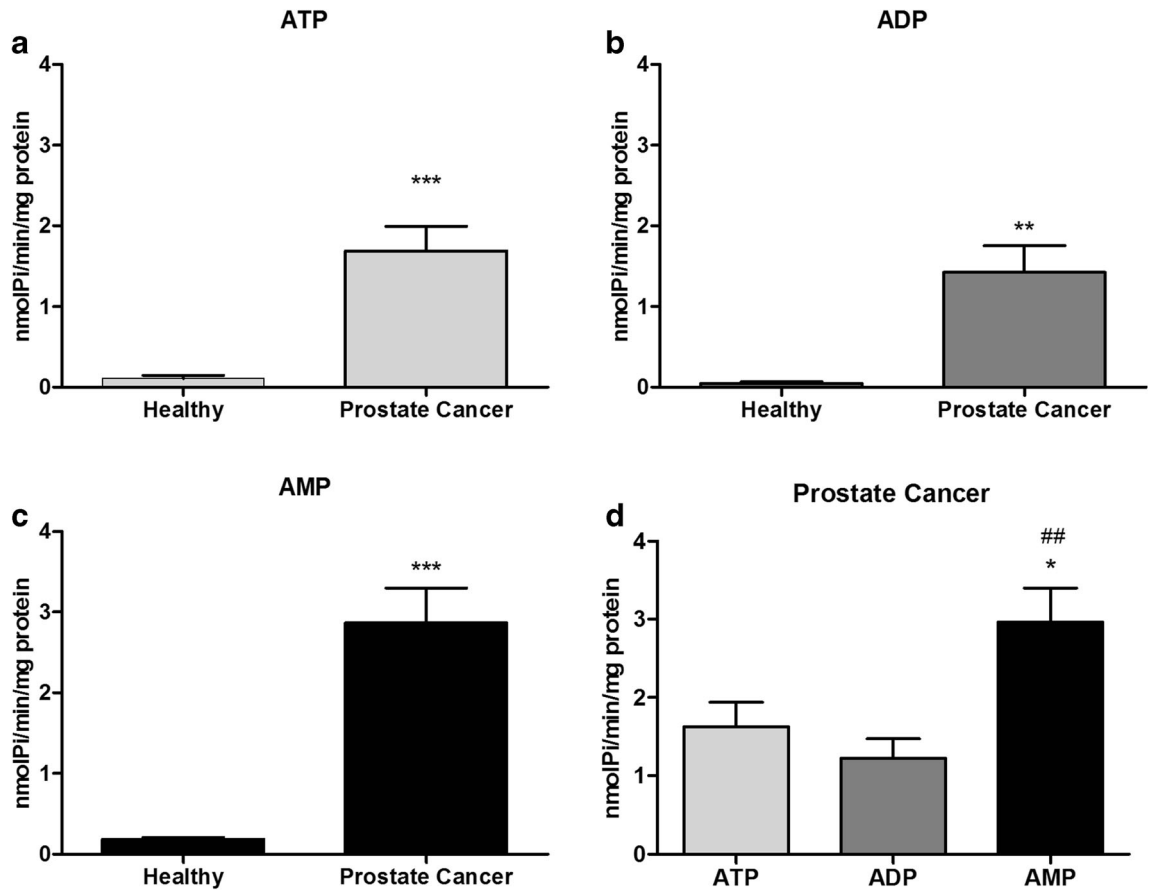

Fig. 1 Comparison of nucleotide hydrolysis between healthy individuals and PC patients. ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis was evaluated in blood plasma as described in the "Methods" section, and final values were represented as nmol Pi/min/mg protein. **d** Demonstrates the evaluation of nucleotide hydrolysis profile between PC patients. The experiments were performed in triplicate. Data were expressed as mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's post-hoc test. The * represents the significant difference in relation to ATP hydrolysis (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.001$), and # represents the significant difference in relation to ADP hydrolysis (## $p < 0.01$)

were centrifuged twice at 16,000 ×*g* for 30 min at 4 °C. The supernatant was collected, and 20 μL was applied to a reverse-phase HPLC (Shimadzu, Japan) using Ultra C18, 25 cm, 94.6 mm 95 lm (Restek-18, USA). The elution was carried out by applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium chloride, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30-min period (flow rate at 1.2 mL/min), according to a previously described method [25]. The amounts of purines were measured by absorption at 254 nm. The retention times of standards were used as parameters to identification and quantification of the samples. Purine concentrations are expressed in micromolar (μM).

