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The signaling effects of ATP on melanoma-like skin cancer

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ABSTRACT

Melanoma is a type of skin cancer originated by the malignant transformation of melanocytes. Increasing incidence and mortality require efforts focused on studies and research about this cancer. Its microenvironment is rich in extracellular ATP, but there are no studies evaluating the ectonucleotidases and ATP effects on tumor-derived melanoma cells with known amounts of ATP. This way, the objective of this work was to evaluate the purinergic signaling in the pathophysiology of *in vivo* melanoma and the *in vitro* effects of ATP signaling. We found increased and effective extracellular ATP hydrolysis in platelets and a significant decrease of extracellular ATP levels and adenosine hydrolysis. In addition, we cultured PBMCs of melanoma patients and used ATP salt with specific concentrations to evaluate its signaling effects. The enzymatic activity analysis revealed that even with higher ATP doses cells metabolize adenine nucleotides less efficiently, and present low ATP, ADP and AMP hydrolytic activity in CM compared to CT cells. In summary, we showed for the first time important data about the purinergic signaling in the pathophysiology of melanoma and ATP signaling exercising immunosuppressive effects. Therefore, as already shown for other tumors, the purinergic signaling should be considered a potential target for melanoma management and treatment and could offer novel therapeutic prospects.

1. Introduction

Cutaneous melanoma (CM) or only melanoma is a neoplasm generated through the malignant transformation of epidermal melanocytes, characterized by insidious and fast progression, heterogenic evolution among patients, and significant resistance to diverse therapeutic strategies. Its incidence has been rising worldwide in the last 30 years and although it represents only 4–7% of skin cancers, this type of skin cancer causes approximately 80% of cancer deaths [1].

In recent years, there has been a growing interest in the potential of purinergic signaling for cancer because it plays an important role modulating the inflammatory and immune responses by extracellular biomolecules such as adenine nucleotides (adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) and their derived nucleoside adenosine [2–4]. Their effects depend on nucleotide concentration, expression pattern of purinergic receptors and enzymes, and general dynamics of their synthesis and degradation [5,6].

Levels of extracellular nucleotides and nucleosides are controlled by ecto-enzymes such as ecto-nucleoside triphosphate diphosphohydrolase (*E*-NTPDase; CD39; EC 3.6.1.5) and ecto-adenosine deaminase (E-ADA; EC 3.5.4.4), which are anchored in the cellular surface with their active site facing the extracellular environment [7]. Together, these enzymes constitute a system for the regulation of nucleotide-mediated signaling by controlling the rate, degradation, and formation of nucleosides [8]. In this context, due to this important physiological role, ectonucleotidases have been studied in different pathological and experimental conditions by our research group [9–15].

Among the extracellular purines, ATP is a key extracellular signaling molecule that participates in several physiological processes such as immune response, neurotransmission, vascular tonus, pain sensation, cell proliferation, differentiation, development, and death [10,16–20]. The tumor microenvironment is rich in extracellular ATP [21–23] and its effect depends on both ATP concentration and rate of degradation to adenosine by ecto-nucleotidases. Accumulation of this nucleotide can reflect an active signaling process with relevant

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pathophysiological implications [24].

Previous studies demonstrated that even after surgical excision, melanoma patients showed an increase in extracellular ATP levels [10]. In this case, ATP signaling increases tumor cells and immune cells interaction, causing an immune suppression. ATP effects can justify some of CM aggressiveness mechanisms. While these data indicate that the purinergic system is a strong pivot of melanomagenesis, the understanding of its effects on the pathophysiology of melanoma needs to be better elucidated, mainly in patients not undergoing treatment. Furthermore, increased ATP levels in the tumor microenvironment contribute to cellular and biochemical composition in different ways. Thus, in this work we demonstrated the involvement of signaling effects of ATP in the pathophysiology of melanoma patients (before any treatment intervention) and in cell culture.

2. Materials and methods

2.1. Chemicals and equipment

Chemicals were of the highest available purity and purchased from Sigma (St Louis, MO, USA) or Merck (Darmstadt, Germany) unless otherwise stated. Aqueous solutions were prepared using deionized, filtered water. The centrifuge used was the refrigerated Sigma 3 k-16® and the rotors were changed depending on the samples.

2.2. Patients and samples

Samples consisted of 20 patients with CM and 20 healthy subjects as the control (CT) group. All participants in the CT group were volunteers, free from pathologies that could compromise the research data, with similar gender and age to the CM patients. Patients with decompensated or ischemic heart disease, renal or hepatic impairment, decompensated diabetes, HIV-positive patients, patients with autoimmune diseases, pregnant women and drug users were excluded from the study. Ten milliliters of blood were obtained from each patient at the time of initial diagnosis and used for separation of platelets and lymphocytes, cell culture and biochemical analyses. The same procedure was carried out for the control group.

2.3. Experimental design

Patients with CM were selected according to the International Classification of Diseases (ICD) before surgical removal or any treatment. Control patients in the study were those who had no acute or chronic pathology or CM history in addition to having normal blood pressure and not undergoing any drug therapy. All subjects gave written informed consent to participate in the study. The Human Ethics Committee of Universidade Federal da Fronteira-Sul, Brazil, approved the protocol number 822.782. All analyses were developed in triplicates to ensure reliability of results.

2.4. Platelets and lymphocytes separation

Platelet-rich plasma was prepared by the method of Pilla and col. [25] modified by Lunkes and col. [26]. Total blood was collected with sodium citrate as anticoagulant and centrifuged at 1500 rpm for 10 min. After, the platelet-rich plasma was centrifuged at 5000 rpm for 30 min and washed with 3.5 mM HEPES buffer, pH 7.0 at least twice. The platelet pellets were suspended in HEPES buffer and protein was adjusted to 0.4–0.6 mg/mL.

The mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficoll-Histopaque density gradients as described by Bo¨yum [27]. Due to the fact that the methodology described above is employed for separating mononuclear cells, the study performed by Jaques et al. [28] demonstrated a high incidence of lymphocytes (95%) in these samples and a practically insignificant amount of monocytes. For this reason, we treat the samples as containing only lymphocytes.

2.5. Protein determination

Protein was measured by the method of Bradford [29] using bovine serum albumin as standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein, and this binding is accompanied by measuring the maximum absorbance of the solution at 595 nm.

2.6. E-NTPDase and E-5′-nucleotidase assays

Twenty microliters of platelet-rich plasma preparation (0.4–0.6 mg/mL protein) were added to the reaction mixture of E-NTPDase or E-5'nucleotidase and preincubated for 10 min at 37 °C, to a final volume of 200 μL. E-NTPDase activity was determined by the method of Lunkes and col. [26]. The reaction was started by the addition of ATP or ADP as substrate at a final concentration of 1.0 mM. E-5′-nucleotidase, determined by the method of Heymann and col. [30]. Phosphate released by ATP, ADP and AMP hydrolysis was measured using KH_2PO_4 as standard. Controls were prepared to correct for nonenzymatic hydrolysis, and all samples were analyzed in triplicate. Specific enzyme activities are reported as nmol Pi released/min/mg of protein.

2.7. Quantitative ATP determination

The quantitative ATP determination was developed using a commercial kit for bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin. The assay is based on luciferase's requirement for ATP in producing light – emission maximum ~560 nm at pH 7.8 [31]. This assay is extremely sensitive.

We combined the components of the reaction as follows in order to make a standard reaction solution and adjust the volumes according to particular requirements. Each reaction contained 1.25 μg/mL of firefly luciferase, 50 μM D-luciferin and 1 mM DTT in 1× Reaction Buffer. After a 15-min incubation, luminescence was measured. For determination of ATP levels in PBMCs, ATP concentration was normalized to cell number.

2.8. Adenosine deaminase (ADA) determination

ADA activity from platelets and lymphocytes was determined according to Giusti and Galanti [32] based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Briefly, 50 μL of cells reacted with 21 mmol/L of adenosine, pH 6.5 was incubated at 37 °C for 60 min. Afterwards, the reaction was stopped by adding a solution of 106.2 mM phenol and 167.8 mM sodium nitroprusside as well as a hypochlorite solution. The amount of ammonia produced was measured at 620 nm and the results were expressed in units per liter (U/L).

2.9. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells were isolated from fresh blood from healthy participants and melanoma patients within 1–2 h after collection using density medium centrifugation and Ficoll-Paque PLUS (GE Healthcare Bio-Science, Darmstadt, Germany). Blood samples were collected in EDTA medium. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS), carefully layered onto Ficoll-Paque PLUS, and centrifuged at 400 *g* for 40 min.

Isolated PBMCs were carefully collected (2–4 mL), resuspended in 15 mL PBS, and centrifuged at 500 *g* for 15 min. The supernatant was removed, and the pellet was resuspended in 15 mL PBS and centrifuged at 500 *g* for 10 min [GE Healthcare info]. The supernatant was removed,

and the pellet was resuspended in 1 mL RPMI medium (11.1 mM glucose, supplemented with 3% FBS, 50 units/ml penicillin, 50 g/mL streptomycin) [1].

PBMCs were cultured in RPMI-1640 (Biochrom AG, Berlin, Germany), supplemented with 100 U/mL penicillin (Gibco, USA) and 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany). Cells were adjusted to a concentration of 500.000 cells/mL/well on a 24-well plate. After settling for a few hours, different concentrations of ATP (0.05, 0.5, 5, 10 and 50 μM) were made in culture medium, added to the PBMCs for 24 and 48 h. All cell cultures were incubated at 37 °C in a water jacketed incubator and all cultures and analysis were developed in triplicates. We consider the maximum of 48 h a time for safe and reliable PBMC culture. This way, we analyzed our culture in 24 h (half the time) and in 48 h (maximum time).

2.10. MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water- soluble tetrazolium salt, which is converted to an insoluble purple formazan. Formazan crystals are impermeable to the cell membranes and therefore they accumulate in viable cells.

Cell viability was determined after 24 and 48 h by MTT 3-(4, 5-dimethy thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Briefly, cells were cultured in 96-well plates, and stained for 1 h at 37 °C with MTT reagent (10% concentration) and 5 mg/mL in phosphate-buffered saline (PBS, adjusted pH). Finally, mitochondrial dehydrogenases metabolized MTT to a purple formazan salt that was solubilized by the addition of 100 μL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 560 nm [33,34].

2.11. Statistical analysis

Statistical analyses were performed with GraphPad Prism 7 (Prism 7.03, GraphPad Software, San Diego, CA, USA). Values are presented as the means ± standard error of the mean (SEM), unless otherwise stated. Normality was tested by the Shapiro-Wilk test. The differences between the groups in relation to the studied variables were evaluated through the analysis of unpaired *t*-test and one-way ANOVA. The results were presented as mean and standard deviation. The differences in the probability of rejection of the null hypothesis as being < 5% ($p < .05$) were considered statistically significant. Statistical significance was defined for p values of *p < .05, **$p < .01$ and ****$p < .001$.

