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Cesar Eduardo Jacintho Moritz

EFEITOS DO EXERCÍCIO AERÓBICO AGUDO NAS NUCLEOTIDASES SOLÚVEIS NO SANGUE DE INDIVÍDUOS ADULTOS SEDENTÁRIOS E FISICAMENTE ATIVOS

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Orientador: Prof. Dr. Alvaro Reischak de Oliveira
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“Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.”

Invicuts
William Ernest Henley, 1875.

RESUMO

INTRODUÇÃO: O exercício físico é um importante componente para manutenção da saúde, atuando na prevenção e no tratamento de diferentes doenças. Os benefícios relacionados ao exercício físico são derivados de adaptações teciduais de curto e longo prazo, coordenadas por diferentes mecanismos de comunicação celular. Assim, o sistema purinérgico é um mecanismo de comunicação intercelular que participa da regulação de respostas inflamatórias, coagulatórias, vasculares e cardíacas. Dados prévios sugerem a atuação fisiopatológica e o potencial terapêutico da sinalização purinérgica em doenças cardiovasculares, metabólicas, neurológicas e oncológicas. Mesmo a sinalização purinérgica sendo um importante modulador de funções intimamente vinculadas ao exercício, sua ação nas respostas agudas e crônicas ao exercício é controversa, principalmente em populações com diferentes características antropométricas e metabólicas.

OBJETIVO: 1) Revisar sistematicamente os efeitos de diferentes modalidades de exercício físico, agudo e crônico, na funcionalidade de enzimas purinérgicas de sujeitos saudáveis e não saudáveis; 2) Avaliar os efeitos do exercício aeróbico de intensidade moderada, na funcionalidade de enzimas purinérgicas solúveis e em todo o espectro do metabolismo ATP, no plasma sanguíneo de sujeitos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos. Além disso, investigar possíveis relações entre marcadores cardiometabólicos, inflamatórios e parâmetros purinérgicos.

MÉTODOS: 1) A busca foi realizada em diferentes bancos de dados eletrônicos, *MEDLINE, EMBASE, Cochrane Library* e *Web of Science* para identificar ensaios clínicos randomizados, ensaios clínicos não randomizados, ensaios clínicos não controlados, estudos quase-experimentais e pré e pós-intervenção. Dois revisores independentes realizaram a seleção dos estudos, extração de dados e análise da qualidade metodológica; 2) Vinte e quatro homens (n=24), com idades entre 20 e 30 anos, foram divididos em três grupos: 1) eutrófico sedentário (NWSED, n=8), 26.37±2.97 anos, índice de massa corporal (IMC) 23.40±2.01 Kg/m^2 e consumo de oxigênio de pico (VO_{2PICO}) 43.96±1.09 mL/Kg/min; 2) sobrepeso sedentário (OWSED, n=8), 25.75±2.91 anos, IMC 27.75±1.26 Kg/m^2, VO_{2PICO} 38.82±2.48 mL/Kg/min; eutrófico fisicamente ativo (PHACT, n=8), 23.12±3.18 anos, IMC 23.65±1.20 Kg/m^2, VO_{2PICO} 50.30±2.34 mL/Kg/min. Os voluntários participaram do nosso desenho experimental composto por dois dias; no primeiro dia os participantes realizaram uma entrevista para coleta do histórico clínico, hábitos alimentares e rotina de exercícios semanais, posteriormente era realizada a avaliação antropométrica e o teste cardiopulmonar máximo. No segundo dia, sete dias após a

primeira visita, os participantes eram submetidos a trinta minutos de exercício em esteira rolante e intensidade de 70% VO_{2PICO}. As coletas de sangue eram feitas em jejum ao chegar no laboratório, imediatamente após o fim do exercício e 1h após o fim do exercício. Foram mensuradas atividade das enzimas purinérgicas, níveis de adenosina 5'-trifosfato (ATP) e seu metabólitos, interleucina 1β (IL-1β), fator de necrose tumoral α (TNF-α), interleucina 8 (IL-8), interleucina 10 (IL-10), glicose, colesterol total (CT), triglicerídeos, lipoproteína de alta densidade (HDL) e lipoproteína de baixa densidade (LDL). Todos os resultados estão apresentados em média e desvio padrão (DP) e α=5%.