Statistical analysis

Results were expressed as mean ± standard error (SEM). Statistical analyses were performed using one-way ANOVA followed by a post-hoc Tukey's test or two-way ANOVA followed by a post-hoc Bonferroni's test. The correlation of nucleotide hydrolysis and clinic pathological data was performed through the Wilcoxon–Mann–Whitney with $\alpha = 5\%$ and Kruskall–Wallis analysis, using the program R-3.3.0. The graphics were produced using GraphPad Prism 5.01 (San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

Results

In this study, we analyzed 29 patients with diagnosis of prostate adenocarcinoma. The median age of these patients was 63.3 years, and 20 of them (71.4%) presented PSA levels < 10. According to the Gleason scale (GS), 24 patients (82.7%) were diagnosed as low-grade and 5 (17.3%) presented high-grade GS. These evaluations generated the following clinical stage classification: 1 (3.4%) patient with CS-I; 8 (27.6%) with CS-IIA; 14 (48.3%) with CS-IIB; and 6 (20.7%) with CS-III. Twenty-eight (96.6%) patients underwent surgery, 24 (82.2%) received hormone therapy, and 16 (55.2%) received radiotherapy (Table 1).

We evaluated the nucleotide (ATP, ADP, and AMP) hydrolysis profile in blood plasma of PC patients in comparison to healthy individuals (Fig. 1a–c). The results demonstrated that PC patients presented elevated hydrolysis levels of all nucleotides tested (ATP 1.69 ± 0.31; ADP 1.42 ± 0.33; AMP 2.86 ± 0.43 nmol Pi/min/mg protein) when compared to healthy individuals (ATP 0.109 ± 0.037; ADP 0.046 ± 0.021; AMP 0.185 ± 0.023). When we compared the nucleotide hydrolysis activity profile in PC patients, we observed that there was a significant higher AMPase activity in comparison to the other groups evaluated (Fig. 1d). After, to identify the enzymes responsible for the nucleotide hydrolysis in the plasma of PC patients, we performed an incubation assay at the presence of specific inhibitors. We used ARL67156 at final concentration of 83 μM to exclude NTPDases; APCP at final concentration of 30 μM to exclude CD73; and levamisole (LEV) at final concentration of 1 mM to exclude alkaline phosphatase. As presented in Fig. 2a, ARL67156 and LEV were not able to change ATP hydrolysis. On the other hand, APCP and LEV significantly reduced AMP hydrolysis, indicating the involvement of CD73 and alkaline phosphatase in the AMP hydrolysis (Fig. 2b).

In order to investigate the origin of the enzymes responsible for the nucleotide hydrolysis, we performed the isolation of MVs from plasma samples of healthy individuals and PC patients. In general, MVs from healthy individuals and PC patients present similar mean size