3. Results

3.1. Clinical characteristics

We evaluated the clinical characteristics of the studied groups through a documented interview with each participant. The characteristics of our study groups are summarized in Table 1. In relation to gender, an increased incidence of CM was observed in men (55%) more than women (45%). The average age was similar between CT and CM groups (46,7 ± 16,6 and 56,1 ± 12,8 years old).

For our CM group, some body areas were more affected: lower extremities (35%), upper extremities (25%), trunk (15%) and head/neck (25%). Regarding risk factors for CM development, sun exposure was higher in CM group (60%) than in control group (55%), and both groups showed elevated percentage for Fitzpatrick Phototype I/II (100% for CM and 95% for CT). The main risk factors for the development of CM result from the combination of constitutional/genetic and environmental factors: skin types I/II Fitzpatrick classification, presence of multiple melanocytic nevi, presence of atypical or dysplastic nevi, history of melanoma or other skin cancer and mutations in genes.

Table 1
Clinical characteristics of studied CT and CM patients. The data were obtained through a documented interview with each participant.

	CT (n = 20)	*CM (n = 20)*
Age	46,7 (± 16,6)	56,1 (± 12,8)
Male (%)	55	65
Female (%)	45	35
Tumor location		
Lower extremities	–	7
Upper extremities	–	5
Trunk	–	3
Head/neck	–	5
Sun exposure (%)	55	60
Fitzpatrick skin classification:		
I / II (%)	95	100
III / IV / V (%)	5	0

CT: Control; CM: Cutaneous Melanoma.

3.2. Alteration on purinergic enzymes activity

To evaluate the purinergic enzymes alterations in melanoma, we first analyzed the hydrolysis of nucleotides ATP and ADP in lymphocytes, and ATP, ADP and AMP in platelets of CM and CT groups (Fig. 1).

The hydrolysis of ATP, ADP and AMP in platelets was significantly increased in CM patients when compared to CT group. As for the hydrolysis of ATP and ADP in lymphocytes, no significant differences were shown between the evaluated groups. These results suggest that platelets of CM patients are activated and represent a key factor about cancer cell extravasation.

3.3. Quantitative ATP determination in serum

In order to confirm the increase of purinergic enzymes activity and ATP consumption in extracellular medium, the extracellular ATP concentration was measured in the serum of CM and CT patients using luciferase/ luciferin reagent. The concentration of extracellular ATP was significantly decreased in CM group when compared to control group (Fig. 2) as expected.

3.4. Adenosine deaminase (ADA) activity

High hydrolysis of extracellular nucleotides causes a formation of large amounts of adenosine. Therefore, we evaluated ADA activity in platelets and lymphocytes of CM and CT groups (Fig. 3). As can be observed, ADA activity in platelets was significantly decreased in CM when compared to CT, but different results were observed in lymphocytes. ADA activity in lymphocytes was significantly increased in CM when compared to CT. These results indicate that (possibly activated) adenosine hydrolysis was inhibited in platelets, possibly promoting immunosuppression, because of its pro-cancer roles. Adenosine hydrolysis was increased in lymphocytes, probably due to its being a defense mechanism against high adenosine concentrations.

3.5. PBMCs culture

Given that high ATP levels and its accumulation can lead to an active signaling process with relevant pathophysiological implications on melanoma development and complications, and based on previous results with patients' analyses [10], we cultured the peripheral blood mononuclear cells of CM and CT patients. We used five doses of adenosine 5′-triphosphate disodium salt hydrate (ATP – 0.05, 0.5, 5, 10 and 50 μM) to treat cells for 24 and 48 h. We used increasing doses of ATP salt to evaluate the time and dose-dependent signaling effects. Fig. 4 shows ATP, ADP and AMP hydrolysis in CM and CT cells after 24 h of treatment with ATP.

