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Liliane Zimmermann de Oliveira

ATIVIDADE DAS ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DA ADENINA EM LEUCEMIA LINFOBLÁSTICA AGUDA B DERIVADA

Santa Maria, RS
2017

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração Análises Clínicas e Toxicológicas da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Ciências Farmacêuticas.**

Orientadora: Prof. Dra. Daniela Bitencourt Rosa Leal

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Aprovado em 01 de setembro de 2017:

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Santa Maria, RS
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DEDICATÓRIA

Ao meu filho Eduardo, símbolo
da perseverança que faz vibrar a vida.

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RESUMO

ATIVIDADE DAS ENZIMAS QUE DEGRADAM NUCLEOTÍDEOS E NUCLEOSÍDEO DA ADENINA EM LEUCEMIA LINFOBLÁSTICA AGUDA B DERIVADA

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A leucemia linfoblástica aguda de precursores B (LLA-B derivada) é uma neoplasia caracterizada pela proliferação clonal anômala de células precursoras linfoides B e pela predominância em pacientes pediátricos. A patogênese da LLA-B promove a desregulação das vias de controle de processos fisiológicos e imunológicos. Dentre os mediadores da modulação dos processos imunes, destacam-se os nucleotídeos ATP, ADP, AMP e o nucleosídeo adenosina, cujas concentrações extracelulares são controladas pela atividade das ectoenzimas E-NTPDase (CD39), E-5'-nucleotidase (CD73), ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPP) e ecto-adenosina desaminase (E-ADA). O objetivo deste estudo foi avaliar as atividades da E-NTPDase e E-ADA em linfócitos periféricos, quantificar a expressão de CD39 e CD73 em sangue periférico, determinar no soro as atividades da NTPDase, ADA e xantina oxidase (XO), a concentração de nucleotídeos e nucleosídeos da adenina e a concentração de citocinas, de forma a avaliar a resposta imune em pacientes com LLA-B. Avaliaram-se pacientes pediátricos portadores de LLA–B recém diagnosticados (D0), com 15 dias de tratamento (D15) e um grupo controle pediátrico. Trinta e dois pacientes D0, 14 pacientes D15 e 34 controles sadios (C) foram admitidos. As atividades das ectonucleotidases e das enzimas solúveis NTPDase e ADA foram determinadas em linfócitos periféricos isolados e no soro, respectivamente. Avaliaram-se as expressões de CD39 e CD73 em sangue total lisado e, citocinas no soro, por citometria de fluxo. Quantificaram-se nucleotídeos no soro por CLAE. Os resultados mostraram que, em linfócitos, a atividade da E-NTPDase (substrato ATP), não mostrou variação significativa entre os grupos, enquanto na E-NTPDase (substrato ADP) houve redução no grupo D15 e, na E-ADA, houve aumento em D0 e D15. No soro, as atividades da NTPDase e XO mostraram redução significativa no grupo D0, enquanto a ADA mostrou-se elevada. No grupo D15 houve aumento nas atividades de NTPDase e XO na comparação com o grupo D0. A expressão de CD39 estava reduzida e a de CD73, aumentada, nos blastos do grupo DO, quando comparados aos linfócitos do grupo D0. As dosagens séricas de citocinas diferiram significativamente entre os grupos D0 e controle para IL-6, IL-17 e IL-10. Mostraram-se elevados os níveis dos nucleosídeos adenosina, inosina e xantina, enquanto a hipoxantina estava reduzida. Os resultados refletem um estado inflamatório da BCP-ALL ao diagnóstico e uma possível modulação do sistema purinérgico e das citocinas em linfócitos neoplásicos, influindo no estado de imunossupressão e de alteração na resposta imune, e possibilitando a proliferação de células neoplásicas. Estudos complementares poderiam identificar características individuais da sinalização purinérgica nos pacientes com LLA-B como forma de contribuir na escolha de uma terapêutica mais específica.

Palavras-chave: LLA-B, NTPDase, ADA, CD39, CD73, Linfócitos.

ABSTRACT

ACTIVITY OF ENZYMES THAT DEGRADE ADENINE NUCLEOTIDES AND NUCLEOSIDE ON B-PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplasm characterized by an anomalous clonal proliferation of B lymphoid precursor cells and predominance in pediatric patients. BCP-ALL promotes the deregulation of physiological and immunological processes. Among the immune processes modulators, ATP, ADP, AMP and adenosine stand out. The extracellular concentrations of these nucleotides are regulated by the ectoenzymes E-NTPDase (CD39), E-5'-NT (CD73), and ecto-adenosine deaminase (E-ADA). This study aimed to evaluate the activities of E-NTPDase and E-ADA in peripheral lymphocytes, measure the expression of CD39 and CD73 in peripheral blood, evaluate the activities of NTPDase, ADA and XO, the levels of soluble nucleotides and nucleosides of adenine and the cytokines in the serum, in order to evaluate the immune response in BCP-ALL patients. Thirty-two patients were evaluated at diagnosis (D0), 14 patients 15 days after the beginning of the treatment (D15), and 34 healthy individuals in the control group (C). The activities of ectonucleotidases and soluble enzymes NTPDase and ADA were determined on isolated peripheral lymphocytes and serum, respectively. Flow cytometry method was used to evaluate the expressions of CD39 and CD73 in lysed whole blood and the levels of cytokines in the serum. The nucleotides and nucleosides levels were evaluated in serum by HPLC method. Concerning to the activity on lymphocytes, E-NTPDase (ADP substrate) was reduced in D15 group, while E-ADA activity was increased on D0 and on D15 when compared to the control group. The activities of NTPDase (ATP and ADP substrate) and XO in serum were decreased on D0, whereas ADA activity was significantly increased on D0 compared to the control group. D15 group showed increase on NTPDase and XO activities compared to D0 group. CD39 and CD73 expression levels were distinct according to the cell maturation degree. On D0, lymphoblasts showed lower CD39 and higher CD73 expressions in relation to lymphocytes from the same group. Serum purine levels of inosine and xanthine were significantly increased, whereas of hypoxanthine was decreased on D0. The cytokines IL-6, IL-17 and IL-10 levels were significantly increased on D0 when compared to control group. The results showed an inflammatory status of BCP-ALL at diagnosis, and a possible modulation of the purinergic signaling system and of cytokines on neoplasic lymphocytes, which may influence in the immunosuppression status and the changes in the immune response, making the neoplasic cell proliferation possible. Complementary studies may identify individual patient characteristics in the purinergic signalling, to assist in the choice of a more specific therapy.

Keywords: BCP-ALL, E-NTPDase, E-ADA, CD39, CD73, lymphocytes.

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LISTA DE ABREVIATURAS

Ado	Adenosina
ADP	Adenosina difosfato
AK	Adenilato quinase
AMP	Adenosina monofosfato
AR	Alto risco de recaída
ATP	Adenosina trifosfato
BMME	Microambiente da medula óssea
BR	Baixo risco de recaída
CF	Citometria de fluxo
CLAE	Cromatografia líquida de alta eficiência
DRM	Doença residual mínima
E-ADA	Ecto-adenosina desaminase
E-NPP	Ecto-nucleotídeo pirofosfatase/fosfodiesterase
E-NTPDase	Ecto-nucleotídeo trifosfato difosfoidrolase
E-5'-NT	Ecto-5'- nucleotidase
EROS	Espécies reativas do oxigênio
GBTLI	Grupo Brasileiro para Tratamento de Leucemia da Infância e Adolescêr
IL	Interleucina
IFN	Interferon
INCA	Instituto Nacional do Câncer
LLA	Leucemia Linfoblástica Aguda
LLA-B	Leucemia Linfoblástica Aguda B derivada
LLC	Leucemia Linfocítica Crônica
MDR	Resistência a múltiplas drogas
MDSC	Células supressoras de origem mieloide
MO	Medula óssea
NDPK	Nucleosídeo difosfoquinase
PAP	Polifosfato-AMP fosfotransferase
PNP	Purina nucleosídeo fosforilase
RL	Respondedor lento
RR	Respondedor rápido
SNC	Sistema nervoso central
SP	Sangue periférico
Th	Linfócito T helper
TME	Microambiente tumoral
TMO	Transplante de medula óssea
TNF-α	Fator de necrose tumoral α
Treg	Células T regulatórias
XO	Xantina oxidase

SUMÁRIO

1 INTRODUÇÃO

As neoplasias malignas têm sido objeto de estudo há muitas décadas. O crescimento desordenado das células faz com que invadam tecidos e órgãos, promovendo a formação de tumores que podem se espalhar para diversas partes do organismo. Os fatores desencadeantes podem ser externos, relacionados com o meio ambiente, ou internos, pré-determinados geneticamente e resultantes da capacidade orgânica individual de se defender das agressões externas (BRANDALISE; PINHEIRO; LEE, 2011; POMBO DE OLIVEIRA; KLUMB, 1998). Entre as neoplasias, as leucemias correspondem a cerca de 30% dos casos de câncer pediátrico e a 1% de adultos (PUI et al., 2003; RIBEIRO, 2001). A Leucemia linfoblástica aguda (LLA) tem se destacado no interesse de pesquisadores por ser a de maior incidência em crianças e apresentar decréscimo na mortalidade em decorrência da evolução terapêutica, chegando a beneficiar até 85% de pacientes com cinco anos de sobrevida livre de doença (INCA, 2016; MARTIN, MORGAN, HIJIYA, 2011; PUI, 2003; ZAGO, FALCÃO, PASQUINI, 2001).

As leucemias são neoplasias hematológicas de etiopatogênese heterogênea que afetam indivíduos de todas as faixas etárias. Mundialmente, respondem por cerca de dez por cento das neoplasias humanas, com prevalência discretamente maior em pacientes do sexo masculino. No Brasil, o Instituto Nacional do Câncer estimava para 2016 um risco de incidência de 5,63 casos novos a cada 100.000 homens e, de 4,38 a cada 100.000 mulheres (INCA, 2016).

Estas neoplasias decorrem da expansão clonal maligna de um precursor hematopoiético em determinada etapa da diferenciação celular. De um modo geral, se originam de um evento genético primário seguido de várias alterações secundárias que afetam os mecanismos celulares de controle da diferenciação e da proliferação (RIBEIRO, 2001; SWERDLOW, 2008).

Dentre as leucemias, a Leucemia Linfoblástica Aguda (LLA) envolve a proliferação clonal de células precursoras de linhagem linfoide e afeta pacientes adultos e pediátricos de ambos os sexos, embora seja predominante em crianças. A incidência mundial estimada é de 1-4,75/100.000 pessoas ano (SWERDLOW et al., 2008). Nas últimas décadas, estudos para caracterização de fatores prognósticos e de risco para esta patologia se desenvolveram enormemente, impactando em inúmeros avanços na terapêutica e crescimento dos índices de sobrevida livre de

doença.

O diagnóstico atual considera fatores etiológicos como idade, citomorfologia, leucometria, presença de fenótipos aberrantes, alterações cromossômicas e moleculares, e organomegalias (RIBEIRO, 2001; SWERDLOW et al., 2008). De um modo geral, os pacientes adultos costumam apresentar fatores de risco mais elevados e incapacidade de tolerar os regimes terapêuticos intensos a que são submetidas as crianças. Apesar da boa resposta dos pacientes pediátricos, ainda persiste uma taxa de maus resultados, com chance de refratariedade ou de recaída (MARTIN, MORGAN, HIJIYA, 2011).

O controle da proliferação e da diferenciação celular envolve diversos processos fisiológicos. A apoptose, processo de morte celular programada, é responsável pela eliminação de células não mais necessárias ou defeituosas por meio de uma relação complexa de moléculas pró-apoptóticas, anti-apoptóticas e caspases, considerando um mecanismo inato de defesa. A interação dos processos biológicos de regulação celular com o sistema imunológico apresenta benefício mútuo por meio da modulação das reações imunológicas e inflamatórias por citocinas, nucleotídeos da adenina (ATP, ADP e AMP) e seu nucleosídeo adenosina, bem como pela ação das ectonucleotidases na hidrólise e controle das concentrações dos nucleotídeos extracelulares (BURNSTOCK; DI VIRGÍLIO, 2013; JUNGER, 2011).

A supressão dos processos apoptóticos pode repercutir na proliferação celular desordenada, desencadeamento de neoplasia e resistência à terapia. Fatores adquiridos, como o de resistência a múltiplas drogas (MDR) e a metilação do DNA, também podem estar associados a resultados clínicos desfavoráveis em pacientes portadores de Leucemia Linfoblástica Aguda (MAIA et al., 2012; STOCK, 2010).

Os medicamentos usados na terapia das leucemias atuam, principalmente, no controle da síntese de proteínas e no bloqueio e inibição das enzimas do ciclo celular. As pesquisas atuais têm como alvo o estudo dos fatores prognósticos e biológicos dos resultados desfavoráveis, bem como a síntese de novos medicamentos mais eficazes e menos tóxicos para os tecidos sadios (BRANDALISE; PINHEIRO; LEE, 2011). O entendimento dos eventos envolvidos é determinante para a estratificação do risco e a decisão terapêutica. As técnicas laboratoriais são fundamentais para a identificação e a avaliação dos marcadores biológicos que caracterizam os fenômenos, pois o perfil alterado pode ser usado como marcador

molecular de detecção, de progressão e de predição da resposta aos tratamentos convencionais.

Considerando-se as inúmeras variáveis envolvidas na leucemogênese, a identificação destas proteínas e a inativação de suas funções são estratégias essenciais para a determinação de fatores prognósticos mais fidedignos, para o monitoramento da terapia e para o desenvolvimento de novos fármacos. Dessa forma, contribui com a clínica na investigação de fatores impactantes na resposta aos diferentes protocolos de tratamento, especialmente os relacionados à persistência de células neoplásicas em alguns casos e, à boa resposta, em outros.

1.1 LEUCEMIA LINFOBLÁSTICA AGUDA

As Leucemias são neoplasias hematológicas de etiopatogênese heterogênea, que podem afetar indivíduos de ambos os sexos, em qualquer faixa etária e que, de acordo com a etapa de diferenciação celular em que se desencadeia sua expansão clonal, podem se caracterizar como de evolução crônica ou aguda e, morfologicamente, como linfoide ou mieloide. Registros internacionais de câncer sugerem que a incidência de leucemias exibe variações geográficas, étnicas e socioeconômicas (INCA, 2016; RIBEIRO, 2001; SWERDLOW et al., 2008).

De um modo geral, as leucemias originam-se de um evento genético primário seguido de várias alterações genéticas secundárias, que afetam os mecanismos celulares de controle da diferenciação e da proliferação (POMBO DE OLIVEIRA; KLUMB, 1998; SWERDLOW et al., 2008). O entendimento dos eventos envolvidos é determinante para a estratificação do risco, a decisão terapêutica, bem como para a pesquisa de novos tratamentos no caso de pacientes com doença refratária (RIBEIRO, 2001).

A Leucemia Linfoblástica Aguda (LLA) é uma doença hematológica maligna que envolve a proliferação clonal de células precursoras da linhagem linfoide. Pode se manifestar nas diversas faixas etárias, embora seja uma doença primariamente de crianças, com uma prevalência de 75-80% dos casos de leucemias da infância e, de 20% das leucemias do adulto (PUI et al, 2003; RIBEIRO, 2001; SWERDLOW et al., 2008). A patogênese da doença envolve a desregulação das vias de controle da proliferação celular, da diferenciação e da vitalidade, que são importantes determinantes da resposta ao tratamento (BRANDALISE; PINHEIRO; LEE, 2011).

Conforme Ribeiro (2001), cerca de 75% dos casos de LLA ocorrem em menores de seis anos, dos quais 80-85% são classificadas como B-derivadas. Nas LLA B-derivadas, há comprometimento da medula óssea (MO) e, na grande maioria, do sangue periférico (SP). No SP, a leucometria varia a cada paciente, apresentando-se de diminuída, a normal ou elevada. O envolvimento extramedular pode afetar o sistema nervoso central (SNC), linfonodos, baço, fígado e, nos indivíduos do sexo masculino, os testículos (BRANDALISE; PINHEIRO; LEE, 2011; SWERDLOW et al., 2008).

A leucemia linfoblástica T-derivada, na maioria dos casos, envolve medula óssea e sangue periférico, bem como pode se manifestar primariamente no timo, ou sítios nodais ou extranodais (SWERDLOW et al., 2008). A LLA-T ocorre em cerca de 15% das leucemias pediátricas, sendo mais comum na adolescência, com discreto predomínio em indivíduos do sexo masculino. Afeta cerca de 25% dos adultos com LLA (SWERDLOW et al., 2008). Em geral, a leucometria é elevada e, frequentemente, apresenta massa mediastínica, linfadenopatia e hepatoesplenomegalia. O prognóstico para esta neoplasia é considerado de alto risco (BRANDALISE; PINHEIRO; LEE, 2011; RIBEIRO, 2001).