RESULTADOS: 1) Dos 203 artigos identificados pela estratégia de busca, doze foram incluídos nesta revisão sistemática, respeitando os critérios de inclusão e exclusão. Oito estudos reportaram que o exercício agudo, em sujeitos saudáveis e não saudáveis, elevou a atividade e/ou expressão das nucleotidases. Quatro estudos demonstraram uma diminuição na atividade das nucleotidases em plaquetas e linfócitos de paciente com síndrome metabólica, doença renal crônica e hipertensão, após diferentes protocolos de exercício crônico; 2) Imediatamente após o protocolo de exercício, os participantes apresentaram um aumento na hidrólise de ATP, ADP, AMP e *p*-Nph-5'-TMP, enquanto somente a hidrólise de AMP permaneceu aumentada 1h após o exercício. Os níveis plasmáticos de ATP e ADP diminuíram imediatamente e após 1h de exercício de forma similar em todos os grupos. Por outro lado, os níveis plasmáticos de adenosina e inosina aumentaram após o exercício, porém somente a adenosina permaneceu elevada 1h após o exercício. Em relação aos marcadores inflamatórios, o nosso protocolo de exercício aumentou significativamente os níveis de TNF-α e IL-8 após o exercício, contudo apenas o TNF-α continuou elevado após 1h. Nossas análises indicaram correlações significativas entre a atividade da 5'-nucleotidase, níveis de adenosina, VO_{2PICO}, triglicerídeos, e os níveis de TNF-α e IL-8.

CONCLUSÕES: Os resultados obtidos com essa tese de doutorado indicam que a sinalização purinérgica participa, pelo menos parcialmente, nas respostas inflamatórias, trombóticas e cardiovasculares desencadeadas pelo exercício físico moderado e de alta intensidade. Em relação especificamente aos exercícios de intensidade moderada, as respostas das nucleotidases solúveis são similares entre indivíduos com diferentes características antropométricas e capacidade cardiorrespiratória.

Palavras-chaves: Exercício; ATP; Adenosina; NTPDases; 5'-nucleotidase; Inflamação.

ABSTRACT

INTRODUCTION: Physical exercise is an important element for health maintaining, acting in the prevention and treatment of several diseases. The health benefits related to exercise are derived from short- and long-term tissue adaptations regulated by pathways of cell signaling. Thus, purinergic signaling is an extracellular communication system, that modulated inflammation, coagulation and cardiovascular activity. Previous data showed the significant pathophysiology role and therapeutic potential of purinergic signaling. Therefore, even though purinergic signaling is an important modulator of functions closely linked to exercise, its role in acute and chronic responses to exercise is controversial, especially in relation to populations with different anthropometric and metabolic characteristics.

OBJECTIVE: 1) The objective of this study was to systematically review the effects of different modalities of acute and chronic exercise in the functionality of purinergic enzymes in healthy and unhealthy subjects; 2) To evaluate the effects of acute moderate-intensity aerobic exercise, in the functionality of soluble purinergic enzymes and the whole spectrum of ATP metabolism, in the blood plasma of normal weight sedentary, overweight sedentary and physically active individuals. Moreover, assess possible relationships between cardiometabolic, inflammatory markers and purinergic parameters.

METHODS: 1) The MEDLINE, EMBASE, Cochrane Library and Web of Science databases were searched to identify, randomized clinical trials, non-randomized clinical trials, uncontrolled clinical trials, quasi-experimental, pre- and post-interventional studies that evaluated the acute or chronic effects of exercise on nucleotidases in humans. Two independent reviewers performed the study selection, data extraction and assessment of risk of bias; 2) Twenty-four male volunteers (n=24), age between 20 and 30 years, were divided into three groups: 1) normal weight sedentary (NWSED, n=8), 25.75±2.91 years, body mass index (BMI) 23.40±2.01 Kg/m^2 and peak oxygen uptake (VO_{2PEAK}) 43.96±1.09 mL/Kg/min; 2) overweight sedentary (OWSED, n=8), 25.75±2.91 years, BMI 27.75±1.26 Kg/m^2 and VO_{2PEAK} 38.82±2.48 mL/Kg/min; 3) normal weight physically active (PHACT, n=8), 23.12±3.18 years, BMI 23.65±1.20 Kg/m^2 and VO_{2PEAK} 50.30±2.34 mL/Kg/min. The volunteers participated of our experimental design composed for two days. On the first day (day 1) the participants realized an interview for the collection of clinical data, eating habits, and weekly exercise routine after that was performed the anthropometric evaluation and cardiopulmonary exercise test. On the second day (day 2), seven days after the first visit, the participants were submitted to thirty

minutes of moderate-intensity exercise on the treadmill at 70% of VO_{2PEAK}. Blood samples were taken at baseline, after 8h of fasting, immediately post-exercise and 1h after exercise. Were assessed the activity of purinergic enzymes, ATP levels and metabolites, interleukin 1β (IL-1β), tumor necrosis factor (TNF-α), interleukin 8 (IL-8), interleukin 10 (IL-10), glucose, cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Results are expressed as mean ± standard deviation (SD) and α=5%.