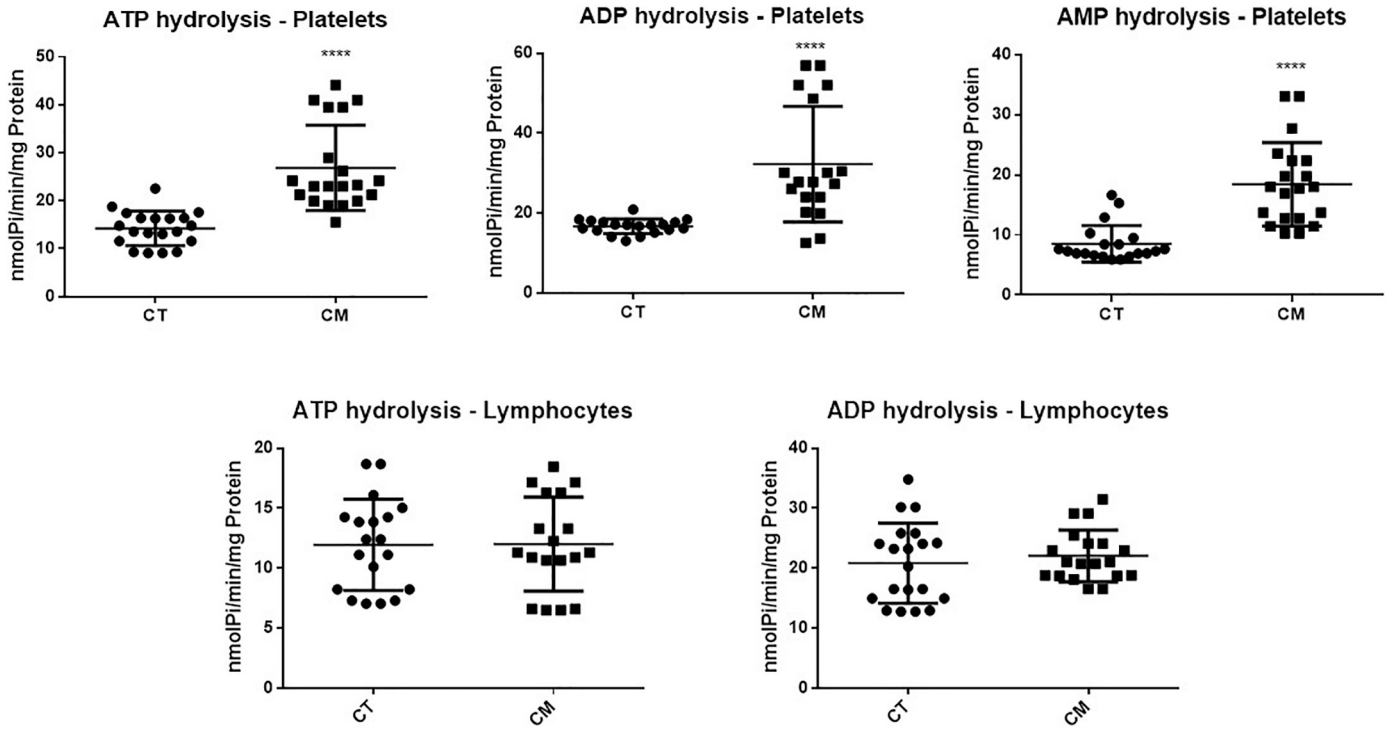

Fig. 1. ATP, ADP and AMP hydrolysis in platelets and lymphocytes in controls (CT) and melanoma patients (CM). E-NTPDase - hydrolyzing ATP and AMP and E-5'-Nucleotidase -hydrolyzing AMP. The assays were followed as described in materials. Data are presented as means ± SEM. ***Indicates a significant difference from the control group (Student's t test, $p < 0.0001$, $n = 20$).

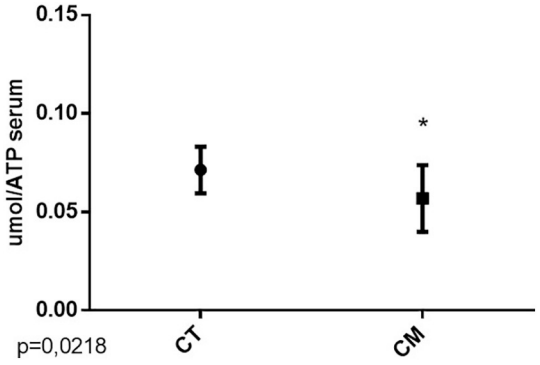

Fig. 2. Quantitative ATP determination in control (CT) and melanoma patients (CM). Extracellular ATP was determined as production of bioluminescence using a luciferin–luciferase reaction system (emission maximum ~560 nm at pH 7.8) through a commercial kit (Invitrogen®). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (Student's t test, $p < 0.05$, $n = 20$).

As can be observed in relation to ATP hydrolysis, when the cells were exposed to 0.05 μM of ATP, hydrolysis decreased only in CM cells when compared to CT cells. In ADP hydrolysis, no statistical difference was observed between groups. As for AMP hydrolysis, similarly to ATP, when the cells were exposed to 10 μM of ATP for 24 h, hydrolysis decreased significantly in CM cells when compared to CT cells.

Cells were similarly treated with ATP for 48 h (Fig. 5). ATP hydrolysis presented a significant decrease in CM group compared to CT group when cells were treated with 0.05 and 5 μM. In relation to ADP hydrolysis, no statistical difference was observed between the groups again, but AMP hydrolysis increased in CM when compared to CT cells when they were exposed to 10 μM of ATP.

Fig. 6 shows the extracellular ATP concentration in PBMCs culture. Similar to what was found in serum, the extracellular ATP concentration in PBMCs was significantly decreased on melanoma cells when compared to CT (both 24 and 48 h). The same occurred when CM cells were stimulated with 0,05 μM of ATP (for 24 and 48 h). Interestingly, in 5 μM and 50 μM concentrations of ATP treatment, the extracellular ATP concentration significantly increased in CM cells after 48 h of treatment.

In relation to ADA activity (Fig. 7), the results showed different changes in 24 and 48 h after ATP treatment. Fig. 7A shows the ADA activity after 24 h of treatment: we observed a significant decrease in CM cells in 0.5 μM, 5 μM, 10 μM and 50 μM groups when compared to CT cells. Interestingly, a similar result of ADA activity in platelets (Fig. 3) was observed: a decrease in ADA activity at different concentrations of ATP treatment.

Different responses to treatment with ATP during 48 h were observed in ADA activity (Fig. 7B). Although the activity in 0.05, 0.5 and 5 μM was significantly decreased in CM cells when compared to CT cells, higher doses of ATP (10 and 50 μM) showed increased ADA activity when compared to other groups.

3.6. Cellular viability assay

In order to demonstrate that the five doses of ATP (0.05, 0.5, 5, 10 and 50 μM) did not exert toxic effects in the cells, we performed the MTT assay to determine cell viability. In summary, ATP treatment did not alter cell viability.