Sob o aspecto morfológico, os linfoblastos da LLA-T e da LLA-B derivada são indistinguíveis. A classificação imunológica é realizada por meio da imunofenotipagem, que caracteriza a linhagem como B ou T derivada, o estágio de diferenciação celular, e a presença ou não de expressão de antígenos aberrantes (BRANDALISE; PINHEIRO; LEE, 2011; SWERDLOW et al., 2008). A análise citogenética possibilita definir alterações numéricas ou estruturais que podem ou não impactarem o prognóstico, possibilitando a classificação de entidades específicas com fenótipo único (SWERDLOW et al., 2008).

Em indivíduos saudáveis, os índices hematimétricos apresentam valores fisiológicos variáveis conforme a idade cronológica (LORENZI, 2006; NATHAN; ORKIN, 1998). Consideram-se, para a classificação do risco, fatores prognósticos pré-tratamento tais como variáveis clínicas e características das células leucêmicas como indicativos de maiores ou menores chances de cura.

A LLA B-derivada apresenta bom prognóstico em crianças, com taxa de remissão completa maior que 95%, porém, é menos favorável em adultos, onde a remissão varia de 60-85%. Na infância, a LLA T-derivada é considerada de risco mais elevado, enquanto os adultos respondem melhor à terapia de indução para

este subgrupo (SWERDLOW et al., 2008).

Com o intuito de debelar a doença, os Protocolos de tratamento recomendam uma combinação de fármacos que tem a função de inibir a proliferação, a diferenciação, a divisão e os processos de biossíntese de compostos, facilitando a morte celular. O tratamento consiste de um regime quimioterápico com múltiplos fármacos que varia conforme o subtipo da leucemia.

A terapia preconizada pelo Grupo Brasileiro para Tratamento da Leucemia da Infância e Adolescência GBTLI LLA 2009 (BRANDALISE; PINHEIRO; LEE, 2011) é administrada por um período de cerca de 30 meses, escalonada em fases sequenciais denominadas, em síntese, de indução, consolidação, intensificação, manutenção e profilaxia do SNC. A indução objetiva reduzir a massa leucêmica de forma que permita o funcionamento normal da medula óssea e a melhoria do estado geral do paciente. Na sequência, as demais fases se constituem de regimes quimioterápicos administrados para eliminar o clone leucêmico (RIBEIRO, 2001). Durante estes períodos, são realizados exames da medula óssea e sangue periférico em datas pré-determinadas a fim de avaliar a resposta ao tratamento e a presença ou não de células residuais por meio do estudo da doença residual mínima (DRM).

O protocolo GBTLI LLA 2009 (BRANDALISE; PINHEIRO; LEE, 2011) considera a idade e os valores de leucometria dos pacientes ao diagnóstico, a classificação imunológica da doença e determinados achados citogenéticos e moleculares como indicadores fortes para prognóstico de sobrevida. No subgrupo com baixo índice de recaída (BR) encontram-se os indivíduos com idade entre 1 e 9 anos, leucometria inferior a 50.000/mm^3, classificação B-derivada, com ausência de acometimento do sistema nervoso central (SNC) e de achados citogenéticos de risco ao diagnóstico. No subgrupo de alto risco (AR) se incluem os pacientes com idade ≥ 9 e < de 18 anos, leucometria inicial ≥ 50.000/mm^3, classificação T-derivada, com acometimento leucêmico do SNC ou com presença de fatores citogenéticos desfavoráveis. A intensidade da resposta ao tratamento inicial mostra dois subgrupos, os respondedores rápidos (RR) e os respondedores lentos (RL), de acordo com o número de células blásticas no sangue periférico no dia D8 do tratamento, o estado leucêmico do mielograma e o percentual de doença residual no dia D15 (DRM/CF) e o resultado do mielograma e da DRM/PCR no dia D35, possibilitando, com estes dados, que sejam feitos ajustes à terapia.

Apesar da boa resposta terapêutica dos pacientes pediátricos, persiste uma taxa de 20% de chances de recaída, mesmo com os melhores protocolos de tratamento (MARTIN, MORGAN, HIJIYA, 2011; RIBEIRO, 2001). Alguns conseguem uma segunda remissão completa, mas há dificuldade na manutenção desta resposta. Para adultos e crianças de pior prognóstico, restam alternativas como o transplante alogeneico de medula óssea e o uso de fármacos em fase mais inicial de pesquisa clínica, especialmente nos casos de alto risco (BARTH et al., 2012; MARTIN, MORGAN, HIJIYA, 2011). Entre os fatores envolvidos na resposta terapêutica, encontram-se os processos fisiológicos de controle da proliferação celular e da regulação dos processos apoptóticos. Desarranjos nestes mecanismos podem levar à persistência de células neoplásicas e à resistência à quimioterapia (HUNTER, LACASSE, KORMELUCK, 2007).

1.2 SISTEMA IMUNOLÓGICO E SINALIZAÇÃO PURINÉRGICA

Os medicamentos usados na terapia das leucemias atuam, principalmente, no controle da síntese de proteínas e no bloqueio e inibição das enzimas do ciclo celular. A função fisiológica do sistema imunológico busca a eliminação de microrganismos e outros antígenos estranhos por meio da ativação de mecanismos efetores que atuam na defesa do hospedeiro (BARTH et al., 2012).

Dentre estes mecanismos, a secreção de citocinas pelas células da imunidade natural e da imunidade adquirida tem a função de mediar e regular as reações imunológicas e inflamatórias. As citocinas estimulam o crescimento, a diferenciação e a comunicação linfocitária, a ativação de diferentes células efetoras para eliminação de antígenos, bem como a estimulação da maturação hematopoiética, constituindo-se de importantes agentes terapêuticos, alvos para antagonistas específicos nas doenças imunológicas e inflamatórias (ABBAS; LICHTMAN; PILLAI, 2011).

Os receptores de citocinas classificam-se em tipo I, II, superfamília das imunoglobulinas e TNF, conforme as homologias estruturais da porção extracelular ligante da citocina e os mecanismos de sinalização intracelular compartilhados. Os receptores transmembrana promovem a ativação celular por meio de alterações no padrão de expressão gênica nas células-alvo, o que desencadeia a síntese (transitória) das citocinas. (SARROUGH et al., 2008) Trata-se de um evento breve e

autolimitado decorrente da instabilidade do RNA mensageiro, que é observado em diversos tipos celulares, com efeitos funcionais antagônicos ou sinérgicos. Nas neoplasias, a avaliação dos níveis de citocinas pro-inflamatórias (TNF-α, IL-1β, IL-6, IL-8, IL-15, IL-17) e anti-inflamatórias (IL-2, IL-4, IL-10, IL-13) fornece subsídios para a intervenção terapêutica (ABBAS; LICHTMAN; PILLAI, 2011).

Além das citocinas, os nucleotídeos da adenina (ATP, ADP e AMP) e seu nucleosídeo adenosina constituem uma classe de moléculas extracelulares que desempenha papel fundamental na modulação da resposta imune. Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações, entretanto, em altas concentrações, o ATP extracelular pode formar poros nas membranas celulares, promovendo mudanças osmóticas na célula e consequente morte celular (DI VIRGILIO et al., 2001).

O ATP possui funções fisiológicas como a neurotransmissão, a inibição da agregação plaquetária, a indução de importantes mediadores pelos linfócitos T e B como TNF-α e IL-2, envolvidos na resposta imune (RALEVIC; BURNSTOCK, 1998; SLUYTER et al., 2001). Nos linfócitos, o ADP não tem papel definido, mas é importante mediador da agregação plaquetária e da tromboregulação após danos teciduais (DI VIRGÍLIO et al., 2001).

Os nucleotídeos extracelulares ATP e ADP e seus metabólitos exercem ações biológicas ao interagirem com duas famílias de receptores purinérgicos presentes na superfície de diversas células denominadas P1 e P2. Os purinoreceptores P2 dividem-se em duas subclasses: os acoplados à proteína G (P2Y) e os ligados a canais iônicos (P2X) (DI VIRGÍLIO et al., 2001; JACOB et al., 2013).

O receptor subtipo P2X7 pode funcionar como um poro iônico não seletivo em mastócitos, plaquetas, macrófagos e linfócitos, além de ser ativado na presença de altas concentrações de ATP extracelular desencadeando eventos pró-inflamatórios (DI VIRGILIO et al., 2001). Chong et al. (2010) observaram que os receptores P2X1, P2X4, P2X5 e P2X7 relacionavam-se à hematopoese e se mostravam super-expressos nas leucemias quando comparados aos controles, enquanto P2X2, P2X3 e P2X6 estavam ausentes ou fracamente expressos em ambos os grupos. O nível mais elevado de P2X7 foi verificado em pacientes em recaída de leucemia.

Burnstock e Di Virgílio (2013) relatam a expressão significativamente mais elevada do receptor P2X7 em células de pacientes com leucemia linfoblástica aguda, bem como nas leucemias mieloides aguda e crônica. Os autores verificaram

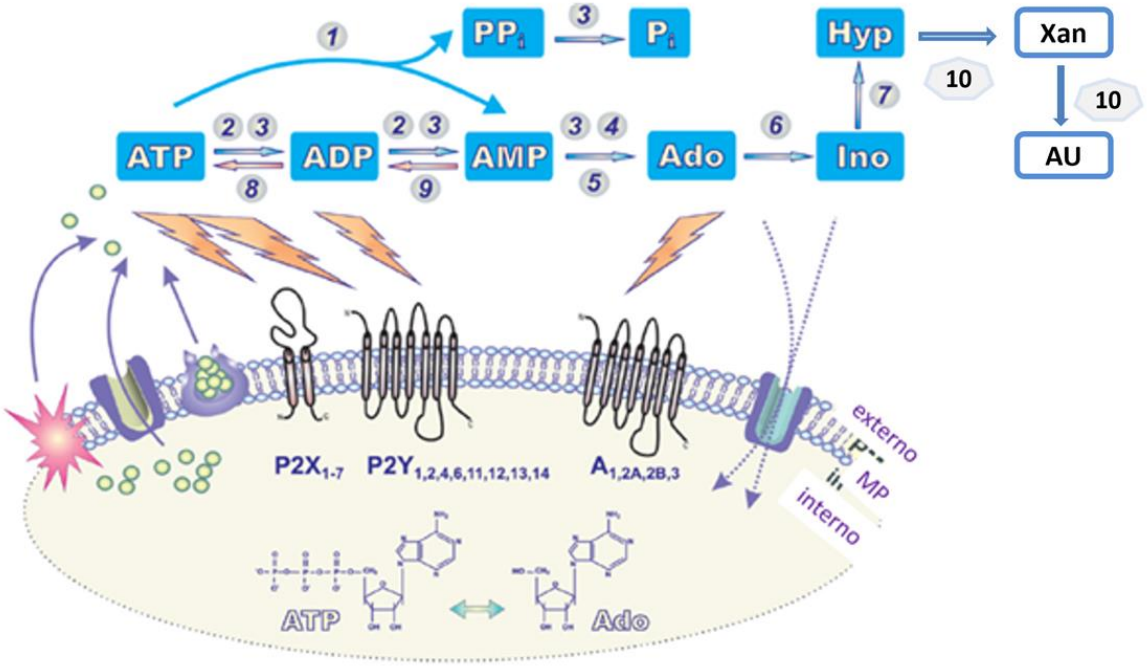
que, em células de linhagem de leucemia U-937, a citotoxicidade induzida pelo ATP mostrou duas etapas, creditando a resposta inicial ao ATP e, a resposta tardia, à adenosina resultante da hidrólise do ATP.

As ectonucleotidases hidrolisam os nucleotídeos extracelulares e regulam as concentrações dos mesmos nos tecidos, por meio de uma cascata enzimática (Figura 1). Constituem-se de E-NTPDases (ecto-difosfoidrolases, apirase, CD39, E.C 3.6.1.5) que catalisa a hidrólise do ATP a ADP e AMP, ecto-nucleotídeo pirofosfatase/fosfodiesterase (E-NPP), e da enzima ecto-5'-nucleotidase (E-5'-NT; CD73; E.C 3.1.3.5), que finaliza a cascata com a hidrólise dos nucleotídeos monofosfatados, liberando a adenosina (ZIMMERMANN, 1996). Essa cascata enzimática é continuada pela ação da ectoenzima adenosina desaminase (E-ADA; E.C 3.5.4.4) a qual catalisa a desaminação irreversível da adenosina e 2' - deoxiadenosina em inosina e 2'- deoxinosina, respectivamente (CRISTALLI et al., 2001; LONGHI et al, 2013).

A E-NTPDase (CD39) inicia a cascata de hidrólise de nucleotídeos da adenina, estando presente em linfócitos, plaquetas e células do endotélio vascular. Esta enzima desempenha importante controle da função dos linfócitos, como o reconhecimento de antígenos, a ativação de funções efetoras das células T citotóxicas e a capacidade de gerar sinais que amplificam interações célula-célula (KACSMAREK et al., 1996; FILIPPINI et al., 1990). A E-NTPDase (CD39) está presente em outros tipos de leucócitos, assim como em células neoplásicas, e esta enzima tem sido estudada em associação com vários tipos de doenças, embora raramente em condições de normalidade, de forma a determinar a expressão e a função "normal" de CD39 (PULTE et al., 2013).

As atividades biológicas e enzimáticas da CD39 estão diretamente relacionadas. Pulte e colaboradores (2007) demonstraram que, em indivíduos normais, os linfócitos B e os monócitos expressam a enzima em mais de 90% das células. A expressão é regulada por linfócitos T ativados e de memória, sugerindo que, além do controle da circulação, tem importante papel na modulação da resposta imune. Os autores observaram um nível de atividade ADPase e ATPase superior nos linfócitos T quando comparados aos linfócitos B. Estes achados mostram que variáveis como a concentração de ATP, estímulo à proliferação linfoide e à liberação de citocinas, bem como a produção de adenosina via CD73 devem ser consideradas quando se avalia a possibilidade de existência de um mecanismo de *feedback* da resposta imune via CD39.

Figura 1 – Visão esquemática da cascata de sinalização purinérgica



Fonte: YEGUTKIN, 2014. Adaptado com autorização RightsLink.
O turnover dos nucleotídeos extracelulares compreende a liberação transitória de ATP endógeno e outros agonistas purinérgicos após dano celular ou por via não-lítica. Esquema de vias de inativação incluem o papel das enzimas NPP(1), NTPDase(2), fosfatases alcalinas(3), ecto-5'-nucleotidase/CD73(4), PAP(5), ADA(6) e PNP(7). No sentido inverso, as atividades de AK(8) e NDPK(9) podem contribuir para a regeneração do ATP extracelular através de reações de fosfotransferência. Xantina oxidase(10).

Por sua vez, a enzima ecto-5'-nucleotidase é um marcador de superfície celular (CD73) indicativo de maturação de linfócitos e com expressão em diversas linhagens tumorais (FILIPPINI et al., 1990). A atividade ecto-5'-nucleotidase do CD73 catalisa a hidrólise do AMP a adenosina que, por ação imunossupressora, facilita o desenvolvimento do tumor por inibir a resposta imune via linfócito T (GHIRINGHELLI et al., 2012; JIN et al., 2010). Wang et al. (2011) consideram que a expressão de CD73 nas células tumorais, quanto nas células hematopoiéticas do hospedeiro, inibem a expansão e ação das células T antitumorais, e que esta característica pode ser usada como estratégia imunoterapêutica no combate ao câncer.

A ação combinada das ectoenzimas CD39 e CD73 é capaz de gerar adenosina extracelular com ação imunossupressora e de remover o ATP extracelular

com função pró-inflamatória. Conigrave et al. (2000) demonstraram que a adenosina contribui para o efeito inibitório do ATP sobre a proliferação celular por meio de receptores de adenosina, enquanto que a indução da diferenciação pelo ATP ocorreria via receptores P2X. A adenosina se liga ao receptor A2AAR, suprimindo a proliferação dos linfócitos T e a secreção de citocinas pró-inflamatórias, ao mesmo tempo que reduz a expressão de receptores de citocinas por meio da elevação dos níveis intracelulares de cAMP via estimulação da adenilciclase (JIN et al., 2010).

A adenosina desaminase (E-ADA) catalisa a desaminação irreversível da adenosina em inosina (RESTA et al., 1998; ROBSON et al., 2006). Está presente no soro e em outros tecidos, particularmente nos tecidos linfoides e é essencial para a função e maturação dos linfócitos T, sendo também requerida para a maturação dos monócitos e macrófagos (ADAMS; HARKNESS, 1976; MACDERMOTT et al., 1980). Alterações em sua atividade são indicadores de distúrbios imunológicos (LONGHI et al., 2013; POURSHARIFI et al., 2009;).