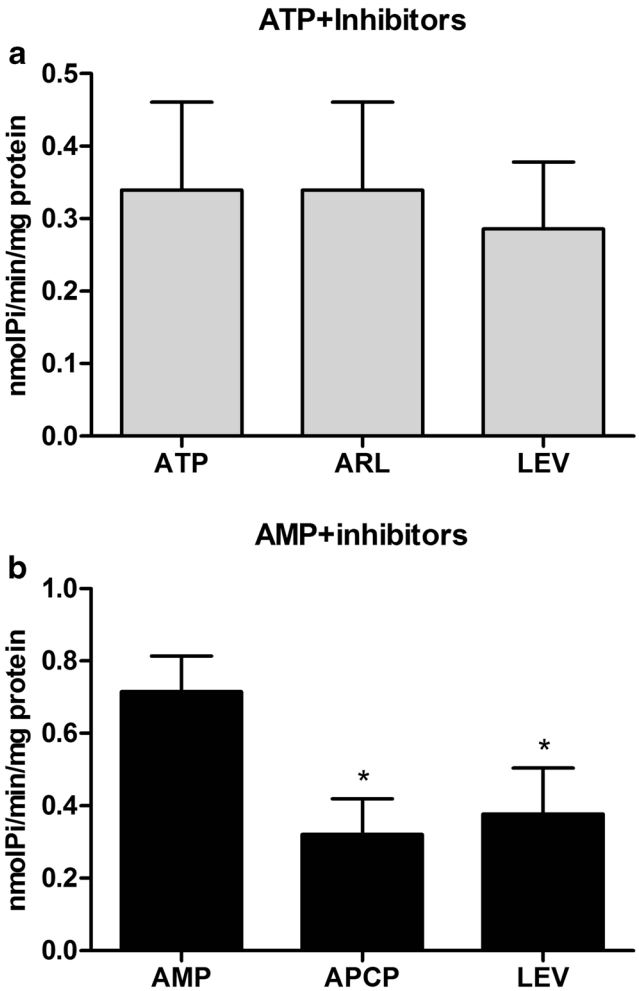

Fig. 2 Effect of specific inhibitors of NTPDases, CD73, and alkaline phosphatase on nucleotide hydrolysis. **a** Incubation of ATP plus ARL67153 (83 μM) and LEV (1 mM). **b** Incubation of AMP plus APCP (30 μM) and LEV (1 mM). The nucleotide hydrolysis was evaluated in the blood plasma as described in the "Methods" section, and final values were represented as nmol Pi/min/mg protein. The experiments were performed in duplicate with $n = 5$. Data were expressed as mean ± SEM and analyzed by one-way ANOVA, followed by Tukey's post-hoc test. * represents the significant difference in relation to AMP hydrolysis (* $p < 0.05$)

(429 ± 303.7 and 393.6 ± 207.7 nm, respectively). In addition, the expression of CD39 and CD73 was evaluated on the membrane of MVs by flow cytometry (Fig. 3). Double labeling with Annexin-V and CD39 antibody showed lower expression on MVs from PC patients than healthy individuals (Fig. 3b). Additionally, MVs from both healthy individuals and PC patients present a very low or absent CD73 expression, respectively (Fig. 3d).

Next, we analyzed the ATP hydrolysis profile of MVs by HPLC (Fig. 4). It was possible to observe that ATP hydrolysis was almost absent after 90 min of incubation (Fig. 4a, b), and the amounts of nucleotides metabolized were not different between healthy individuals and PC patients (Fig. 4c). It is worth to mention that low levels of nucleotide hydrolysis in MVs, evaluated by HPLC, are in agreement with low expression of CD39 and CD73 observed on MV membrane.

Subsequently, we correlated the results of nucleotide hydrolysis obtained in the plasma with the clinical and pathological data of PC patients. No significant differences were found in the correlation analysis of ATP, ADP, and AMP hydrolysis with PSA levels and Gleason score grade (Figs. 5 and 6). Interestingly, in comparison with the patient's clinical stage (CS), it was observed that patients CS-IIB and CSIII presented a significant lower ATPase activity than CS (IIA) (Fig. 7a). When we evaluated the nucleotide hydrolysis profile with each group of CS, it was possible to observe that in the CS-IIA, there were no significant differences among ATP, ADP, and AMP hydrolysis (Fig. 7b). However, for CS-IIB and CS-III, AMP hydrolysis was higher than ATP and ADP hydrolysis (Fig. 7b). Data presented herein lead us to suggest that patients with lower CS presented a higher ATP hydrolysis. On the other hand, AMPase activity was continuously elevated in PC patients, irrespective of their clinical stage. In addition, when we evaluated the nucleotide hydrolysis according to different kinds of treatment, we observed that the patients who did not receive radiotherapy presented