RESULTS: 1) Eight studies reported that acute exercise, in healthy and unhealthy subjects, elevated the activities or expression of nucleotidases. Four studies evaluated the effects of chronic training on nucleotidase activities in the platelets and lymphocytes of patients with metabolic syndrome, chronic kidney disease and hypertension and found a decrease in nucleotidase activities in these conditions; 2) Immediately post-exercise, all subjects showed increases in ATP, ADP, AMP and *p*-Nph-5'-TMP hydrolysis, while AMP hydrolysis remained increased at 1h after exercise. The plasma levels of ATP and ADP decreased at post- and 1h post-exercise in all groups. However, adenosine and inosine levels increased at post-exercise, but only adenosine remained augmented at 1h after exercise in all groups. With regard to inflammatory responses, the exercise protocol increased TNF-α and IL-8 concentrations in all subjects, but only TNF-α remained elevated at 1h after exercise. Our analysis demonstrated significant correlations between the activity of 5'-NT, adenosine levels, $\dot{V}O_{2peak}$, triglyceride, TNF-α and IL-8 levels.

CONCLUSIONS: The results obtained with this doctoral thesis showed that purinergic signaling participates, at least partially, for inflammatory, thrombotic and cardiovascular responses triggered by moderate- and high-intensity exercise. Related to moderate-intensity aerobic exercise, the nucleotidase response are similar between individuals with distinct anthropometric characteristics and cardiorespiratory fitness.

Keywords: Exercise; ATP; Adenosine; NTPDases; 5'-nucleotidase; Inflammation.

LISTA DE ABREVIATURAS

5’-NT – 5’-nucleotidase

ADO – Adenosina

ADA – Adenosina deaminase

ADP – Adenosina 5’-difosfato

AMP – Adenosina 5’-monofosfato

AMPc – Adenosina 3’,5’-monofosfato cíclico

ATP – Adenosina 5’-trifosfato

AU – Ácido úrico

AVC – Acidente vascular cerebral

CEPE – Comitê de Ética em Pesquisa

CT – Colesterol total

DAC – Doença arterial coronariana

DP – Desvio padrão

DVP – Doença vascular periférica

E-5’-NT/CD73 – Ecto-5’-nucleotidase/CD73

E-NTDPase1/CD39 – Ecto-nucleosídeo trifosfato difosfoidrolase 1

E-NTPDase2/CD39L1 – Ecto-nucleosídeo trifosfato difosfoidrolase 2

GUO – Guanosina

HDL – Lipoproteína de alta densidade

HX – Hipoxantina

HPLC – Cromatografia líquida de alta performance

IL-1β – Interleucina 1β

IL-10 – Interleucina 10

IL-8 – Interleucina 8

IMC – Índice de massa corporal

INO – Inosina

LDL – Lipoproteína de baixa densidade

MINORS – Índice metodológico para estudos não randomizados

NAD^+ – nicotinamida adenina dinucleotídeo

NE – Norepinefrina

NPPs – Nucleotídeo pirofosfatases/fosfodiesterases

NT – Transportadores de nucleosídeos

NTPDases – Nucleosídeo trifosfato difosfoidrolases

NWSED – Grupo eutrófico sedentário

O_2 – Oxigênio

OWSED – Grupo sobrepeso sedentário

P1 – Receptores purinérgicos do tipo P1

P2 – Receptores purinérgicos do tipo P2

PA – Pressão arterial

PHACT – Grupo eutrófico fisicamente ativo

Pi – Fosfato inorgânico

PPi – Pirofosfato inorgânico

p-Nph-5'-TMP – *p*-nitrofenil 5'-timinidina monofosfato

TCLE – Termo de consentimento livre e esclarecido

TNF-α – Fator de necrose tumoral α

VEs – Vesículas extracelulares

$VO_{2MÁX}$ – Consumo máximo de oxigênio

VO_{2PICO} – Consumo de oxigênio de pico

XT – Xantina

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1 APRESENTAÇÃO

A presente tese de doutorado foi instigada a partir de questionamentos sobre diferentes aspectos das adaptações celulares e moleculares promovidas pelo exercício físico. Assim sendo, o foco desse trabalho foi esclarecer e desenvolver novos conhecimentos sobre a relação entre o exercício físico e a sinalização purinérgica, em indivíduos com diferentes características biológicas e capacidade cardiorrespiratória. Esse tema é de eminente importância pois a sinalização purinérgica é reguladora de diversas atividades bioquímicas e fisiológicas intimamente ligadas ao exercício físico, assim como: ritmo cardíaco, função vascular, inflamação e coagulação.