Em estudo recente, Kaljas et al. (2017) mostraram que, além de diminuírem os níveis de adenosina por conversão para inosina e se ligarem a diferentes subconjuntos de células imunes, a ADA1 pode atuar como fator de crescimento, enquanto a ADA2 pode suprimir Tregs por meio da adenosina extracelular imunossupressora. As células T regulatórias (Treg) são uma das células imunossupressoras-chave no contexto do câncer. Estas células co-expressam CD39 e CD73 de membrana, e são capazes de transformar o ATP em adenosina, ao mesmo tempo em que exibem função imunossupressora (GUIRINGHELLI et al.,2012).

A transformação dos linfócitos T $CD4^+$ em células Th17 se dá pela ação de TCR em combinação com IL-6 e TGF-β. Conforme Guiringhelli e colaboradores (2012), sua função na imunidade do câncer é controversa, alguns estudos (HE et al. 2010; WANG et al., 2009) sugerem que células Th17 poderiam promover o desenvolvimento de tumores ao promoverem angiogênese e inflamação via STAT3 e mobilização de MDSC. Por outro lado, esta célula teria a capacidade de inibir o crescimento tumoral ao promover a ativação dos linfócitos T citotóxicos (MARTIN-OROZCO et al., 2009). Desta forma, as células Th17 exerceriam funções regulatórias ou inflamatórias no câncer de acordo com as citocinas do microambiente (GUIRINGHELLI et al., 2012).

O estudo das vias intracelulares e dos mecanismos de inibição ou modulação

dos processos celulares pode melhorar o entendimento dos fatores desencadeantes das leucemias e, consequentemente, contribuir para aumentar a efetividade dos fármacos na função de destruir e impedir o desenvolvimento das células neoplásicas. Os novos agentes terapêuticos têm, cada vez mais, a função de atingir seletivamente as células leucêmicas e produzir maiores efeitos tóxicos nestas do que nas células normais (FIGUEIREDO-PONTES et al., 2008; PAREDES-GAMERO et al., 2013; SUÁREZ et al., 2005). No contexto atual, as técnicas laboratoriais são fundamentais para a identificação e a avaliação de marcadores biológicos que caracterizam os fenômenos de apoptose, de multirresistência a drogas (MDR), de imunodeficiência e de citotoxicidade, de modo a fornecer subsídios para a determinação de fatores prognósticos mais fidedignos e possibilitar intervenções terapêuticas mais eficazes.

1.3 OBJETIVOS

1.3.1 Objetivo geral

Avaliar a atividade das enzimas que degradam nucleotídeos e nucleosídeo da adenina para regulação da resposta imune na leucemia linfoblástica aguda B derivada (LLA-B).

1.3.2 Objetivos específicos

- Avaliar a atividade das enzimas E-NTPDase e E-ADA em linfócitos periféricos;
- Avaliar a atividade das enzimas NTPDase, ADA e XO no soro;
- Determinar a expressão de CD39 e de CD73 em linfócitos;
- Quantificar nucleotídeos e nucleosídeo da adenina no soro.
- Determinar as concentrações séricas de citocinas anti-inflamatórias e pró-inflamatórias.
- Relacionar a expressão das diferentes proteínas pesquisadas com a resposta imune e com a resposta ao tratamento quimioterápico.

APRESENTAÇÃO

Os resultados desta tese estão apresentados na forma de dois manuscritos, o primeiro submetido à revista Biomedicine & Pharmacotherapy, os quais se encontram no item **MANUSCRITOS** como “**MANUSCRITO 1**” e “**MANUSCRITO 2**”. As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se nos próprios manuscritos e representam a íntegra do estudo.

Os itens **DISCUSSÃO** e **CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados apresentados nos manuscritos. No item **REFERÊNCIAS** encontram-se as citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

3 MANUSCRITO 1

Ectoenzimas E-NTPDase, 5’-NT e E-ADA: como elas funcionam nos linfócitos periféricos da leucemia linfoblástica aguda B-derivada

Ectoenzymes E-NTPDASE, E-5’-NT and E-ADA: how they function in peripheral lymphocytes of B-precursor acute lymphoblastic leukemia

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Ectoenzymes E-NTPDASE, E-5'-NT and E-ADA: how they function in peripheral lymphocytes of B-precursor acute lymphoblastic leukemia

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ABSTRACT

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplasm characterized by an anomalous clonal proliferation of B lymphoid precursor cells and predominance in pediatric patients. BCP-ALL promotes the deregulation of physiological and immunological processes. Among the modulators of the immune processes, one can highlight the nucleotides ATP, ADP, AMP, and the nucleoside adenosine, whose extracellular concentrations are regulated by the ectoenzymes E-NTPDase (CD39), E-5'-NT (CD73), and ecto-adenosine deaminase (E-ADA). To understand the implication of these enzymes, this study aimed to evaluate the activities of E-NTPDase and E-ADA, as well as the expressions of CD39 and CD73 in peripheral lymphocytes and in peripheral blood, respectively, of pediatric BCP-ALL patients at diagnosis (D0) and after 15 days of treatment (D15), in addition to the control group. In this manner, 32 D0 patients, 14 D15 patients, and 34 healthy controls (C) were evaluated. The determination of E-NTPDase and E-ADA activities was performed on isolated peripheral lymphocytes, and the expressions of CD39 and CD73 were assessed by flow cytometry in lysed total peripheral blood. The activity of E-NTPDase (ADP substrate) was reduced in D15 (-51%), while E-ADA activity was increased in D0 (135%) and in D15 (82%) in relation to the control group. The evaluation of the expressions of CD39 and CD73 showed distinct results according to the degree of cell maturation. At D0, lymphoblasts showed lower CD39 (-54%) and higher CD73 (70%) expressions in relation to lymphocytes from the same group. However, expression in lymphocytes did not show a significant difference between D0 and the control groups. Correlation tests between the number of leukocytes and lymphoblasts with the expressions of CD39 and CD73, as well as with E-NTPDase and E-ADA activities showed the heterogeneity of the enzymatic patterns of the purinergic cascade in mature and immature lymphocytes. Variations in ectoenzyme activities suggest a possible modulation of the purinergic system in neoplastic lymphocytes, which may be reversed by treatment with consequent reduction in the number of lymphoblasts and leukocytes.

Keywords: BCP-ALL, E-NTPDase, E-5’-NT, E-ADA, lymphocytes, lymphoblasts

1. Introduction

Acute Lymphoblastic Leukemia (ALL) is a hematological neoplasm caused by the anomalous clonal proliferation of precursor cells of the lymphoid lineage. It is a heterogeneous disease in terms of clinical presentation, immunophenotype, genetic abnormalities, prognosis, and response to treatment [1–3]. The proliferative advantage of leukemic cells over normal cells affects the hematopoietic system, promoting effects such as anemia, thrombocytopenia, neutropenia, and decreased cell-mediated immunity [1, 4]. Among the lymphoblastic leukemias, the B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has prevalence of 75-80% of the cases of childhood leukemia [1, 4, 5], in addition to having one of the highest healing rates [3].

Studies that characterize prognostic and risk factors for this pathology have developed enormously. Numerous advances in therapeutics as well as decreased mortality and a rate reaching up to 85% of pediatric patients with five years disease-free survival have been observed [4, 6–8]. The treatment promotes pharmacological action on physiological processes such as the modulation of immune and inflammatory response, control of cell proliferation and differentiation, and regulation of apoptotic processes [3, 9].

In a great number of biochemical processes, nucleotides play key roles in DNA and RNA synthesis, energy supply, the action of coenzymes, and metabolic regulation [2]. The ratios of nucleoside mono-, di-, and triphosphates as well as the eNTP receptors determine the shift towards nucleotide synthesis or degradation through mediation of distinct processes such as growth stimulation, apoptosis, chemotaxis, cell differentiation, cytokine release, and neurotransmission [10, 11]. The turnover of extracellular nucleotides and nucleosides, as well as the up-regulation of ectoenzymes are crucial for providing the microenvironment for tumor development [12]. The ectoenzymes E-NTPDases, E-5'-NT, and E-ADA hydrolyze the extracellular nucleotides and their derivatives and regulate their concentrations by means of an enzymatic cascade [13–15].

The E-NTPDase (CD39) initiates the cascade of the nucleotides of adenine hydrolyzing ATP and ADP to AMP [16, 17]. In human lymphocytes, CD39 activity controls the interaction of ATP in the membrane to trigger a proinflammatory response and exerts immunoregulatory functions of cytokine expression, cell-cell adhesion, cell proliferation, and apoptosis [18]. Moreover, the expression and activity

of CD39 can vary from high levels in resting B cells and in more differentiated leukemic cells, such as CLL, to unidentified levels in normal hematopoietic stem cells or in Jurkat cell line (ALL) [18].

The enzyme ecto-5'-nucleotidase (CD73) catalyzes the AMP hydrolysis to adenosine (Ado) [19]. On the cell surface, CD73 is related to maturation, activation, and transmigration of lymphocytes in the microenvironment of several tumor lines. It is also related to lower survival rates in patients with high expressions of this molecule [20]. The action of Ado is controversial, and some studies have shown that immunosuppression plays a cytoprotective role by inhibiting the T lymphocyte immune response. On the other hand, other studies have shown that Ado reduces viability and cell cycle inducing apoptosis, both effects of which are mediated by purinergic receptors [12].

The enzymatic cascade is continued by the action of the ectoenzyme adenosine deaminase (E-ADA), which catalyzes the irreversible deamination of adenosine in inosine [21–24]. High E-ADA activity in lymphoid tissues may balance adenosine immune suppression and sustain the activation of T cells during inflammation. Additionally, changes in activity may be indicators of immune disorders [25].

The chemotherapy regimen for the treatment of ALL consists of multiple drugs that act mainly in the control of protein synthesis and blockade and inhibition of the enzymes from the cell cycle [26]. The therapeutic protocols consider age, leukometry and immunological classification of the disease as some characteristics of the diagnostic and cytogenetic of the disease and molecular findings as indicators of prognosis, risk of relapse and survival [7, 27, 28].

B-cell precursor acute lymphoblastic leukemia is a disease caused by the anomalous clonal proliferation of the lymphoid tissue. In this manner, the purinergic system has important action on lymphocytes by modulating inflammatory and immunological processes. Hence, the evaluation of the enzymes E-NTPDase and E-ADA in lymphocytes as well as the expression profiles of CD39 and CD73 in peripheral blood of patients with BCP-ALL at diagnosis and under treatment is of special relevance. These evaluations may contribute to the identification of biological markers that may characterize the disease.

2. Materials and methods

2.1 Chemicals

The substrates ATP, ADP, adenosine, Trizma base, Comassie Brilliant Blue G, and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO, USA). K_2HPO_4 was obtained from Reagen. All other chemicals used in this experiment were of the highest purity.

2.2 Patients and samples

The sample consisted of 32 BCP-ALL pediatric patients, mean age 8.4 years old and 34 healthy pediatric subjects, mean age 8.7 years old as a control group. The diagnosis of BCP-ALL was based on International Statistical Classification of Diseases and Related Health Problems ICD10 (C91.0). The parents or guardians of the patients and healthy subjects gave written informed consent to participate in this study. The Human Ethics Committee of the Federal University of Santa Maria approved the protocol under No. 16689613.0.0000.5346. To be eligible for this study, subjects had to be newly diagnosed for BCP-ALL but not receiving any type of chemotherapy or previous corticosteroid before the sample collection. The subjects of the control group (C) must not have had any malignant neoplasia, inflammatory or immune diseases and must not have taken any corticotherapy before sample collection. The samples from the BCP-ALL subjects were obtained in two steps: D0 (at diagnosis) and D15 (after fifteen days of induction treatment according to the Brazilian Cooperative Group for Childhood ALL Treatment (GBTLI) ALL-2009 protocol (GBTLI ALL-2009) [28]. The patients included in D15 group consisted of the D0 group patients from whom it was possible to obtain peripheral blood at D15 treatment checkpoint.

The samples were obtained from peripheral blood with anticoagulant (EDTA K_2) in order to perform enzymatic activity and expression assay in lymphocytes. Samples from the healthy (C) and BCP-ALL (D0 and D15) subjects were matched by age group. D15 group was represented by a smaller number of samples due to reduced leukometry.

2.3 Hematological parameters

Hematological parameters were performed in peripheral blood samples anticoagulated with EDTA K_2 and processed on a SYSMEX XT-1800I, Roche

Diagnostic (USA) equipment.

2.4 Isolation of lymphocytes from human blood

Lymphocytes-rich mononuclear cells were isolated from peripheral human blood collected with anticoagulant and separated on a Ficoll-Histopaque density gradient (D=1.077 g/mL) as described by Böyum [29]. The percentage of lymphocytes was above 94% as previously outlined [30].

2.5 Protein determination

Protein was measured by the Coomassie Blue method according to Bradford [31] using serum albumin as standard.

2.6 E-NTPDase-1 activity determination in peripheral lymphocytes

The E-NTPDase-1 activity in lymphocytes was determined as previously described by Leal et al.[15], in which the reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris-HCl buffer at pH 8.0, with a final volume of 200 µL intact mononuclear cells suspended in saline solution was added to the reaction medium (2–4 µg of protein), and pre-incubated for 10 min at 37°C. Incubation proceeded for 70 min. The reaction was initiated by adding substrate (ATP or ADP) at a final concentration of 2.0 mM, and stopped with 200 µL of 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was assayed by the method previously described by Chan et al. [32]. All samples were analyzed in triplicate and the activity was reported as nmol of Pi released/min/mg of protein. The E-NTPDase-1 activity was evaluated in samples of BCP-ALL D0, D15 groups, and the control group.

2.7 E-ADA activity determination in peripheral lymphocytes

E-ADA activity in lymphocytes was measured by the method by Giusti and Galanti [33], which is based on the direct measurement of the formation of ammonia produced when ADA acts in the excess of adenosine. All experiments were performed in triplicate and the values were expressed in U/mg protein for ADA activity. The E-ADA activity was evaluated in samples of BCP-ALL D0, D15 groups, and the control group.

2.8 E-NTPDase (CD39) and E-5'-NT (CD73) expression determination in peripheral lymphocytes by multiparametric flow cytometry

The expressions of CD39 and CD73 were assessed in peripheral blood by flow cytometry lyse/wash method (FACS Lysing®, BD). Then, 100 µL of EDTA whole blood (1.10^6 cells/tube) was incubated with monoclonal antibodies: 10 µL anti-CD39 (PE; BD Pharmingen), 2.5 µL anti-CD73 (APC; BD Pharmingen) and 2.5 µL anti-CD45 (FITC; Beckman Coulter) for 25 min at room temperature in the dark. Afterwards, 2 mL of lysing solution was added and incubated, for 15 min at room temperature (RT) in the dark. In the next step, the mixture was centrifuged at 120 g for 5 min. The supernatant was discarded and 2mL of PBS (with 0.1% azide and 1% fetal bovine serum) was added. The washing process was repeated twice, and the cells were resuspended in 1 mL of PBS (with 0.1% azide and 1% fetal bovine serum) and analyzed at medium speed using a BD FACS Calibur Flow Cytometer® and FACS FLOW PRO software®. The expressions of CD39 and CD73 were evaluated in the samples from the BCP-ALL D0 and the control group.

2.9 Statistical analysis

The Shapiro-Wilk test was used to determine data normality. The student t test was used to compare the control, D0, and D15 groups when data were considered normal. Since the data were non-parametric, the Mann-Whitney test was used. For correlation analysis, Pearson or Spearman correlation coefficients was used, considering that the data were parametric or non-parametric, respectively. The correlation assortment was performed according to Pestana [34]. Calculations and graphs were performed and generated using the SPSS 23 version software (IBM) as a computational tool for statistical data analysis. Results were expressed as mean ± Standard Error of Mean (SEM). Statistical significance was considered at $P<0.05$.