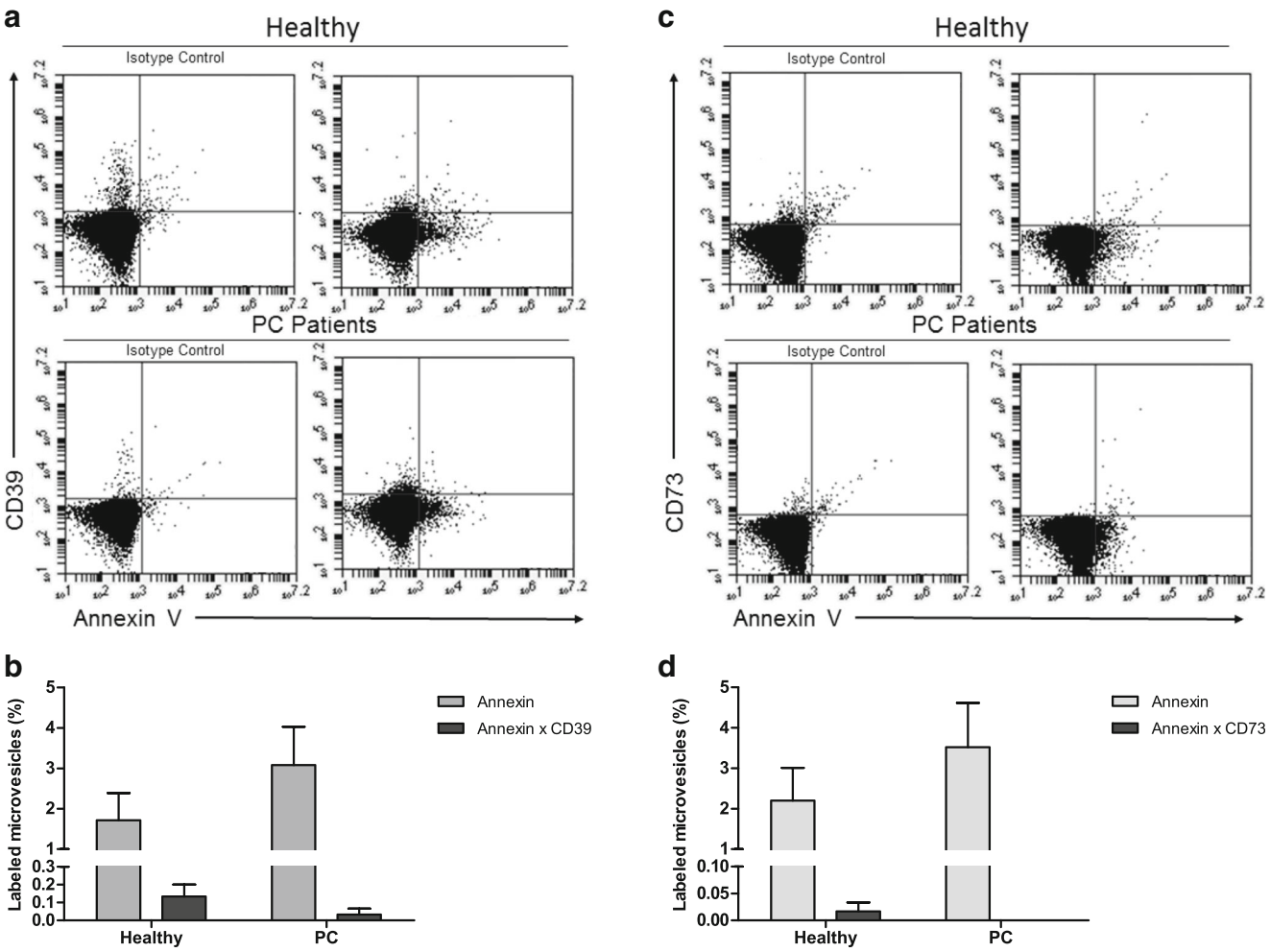

Fig. 3 The expression of CD39 and CD73 on MVs was evaluated by flow cytometry. Representative flow cytometry dot-plots showed the MVs labeled to CD39 (**a**) and CD73 (**c**). The percentage of labeling was demonstrated at (**b**) and (**d**), respectively. Annexin-V was used to identify phosphatidylserine that is present at the MV membrane [20]. Data were expressed as mean ± SEM ($n = 6$) and analyzed by two-way ANOVA, followed by Bonferroni's post-hoc test