Posto isto, esta tese está estruturada da seguinte forma:

1) Introdução: uma breve revisão bibliográfica sobre os temas abordados na tese e justificativa para a realização da pesquisa.
2) Capítulo I (artigo 1 – revisão sistemática): *Effects of physical exercise on the functionality of human nucleotidases: A systematic review*, submetido à revista *Purinergic Signalling*.
3) Capítulo II (artigo 2 – artigo original): *Exercise promotes purinergic and inflammatory responses in sedentary, overweight and physically active subjects,* aceito para publicação na revista *Experimental Physiology*.
4) Conclusões: aspectos finais da tese, apanhado geral de resultados e contribuição do trabalho para o desenvolvimento de novos conhecimentos na área de ciências do movimento humano e saúde.
5) Perspectivas futuras: trabalhos subsequentes para o desenvolvimento do tema.

O documento está de acordo com o regimento interno do Programa de Pós-Graduação em Ciências do Movimento Humano, a partir da resolução 10/2014 do Comitê de Ética em Pesquisa (CEPE)/UFRGS. O projeto foi aprovado pelo CEPE/UFRGS (CAAE 79422417.2.0000.5347), com o título “Efeitos do exercício aeróbico agudo e crônico nas nucleotidases solúveis e associadas a microvesículas no sangue de indivíduos adultos sedentários e fisicamente ativos”. Todos os participantes assinaram o termo de consentimento livre e esclarecido para participação no estudo (TCLE).

2 INTRODUÇÃO

O conceito sobre um sistema de comunicação extracelular envolvendo nucleotídeos e nucleosídeos começou a ser moldado a partir de 1929, quando foi publicado o primeiro trabalho demonstrando principalmente a ação da adenosina (ADO) sobre o sistema cardiovascular, provocando bradicardia, vasodilatação coronária e diminuição da pressão arterial (PA) (DRURY; SZENT-GYÖRGYI, 1929). Apesar disso, somente em 1972 que nucleotídeos e nucleosídeos, como adenosina 5'-trifosfato (ATP) e ADO, respectivamente, foram sugeridos como moléculas biossinalizadoras (BURNSTOCK, 1972).

Atualmente a sinalização purinérgica é considerada um elegante e complexo mecanismo de comunicação intercelular, influenciando aspectos relacionados à saúde e doença, identificado em diferentes tipos celulares, incluindo, cardiomiócitos, plaquetas, linfócitos, endotélio, neurônios e astrócitos (BURNSTOCK, 2017; HECHLER; GACHET, 2015; KÖHLER *et al*., 2007; LEDDEROSE *et al*., 2016). No entanto, até o início da década de 90, a concepção sobre sinalização purinérgica não era efetivamente aceita pela comunidade científica. Assim, somente após a completa caracterização dos receptores purinérgicos, e suas respectivas subdivisões, que os potenciais bioquímicos, fisiológicos, fisiopatológicos e terapêuticos aplicados ao sistema purinérgico foram explorados, com o crescente número de trabalhos publicados (BURNSTOCK, 2017; RALEVIC; BURNSTOCK, 1998).

Assim como outros sistemas de comunicação celular, o sistema purinérgico é composto moléculas biossinalizadoras, enzimas reguladoras e receptores específicos. Nesse sentido, além do ATP e ADO, outros nucleotídeos e nucleosídeos podem exercer a função de biossinalizadores extracelulares, como adenosina 5'-difosfato (ADP), inosina (INO), guanosina 5'-trifosfato (GTP) e guanosina (GUO) (YEGUTKIN, 2008). Para que desencadeiem seus efeitos, essas moléculas precisam ligar-se a receptores purinérgicos, os quais são divididos amplamente em P1 e P2. Os nucleotídeos exercem seus efeitos via receptores P2, já os nucleosídeos, como ADO e INO, ligam-se especificamente aos receptores P1, subdivididos em A_1, A_{2A}, A_{2B} e A_3, todos esses receptores metabotrópicos, ligados à proteínas G. Em contrapartida, os receptores P2, ativados por ATP e ADP, são primariamente divididos em P2X e P2Y. Os receptores P2X são ionotrópicos, de ação rápida, e subdividem-se em $P2X_{1\text{-}7}$; adicionalmente, os receptores P2Y são divididos em $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$, todos metabotrópicos, ligados às proteínas G (HECHLER; GACHET, 2015; RALEVIC; DUNN, 2015).