3 Results

3.1 General profile of the patients and hematological parameters

The sample consisted of a control group represented by 34 healthy subjects with a mean age of 8.7 years old (range: 2-18 years) of whom 14 were male and 20 female. The BCP-ALL subjects constituted two distinct groups, the first by the newly diagnosed D0 group (untreated) consisting of 32 patients with a mean age of 8.4 years old (range: 1.9-18 years) of whom 20 were male and 12 female. The second,

which was the D15 group (15 days induction phase treated), consisted of 14 patients from the D0 group from which it was possible to obtain samples at D15 treatment checkpoint. Patients were treated according to the Brazilian Cooperative Group for Childhood ALL Treatment (GBTLI) ALL-2009 protocol [28]. The hematological parameters of D0, D15, and the control group are shown in Table 1. The results obtained for WBC count in the D0 group (P<0.01, n = 32) were increased by 4.3 folds when compared to the control group. On the other hand, the results of hemoglobin (P<0.001, n=32) and platelet (P<0.001, n=32) counts were lower compared to control. After 15 days of induction phase treatment, the WBC (P<0.001, n=14), Hb (P<0.001, n=14), and platelet (P<0.001, n=14) counts of the D15 group were reduced when compared to the control group, whereas the lymphocyte count (P<0.05, n = 14) increased by 76.84% when compared to the control group. It was also possible to observe an increase of 106% in the D15 group in the lymphocyte count and a reduction of 98.87% in the lymphoblast count when compared to the D0 group.

3.2 E-NTPDase activity determination

The results of E-NTPDase activity to ATP and ADP hydrolysis in lymphocytes from BCP-ALL patients and the control group are shown in Figure 1. The activity of E-NTPDase using ATP as substrate did not show significant difference between groups D0 (mean=33.73 ± 6.60 nmol Pi/min/mg protein, n=28), D15 (29.23 ± 6.20 nmol Pi/min/mg protein, n=12), and the control group (mean=29.51 ± 2.71 nmol Pi/min/mg protein, n=30) (P>0.05) (Fig. 1A). However, reduction of E-NTPDase activity was observed using ADP as substrate in D15 (mean=21.80 ± 4.70 nmol Pi/min/mg protein, n=14) when compared to the control group (mean=44.51 ± 6.70 nmol Pi/min/mg protein, n=30) (p<0.01). The E-NTPDase activity for ADP hydrolysis in D0 group (mean=28.39 ± 4.10 nmol Pi/min/mg protein, n=28) was not altered when compared to groups control and D15 (P>0.05) (Fig. 1B). The comparative analyzes between D0 and D15 were performed only between the patients of whom it was possible to match the sample data at diagnosis and 15 days after.

3.3 E-ADA activity determination

Regarding adenosine deamination, a significant increase in the activity of the enzyme E-ADA was observed in the comparison to D0 (mean=1.34 ± 0.21 U/mg protein, n=24) with the control group (mean=0.57 ± 0.07 U/mg protein, n=30)

(*P*<0.001), as well as in the comparison of D15 (mean=1.04 ± 0.24 U/mg Protein, n=13) and the control group (*P*<0.05). However, when D0 and D15 were compared, there was no significant change (*P*>0.05) in adenosine deamination after 15 days of treatment (Fig. 2).

3.4 The expressions of CD39 and CD73 in peripheral lymphocytes

The expressions of CD39 and CD73 were evaluated in D0 and the control groups. Their expressions in lymphocytic and lymphoblastic populations exhibited distinct results and are presented in Figure 3. Considering the lymphocytes, no difference in CD39 expression was observed between D0 (mean= 59.41%± 14.60; n=15) and the control groups (mean= 70.90% ± 10.50; n=14) (*P*>0.05). However, lymphoblasts showed a reduction of 54% in the expression of CD39 in D0 (mean= 26.81% ± 8.90, n=15) when compared to the lymphocytes of the same group (*P*<0.01). Regarding the expression of CD73 in lymphocytes, D0 (mean=39.20% ± 2.40) and the control group (mean=37.50% ± 7.40) showed similar results (*P*>0.05). On the other hand, CD73 expression in lymphoblasts of D0 (mean=66.80% ±14.50) was 70.40% higher (*P*<0.05) when compared to the lymphocytes of the same group.

3.5 Results of the correlation between leukocyte count or lymphoblast count and E-NTPDase or E-ADA activities and CD39 or CD73 expression in D0 and D15

The data were stratified by correlating the samples from D0 (diagnosis) and control groups, as well as those from the D0 and D15 groups. The parameters age, hemoglobin, and platelet counts did not show significant statistical difference when correlated with enzymatic determinations (*P*> 0.05). On the other hand, leukometry showed significant difference in the correlation between groups (*P* <0.001). Correlation analyses were performed in D0, n=14 (leukocyte count, lymphoblast count, E-NTPDase or E-ADA activities, CD39 or CD73 expression) and in D15, n=14 (leukocyte count, E-NTPDase or E-ADA activities).

A negative correlation was observed (Table 2) between the leukocyte count and E-NTPDase activity for ATP hydrolysis in D0 (r_s= -0.4833; *P*<0.01). Conversely, the correlation was not significant between leukocyte count and E-NTPDase activity for ADP hydrolysis or between leukocyte count and E-ADA activity in adenosine deamination. A negative correlation was observed between the lymphoblast count in

D0 and E-NTPDase activity for ATP hydrolysis (r_s= -0.5748; *P*<0.01). Nevertheless, the correlations between lymphoblast count and E-NTPDase activity for ADP hydrolysis and between lymphoblast count and E-ADA activity in adenosine deamination were not significant.

Regarding the expressions of CD39 and CD73 in D0 (Table 2), the results of the correlations varied according to the evaluated cell population, lymphoblasts or lymphocytes. A significant negative correlation was observed between peripheral lymphoblast count and the expression of CD39 in lymphoblasts (r_s= -0.7542; *P*<0.05). A negative correlation between the leukocyte count and the expression of CD39 in lymphoblast (r_s= -0.6804; *P*<0.05) were observed. Notwithstanding, the correlation between lymphoblast count and the expression of CD39 in lymphocytes both between leukocyte count and CD39 expression in lymphocytes were not significant.

A positive correlation was observed between the peripheral lymphoblast count and CD73 expression in lymphoblasts (Table 2) as well as between the leukocyte count and expression of CD73 in lymphoblasts. Nonetheless, the correlation between lymphoblast count and the expression of CD73 in lymphocytes, between leukocyte count and CD73 expression in lymphocytes were negative. All correlations with CD73 were not statistically significant.

The correlations of D15 group were negative, weak and not significant between peripheral leukocyte count and E-NTPDase activity to ATP hydrolysis (r = - 0.3326, P>0.05), E-NTPDase activity to ADP hydrolysis (rs = - 0.1047, P>0.05), and E-ADA activity in adenosine deamination (r = - 0.1440; *P*>0.05) (data not shown).

4 Discussion

B-cell precursor acute lymphoblastic leukemia is a hematological neoplasm characterized by uncontrolled production of hematopoietic B-precursor cells within the bone marrow (BM). A very updated subject has been the interaction of purine pathway and new drugs for cancer therapy. In this context we highlight the variables involved in purinergic signaling both in physiological and pathological microenvironment. Nevertheless, BCP-ALL presents immunobiological heterogeneity regarding differentiation and functional properties of the lymphoid tissue [28]. Variables that have the greatest impact on prognosis include age of diagnosis, initial leukometry, leukemia cell genetics, and initial response to treatment [3, 27].

Among the variables evaluated in the present study, the initial leukometry and the number of lymphoblasts in peripheral blood had the greatest impact on correlation strength. Comparing our leukometry results with the pediatric reference values, the data herein showed that patients with BCP-ALL have extremely variable levels, ranging from what is considered normal for the age group up to hundreds of folds higher [35,36]. A study by Vilchiz-Ordoñez et al. [37] showed that the inflammatory ALL bone marrow microenvironment could impair normal hematopoietic differentiation and promote neoplastic cells development according to the cytokines and growth factor receptor stimuli. A recent study in human lymphoblastic T cell line Jurkat pointed to high mitochondrial metabolism of the autocrine purinergic signaling the increase of extracellular ATP levels, which trigger P2X receptor stimuli and promotes unimpeded proliferation of cancer cells [38].

The present study showed in the D0 group significant negative correlation between the level of E-NTPDase-1 activity for ATP hydrolysis in lymphocytes and the initial leukometry as well as between the E-NTPDase-1 activity and lymphoblast count. The opposite was observed in a study by Schetinger et al. [39], in which the E-NTPDase activity for ATP and ADP hydrolysis in B lymphocytes from CLL were increased at all stages of the disease, with higher levels in the more advanced stage group. The D15 group showed a reduction of ADP hydrolysis when compared to the untreated group or healthy controls. A study by Sakowicz et al. [40] observed the ATP and adenosine actions in immune cells may be simultaneous and interdependent, therefore, during increases in extracellular nucleotide level, the purine cascade may be redirected through the ecto-adenylate kinase (AK) activity that phosphorylates AMP back into ADP.

Our data showed a significant increase in E-ADA activity in lymphocytes of both BCP-ALL groups (D0 and D15) when compared to healthy controls. Considering that adenosine suppresses T-cell proliferation by A2A receptor and modulates the expression of pro-inflammatory cytokine receptors [41], we suggest that increased E-ADA activity may imply in a mechanism to increase the adenosine deamination and to reduce its cellular suppression. Previous studies have proven that the direction of the cascade pathway depends on the adenosine uptake in the cell such as by the level of adenosine released into the extracellular medium by either phosphorylation to AMP or by deamination to inosine [10, 27, 42–44]. Yegutkin et al. [10] showed that lymphoid cells with low or no ecto-nucleotidase activities and relatively high

expression of ecto-adenosine deaminase (ADA) are characterized by “ATP-generating/adenosine-eliminating” phenotype, enabling the deviation of the lymphotoxic effects of adenosine.

In accordance with this data, we found significant higher levels of inosine ($P<0.05$) and lower levels of hypoxanthine ($P<0.01$) in the serum of BCP-ALL patients at diagnosis than in healthy controls (data not shown). Since inosine is an anti-oxidant agent, we suggest its accumulation may protect the DNA of the lymphoblasts from damage, thus elevating the chance of cell survival. The significantly lower levels of hypoxanthine may be due to decreased activity of purine nucleoside phosphorylase (PNP), although this is just speculation since PNP was not measured.

Notably, the evaluation of the expressions of CD39 and CD73 showed distinct results according to the degree of cell maturation. It was also possible to observe a significantly negative correlation between the expression of CD39 in lymphoblasts and the peripheral lymphoblast count ($P<0.05$), as well with the initial leukometry. In accordance with our results, Pulte et al. [18] showed that the expression of CD39 was up to 90% in resting B cells, although it was unidentified in the cell line Jurkat (ALL) or in normal hematopoietic stem cells. However, CD39 was observed in later bone marrow precursors and in more differentiated leukemic cells, such as CLL.

The determination of the expression of CD73 exhibited significantly higher levels in lymphoblasts than in lymphocytes in the present study. The CD73 expression levels in the lymphoblasts showed not statistically significant correlation between the initial leukometry as well with the lymphoblast count at diagnosis (D0). Previous studies have shown that CD73 present in the neoplastic cells promotes the suppression of the antitumor immune response by inhibiting the activation, clonal expansion, and homing of tumor-specific T cells as well as by the action of adenosine [45, 46]. Studies have also pointed to the use of monoclonal antibodies that use the CD73 molecule as a therapeutic target in order to reduce immunosuppression and, consequently, the likelihood of metastases [46, 47].

Nevertheless, after the first 15 days of treatment, a reduction in the leukocyte and lymphoblast count was observed when compared to the untreated group or healthy controls. In addition, a reduction of the E-NTPDase-1 activity for ADP hydrolysis was observed when compared to healthy controls. One of the effects of induction therapy is the inhibition of neoplastic lymphoid tissue proliferation and the

reduction of lymphoblasts by cell lysis [28]. The aim of cancer therapy is to increase the efficiency toward the malignant cells and decrease the associated side effects [48].

In conclusion, the changes observed in the activities of E-NTPDase and E-ADA at diagnosis demonstrate the strong contribution of the purinergic pathway in modulating the immune cells to balance the immunosuppressive and the inflammatory profiles as a result of acute lymphoblastic leukemia. There was, at first, a proinflammatory microenvironment, which may have facilitated the proliferation of neoplastic cells. At the same time, differentiated expressions of CD39 and CD73 in lymphocytes and lymphoblasts of BCP-ALL patients corroborate the relationship of these molecules with the clonal development of neoplastic cells and the physiological pathway. Results suggest a possible modulation of the purinergic system during the development of leukemic cells that may be reversed in part by initial therapy and consequent reduction in the leukocyte and lymphoblast count. The advances in the study of purinergic pathway highlight a great number of molecules and enzymes that may be now used as therapeutic target. These molecules may alter the pathophysiology of leukemia and serve to monitor the effects of treatment as minimal residual disease and to deviate pathway either inhibiting or stimulating enzymes to blockade the cancer cell development.

Conflict of interest

The authors declare that they have no conflicts of interest.

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TABLES

Table 1 - Hematological parameters in patients with B-cell precursor acute lymphoblastic leukemia (BPC-ALL) in D0, D15 and control groups

Parameters	Control	D0	D15
WBC (x10^3/µL)	7.20 ± 0.33	31.10 ± 6.80**	2.10 ± 0.38**
Lymphoblasts (%)	Absent	49.59 ± 6.50	0.56 ± 0.44
Lymphocytes (%)	39.33 ± 3.03	33.66 ± 5.44	69.55 ± 6.78*
Hb (g/dL)	12.60 ± 0.22	8.50 ± 0.35**	8.60 ± 0.26*
Platelets (x10^3/µL)	283.20 ± 13.40	100.10 ± 24.30***	99.60 ± 24.20**

Continuous variables are presented as mean ± SEM. Groups: control, D0 (newly diagnosed) and D15 (treated) with n=34; n=32; n=14, respectively. (*) (**) (***) indicate a statistically significant difference with $P<0.05$; $P<0.01$; $P<0.001$, respectively, when compared to control group. WBC: white blood cells; Hb: hemoglobin. (Student t /Mann-Whitney test)

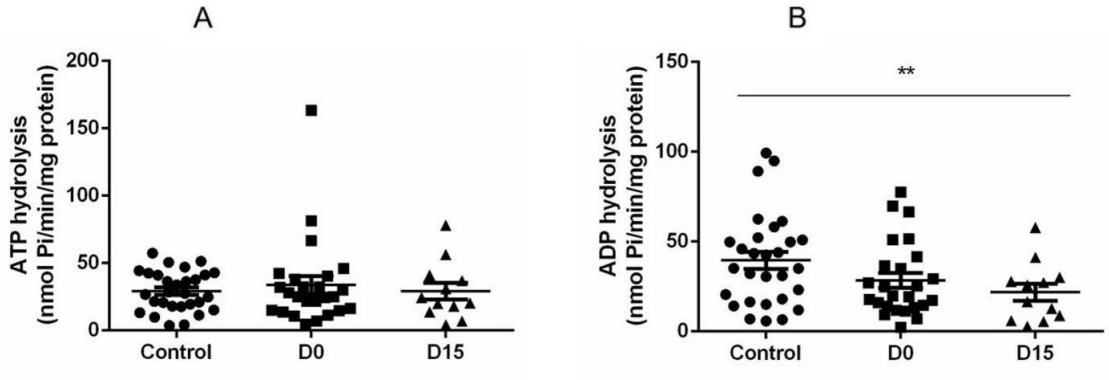
Table 2 - Correlation of leucocytes and lymphoblasts count with E-NTPDase-1 (ATP, ADP) and E-ADA (Ado) activities, CD39 (E-NTPDase-1) and CD73 (E-5'-NT) expressions in lymphocytes and lymphoblasts of BCP-ALL patient peripheral blood at diagnosis (D0)

Variable	Value of r	*P* value
Le X hydrolysis ATP	-0.483 (*rs*)	*P*<0.01**
Le X hydrolysis ADP	-0.332 (*rs*)	*P*>0.05
Le X deamination Ado	-0.278 (*rs*)	*P*>0.05
Bl X hydrolysis ATP	-0.574 (*rs*)	*P*<0.01**
Bl X hydrolysis ADP	-0.198 (*rs*)	*P*>0.05
Bl X deamination Ado	0.214 (*rs*)	*P*>0.05
Le X CD39 (bl)	-0.680 (*r*)	*P*<0.05*
Le X CD39 (ly)	-0.339 (*rs*)	*P*>0.05
Bl X CD39 (bl)	-0.754 (*r*)	*P*<0.05*
Bl X CD39 (ly)	-0.373 (*rs*)	*P*>0.05
Le X CD73 (bl)	0.583 (*rs*)	*P*>0.05
Le X CD73 (ly)	-0.515 (*r*)	*P*>0.05
Bl X CD73 (bl)	0.617 (*rs*)	*P*>0.05
Bl X CD73 (ly)	-0.559 (*r*)	*P*>0.05

Continuous variables are presented as Group: D0 (diagnosis) with n=32. (*) (**) indicate a statistically significant difference between the groups, with $P<0.05$; $P<0.01$, respectively.
Le: leukocytes count; Bl: lymphoblasts count; ly: lymphocytes (expression); bl: lymphoblast (expression); *r*: Pearson; *rs*: Spearman correlation.