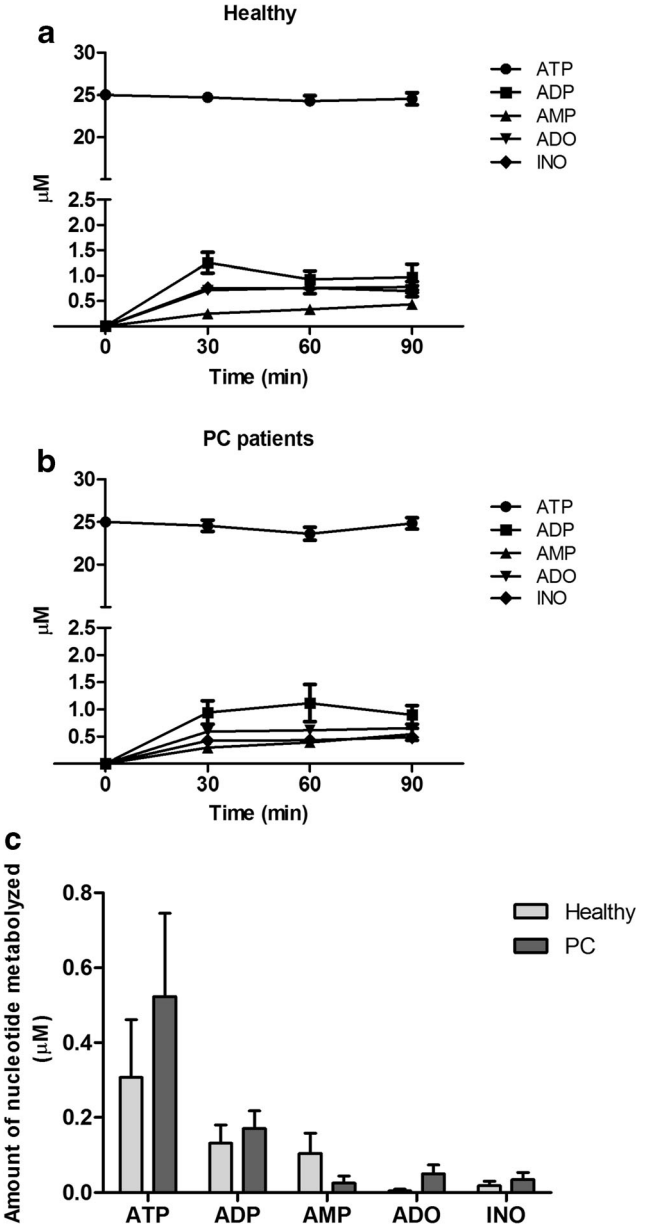

Fig. 4 HPLC analysis of ATP hydrolysis by intact MVs. Levels of nucleotides and nucleosides were evaluated after 30, 60, and 90 min of ATP incubation in MVs isolated from blood plasma of healthy individuals (**a**) and prostate cancer patients (**b**). The amount of nucleotide hydrolyzed was expressed in micromolar (μM) after 60 min of incubation (**c**). Data were expressed as mean ± SEM ($n = 9$) and analyzed by two-way ANOVA, followed by Bonferroni's post-hoc test

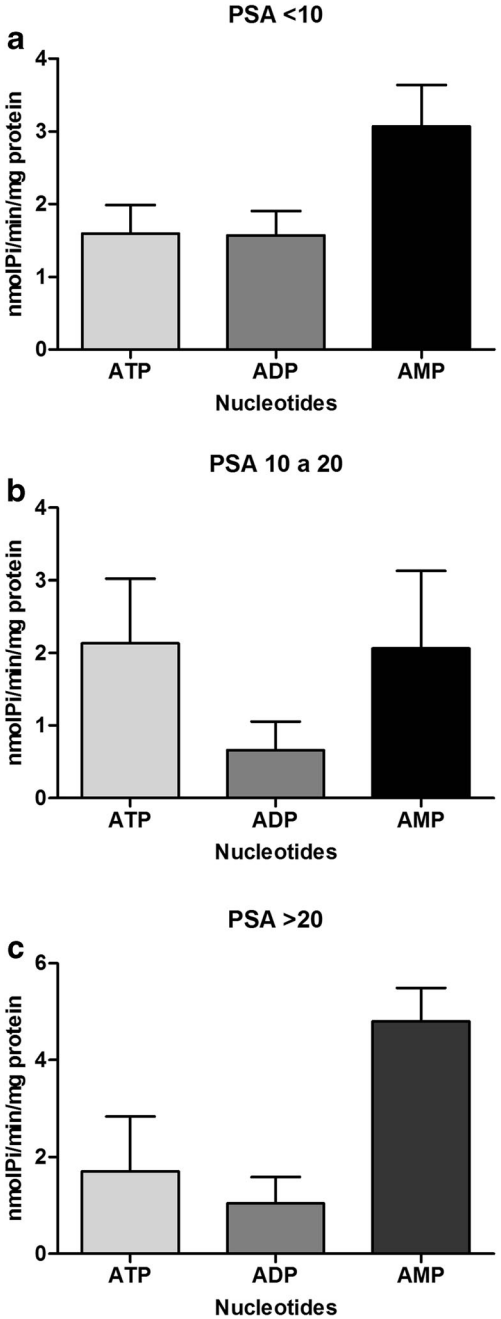

Fig. 5 Correlation data between nucleotide hydrolysis and PSA levels. The nucleotide hydrolysis was considered in accordance with the PSA levels. **a** PSA < 10; **b** PSA 10 to 20; **c** PSA > 20. Data were expressed as mean ± SEM and analyzed by Wilcoxon–Mann–Whitney with $\alpha = 5\%$ using Kruskall–Wallis analysis, followed by Dunn's post-hoc test

higher AMP hydrolysis than those patients who received this type of treatment (Table 2).

Discussion

Data from the International Agency for Research on Cancer (IARC) and World Health Organization (WHO) [3] showed that cancer is an unquestionable public health problem and there is need of innovation in strategies for tracking, treating, and following the patients [12, 13]. Among the countless alternatives that have been studied in the last years, the components of the purinergic system are highlighted [27]. It is well known that ATP and ADO act in an orchestrated manner on antitumor immune response, resulting in pro-tumor benefits [17]. Here, we demonstrated that samples of plasma of PC