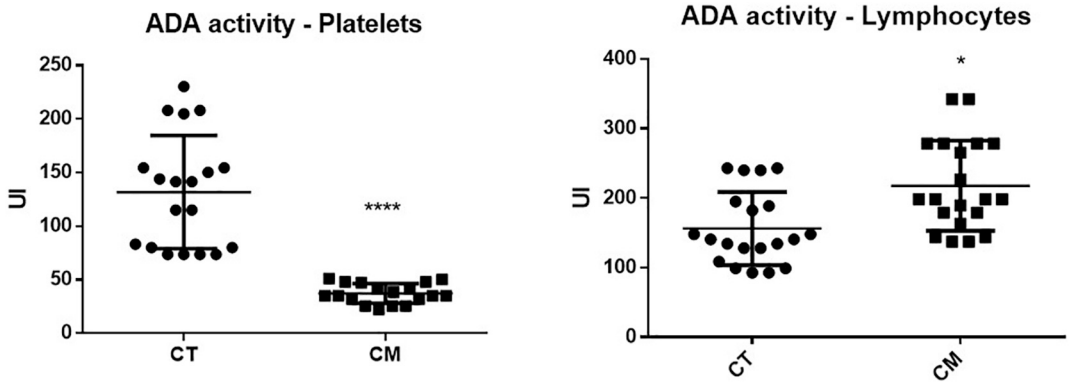

Fig. 3. ADA (Adenosine desaminase – hydrolyzing adenosine) in platelets and in lymphocytes of control (CT) and melanoma (CM) groups. The assay is based on the direct measurement of ammonia produced when adenosine deaminase acts in excess of adenosine. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol the ammonia per minute from adenosine at standard assay conditions. Data are presented as means ± SEM.*Indicates a significant difference from the control group (Student's t test, $p < 0.05$, $n = 20$). ***Indicates a significant difference from the control group (Student's t test, $p < 0.0001$, $n = 20$).

4. Discussion

This study clarifies the effects of ATP as an immunosuppressive signaling molecule in melanoma skin cancer. The extracellular ATP acts actively in tumor environments through its concentration and/or degradation rate for molecules with two or one phosphate – ADP and AMP [24]. By binding to their specific receptors, the purinergic signaling promotes regulatory T cell proliferation and immunosuppression [6,13,35,36].

We evaluated the activity of the purinergic system enzymes in platelets and an increased and effective extracellular ATP hydrolysis was observed, evidenced by a significant decrease on extracellular ATP levels and adenosine hydrolysis. To better understand this finding, for the first time we cultured the PBMCs of these patients and used ATP salt in these cells, given that previous studies demonstrated it is the cause of deleterious changes in melanoma, evidenced by the uncompensated inflammatory profile that it signals. [10,37–39]. The main and novel discovery of this study is that extracellular ATP as well as the ectonucleotidases activities have an important role in signaling the melanoma pathophysiology, with promising therapeutic prospects.

The increase of nucleotide hydrolysis in platelets (Fig. 1) and lower ATP levels in serum (Fig. 2) found in melanoma patients can be a

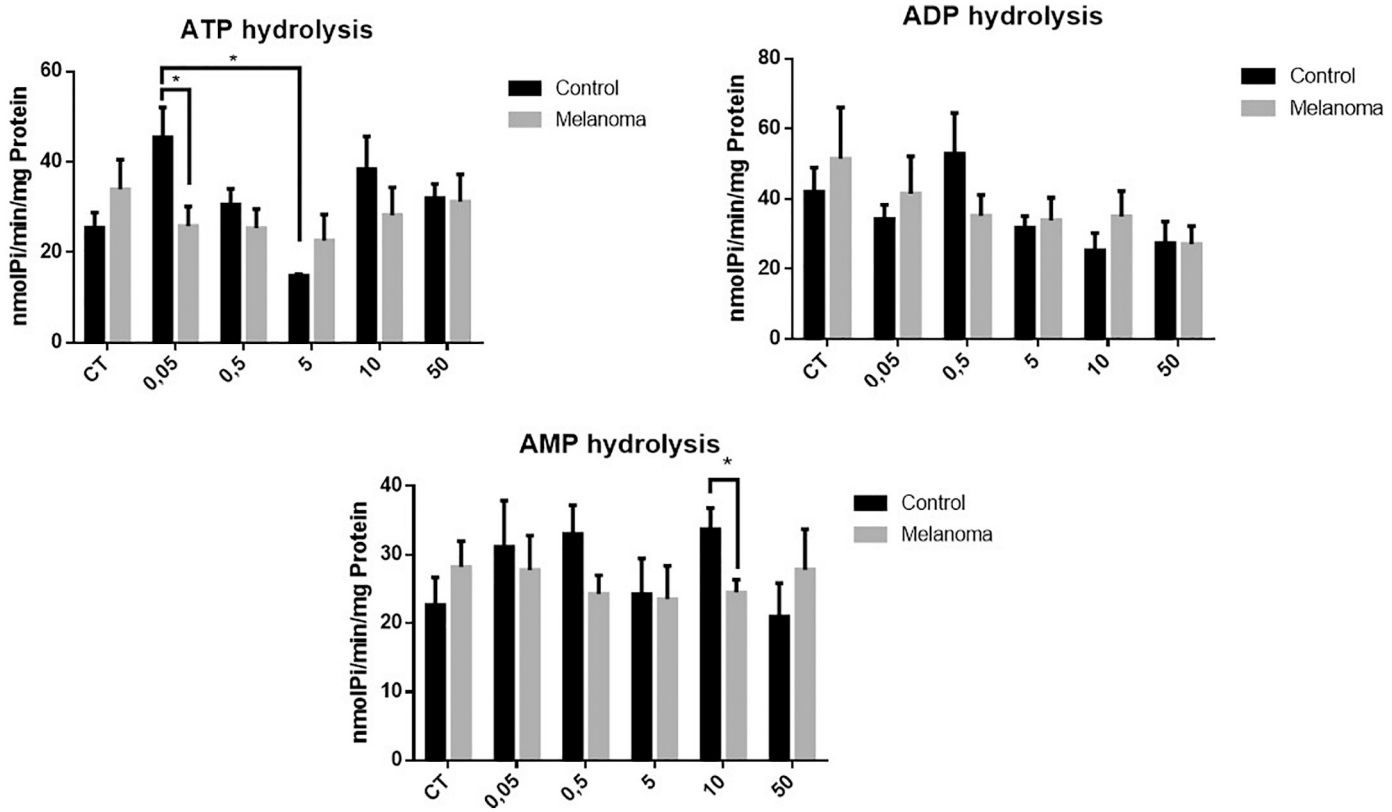

Fig. 4. Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for 24 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$).