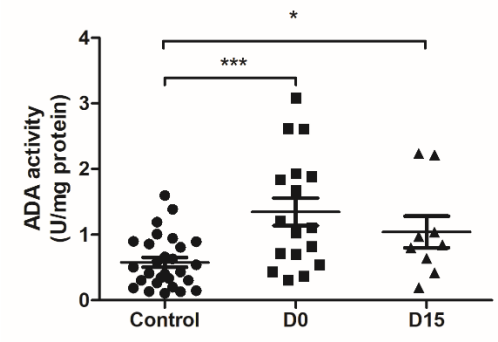
FIGURES

Figure 1 – ATP(A) and ADP(B) hydrolysis in lymphocytes of BCP-ALL D0, D15 and control groups.



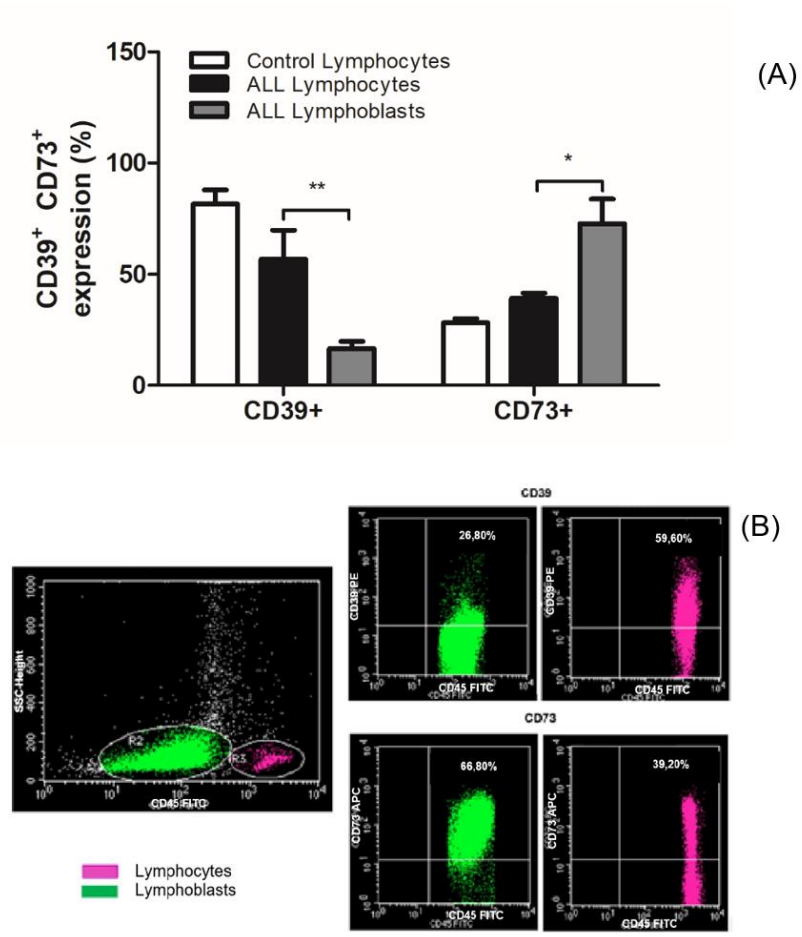
Enzyme activities are reported as nmol of Pi released/min/mg of protein. Variables are expressed as mean ± standard error of the mean (SEM). Bars represent mean ± SEM. (**) indicates a significant difference *P*<0.01, with n=30 (control); n=28 (D0); n=14 (D15). (Mann-Whitney test).

Figure 2 – Adenosine deaminase activity in lymphocytes of BCP-ALL D0, D15 and control groups.



Enzyme activities are reported as U/mg protein. Variables are expressed as mean ± standard error of the mean (SEM). Bars represent mean ± SEM. (*)(***) indicate a statistically significant difference *P*<0.05 *and P*<0.001, respectively, with n=30 (control); n=24 (D0); n=12 (D15). (Mann-Whitney test).

Figure 3 – CD39 and CD73 expression in peripheral lymphocytes and lymphoblasts of BCP-ALL D0 and lymphocytes of the control group



Enzyme expression is reported as percentage of positive events regarding the entire population of lymphocytes or lymphoblasts at CD45 *gate*. (A) Bars represent mean ± SEM. (*) and (**) indicate a significant $P<0.05$ and $P<0.01$, respectively; with n=14 (control); n=15 (D0). (Mann-Whitney test). (B) The dot plot represents the mean results of a patient of BCP-ALL D0 group. Enzyme expression is reported as percentage of positive cells regarding the entire CD45+ population at gate evaluated (lymphocytes or lymphoblasts).

3 MANUSCRITO 2

Catabolismo dos nucleotídeos extracelulares: o perfil no soro de leucemia linfoblástica aguda B-derivada

Extracellular nucleotide catabolism: the profile in serum of B-cell precursor acute lymphoblastic leukemia (BCP-ALL)

Liliane Z. Oliveira, Pedro H. Doleski, Daniela F. Passos, Clandio T. Marques, Cesar E. J. Moritz, Emerson A. Casali, Daniela B.R. Leal

Extracellular nucleotide catabolism: the profile in serum of B-cell precursor acute lymphoblastic leukemia (BCP-ALL)

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ABSTRACT

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a lymphoid neoplasm that impairs normal differentiation of B lymphoid cells. Affecting the immune system, this is the most common childhood malignancy. Adenine nucleotides (ATP, ADP and AMP) and their nucleoside adenosine (Ado) play multiple roles in the immune system. These nucleotides are regulated by ectoenzymes named NTPDase, 5'-NT and adenosine deaminase (ADA). The aim of this study was to investigate the possible effects of these enzymes in the extracellular milieu on soluble nucleotides and nucleosides in the serum of BCP-ALL patients, as well as their relationship with the cytokine profiles. Twenty-four pediatric patients were evaluated at diagnosis (D0), of which 7 patients were also evaluated at day 15-treatment checkpoint (D15), in addition to 30 healthy subjects (control group). NTPDase activity for ATP and ADP hydrolysis was decreased on D0 when compared to the control group, and increased on D15 when compared to D0. ADA activity was significantly increased in D0 compared to the control group. Xanthine oxidase activity in D0 was significantly decreased comparing to the control group and significantly increased after treatment (D15). Serum purine levels exhibited no significant statistical difference between the groups, except for inosine and xanthine, which were significantly increased, and hypoxanthine, which was decreased in D0. The cytokines IL-6, IL-17 and IL-10 levels were significantly increased on D0 when compared to control group. Correlations performed between the lymphoblast count and/or leukocyte count at diagnosis and the serum activities of NTPDases, adenosine deaminase and xanthine oxidase showed distinct relationship. Our results showed an extracellular catabolic activity which reflects the inflammatory status of BCP-ALL at diagnosis, while exhibiting distinct results under the effect of initial oncologic therapy. Complementary studies may identify individual patient characteristics in the purinergic signalling, to assist in the choice of a more specific therapy.

Keywords: BCP-ALL; NTPDase; ADA; XO; Nucleotides.

1 Introduction

Acute lymphoblastic leukemia (ALL) is a lymphoid neoplasm caused by genetic alterations that impair normal differentiation of lymphoid cells [1,2]. These changes affect proliferation and differentiation control, triggering the clonal proliferation of precursor cells of the lymphoid lineage [2,3]. B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common childhood malignancy, and has the highest rates of healing among pediatric leukemia patients [4]. The variables that have the greatest influence on prognosis are the age at diagnosis, initial leukocyte count, leukemic cell genetics and initial response to treatment [3,5].

The bone marrow microenvironment (BMME) plays a key role in supporting survival and proliferation of cancer cells, as well as drug resistance [3,4,6]. BMME is composed by an interacting functional network of cytokines, growth factors and adhesion molecules that may influence stromal cells by altering the cellular functions [7–9]. Nucleotidases and other enzyme families, that hydrolyze adenine nucleotides (ATP, ADP and AMP) and their nucleoside adenosine (Ado), constitute a class of extracellular molecules that play multiple roles in the immune system [10,11]. Under physiological conditions, nucleotides are found in the extracellular milieu–at low concentrations, but upon stimulation or in pathological conditions, high concentrations of extracellular ATP can promote osmotic changes in the cell and behave as stimulants of lymphocyte proliferation either as pro-inflammatory mediators or as immunosuppressants [10–14]. Purinergic signaling is based on the integration of signals to the immune cells, mediated by ATP/ADP to receptor type-P2 or Ado to receptor type-P1 [10,15].

Soluble forms of nucleotidases can be found in serum and act on nucleotides through an extracellular hydrolysis cascade and subsequent formation of respective nucleoside and free phosphate [10,13,16]. The sequential activities of the nucleotidases NTPDase and 5’-NT in the hydrolysis of extracellular ATP/ADP to AMP, and AMP to adenosine, respectively, are the key modulators of the biochemical composition of the microenvironment and the major source of extracellular adenosine[10,13,15,17,18]. Di Virgilio and Adinolfi [15] consider adenosine one of the most pleiotropic constituents of tumor microenvironment (TME) because it can affect both host and tumor responses. The activity of adenosine in host cells is usually anti-inflammatory or immunosuppressant, while in tumor cells its function depends on the specific adenosine receptors (AR) expressed by the tumor cells, leading to opposite

effects as stimulating or inhibiting tumor growth [15,19–21]

The nucleotide degradation continues by adenosine deaminase (ADA) which catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine [11,16,22–24]. Purine nucleoside phosphorylase (PNP) phosphorylates (deoxy) inosine and (deoxy) guanosine, converting them to hypoxanthine and guanine, respectively [16]. Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine and this to uric acid, a more stable element, with superoxide anion forming and generation of hydrogen peroxide [10].

Inflammatory conditions and soluble hematopoietic growth factors create a bone marrow microenvironment on the host that favors hematopoietic precursors leukemogenesis [9,25]. The inflammatory response in patients with ALL includes cytokines and cytokine receptors and their close relationship between the innate and the adaptive immune responses through T-cells [26]. Cytokines are important mediators of immune responses, regulating cell differentiation, stimulating or inhibiting cell growth, inducing cell chemotaxis and modulating the expression of other cytokines [2,8,26]. Apoptosis may be prevented if an inadequate expression of cytokines and/or abnormal activation of their receptors deviate the normal cell growth and favor the malignant clonal expansion [2,25].

Studies indicate that the use of cytokine assays help with the diagnosis and prognosis in newly diagnosed acute leukemia patients [8,27,28]. Nevertheless, others focus on regulation of extracellular nucleotides and nucleosides levels to evaluate neoplastic disease states and even the use of enzymes of the purine metabolism, as well cytokines, as targets in cancer therapy [8–11,29]. Considering that ALL is an inflammatory neoplasm, we suppose that soluble NTPDases and adenosine deaminase may modulate the adenine nucleotides in serum of BCP-ALL patients. To evaluate this, we investigated the possible effects of these enzymes in soluble nucleotides and nucleosides in serum of BCP-ALL patients, as well as their relationship with the cytokine profiles.

2. Material and methods

2.1 Chemicals

The substrates ATP, ADP, adenosine, hypoxanthine as well as Trizma base, Coomassie Brilliant Blue G and bovine serum albumin were obtained from Sigma

Chemical Co (St. Louis, MO, USA) and K_2HPO_4, from Merck (Darmstadt, Germany). All other reagents used in this experiment were of analytical grade and of highest purity.

2.2 Patients and samples

The sample consisted of 24 BCP-ALL pediatric patients and 30 healthy pediatric subjects as a control group. The diagnosis of BCP-ALL was based on International Statistical Classification of Diseases and Related Health Problems ICD10 (C91.0). The parents or guardians of the patients and healthy subjects gave written informed consent to participate in this study. The Human Ethics Committee of the Health Science Center from the Federal University of Santa Maria approved the protocol under number 16689613.0.0000.5346. To participate in this study, patients must be newly diagnosed for BCP-ALL and must not have had any type of chemotherapy or corticotherapy before the collection of the sample. The samples from BCP-ALL patients were obtained in two timepoints, D0 (at diagnosis) and D15 (after fifteen days of induction treatment according to the Brazilian Cooperative Group for Childhood ALL Treatment (GBTLI) ALL-2009 protocol (GBTLI ALL-2009) [5]. To participate in the control group (C) the subjects who have had any type of malignant neoplasia, inflammatory or immune diseases, or taken any corticotherapy before the collection of the peripheral blood were excluded. D15 group was represented by DO group patients from whom it was possible to obtain serum at D15 treatment checkpoint. This group had a smaller number of patients since a few patients were excluded due to the reduced leukometry or other exclusion criteria. All samples were matched by age group.

2.3 Hematological parameters

The hematological parameters were performed in peripheral blood samples anticoagulated with EDTA K_2 and processed on SYSMEX XT-1800I, Roche Diagnostic, USA equipment.

2.4 Separation of blood serum

The samples were obtained from peripheral blood in tubes without anticoagulant to all determinations and after the clot formation, they were centrifuged

at 1400xg for 15 min at room temperature. The resultant serum samples were aliquoted in microtubes and frozen until the assays had been performed.

2.5 Protein determination

Protein concentration was determined by the Coomassie blue method according to Bradford [30], using bovine serum albumin as standard.

2.6 Determination of NTPDase activity in serum

NTPDase activities in serum were determined as previously described by Oses et al. [31]. A reaction mixture containing 3 mM of ATP or ADP as substrate and 112.5 mM Tris-HCl (pH 8,0) were incubated with approximately 1.0 mg of homogenized serum protein at 37ºC for 40 min in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid (TCA). The samples were centrifuged at 5000xg for 5 minutes to eliminate precipitated protein and the supernatant was used for the colorimetric assay. The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan et al.[32]. In order to correct non-enzymatic hydrolysis, proper controls were carried out by adding the serum after the reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein (nmol of Pi/min/mg protein).

2.7 Determination of ADA activity in serum

ADA activity in serum was measured by spectrophotometric method described by Giusti and Gakis [33]. The methodology is based on the direct measurement of ammonia produced once the enzyme acts in presence of Ado. The enzymatic reaction started when 475 µL of 21 mM adenosine as substrate was added into 25 microliters of serum, and incubated for 1 h at 37 °C. The reaction was stopped by adding 1.5 mL of 106/0.16 mM phenol–nitroprusside solution to the reaction mixture, which was immediately mixed with 1.5 mL of 125/11 mM alkaline hypochlorite solution (sodium hypochlorite). The ammonia released reacts with alkaline-hypochlorite and phenol in the presence of a catalyst – sodium nitroprusside – to produce indophenol (a blue color). The concentration of ammonia is directly proportional to the indophenol and the absorbance was read at 650 nm. Seventy-five

micromolar of ammonium sulfate was used as ammonium standard. All experiments were performed in triplicate and the values of ADA activity in serum were expressed as U/L.

2.8 Determination of Xanthine oxidase activity in serum

The xanthine oxidase assay was performed by quantifying the rate of urate formation. The reaction mixture was prepared by mixing 0.1 mL serum with 1.9 ml of 0.05 mol/L potassium phosphate buffer, pH 7.5 and 1mM of xanthine as substrate. The rate of urate formation from xanthine degradation was determined by measuring the increased absorbance at 290 nm against blank. One international unit of XO will metabolize one micromole of Xanthine per minute [34]. All experiments were performed in triplicate and the activity of XO was expressed as U/L in serum.

2.9 Analysis of purine levels

The ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine and uric acid in sera of BCP-ALL DO patients and healthy individuals were analyzed by high-pressure liquid chromatography **(**HPLC). The denaturation of proteins was performed using 0.6 mol/L perchloric acid. All samples were then centrifuged (14.000 x *g* for 10 min at 4º C), supernatants were neutralized with 4.0N KOH and clarified with a second centrifugation (14.000 x *g* for 30 min at 4ºC). After second centrifugation, the supernatants were collected and centrifuged again (14.000 x *g* for 30 min at 4ºC). Aliquots of 20 µL were applied to a reversed-phase HPLC (Shimadzu, Japan) using a C_{18} column (Ultra C18, 25 cm x 4.6 mm x 5 µm, Restek – USA). The elution was carried out applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30 min period (flow rate at 1.4 mL/min) according to the method previously described by Voelter et al. [35]. The amounts of purines were measured by absorption at 254 nm. The retention time of standards was used as parameter for identification and quantification. Purine concentrations were expressed as nmol of compound per mL of serum (nmol/mL).

2.10 Cytokines measurement

Serum cytokines were measured by flow cytometry by means of Cytometric Bead Array (CBA). The human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose,

CA, USA) was applied simultaneously following manufacturer instructions. Quantitative results were generated using BD Accuri™ C6 flow cytometer and FCAP Array™ software. All experiments were performed in duplicate and the results were expressed as pg/mL.