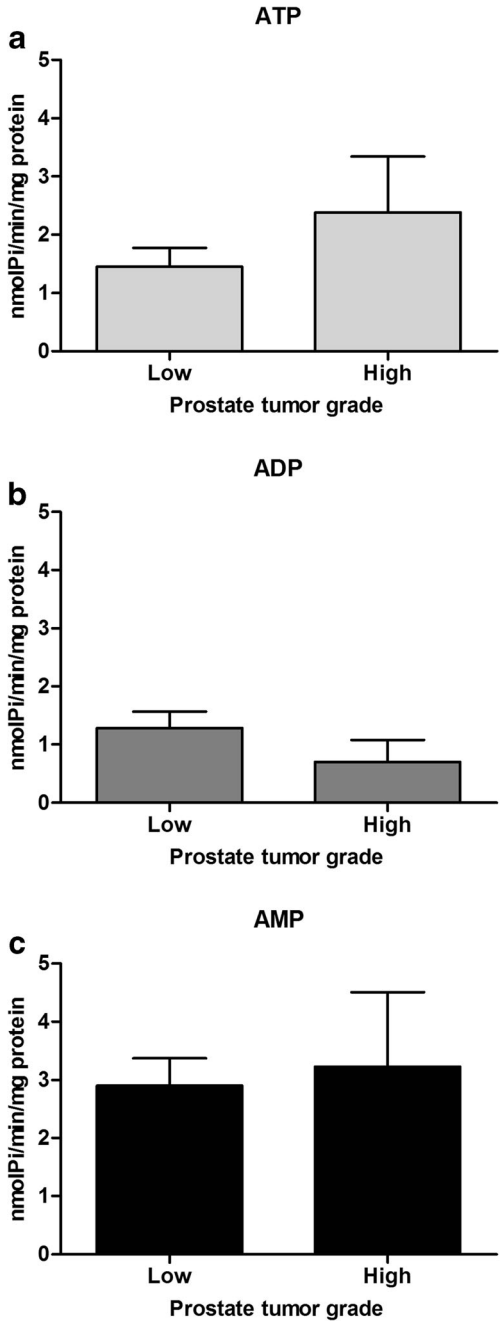

Fig. 6 Correlation data between nucleotide hydrolysis and Gleason score. The nucleotide hydrolysis. **a** ATP, **b** ADP, and **c** AMP were considered in accordance with the Gleason score. Patients were divided as low grade (Gleason 1 to 3) and high grade (Gleason 4 and 5). Data were expressed as mean ± SEM and analyzed by Wilcoxon–Mann–Whitney with $\alpha = 5\%$ using Kruskall–Wallis analysis, followed by Dunn's post-hoc test

patients presented higher ATP, ADP, and AMPase activities when compared to healthy individuals (Fig. 1), showing that this malignancy promotes alteration in nucleotide metabolism and produces modified levels of circulating adenosine. Also, we showed that ATPase activity was not altered by ARL67156, an inhibitor of NTPDases, and by LEV, a specific inhibitor of alkaline phosphatases, suggesting that other enzymes could also be acting. However, AMPase activity was reduced at the presence of APCP and LEV, the inhibitors of CD73 and alkaline phosphatase, respectively, indicating that these enzymes are responsible for the AMP hydrolysis in the plasma of PC patients.

In the tumor microenvironment, the extracellular ATP acts as a chemoattractant to immune cells [16]. Its extracellular levels can reach micromolar concentrations promoting tumor cell death [28]. In this way, ecto-ATPases, such as NTPDases, phosphodiesterases, and/or alkaline phosphatases, are required to maintain the tumor homeostasis by hydrolyzing ATP and ADP and, subsequently, AMP, which is substrate to alkaline phosphatase or to CD73 that is considered the main producer of extracellular ADO [29]. In the inflamed tumor microenvironment, ADO promotes immunosuppression, protecting the tumor cells [16]. Beyond that, this nucleoside can promote cancer cell proliferation, differentiation, and apoptosis of healthy stroma cells, favoring angiogenesis [30]. It is described that CD39 and CD73 work in a synchronize manner to promote a pro-tumoral niche [28]. In fact, CD39 expression is altered in different kinds of tumors when compared to the respective normal tissues, including prostate cancer [31]. The same is demonstrated regarding the enzyme CD73 that presents a high expression in solid tumors [30] and is related to high malignancy grade and tumor progression [16, 32]. In addition to its function in the tumor microenvironment, CD39 and CD73 are described to be secreted in the blood stream, associated to extracellular vesicles or in a soluble form [20, 33].

Furthermore, our results demonstrated a low expression of CD39 on MV membrane, which was accompanied by a low ATPase activity. Since LEV also did not change ATP hydrolysis, we excluded the involvement of the alkaline phosphatase. In fact, Battisti et al. [27], showed that PC patients have an elevated phosphodiesterase activity in comparison to healthy individuals and demonstrated that this activity was correlated with low Gleason grade. In this way, we can deduce that the ATPase activity evaluated in our work might be attributed also to a phosphodiesterase.