Estímulos mecânicos, hipóxia e alterações na pressão hidrostática contribuem para a liberação dessas moléculas sinalizadoras para o ambiente extracelular, possibilitando que elas se liguem à receptores e sejam subsequentemente degradadas por ação de enzimas específicas descritas abaixo. O ATP pode ser liberado via necrose ou apoptose, mecanismos de lise celular, sinalizando dano ou morte celular, sendo essencial para o desenvolvimento da resposta inflamatória (FAAS; SÁEZ; DE VOS, 2017). Outros mecanismos relevantes para liberação de nucleotídeos e nucleosídeos são através de canais ionotrópicos voltagem e mecano-depedentes, difusão facilitada, exocitose e canais de conexina e panexina (mecanismos não dependentes de lise celular) (YEGUTKIN, 2008; LOHMAN; ISAKSON, 2014). Adicionalmente, os canais de conexina e panexina destacam-se por contribuírem para liberação de outras moléculas sinalizadoras, como o glutamato, glutationa, nicotinamida adenina dinucleotídeo (NAD^+) e prostaglandina E2 (CHEUNG; CHEVER; ROUACH, 2014).

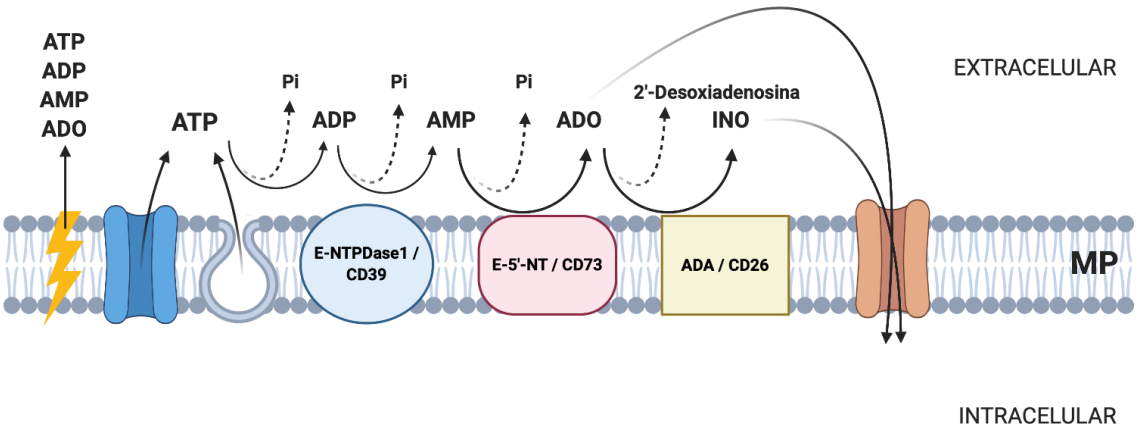
As enzimas purinérgicas, genericamente denominadas como nucleotidases, são responsáveis por modular a magnitude e a duração das respostas purinérgicas, através da regulação dos níveis extracelulares de nucleotídeos e nucleosídeos (SCHETINGER *et al.*, 2008). As nucleotidases podem ser encontradas em diferentes formas, principalmente ligadas à membrana plasmática de diferentes células com seu sítio catalítico voltado para o ambiente extracelular, nomeadas nessa forma como ectonucleotidases. Adicionalmente, as nucleotidases são encontradas em forma solúvel nos fluídos biológicos, principalmente na corrente sanguínea, contribuindo fortemente para o metabolismo nucleotídeos e nucleosídeos no sangue (YEGUTKIN, 2014; OSES *et al.*, 2004). Por último, dados recentes da literatura demonstram a presença de diferentes ectonucleotidases acopladas à membrana de microvesículas e exossomos (SMYTH *et al.*, 2013; JIANG *et al.*, 2014).

As nucleotidases com maior notoriedade na regulação das respostas purinérgicas são as famílias das nucleosídeo trifosfato difosfoidrolases (NTPDases), nucleotídeo pirofosfatases/fosfodiesterases (NPPs), 5'-nucleotidase (5'-NT) e a adenosina deaminase (ADA), as quais podem ser ligadas à membrana ou solúveis, como comentado anteriormente (YEGUTKIN, 2014). Assim, considerando toda a complexa rede de enzimas purinérgicas, é essencial destacar as vias de síntese, degradação e ressíntese de ATP coexistindo simultaneamente (Figura 1), modulando inflamação, coagulação e atividade vascular (YEGUTKIN, 2002).