2.11 Statistical analysis

For statistical analysis, the Shapiro-Wilk test was used to verify the normality of the data. To compare Control, D0 and D15 groups, when they were normal, ANOVA with Tukey *post hoc* test was used; as the data were no parametric, Kruskall-Wallis test was used. To compare Control and D0 groups, when they were normal, Student *t* test was used; as the data were no parametric, Mann-Whitney test was used. For the correlation analysis Pearson or Spearman correlation coefficients was used, as the data were parametric or no parametric, respectively. The correlation assortment was performed according to Pestana [36]. The calculations and graphs were performed and generated using the SPSS 23 version software (IBM) as computational tool to statistical analysis of data. Variables were expressed as mean ± standard error of mean (SEM). Differences were considered significant for $P<0.05$.

3. Results

3.1 General characteristics of the patients

The control group (C) was represented by 30 healthy individuals, with a mean age of 8.2 years old (range: 1.3 - 17ys), of which 17 were male and 13 were female. The BCP-ALL subjects constituted two distinct groups. The first group consisted of newly diagnosed patients (D0 group), untreated, represented by 24 newly diagnosed subjects with a mean age of 8.7 years old (range: 1.9 - 18ys), of whom 16 were male and eight were female. The second group, D15, consisted of seven patients of D0 group from whom it was possible to obtain samples at day 15-treatment checkpoint. Patients were treated according to the Brazilian Cooperative Group for Childhood Treatment (GBTLI) ALL-2009 protocol [5]. The mean WBC count was significantly increased (279% higher, $P<0.01$) in D0 (30.42/mm^3, SEM ± 7.29, n=24), and significantly decreased (32% lower, $P<0.001$) in D15 (2.49/mm^3, SEM ± 0.69, n=7) when compared to the control group (8.02/mm^3, SEM ± 0.47, n=30). The mean lymphoblast count in D0 group represented 53.54%, (SEM ± 7.27, n=24) of nucleated

cells in peripheral blood. The mean lymphocyte count in D15 group represented 91.14% (SEM ± 2.65, n=7) of nucleated cells in peripheral blood. (Data not show).

3.2 NTPDase activities in serum

The results of the ATPase and ADPase activities in serum of control subjects and BCP-ALL D0 and D15 patients are graphically represented in Figure 1A and 1B, respectively. NTPDase activity for ATP hydrolysis was significantly decreased (45% lower, *P*<0.01) in D0 (2.25 nmol of Pi/min/mg of protein, SEM ± 0.34, n=11) compared to the control group (4.08 nmol of Pi/min/mg of protein, SEM ± 0.44, n=7). In contrast, D15 group (3.90 nmol of Pi/min/mg of protein, SEM ± 0.28, n=7) exhibited significantly increased activity (73% higher, *P*<0.01) compared to D0 group. However, the NTPDase activity for ADP hydrolysis was significantly decreased in D0 (29% lower, *P*<0.001) (3.07 nmol of Pi/min/mg of protein, SEM ± 0.184, n=11) compared to the control group (4.31 nmol of Pi/min/mg of protein, SEM ± 0.16, n=7). In contrast, D15 group (4.14 nmol of Pi/min/mg of protein, SEM ± 0.34, n=7) exhibited significant increased activity (34% higher, *P*<0.001) after treatment compared to D0 group.

3.3 ADA activity in serum

The results obtained for ADA enzyme activity in serum are presented in Figure 2. ADA activity in adenosine deamination was significantly increased (126% higher, *P*<0.01) in D0 (78.82 U/L, SEM ± 11.63, n=11) compared to the control group (34.84 U/L, SEM ± 3.77, n=7), however, comparing D15 to D0 there was no significant statistical difference between the groups.

3.4 XO activity in serum

The results obtained for XO activity in serum are presented in Figure 3. The xanthine oxidase activity in D0 (2.71 U/L, SEM ± 0.20, n=11) was significantly decreased (46% lower, *P*<0.001) compared to the control group (5.01 U/L, SEM ± 0.56, n=7). In contrast, D15 group (3.89 U/L, SEM ± 0.89, n=5) exhibited significant increased enzymatic activity (43% higher, *P*<0.001) after treatment compared to D0 group.

3.5 Serum purine levels

The purine levels were measured in serum of both control (n=10) and D0 (n=10) groups by HPLC and the results are shown in Table 1. The levels of ATP, ADP, AMP and uric acid exhibited a non-significant statistical difference between the groups (P>0.05). However, the levels of adenosine were significantly increased (155% higher, P<0.05) in D0 compared to the control group, as well as of inosine (317% higher, P<0.05) and of xanthine (118% higher, P<0.05). Conversely, the levels of hypoxanthine were significantly decreased in D0 (53% lower, P<0.01) compared to the control group.

3.6 Serum cytokine levels

The serum levels of cytokines IFN-γ, TNF-α, IL-2 and IL-4 showed no significant statistical difference (Figure 4,) between D0 (P>0.05, n=24) and the control group (n=30). Furthermore, the levels of cytokines IL-6, IL-17 and IL-10 exhibited significantly distinct results. The levels of IL-6 in D0 (22.15 pg/mL, SEM ± 3.57) were statistically increased (490% higher, P<0.001) compared to the C group (3.83 pg/mL, SEM ± 0.66). IL-17 levels in D0 (50.65 pg/mL, SEM ± 8.51) were significantly increased (110% higher, P<0.05) compared to group C (24.02 pg/mL, SEM ± 2.94). The evaluation of IL-10 levels in D0 (8.99 pg/mL, SEM ± 2.47), compared to C group (1.96 pg/mL, SEM ± 0.24), showed a statistically significant increase of 358% higher (P<0.01).

The cytokine assay results for D15 group did not show significant decrease in IFN-γ, TNF-α, IL-4, Il-6, IL-10 and IL-17 levels (Figure 4). Though a significant statistical difference (P<0.001, n=5) was found between IL-6 in D15 group (2.47 pg/mL, SEM ± 0.86) and D0 group (22.15 pg/mL, SEM ± 0.86).

3.7 Results of the correlation between leukocyte count or lymphoblast count and NTPDase, ADA and XO serum activities or levels of nucleotides and nucleosides of adenine in D0 group

Data was stratified by correlating the samples from D0 (diagnosis) and control groups, and the parameter leukometry showed a statistically significant difference in the correlation between groups (P <0.001). All correlation analyses were performed in D0 group (n=10) and matching the following parameters: leukocyte count; lymphoblast count; NTPDase (ATP, ADP), ADA and XO activities; ATP, ADP, Ado,

INO, HYPO, XAN, UA nucleotides or nucleosides levels, the results are represented in Table 2. The correlations performed between leukometry and enzymatic activities of serum NTPDase, adenosine deaminase and xanthine oxidase showed negative relationship, except ADPase that showed a positive result. The results of the correlations performed between the leukometry and the nucleotides or nucleosides were negative, except hypoxanthine that showed a positive result. Neverthless, all weak or moderate correlations with lymphocyte count were not statistically significant (P >0.05).

Considering the correlations performed between the lymphoblast count at diagnosis and the enzymatic activities of serum NTPDase, adenosine deaminase and xanthine oxidase, our data exhibited negative relationship, except ADPase and ADA that showed positive results. The results for lymphoblast count and nucleotides or nucleosides levels showed weak correlations. However, all correlations with lymphoblast count were not statistically significant (P >0.05) (Table 2).

4. Discussion

The studies in BCP-ALL have improved the understanding of pathophysiology and genetics of the neoplastic cells, pharmacokinetic and mechanisms of drug resistance, reaching out five-year event-free survival rates to more than 85% in several clinical trials [3]. Despite these findings, there are still 20% of patients whose treatment fails or disease relapses [3,5]. Many researchers have pointed out that tumor microenvironment plays a key role in supporting survival and expansion of cancer cells [2,4,9,37,38].

Leukemic microenvironment encompasses key components of the purine pathways that modulate reactions on stem cells as well as participate in inflammation and immunity processes [37]. To understand the immunomodulatory effects of purines in the extracellular milieu of pediatric BCP-ALL patients, we evaluated the levels of nucleotidase activities and cytokines, at diagnosis and 15 days after the initial treatment (D15), and of serum nucleotides at diagnosis. Regarding to extracellular nucleotidases (eNTPs), our results suggest that there is an activation of the enzymes of the catabolic pathway.

NTPDase activity in serum of D0 group was lower than control and D15 group in both ATP and ADP hydrolysis reactions. The activity of NTPDase in the serum is related with the release of this enzyme by cells, or by the turnover of leukocytes in

the blood flow [16]. Interestingly, our data showed a negative ATP hydrolysis and a positive ADP hydrolysis correlation between the leukocyte and lymphoblast counts in D0 group. In this way, it was possible that the lower NTPDase activity found in D0 group was related to the increase and survival of leukocytes in the blood flow. The reduced turnover of leukocytes in BCP-ALL patients at diagnosis may reduce the release of ATP to the extracellular environment and consequently decrease the hydrolysis of this nucleotide in the serum. However, the positive correlation in the ADP hydrolysis may be related to the intention of reducing the interaction with the P2Y receptors and increasing the production of extracellular adenosine.

ATP shows affinity to P2X, and the activation of this receptor has been related to the expansion of hematopoietic stem cells (HSCs) [39]. The analysis of extracellular purines did not show differences on ATP, ADP or AMP levels in the serum of BCP-ALL patients. However, Burnstock and Di Virgilio [40] reported a significantly higher expression of the P2X7 receptor in cells of patients with acute lymphoblastic leukemia. Chong et al. [41] found P2X1, P2X4, P2X5 and P2X7 are over expressed in the leukemia, being that P2X7 may be used to monitor the follow-up of therapy for pediatric leukemia, as the highest levels of P2X7 were detected in relapsed patients and decreased expression was observed in complete remission status.

A study of Kaljas et al. [42] showed that adenosine deaminase ADA1 and ADA2 bind to different subsets of immune cells, regardless both decrease the levels of adenosine by converting to inosine; ADA1 can act as growth factor, while ADA2 might suppress Tregs by immunosuppressive extracellular adenosine. Within an immune-tolerant microenvironment, the CD39/CD73 complex and the extracellular adenosine may alter the functions of tumor-specific T cells, favoring the suppressive state of Treg and Th17 cells, and prompting the Th2 promoting tumor response [43]. Corroborating to this data, our results showed higher levels of extracellular adenosine deaminase activity in D0 and D15. In accordance, studies point to the need of the extracellular nucleotides to be rapidly inactivated into adenosine [16]. In this way, the high levels of ADA activity found in the study suggest it was in accordance with the high levels of the adenosine to be metabolized and the pathway direction.

We observed higher levels of inosine (Ino) in the serum of the BCP-ALL patients comparing to the control group. Ino is a product of the adenosine deamination by ADA that serves as substrate to the enzyme purine nucleoside

phosphorylase (PNP) and, within the purine pathway, works as a negative feedback mechanism [16]. Since Ino is an anti-oxidant agent, we suggest its accumulation may protect the DNA of the lymphoblasts from damage, thus elevating the chance of cell survival. PNP converts inosine (Ino) and guanosine (dGUO) into hypoxanthine (Hx) and guanine. In the absence of PNP, dGUO is phosphorylated to dGTP [16,29]. It was reported by Kartajian [29], that dGTP accumulation, promoted by high dGUO phosphorylation capacity of T cells, may lead to a susceptibility to apoptosis by inhibiting DNA synthesis.

At usual conditions, the PNP product, hypoxanthine, enters the bloodstream at high levels after cell death and is catabolized by xanthine oxidase (XO) to xanthine (Xan) and uric acid (UA) [29,44]. In our study, Hx presented low levels, however Xan showed higher levels. These data added to the fact that XO activity was reduced compared to the control group, allows us to assume that xanthine comes from a source other than hypoxanthine. It may come from a salvage pathway like guanine by guanine deaminase activity. The low activity of XO may not be able to sufficiently catalize the oxidation of xanthine to uric acid. The enzyme activity of XO can increase the levels of free radicals which in high levels can increase the cell death and the pro-inflammatory responses [44,45]. However, it may possible that the reduced XO activity, found in BCP-ALL patients, may induce a protective microenvironment, whereas consequently can be related with the increased malignant cell survival in these patients at D0.

Some studies have shown a pro-inflammatory state and significant alterations in the bone marrow of patients with BCP-ALL at diagnosis that lead to survival and proliferation of the malignant cells and to impair the hematopoiesis [4,26]. The present study showed both increased leukometry and lymphoblast count in peripheral blood of BCP-ALL patients at diagnosis (D0) corroborating the proliferative state of the bone marrow and the prevalence of more immature forms from the lymphoid lineage.

Considering that the most cytokines act locally at short distances, Park et al.[46] found that lymphocytes in the bone marrow could play a more important role in anti-leukemic immunity than the lymphocytes in the peripheral blood, because they are mixed with leukemic cells and they increase the availability of cytokines at the target site. In the present study, the measurement of serum cytokines showed significant higher IL-6 levels in D0 when compared to the control group. IL-6 is

important in the maintenance of cancer stem cells in the neoplastic microenvironment, moreover, IL-6 has been reported as a sensitive predictor of bacterial infection in neutropenic as well as non-neutropenic febrile children with ALL [26].

The inflammatory IL-6 response stimulates IL-17[47]. We found significantly higher IL-17 levels in D0 when compared to the control group. In accordance with our findings, BI et al. [48] demonstrated that increased Th17 cells and decreased Th1 cells were present in peripheral blood and bone marrow from newly diagnosed B-ALL patients. They also suggested that elevated Th17 cells secrete IL-17A promoting the proliferation and resistance to daunorubicin, a chemotherapy for B-ALL. Th17 cells have controversial functions in cancer immunity, while IL-17 promotes angiogenesis and inflammation, tumor-specific Th17, on the other hand, could regulate the tumor growth by promoting cytotoxic T cells activation according the cytokine microenvironment [21].

The levels of IL-10 were significantly increased in D0 when compared to the control group. IL-10 has been associated with suppression of the immune reactions, escape of leukemia cells from immune surveillance and, at high levels, with recurrent ALL, highlighting the importance of these data as prognostic value and as markers of the efficacy of therapy [2,45]. The presence of still high levels of IL-10 on D15 group showed the initial treatment was not enough to reverse the anti-inflammatory environment of IL-10 and normalize the results.

The results from studies involving cytokines in ALL have been controversial. According to Park et al. [46], the inadequate expression of cytokines and/or abnormal activation of their receptors may trigger the clonal expansion and proliferation of lymphatic precursor cells in ALL, the prevention of apoptosis and an uncontrolled of immune modulation. After the initial therapy we found in D15 a reduction of IL-6 and IL-17. Ordonez et al. [9] showed in a study that were two groups accordingly their concentration of cytokines, one with high amounts of growth factors, interferons and proinflammatory factors; whereas the other with low or normal amounts of proinflammatory factors. Thus, the manipulation of the tumor microenvironment may auxiliary the design of childhood ALL therapeutic strategies [48].

In conclusion, the activities of the enzymes are closely related to the level of nucleotides or nucleosides in the extracellular milieu, as well to the profile of cytokines. At diagnosis, the low activities of NTPDase and xanthine oxidase, and the

high levels of adenosine deaminase activity showed that inosine levels increased as ADA sought to reduce adenosine levels, one of the most important product of the purine metabolism jointly with the ATP to modulate the immune responses. After therapy started, the increased number of lymphoblasts and leucocytes provides a great number of cells to enter in apoptosis, releasing ATP and other soluble products into the extracellular milieu so to stimulate the production of cytokines and other products, providing an environment that is detrimental to the survival of malignant cells. The enriched environment of acute leukemia has become an excellent therapeutic target to the investigation and development of drugs that may alter the host responses, as well of the leukemic cells, to improve the response of those patients with refractory leukemia or relapse.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Tables

Table 1 - Nucleotides and nucleosides metabolites concentrations in serum of BCP-ALL patients and control group.

Nucleotide/ metabolites	**Control** (nmol/mL)	**D0** (nmol/mL)
ATP	0.24 ± 0.08	0.31 ± 0.15
ADP	0.89 ± 0.41	0.44 ± 0.15
AMP	1.32 ± 0.15	1.78 ± 0.56
Adenosine	9.78 ± 0.56	24.99 ± 6.91*
Inosine	0.73 ± 0.21	3.05 ± 1.29 *
Hypoxanthine	14.61 ± 1.91	6.94 ± 1.57 **
Xanthine	24.13 ± 2.81	52.78 ± 9.51 *
Uric acid	22.95 ± 1.95	23.68 ± 3.40

Data are reported as mean ± SEM. Groups: Control (healthy subjects, n=10), D0 (newly diagnosed, n=10). BCP-ALL: B-cell precursor acute lymphoblastic leukemia. (*) and (**) indicates statistically significant differences with $P<0.05$ and $P<0.01$, respectively when compared D0 to control group. Mann-Whitney tests were used for statistical analysis.