In this study, we also evaluated the expression of CD73 on MVs by flow cytometry. As mentioned before, MVs of PC patients showed a very low expression of CD73 and a low AMP metabolism. However, the total plasma samples of PC patients presented an elevated AMP hydrolysis, and this activity was modulated by APCP and LEV. So, we can conclude that the CD73 and phosphatase alkaline, in a soluble form, are responsible for the AMPase activity founded here. CD73 is well described as an important factor correlated with different kinds of cancer development, including prostate cancer [34]. In addition, AMPase activity in serum of patients with advanced melanoma has been attributed to

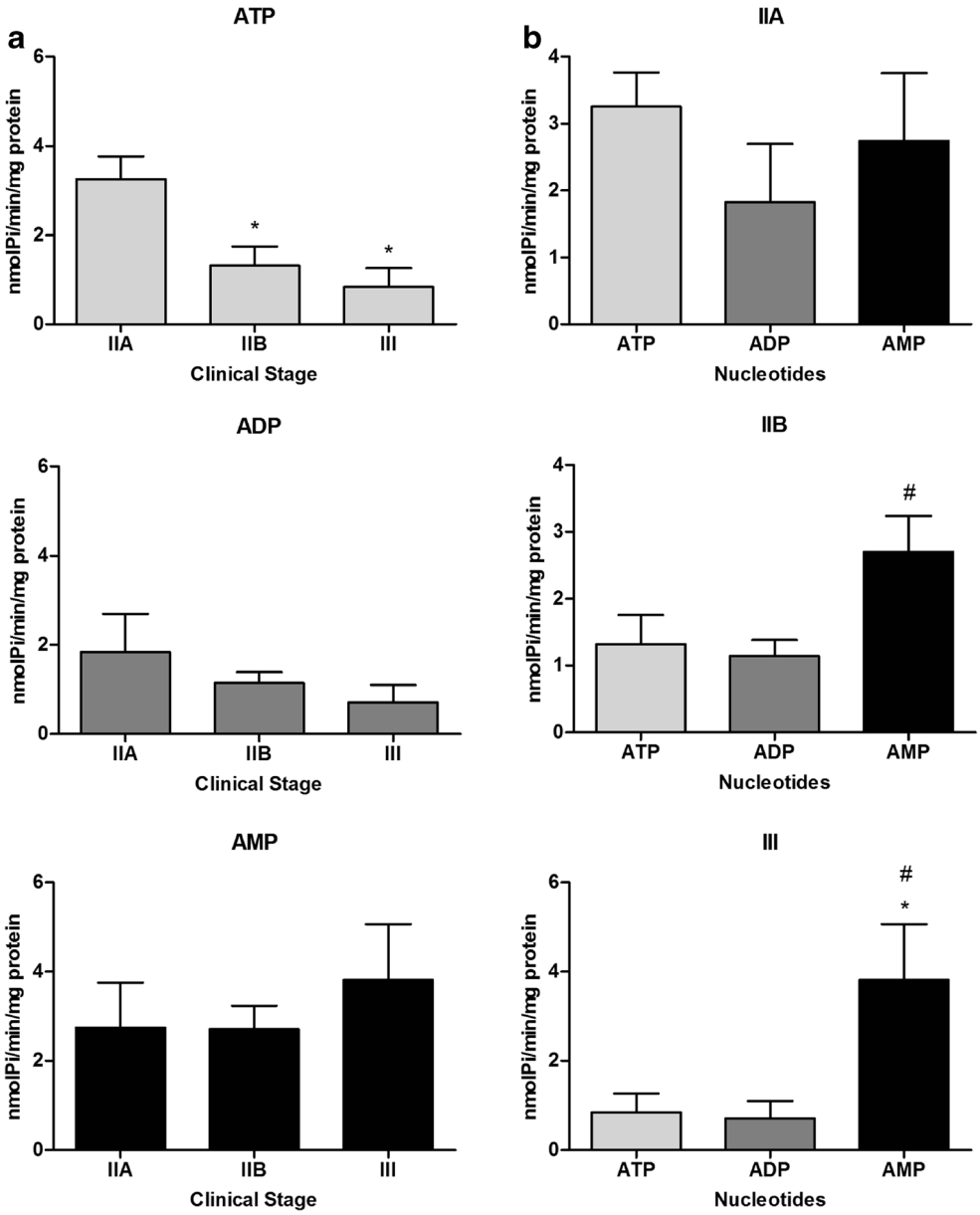

Fig. 7 Evaluation of nucleotide hydrolysis considering the clinical stage (CS) of PC patients. **a** Analysis of each nucleotide hydrolysis profile between the different CS (IIA, IIB, and III). The * represents the significant difference in relation to the CS-IIA group (* $p < 0.05$). **b** Profile of nucleotide hydrolysis in the side of each CS group. The * represents the significant difference in relation to ATP hydrolysis (* $p < 0.05$) and # represents the significant difference in relation to ADP hydrolysis (# $p < 0.05$). The experiments were performed in triplicate, and data were expressed as mean ± SEM and analyzed by Wilcoxon–Mann–Whitney with $\alpha = 5\%$ using Kruskall–Wallis analysis followed by Dunn's post-hoc test

soluble CD73, and it was suggested as a serum prognostic marker to this malignancy [35]. Additionally, elevated alkaline phosphatase activity in serum was related to worst prognostic to colorectal, gastric, and bladder cancer, and a recent study of meta-analysis suggested that the elevated serum activity of this enzyme can be considered a good prognostic factor to prostate cancer [36].

Regarding the results obtained, only the correlation of the nucleotide hydrolysis and CS showed significant results. In an interesting way, patients with CS-IIA presented higher ATPase activity than CS-IIB and CS-III patients. On the other hand, ADPase and AMPase activities did not show significant differences among the CS. When we evaluated the nucleotide hydrolysis profile for each group of CS, it was possible to see that in patients with CS-IIA, the hydrolysis of ATP, ADP, and AMP presented similar levels. However, in the CS-IIB and CS-III, we observed that AMP hydrolysis was higher than ATP and ADP. These hydrolysis profiles lead us to suggest that high ATPase activity observed in initial CS was involved in the initial tumor development, where