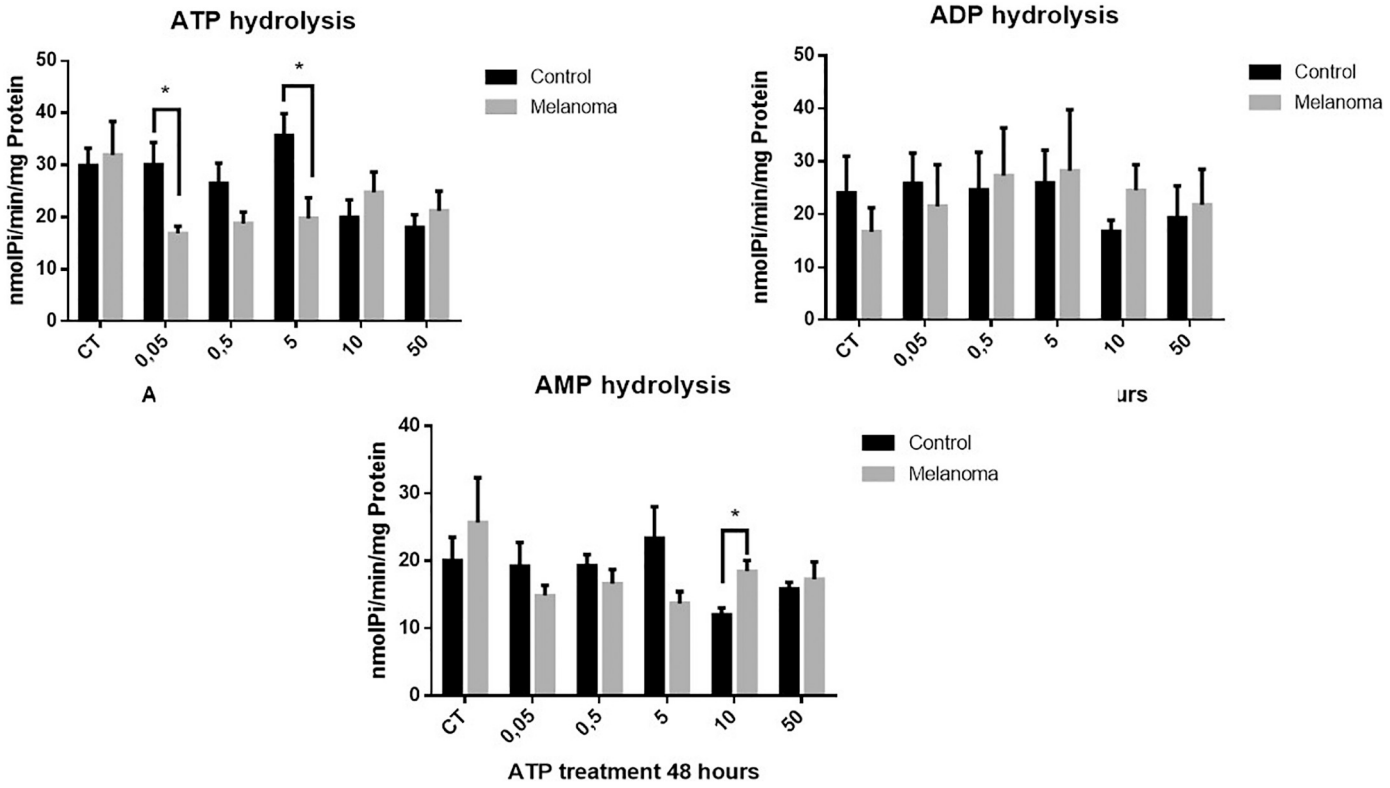

Fig. 5. Effects on ATP, ADP and AMP hydrolysis after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for 48 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients. The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$).

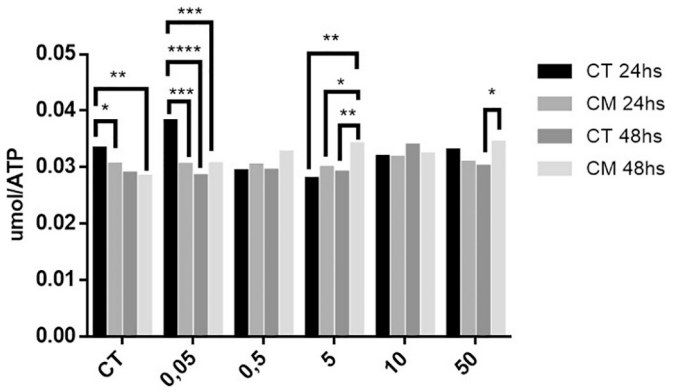

Fig. 6. ATP quantification in control (CT) and melanoma (CM) PBMCs cells after treatment with Adenosine Triphosphate (ATP) with 0,05, 0,5, 5, 10 and 50 μM) for 24 and 48 hours. The assay was followed as described in commercial kit (Invitrogen®). Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

possible mechanism of immunosuppression: high ATP hydrolysis could lead to the formation of large amounts of adenosine, therefore developing immunosuppression [24]. Interestingly, previous studies of our group with patients who underwent the surgical removal of melanoma showed a decrease of ATP and ADP hydrolysis in platelets and lymphocytes, as well as a decrease of AMP hydrolysis in platelets [10]. This difference between pre and post-treatment of melanoma patients can be a result of a chronic phase of purinergic signaling that can occur from days to months after treatment and lead to an extracellular ATP accumulation for extended periods [14,40], contributing to tissue damage and inflammation.