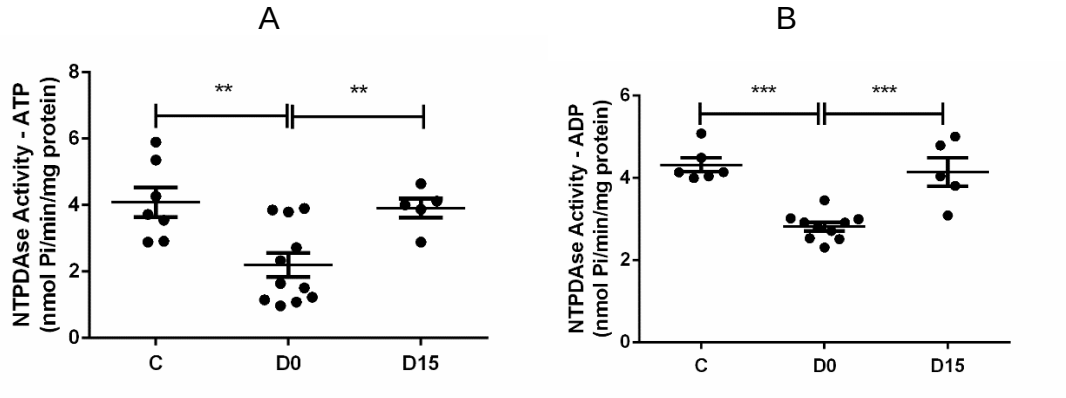
Table 2 - Correlation between leucocyte count or lymphoblast count with NTPDase (ATP, ADP), ADA and XO serum enzyme activities or nucleotides and nucleosides of adenine in serum of BCP-ALL patients at diagnosis (D0)

Variable	**Value of r**
Le X hydrolysis ATP	-0.027 (Pe)
Le X hydrolysis ADP	0.405 (Pe)
Le X deamination Ado	-0.098 (Pe)
Le X oxidation Xan	-0.489 (Pe)
Le X ATP	-0.267 (Sp)
Le X Ado	-0.433 (Sp)
Le X Ino	-0.133 (Sp)
Le X Hx	0.016 (Sp)
Le X Xan	-0.416 (Sp)
Bl X hydrolysis ATP	-0.352 (Pe)
Bl X hydrolysis ADP	0.336 (Pe)
Bl X deamination Ado	0.207 (Pe)
Bl X oxidation Xan	-0.503 (Pe)
Bl X ATP	0.067 (Sp)
Bl X Ado	-0.167 (Sp)
Bl X Ino	0.276 (Sp)
Bl X Hx	0.141 (Pe)
Bl X Xan	0.337 (Pe)

Variables are represented as D0 group (diagnosis) with n=10. All the data presented no significant statistical difference between the groups. Le: leukocyte count; Bl: lymphoblast count; Enzymes: NTPDase (ATP/ADP hydrolysis), ADA (adenosine deaminase), X0 (xanthine oxidase); Nucleotides e nucleosides: ATP, ADP, Ado, Ino, Hx, Xan, UA; Pe: Pearson; Sp: Spearman correlation. All correlations mentioned above were not statistically significant (P >0.05).

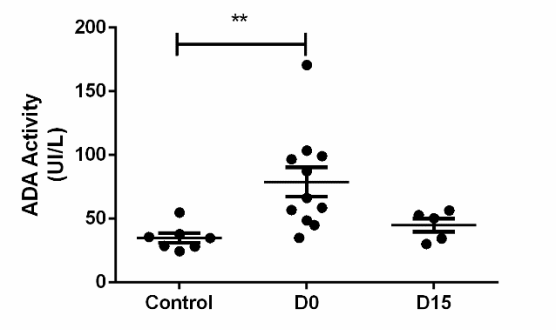
FIGURES

Figure 1 - ATP (A) and ADP (B) hydrolysis in serum of control, BCP-ALL patients D0 and D15 groups.



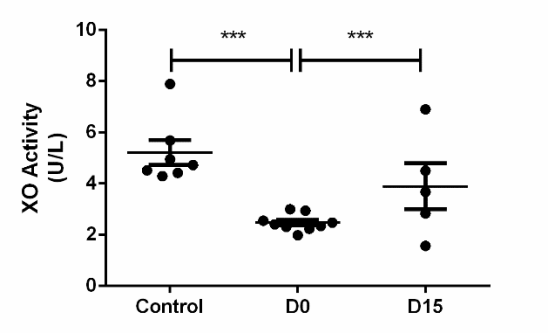
Enzyme specific activities were reported as nmol of Pi released/min/mg of protein. Variables were expressed as mean ± standard error of the mean (SEM). Bars represent mean ± S.E.M. (**), (***) indicates a significant (P<0.01, P<0.001) statistical difference, respectively, between the D0 (n=11), D15 patients (n=7) and control (n=7). One-way ANOVA with Tukey *post hoc* was used for all analyses.

Figure 2 - Adenosine activity in serum of control and BCP-ALL patients D0 and D15 groups.



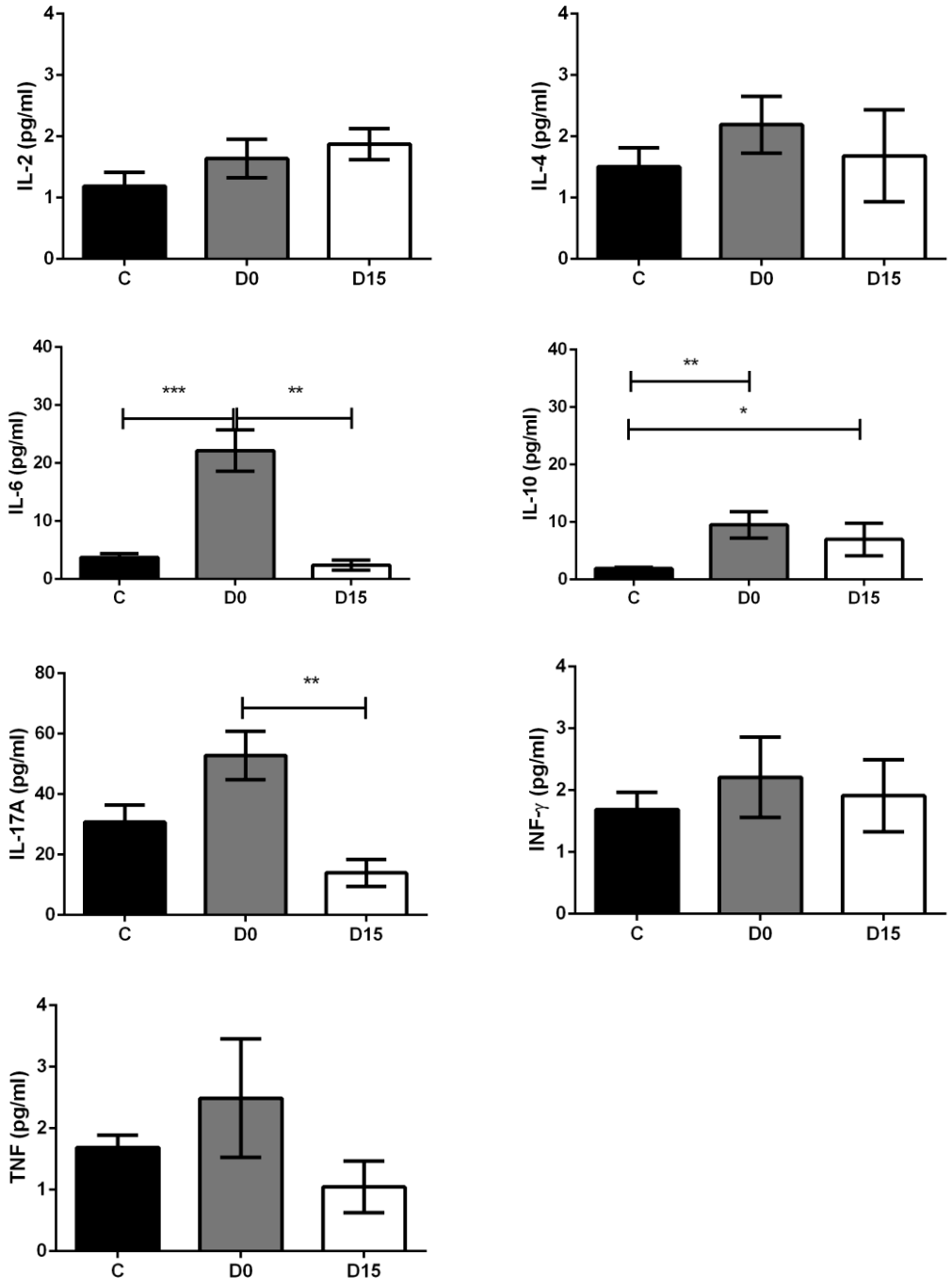
Enzyme activity was reported as U/L. Variables were expressed as mean ± standard error of the mean (SEM). Bars represent mean ± S.E.M. (**) indicates a significant difference (P<0.01) between D0 (n=11), D15 (n=7) and control (n=7) groups. One-way ANOVA with Tukey *post hoc* was used for all analyses.

Figure 3.- Xanthine oxidase activity in serum of control and BCP-ALL patients D0 and D15 groups.



Enzyme activities were reported as U/L. Variables were expressed as mean ± standard error of the mean (SEM). Bars represent mean ± S.E.M. (***) indicates a significant difference (P<0.001) between D0 (n=11), D15 (n=7) and control (n=7) groups. One-way ANOVA with Tukey *post hoc* was used for all analyses.

Figure 4 - Cytokines levels in serum of control and BCP-ALL patients D0 and D15 groups.



The cytokines levels were reported as pg/mL Bars represent mean ± S.E.M. (*) (**) and (***) indicate a statistically significant difference $P<0.05$, $P<0.01$ and $P<0.001$ respectively, between D0 (n=24), D15 (n=7) and control (n=30) groups. One-way ANOVA with Tukey *post hoc* or Kruskal-Wallis were used for statistical analyses.

4 DISCUSSAO

A leucemia linfoblástica aguda de células precursoras B (LLA-B) é uma neoplasia que apresenta heterogeneidade imunobiológica em relação à diferenciação e propriedades funcionais do tecido linfoide (BRANDALISE; PINHEIRO; LEE, 2011; PUI, 2003; STOCK, 2010;). Considerada uma neoplasia prevalente em crianças, os estudos em LLA-B melhoraram a compreensão da fisiopatologia e genética das células neoplásicas, da farmacocinética e dos mecanismos de resistência aos medicamentos, impactando nos índices de sobrevida livre de eventos (PUI et al., 2013). Apesar destes achados, para cerca de 20% dos pacientes o tratamento falha, ou a doença recai (BRANDALISE; PINHEIRO; LEE, 2011; PUI, 2013).

Neste contexto, um tema recorrente tem sido a interação da via das purinas e novos medicamentos para a terapia do câncer. O microambiente leucêmico envolve componentes-chave da via das purinas que modulam reações em células precursoras da medula óssea, além de participarem de processos inflamatórios e imunológicos (BURGLER et al., 2017; FIGUEROA et al., 2015; ORDONEZ et al., 2015; WU et al., 2005). Buscando compreender os efeitos imunomoduladores das purinas no microambiente leucêmico e no sangue periférico dos pacientes pediátricos com LLA-B, avaliou-se a atividade das enzimas NTPDase e ADA em linfócitos periféricos e no soro, XO no soro, a expressão de CD39 e CD73 em sangue periférico total e os níveis séricos de nucleotídeos*, nucleosídeos* e citocinas, ao diagnóstico (D0*) e 15 dias após o início do tratamento (D15), utilizando-se como parâmetro de comparação um grupo controle pediátrico.

A atividade de E-NTPDase nas reações de hidrólise de ATP e ADP nos linfócitos não mostrou variação em D0 na comparação com os grupos controle e D15, entretanto houve redução na atividade do grupo D15 na hidrólise do ADP na comparação com o grupo controle. Os resultados mostraram uma correlação negativa da hidrólise de ATP com as contagens leucocitária e linfoblástica no grupo D0. A baixa variabilidade na atividade de E-NTPDase no grupo D0 poderia estar relacionada a uma tolerância imunológica de supressão dos linfócitos Treg e a preservação da proliferação dos linfoblastos.

Resultados semelhantes foram encontrados por MORSCH (2006), que demonstrou que a atividade de hidrólise do ATP e do ADP pela NTPDase-1 variava

conforme a fase do tratamento da LLA, embora não apresentasse diferença significativa em relação ao grau de risco de recaída da doença. Efeito inverso foi observado por SCHETINGER et al. (2007) quando avaliaram pacientes com leucemia linfoide crônica (LLC-B), nos quais a hidrólise do ATP e do ADP estava aumentada em todos os estágios da doença, com níveis mais elevados no grupo em estágio avançado. A atividade alterada em linfócitos de pacientes com LLA (linfoblastos) e LLC B (linfócitos) evidenciam uma modulação do sistema purinérgico durante o desenvolvimento da leucemia relacionada ao grau de maturação celular.

Curiosamente, a avaliação das expressões de CD39 e CD73 mostrou resultados distintos de acordo com o grau de maturação celular. Também foi possível observar uma correlação negativa entre a expressão de CD39 em linfoblastos de D0 e a contagem de linfoblastos periféricos, bem como com a leucometria inicial. Em concordância com nossos resultados, PULTE et al. (2007) mostraram que a expressão de CD39 era de até 90% nas células B em repouso, embora não fosse identificada na linha celular Jurkat (LLA) ou em células estaminais hematopoiéticas normais. Contudo, o CD39 foi observado em precursores da medula óssea e em células leucêmicas mais diferenciadas, como LLC. Desta forma, há que se ter em conta a variabilidade no grau de expressão e nos tipos celulares envolvidos para considerar as alterações na atividade e na expressão em estudos de doenças em particular.

O antígeno de diferenciação linfoide CD73 funciona como molécula de adesão para a ligação do linfócito ao endotélio e, como sinalizadora, na ligação dos linfócitos T aos anticorpos (ANTONIOLI et al., 2016; WIETEN et al., 2011). No presente estudo, a determinação da expressão de CD73 exibiu valores significativamente mais elevados nos linfoblastos do grupo D0 quando comparados aos linfócitos do próprio grupo. Também foi possível observar uma correlação positiva entre a expressão de CD73 em linfoblastos e negativa entre a expressão de CD73 em linfócitos e a contagem de linfoblastos periféricos, bem como com a leucometria inicial. WIETEN et al. (2011), evidenciaram que a expressão de CD73 na LLA da infância varia de baixo ou ausente a valores muito elevados conforme os subgrupos imunológicos, mais maduros ou mais imaturos, respectivamente, sendo que a expressão elevada poderia estar relacionada à sensibilidade ao fármaco 6-mercaptopurina. Estudos apontaram o uso da molécula CD73 como alvo terapêutico de anticorpos monoclonais a fim de reduzir a imunossupressão e, consequentemente, a probabilidade de metástases (WANG et al., 2011; WIETEN et

al., 2011).

Yegutkin et al. (2002) mostraram que as células linfoides com atividade baixa ou inexistente de ecto-nucleotidases e expressão relativamente alta de ecto-adenosina desaminase (E-ADA) são caracterizadas pelo fenótipo “sintetiza ATP/elimina adenosina”. No estudo, a atividade da E-ADA nas reações de desaminação da adenosina se mostrou aumentada nos linfócitos dos grupos D0 e D15 na comparação com o controle. Não foram observadas correlações significativas entre o número de leucócitos e de linfoblastos com a desaminação da adenosina em linfócitos nos grupos D0 e D15. Os dados corroboram a modulação do sistema purinérgico sobre os linfócitos periféricos no sentido de reduzir os níveis de adenosina.

No ambiente extracelular, verificou-se que a atividade da NTPDase nas reações de hidrólise de ATP e ADP no soro foi menor em D0 do que nos grupos controle e D15. Embora não significativos, os dados mostraram correlação negativa da hidrólise do ATP e positiva da hidrólise do ADP com as contagens leucocitária e linfoblástica no grupo D0. A atividade da NTPDase no soro está relacionada com a liberação desta enzima por células, ou pelo turnover de leucócitos no fluxo sanguíneo (YEGUTKIN, 2008). Desta forma, parece possível que a menor atividade de NTPDase encontrada no grupo D0 esteja relacionada ao aumento e sobrevivência de leucócitos no fluxo sanguíneo. O turnover reduzido de leucócitos em D0 pode reduzir a liberação de ATP para o espaço extracelular e, consequentemente, diminuir a hidrólise desse nucleotídeo no soro. No entanto, a correlação positiva na hidrólise do ADP pode estar relacionada ao propósito de reduzir a interação com os receptores P2Y e aumentar a produção de adenosina extracelular.