A família das NTPDases (Figura 1A) hidrolisa nucleotídeos tri- e difosfatados (especialmente ATP e ADP), formando como produtos nucleotídeos monofosfatados, como a adenosina 5'-monofosfato (AMP). Atualmente, oito membros das NTPDases foram

identificados em mamíferos (NTPDase1-8) as quais são expressas em quase todos os tecidos humanos (ROBSON; SÉVIGNY; ZIMMERMANN, 2006). Similarmente, a família das NPPs (Figura 1B) é composta por sete enzimas (NPP1-7), no entanto somente as NPP1, 2, 3 e 4 possuem relevância para a sinalização purinérgica (LOPEZ *et al.*, 2020). Em relação aos substratos, as NPPs são versáteis, hidrolisam ligações pirofosfato inorgânico (PPi) e fosfodiéster; diferentemente das NTPDases, as NPPs degradam ATP diretamente à AMP e PPi (ATP → AMP + PPi); ADP à AMP e fosfato inorgânico (Pi) (ADP → AMP + Pi); adenosina 3',5'-monofosfato cíclico (AMPc) à AMP; NAD^+ à AMP e nicotinamida mononucleotídeo (BAGATINI *et al.*, 2018). Adicionalmente, a enzima 5'-NT (Figura 1A e B) possui sete subtipos caracterizados, cinco localizam-se no citosol, uma na matriz mitocondrial e uma em forma de ecto-enzima, associada à membrana plasmática, denominada ecto-5'-nucleotidase ou CD73 (E-5'-NT/CD73). Assim como a ecto-nucleosídeo trifosfato difosfohidrolases 1 ou CD39 (E-NTDPase1/CD39), a E-5'-NT/CD73 pode ser clivada da membrana plasmática e liberada na corrente sanguínea ou em outros fluídos biológicos (SCHNEIDER *et al.*, 2019). Nesse sentido, a principal função da 5'-NT é a formação de ADO via hidrólise de AMP, que pode exercer suas funções via receptores P1 ou ser captada para o interior das células por meio de transportadores de nucleosídeos (NT) (YEGUTKIN, 2008). Parte da cascata de sinalização purinérgica encerra-se pela ação da ADA, que converte irreversivelmente a ADO em INO. Semelhante às NTPDases, a ADA está virtualmente presente em todos tecidos humanos e possui duas isoformas, ADA1 e ADA2, porém somente a ADA1 é relevante ao sistema purinérgico, presente solúvel no sangue e ligada à membrana plasmática acoplada à CD26 ou em receptores P1 (KUTRYB-ZAJAC *et al.*, 2020).