A study with breast cancer patients clearly demonstrated that hydrolysis of nucleotides by platelets is changed, with significant increase in NTPDase1 activity [41]. In lung cancer, an increase in E-5′-Nucleotidase activity was observed [9], and patients with thyroid cancer demonstrated a post-thyroidectomy increase in all purinergic system enzyme activities [42]. Furthermore, other types of cancer were studied [23,24,43–45] and displayed altered purinergic enzymes activity, what reinforces the importance of studying the mechanisms related to the purinergic system as well as diseases. Besides its immunosuppressive signaling effects, the increase in ATP hydrolysis can rise extracellular concentrations of adenosine, which is linked to tumor progression, chemotaxis, migration, invasion, and metastasis formation – one of the strongest immunosuppressive molecules [45]. Our results showed that in platelets, cells known for promoting metastasis dissemination [24], adenosine hydrolysis was decreased in CM patients, but in lymphocytes, known for immune functions [46], an increase in ADA activity was observed (Fig. 3). The high ADA activity seen in lymphocytes could be explained by the high concentrations of adenosine generated by the increased hydrolysis of ATP, ADP and AMP by platelets. Although the enzyme behavior is different in other diseases such as prostate cancer [24], the same augment in ADA lymphocytes was found in melanoma patients after surgical removal of the tumor, as well as sickle cell anemia and toxoplasmosis [47,48]. Therefore, we can suggest a stronger evidence of a possible immunosuppression caused by ATP signaling.

While immunosuppression caused by differences in ATP and adenosine levels acts in tumor progression [24,49], pharmacological modulation of nucleotide and nucleoside degrading enzymes in the tumor microenvironment can provide efficient means to revert this condition [49]. Thus, based on previous works [22,50–53], we cultured PBMCs from melanoma patients as well as control group and treated

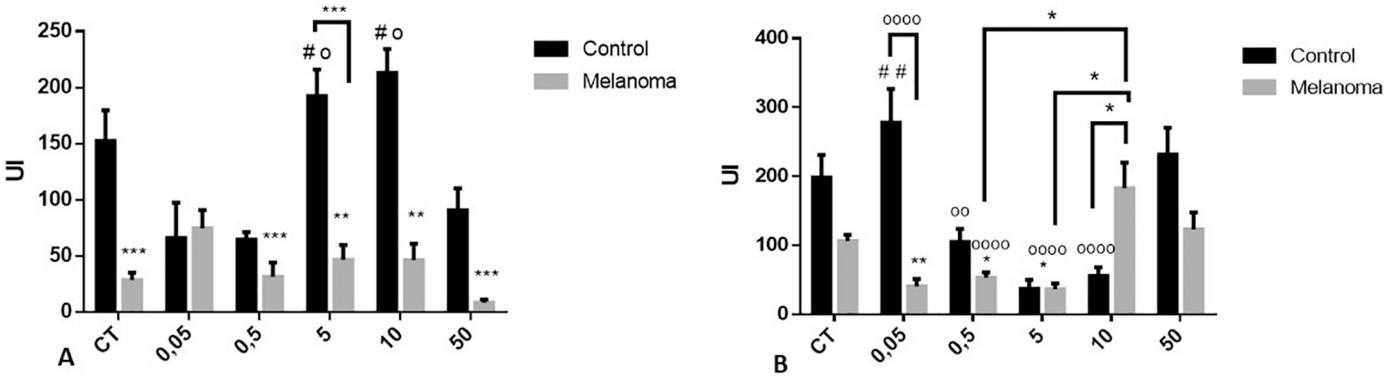

Fig. 7. Effects on ADA (Adenosine desaminase – hydrolyzing adenosine) activity after treatment with Adenosine Triphosphate (ATP) in different concentrations: 0,05, 0,5, 5, 10 and 50 μM for (A) 24 hours and (B) 48 hours, in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$); #Indicates a significant difference from the control melanoma group (One-Way ANOVA, $p < 0.05$, $n = 20$). ooIndicates a significant difference from the 0,05 CM group (One-Way ANOVA, $p < 0.001$, $n = 20$). ?Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

with different concentrations of ATP, in order to elucidate the possible signaling mechanism of this molecule through time and dose-dependent alterations in the purinergic system.

ATP concentrations were selected based on studies on cell lines and mice [10,22,23,51]. In general, it was observed that PBMCs had a similar behavior to the one found in platelets for both 24 (Fig. 4) and 48 h (Fig. 5). However, the enzymatic activity analysis revealed that even with higher ATP doses, cells metabolized adenine nucleotides less efficiently and presented low ATP, ADP and AMP hydrolytic activity in CM compared to CT cells. In human cervical cancer, lower ATP and ADP hydrolysis were observed in cell lines [54]. In this case, the signaling of ATP in these rates could not have any effects on the purinergic system enzymes activity.