hypoxic environment induces an elevated ATP secretion, which promotes the recruitment of the immune system to tumor host [16] and protecting the tumor mass of the cytotoxic effect promoted by elevated ATP concentrations [37]. In agreement with previous results, data presented in Table 2 pointed out that those patients without radiotherapy presented a higher AMPase activity than patients who received this treatment. These results could indicate that the immunosuppression is promoted by ADO, which is a product of AMP hydrolysis, evidenced in all CS groups and in patients who did not receive radiotherapy.

Table 2 Evaluation of nucleotide hydrolysis of prostate cancer patients who undergone different kinds of treatments

Treatment	Nucleotide hydrolysis (nmol Pi/min/mg PTN)		
	ATP	ADP	AMP
Transurethral resection of the prostate (TURP)			
Yes ($n = 17$)	2.07 ± 1.68	1.34 ± 1.39	2.65 ± 1.96
No ($n = 10$)	0.87 ± 1.30	1,0.3 ± 1.10	3.49 ± 2.77
Radical prostatectomy			
Yes ($n = 16$)	1.20 ± 1.61	1.42 ± 1.51	2.90 ± 2.62
No ($n = 11$)	2.25 ± 1.52	0.95 ± 0.84	3.06 ± 1.80
TURP + Prostatectomy			
Yes ($n = 7$)	1.49 ± 1.98	1.76 ± 1.95	2.15 ± 2.10
No ($n = 20$)	1.68 ± 1.55	1.04 ± 0.95	3.25 ± 2.32
Radiotherapy			
Yes ($n = 15$)	1.87 ± 1.76	1.24 ± 1.49	2.18 ± 1.79
No ($n = 12$)	1.32 ± 1.48	1.20 ± 1.02	3.94 ± 2.52[a]
Hormone therapy			
Yes ($n = 23$)	1.62 ± 1.68	1.28 ± 1.34	2.79 ± 1.14
No ($n = 4$)	1.67 ± 1.56	0.89 ± 0.93	3.94 ± 3.17

[a] Demonstrates statistical difference in relation to the group of patients who received radiotherapy. The values represent mean ± SD. These analyses were performed by SPSS statistical program, using one-way ANOVA, followed by Tukey's post-hoc test

Finally, we can suggest that the coordinated action of NTPDases, including CD39, phosphodiesterases, CD73, and alkaline phosphatase, observed in plasma of PC patients studied, could modulate the immune system [30] and, thus, assure a favorable environment to PC progression. Additional experiments are necessary to corroborate this hypothesis demonstrating the expression of these enzymes in the blood plasma and biopsies of PC patients.

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Compliance with ethical standards

Conflicts of interest notification The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethical Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (CAAE: 62424416.0.0000.5336) and by the Ethical Council of the Hospital São Vicente de Paulo-CACON, Cruz Alta, RS (2017-001).

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

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