A



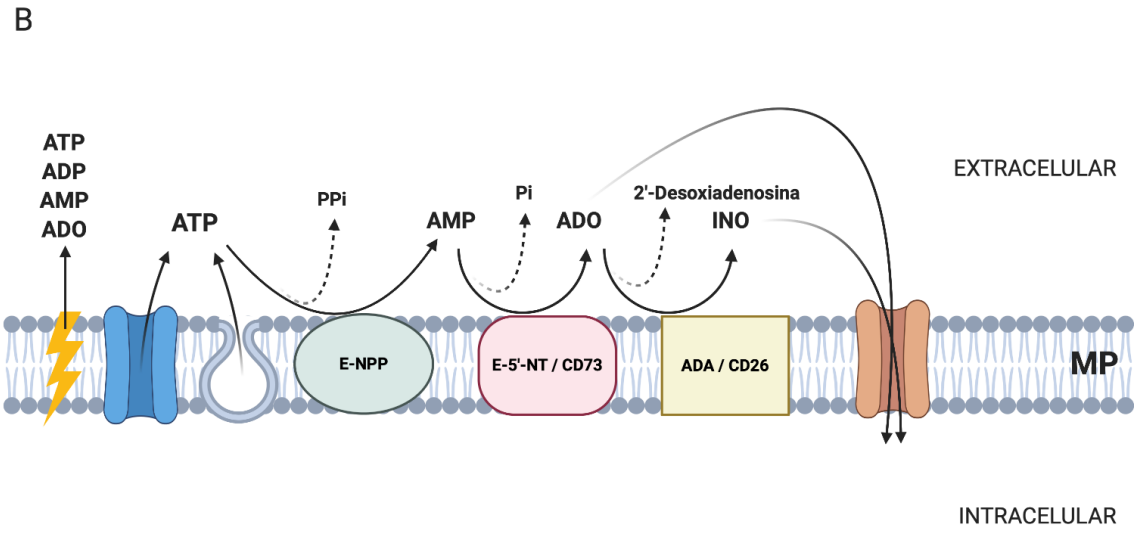


Figura 1. Resumo do metabolismo extracelular de nucleotídeos e nucleosídeos, baseado em enzimas purinérgicas acopladas à membrana plasmática (MP). A liberação de nucleotídeos e nucleosídeos ocorre através de lise celular ou mecanismos não dependentes de lise celular. (A) Via para a hidrólise de ATP – ADO envolvendo E-NTPDase1/CD39, E-5'-NT/CD73 e ADA/CD26. (B) Via para hidrólise de ATP – ADO envolvendo E-NPP, E-5'-NT/CD73 e ADA/CD26. Ambas as representações vão até a recaptação de ADO e INO por transportadores de nucleosídeos (NT), entrando na rota de salvação de purinas. Os meios aqui apresentados para o metabolismo de nucleotídeos e nucleosídeos coexistem simultaneamente.

As NTPDases e a 5'-NT são fundamentais para a modulação da inflamação, coagulação, atividade vascular e cardíaca; esses processos modulatórios ocorrem primariamente pelo controle dos níveis extracelulares de ATP, ADP, AMP e ADO (SCHETINGER *et al.*, 2007). Consequentemente, no decorrer de um processo inflamatório, os níveis de ATP e ADO são precisamente controlados por essas enzimas, o "perfeito" equilíbrio entre os níveis dessas moléculas permite que a inflamação transcorra normalmente para sua conclusão (Figura 2A e B). Assim, quando liberado para ambiente extracelular, o ATP possui caráter pró-inflamatório, promovendo a quimiotaxia de células imunes, produção de radicais livres por neutrófilos, liberação de citocinas pró-inflamatórias e induzindo apoptose (FAAS; SÁEZ; DE VOS, 2017). Em contraste, a ADO promove ações anti-inflamatórias contrárias ao ATP: inibe a adesão em células endoteliais, liberação de superóxido por neutrófilos e fator de necrose tumoral α (TNF-α), e estimula a liberação de interleucina 10 (IL-10) (LEDDEROSE *et al.*, 2016).