It was possible to note that the ATP concentrations used caused diverse effects on cells, not often following a pattern. Therefore, we propose that PBMCs comprise a heterogeneous population that responds differently to extracellular ATP according to the level of P2X7 receptor present in the cell membrane and according the time of exposure [55]. Thus, we shed light on how melanoma cells respond to different extracellular ATP signaling effects.

Knowing that alterations on ATP concentrations lead to increased tumor growth and increased invasiveness of malignancies [56], we analyzed the extracellular ATP levels in cell culture (Fig. 6), and observed higher ATP metabolization in CM cells than CT cells. However, when adding ATP as a signaling molecule, there was an increase in ATP levels in melanoma cells (which can be observed at concentrations of 5 and 50 μM). This effect of high ATP levels in cancer cells was confirmed by a study with ATP infusion in patients with advanced non-small-cell lung cancer, where this molecule could reduce weight loss, increase muscle strength and improve the overall quality of life. These observations were confirmed by the incubation of whole human blood with low/medium ATP (100–500 mM), impairing LPS-stimulated IL-12 and IFNg secretion [57,58].

Studies with other cancer models tried to explain the signaling effects of this molecule. For example, when using breast cancer cells and bone migration of breast cancer cells [59]. However, in nasopharyngeal carcinoma cells the increase of extracellular ATP inhibited the growth and migration of this cell line [39]. This way, ATP released into the tumor microenvironment is considered a biochemical hallmark and could offer novel therapeutic prospects [24] through its signaling mechanism.

To understand the complete purinergic pathways, we performed the ADA activity of PBMCs culture for 24 and 48 h (Fig. 7) to investigate whether hydrolysis of extracellular adenosine formed by ATP degradation was altered after ATP stimuli. We found a different pattern of this enzyme activity depending on the dose and the time of exposure. We were able to note that ADA activity was decreased in cells of melanoma patients even in the control group (without ATP treatment). Except when the treatment dose was 10 μM for 48 h, all other CM groups showed lower ADA activity. These results are in accordance with

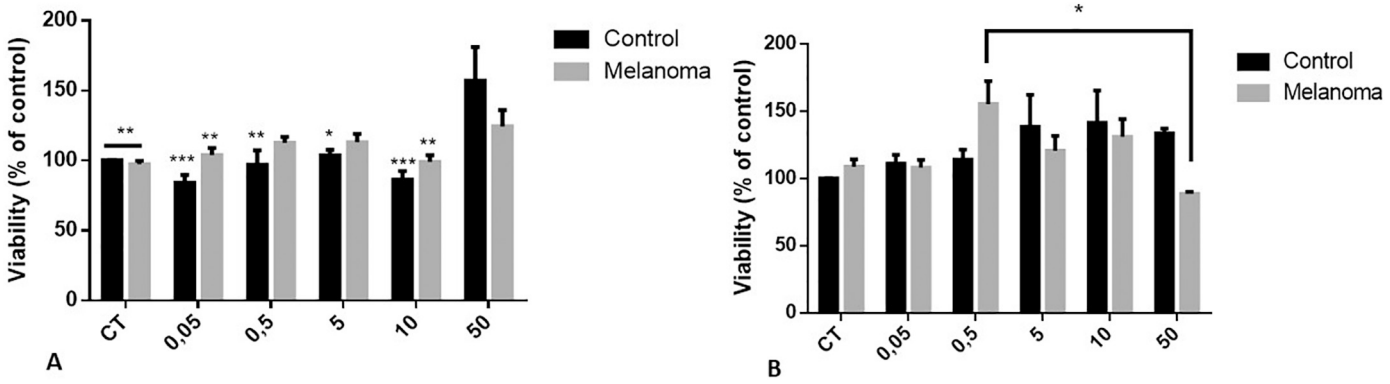

Fig. 8. Cell viability determined after 24 hours (A) and 48 hours (B) by MTT assay in mononuclear cells of human peripheral blood (PBMCs) of control (CT) and melanoma patients (CM). The assay was followed as described in materials. Data are presented as means ± SEM. *Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.05$, $n = 20$). **Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.001$, $n = 20$). ***Indicates a significant difference from the control group (One-Way ANOVA, $p < 0.0001$, $n = 20$).

the data obtained from the platelets of these patients, where an intense decrease of adenosine hydrolysis in the CM group was also observed in the CT group.

"The difference between time points means observed by different ATP concentrations in PBMCs could be explained by their P2 receptors binding and activation. Some reports on P2 receptors in cancer cells indicate that ATP in high concentrations might enhance cancer growth and contribute to malignancy [60]. Thus, our results showed that ATP presented different levels of activation of these receptors when cells to different salt concentrations.

Another possible explanation is that extracellular ATP is rapidly degraded to ADP, AMP and adenosine by ectonucleotidases, and these metabolites also modulate the cancer microenvironment by activation of purinergic or adenosinergic signaling. The enzymatic activity analysis revealed that our PBMCs metabolized adenine nucleotides according to different patterns when exposed to different concentrations of ATP and when compared to other tumoral cells [43,61–63]. In addition to all these effects, MTT assays showed that ATP treatment did not alter cell viability, so it was not toxic and did not kill the cells Fig. 8.

5. Conclusion

In summary, we showed for the first time important data about the purinergic signaling in the pathophysiology of *in vivo* melanoma and the effects of *in vitro* ATP signaling. Such understanding allows us to infer that ATP signaling exerts immunosuppressive effects and may modulate the purinergic signaling cascade in an upstream manner, thereby offering new avenues for drug therapies.

Conflicts of interest

None.

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