O ATP mostra afinidade com o P2X e a ativação desse receptor tem sido relacionada à expansão de células precursoras hematopoiéticas (HSCs) (FENG et al., 2015). A avaliação de purinas extracelulares não mostrou variação nos níveis de ATP, ADP ou AMP no soro de pacientes com LLA-B. No entanto, BURNSTOCK, DI VIRGILIO (2013) relataram expressão significativamente maior do receptor P2X7 em células de pacientes com LLA ao diagnóstico. CHONG et al. (2010) evidenciaram uma maior expressão de P2X1, P2X4, P2X5 e P2X7 em leucemias, sendo que o P2X7 pode ser usado no monitoramento da terapia para leucemia pediátrica, pois os

níveis mais altos de P2X7 foram detectados em pacientes com recaída e a diminuição da expressão foi observada na fase de remissão completa.

No presente estudo, a atividade da ADA extracelular se mostrou aumentada no soro do grupo D0 na comparação com o controle. Entretanto, não foram observadas correlações significativas entre o número de leucócitos e de linfoblastos com a desaminação da adenosina no soro nos grupos D0 e D15. Estudos apontam para a necessidade de inativação dos nucleotídeos extracelulares rapidamente à adenosina (YEGUTKIN, 2008), portanto, o aumento da atividade da ADA no soro evidenciaria uma regulação do receptor P1 para reduzir os níveis de adenosina no soro.

As ações do ATP e da adenosina em células imunes podem ser simultâneas e interdependentes. Portanto, durante os aumentos nos níveis de nucleotídeos extracelulares, a cascata de purinas pode ser redirecionada através da fosforilação do AMP a ADP pela ecto-adenilato quinase, ou por desaminação para inosina, pela ADA (LONGHI et al., 2013; SAKOWICZ et al., 2010; YEGUTKIN et al., 2002; ZIMMERMANN, 2000;). Encontramos níveis mais elevados de adenosina, inosina e xantina, e mais reduzidos de hipoxantina, no soro de pacientes com LLA-B ao diagnóstico (D0) do que em controles saudáveis. Uma vez que a inosina é um agente antioxidante, sugere-se que sua acumulação pode proteger o DNA dos linfoblastos de danos, elevando assim a chance de sobrevivência das células neoplásicas.

Os níveis significativamente mais baixos de hipoxantina poderiam ser decorrentes da diminuição da atividade da purina nucleosídeo fosforilase (PNP), embora esta seja apenas especulação, já que a PNP não foi medida. A hipoxantina entra na corrente sanguínea em níveis elevados após a morte celular e é catabolizada pela xantina oxidase para xantina e ácido úrico, um produto nefrotóxico quando em altos níveis (YAMAGUCKI, 2013). Surpreendentemente, os níveis de xantina sérica mostraram-se aumentados em D0, enquanto a atividade da xantina oxidase encontrava-se diminuída, sugerindo-se que a xantina poderia provir de outra fonte que não a hipoxantina. A xantina é considerada um ponto de convergência do metabolismo das purinas, inferindo-se que uma das possibilidades poderia ser atribuída à guanina por meio da ação da guanina desaminase (SNYDER et al., 2002; STOYCHEV, KIERSDAZUK, SHUGAR, 2002). A atividade da XO pode aumentar os níveis de radicais livres que, quando elevados, aumentam a morte celular e as

respostas pró-inflamatórias (YEGUTKIN, 2008). É possível que a atividade reduzida da XO possa induzir um microambiente protetor que, consequentemente, estaria relacionado com o aumento da sobrevida das células neoplásicas nesses pacientes.

O sistema purinérgico e as citocinas têm uma estreita relação na modulação da resposta imune e inflamatória. Durante a hematopoiese, as citocinas e seus receptores contribuem para a sobrevivência celular, a proliferação e a diferenciação por meio de uma rede funcional (RUSSELL et al., 2009).

A avaliação das citocinas séricas apresentou níveis significativamente aumentados de IL-6, IL-17 e IL-10 em D0 quando comparados ao grupo controle. A IL-6 está relacionada a um estímulo pró-inflamatório, sendo importante na indução das proteínas de fase aguda e preservação das células no microambiente neoplásico. A resposta pró-inflamatória da IL-6 estimula a produção da IL-17 (FAYAD et al., 2001; FIGUEROA, 2015). Esta via apresenta funções controversas. Enquanto a IL-17 estaria associada à proliferação de células B, os linfócitos Th17 promoveriam a ativação de células T citotóxicas conforme a constituição do microambiente de citocinas (BI et al., 2016; GHIRINGHELLI et al., 2012).

Numa mostra da dinâmica das citocinas, os níveis elevados de IL-10 com função anti-inflamatória, têm sido associados à supressão das reações imunes, ao escape das células leucêmicas à vigilância imunológica e à recaída medular na LLA (PARK, 2006; WU et al., 2005). Este efeito induz a proliferação e a diferenciação dos linfócitos B via inibição de citocinas do complexo Th1 (FAYAD et al., 2001). Considerando o grupo D15, a presença de ainda altos níveis de citocinas sugere que o tratamento inicial ainda não foi suficiente para normalizar os resultados. Ordoñez et al. (2015), identificaram uma estreita associação entre o microambiente inflamatório e o desenvolvimento da LLA, que poderia permitir a manipulação como estratégia terapêutica.

Estudos mostram que um estado pró-inflamatório e alterações significativas na medula óssea de pacientes com LLA-B ao diagnóstico favorecem a sobrevivência e a proliferação das células neoplásicas e prejudicam a hematopoiese normal (BURGLER et al., 2017; FIGUEROA et al., 2015). Dentro de um microambiente imunotolerante, o complexo CD39/CD73 e a adenosina extracelular podem alterar as funções das células T específicas de tumor, favorecendo o estado supressor das células Treg e Th17 e levando à resposta tumoral Th2 (Antonioli, 2013). O presente estudo mostrou aumento da leucometria e dos linfoblastos no sangue periférico de pacientes LLA-B ao diagnóstico (D0), corroborando o estado proliferativo da medula óssea e a prevalência de formas mais imaturas da linhagem linfoide.

O estudo desenvolvido para avaliação da atividade e da expressão de enzimas da sinalização purinérgica, associado à avaliação dos níveis de citocinas em leucemia linfoblástica aguda B derivada (LLA-B) permitiu concluirmos que as mudanças observadas nas atividades de E-NTPDase e E-ADA ao diagnóstico demonstram a forte contribuição da sinalização purinérgica na modulação das células imunes, de modo a equilibrar os perfis imunossupressor e inflamatório resultantes da leucemia linfoblástica aguda. No início, havia um microambiente pró-inflamatório, que pode ter facilitado a proliferação de células neoplásicas. Ao mesmo tempo, expressões diferenciadas de CD39 e CD73 em linfócitos e linfoblastos de pacientes com LLA-B corroboram a relação dessas moléculas com o desenvolvimento clonal de células neoplásicas e a via fisiológica. Os resultados sugerem uma possível modulação do sistema purinérgico durante o desenvolvimento de células leucêmicas que podem ser revertidos, em parte, pela terapia inicial e consequente redução na contagem de leucócitos e linfoblastos.

5 CONCLUSÃO

- A atividade da E-NTPDase não apresentou variação significativa na hidrólise do ATP e do ADP em linfócitos, exceto após 15 dias de tratamento onde houve uma redução na hidrólise do ADP. Observou-se que a hidrólise do ATP tem correlação negativa e significativa com o número de leucócitos assim como com o número de linfoblastos no sangue periférico. A atividade da E-ADA em linfócitos mostrou aumento significativo ao diagnóstico e após 15 dias de tratamento. Considerando-se o efeito sobre as células imunes, parece ter havido uma reação do sistema purinérgico no sentido de reverter o estado anti-inflamatório e imunossupressor promovido pela adenosina e facilitar uma resposta anti-neoplásica do sistema imune.
- A expressão de CD39 e de CD73 mostraram resultados distintos conforme o tipo celular avaliado. Linfoblastos exibiram menor expressão de CD39 e maior de CD73. As expressões em linfócitos e linfoblastos de pacientes com LLA têm relação com a contagem de leucócitos e de linfoblastos no sangue periférico, refletindo o estado de maturação das células, mesmo nas amostras de pacientes que co-apresentam populações normais e patológicas.
- As atividades da NTPDase e xantina oxidase mostraram-se reduzidas no soro ao diagnóstico, enquanto a da ADA apresentou-se aumentada. A atividade reduzida pode estar relacionada à baixa liberação de ATP para o espaço extracelular decorrente do baixo turnover de leucócitos ao diagnóstico. A elevação da atividade da ADA parece estar relacionada aos níveis de adenosina no meio extracelular, que podem ser provenientes de outras fontes que não leucócitos.
- Os níveis de adenosina e inosina aumentados refletem as atividades enzimáticas, enquanto os níveis elevados de xantina podem estar relacionados à atividade catabólica de outras vias produtoras de xantina, uma vez que a hipoxantina mostrou-se diminuída e a atividade da xantina oxidase, reduzida.
- A relação das citocinas pró-inflamatórias IL-6 e IL-17 e anti-inflamatória IL-10 que estão aumentadas ao diagnóstico parece contribuir para o estado de ineficiência da resposta imune, o que possibilitaria a proliferação das células neoplásicas neste estágio da doença. Os resultados em D15 refletem sinais do tratamento inicial sobre as células neoplásicas, como a apoptose e a redução no número de

leucócitos, desencadeando redução de citocinas pró-inflamatórias e estimulação de outras células do sistema imune pela liberação de ATP e outros produtos solúveis no meio extracelular.

6 PERSPECTIVAS FUTURAS

Este estudo forneceu subsídios importantes para o esclarecimento da relação entre as enzimas do sistema purinérgico e a leucemia linfoblástica aguda B derivada de pacientes pediátricos. No entanto, alguns aspectos precisam ser esclarecidos.

Dessa forma, para complementar os estudos pretende-se:

- Determinar a expressão do receptor P2X7, por Western blotting, buscando complementar o estudo das alterações detectadas, assim como os fatores que contribuíram para o desfecho.
- Determinar a atividade das enzimas 5’-NT e PNP nas amostras utilizadas no estudo.
- Realizar experimento in vitro para analisar o efeito da terapia medicamentosa na inibição das enzimas da via das purinas.
- Avaliar as proteínas de apoptose Bax e Bcl-2, por citometria de fluxo, em linfócitos/linfoblastos de amostras utilizadas no estudo e que estão armazenadas sob congelamento.
- Quantificar a atividade das caspases 1, 3, 8, 9 em amostras utilizadas no estudo e que estão armazenadas sob congelamento.

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Apêndice A - Termo de consentimento livre e esclarecido

Título do projeto: **"Leucemia linfocítica aguda: estudo da expressão de proteínas relacionadas à apoptose, resistência a múltiplas drogas e sinalização purinérgica"**
Pesquisador responsável: **Prof. Dra. Daniela Bitencourt Rosa Leal**
Instituição: **Universidade Federal de Santa Maria – Centro de Ciências da Saúde**
Pesquisadores participantes: Ms. Liliane Zimmermann de Oliveira, Prof. Dr. José Edson Paz da Silva, Dra. Virginia Maria Cóser, Ms. Pâmela Brum Soares, Ms. Cláudia Bertoncelli, João Felipe Peres Rezer, Pedro Henrique Doleski
Telefone para contato: (55) 96236614 (Liliane) ou (55) 32209581 (Daniela)

Local da coleta dos dados: _____
Nome do paciente: _____Idade: _____Sexo:_____
Responsável legal: _____

Objetivo do estudo/Riscos/Procedimentos/ Benefícios/Sigilo:

Você está sendo convidado(a) a participar como voluntário de uma pesquisa, tendo o direito de desistir a qualquer momento sem punição.

Objetivo: a pesquisa buscará informações sobre os aspectos laboratoriais de pacientes adultos e pediátricos portadores de Leucemia Linfocítica Aguda (LLA) e suas implicações na evolução da doença.

Procedimento e riscos: os exames laboratoriais realizados por você no HUSM serão avaliados pelos pesquisadores responsáveis por meio dos registros no Serviço de Hematologia e Oncologia. Para o estudo, será analisado o material biológico excedente (sangue e/ou medula óssea) coletado de você por ocasião da investigação para o diagnóstico. As amostras coletadas por profissionais capacitados fazem parte da rotina para o diagnóstico e tratamento da Leucemia Linfocítica Aguda no Serviço de Hematologia e Oncologia do HUSM, havendo o risco inerente ao procedimento de coleta do material, como desconforto pela picada da agulha, podendo o local ficar dolorido ou arroxeado, porém com retorno ao normal em poucos dias, sem prejuízo para a saúde. O tempo de duração previsto para a sua participação na pesquisa será durante esta análise.

Benefícios: os resultados do estudo não trarão benefícios diretos, porém sua contribuição é importante e consiste apenas para ajudar novos estudos sobre a evolução e tratamento da doença. O projeto não lhe trará custos financeiros, não haverá recompensa pela sua participação e não ocorrerá penalidade caso você não aceite participar da pesquisa.

Confidencialidade: sua identidade e dados pessoais não serão revelados, nem divulgados sem a sua autorização. Apenas os pesquisadores terão acesso às informações pessoais e aos resultados dos exames, os quais serão utilizados em conjunto com os dados de outros pacientes para a avaliação do estudo e em publicações científicas.

Eu _____ (paciente ou responsável), após ler/ouvir as informações sobre a pesquisa e esclarecer minhas dúvidas, concordo voluntariamente em participar desse estudo. Discuti com o pesquisador _____ sobre a minha decisão em participar dessa pesquisa, ficando claro para mim os propósitos do estudo, os procedimentos a serem realizados, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades, prejuízos ou perda de qualquer benefício que eu possa ter adquirido no meu tratamento neste serviço.

Santa Maria, ___ de _____ de 20 ___.

Paciente ou responsável legal: _____RG: _____

(para casos de pacientes menores de 18 anos, analfabetos, semianalfabetos ou portadores de deficiência auditiva ou visual).

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste sujeito da pesquisa ou representante legal para a participação neste estudo.

Pesquisador responsável: _____

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato: Comitê de Ética em Pesquisa – UFSM - Cidade Universitária - Bairro Camobi, Av. Roraima, nº1000 - CEP: 97.105.900 Santa Maria – RS. Telefone: (55) 3220-9362 – Fax: (55)3220-8009 Email: comiteeticapesquisa@smail.ufsm.br. Web: www.ufsm.br/cep

Apêndice B - Termo de confidencialidade

Título do projeto: **LEUCEMIA LINFOCÍTICA AGUDA: ESTUDO DA EXPRESSÃO DE PROTEÍNAS RELACIONADAS À APOPTOSE, RESISTÊNCIA A MÚLTIPLAS DROGAS E SINALIZAÇÃO PURINÉRGICA**

Pesquisador responsável: **Prof. Dra. Daniela Bitencourt Rosa Leal**

Instituição: **Universidade Federal de Santa Maria – Centro de Ciências da Saúde**

Pesquisadores participantes: Ms. Liliane Zimmermann de Oliveira, Prof. Dr. José Edson Paz da Silva, Dra. Virginia Maria Cóser, Ms.Pâmela Brum Soares, Ms.Cláudia Bertoncelli, João Felipe Peres Rezer, Pedro Henrique Doleski

Telefone para contato: (55) 96236614 (Liliane) ou (55) 32209581 (Daniela)

Os pesquisadores do presente projeto assumem o compromisso de preservar a privacidade dos pacientes cujos dados serão coletados em laudos dos exames realizados no Hospital Universitário de Santa Maria e concordam igualmente, que estas informações serão utilizadas única e exclusivamente para execução do presente projeto. As informações somente poderão ser divulgadas de forma anônima e serão mantidas na sala número 4102 do Departamento de Microbiologia e Parasitologia da UFSM, prédio 20, por um período de dois anos após o término da pesquisa, sob a responsabilidade da Pesquisadora responsável. Após este período, os dados serão destruídos.

Este projeto de pesquisa foi revisado e aprovado pelo Comitê de Ética em Pesquisa da UFSM em __/__/20__, com o número do CAAE: _____.

Santa Maria, ___ de _____ de 20___.

Pesquisador responsável: _____
Prof. Dra. Daniela Bitencourt Rosa Leal