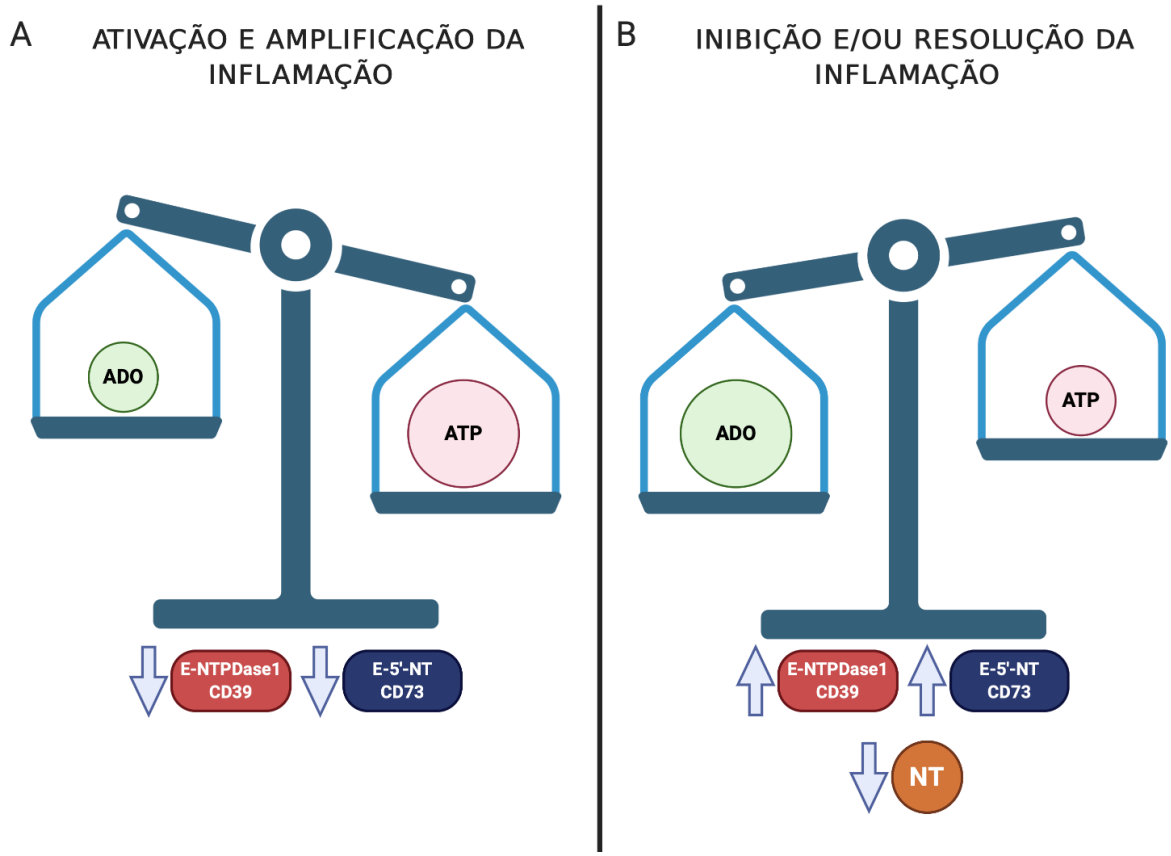


Figura 2. Regulação da inflamação via ATP e ADO. (A) Durante a inflamação altos níveis de ATP são liberados no sítio inflamatório, aumentando o recrutamento de células inflamatórias e a secreção de citocinas pró-inflamatórias que inibem a atividade da E-NTPDase1/CD39 e E-5'-NT/CD73. (B) O fim do processo inflamatório é induzido pelo aumento dos níveis extracelulares de ADO, que ocorre pela desfosforilação do ATP pela E-NTPDase1/CD39 e E-5'-NT/CD73. A inibição dos transportadores de nucleosídeos (NT) também é necessária para que os níveis de ADO permaneçam elevados, promovendo ações anti-inflamatórias para resolução ou inibição da inflamação.

A sinalização purinérgica possui papel central na regulação da atividade cardiovascular e seu potencial terapêutico adquire gradual importância clínica (BIRKENFELD et al., 2019). Primeiramente, a E-NTPDase1/CD39 é a principal ectonucleotidase presente no endotélio, controlando os níveis de ATP e ADP, apresentando papel central na tromborregulação, juntamente com a ecto-nucleosídeo trifosfato difosfohidrolases 2 (E-NTPDase2/CD39L1). Nesse sentido, é amplamente descrito o papel pró-coagulatório do ADP, promovendo o recrutamento plaquetário via receptores $P2Y_1$ e $P2Y_{12}$, sendo um elemento chave em toda cascata de coagulação (DEAGLIO; ROBSON, 2011). Assim, para manter um rigoroso controle da coagulação, os níveis de ATP e ADP são regulados por NTPDases e NPPs, promovendo a formação de AMP e consequentemente ADO pela ação da 5'-NT. Por sua vez, a ADO é um

dos mais potentes inibidores da atividade plaquetária, exercendo esses efeitos ao ligar-se nos receptores A_{2A} e A_3 (ATKINSON *et al.*, 2006).

Conforme supracitado, NTPDases e 5'-NT possuem função central na saúde cardiovascular e são sugeridas como cardioprotetoras. A E-NTPDase1/CD39 inibe a ativação plaquetária, tendo potencial terapêutico em pacientes que sofreram acidente vascular cerebral (AVC), doença arterial coronariana (DAC) e doença vascular periférica (DVP). Além disso, as NTPDases participam do controle da transdução da sinalização cardíaca, tendo em vista que o ATP e a norepinefrina (NE) são secretados simultaneamente por nervos adrenérgicos. Em altas concentrações o ATP ativa receptores P2X, facilitando a liberação de NE via *feedback* positivo; contrariamente, em baixas concentrações, o ATP ativa receptores P2Y inibindo a secreção de NE por *feedback* negativo. As NTPDases realizam o controle desse sistema, metabolizando o ATP liberado e, como resultado, regulando seus níveis (MARCUS *et al.*, 2005; MATHIEU, 2012). Complementando as ações das NTPDases, a 5'-NT é a principal responsável pela formação de ADO no sistema cardiovascular, exercendo um potente efeito vasodilatador coronário e angiogênico (BURNSTOCK, 2017). Em condições normais ou de injúria moderada, a ADO produzida via 5'NT, participa da regulação da atividade cardíaca para garantir o fornecimento adequado de oxigênio (O_2) e substratos energéticos (HEADRICK *et al.*, 2011). Paralelamente, em situações de injúria isquêmica avançada, a atividade da E-5'-NT/CD73 no tecido cardíaco é aumentada, promovendo maior formação de ADO e ativação de receptores A_{2B}, como uma estratégia para atenuação da lesão miocárdica (ECLKE *et al.*, 2007). Por fim, estados metabólicos de hipercolesterolemia e hiperglicemia promovem uma regulação positiva na atividade de E-NTPDases e E-5'-NT/CD73 plaquetária, sugerindo uma possível atividade enzimática compensatória para modular estados pró-inflamatórios e pró-trombóticos encontradas nessas circunstâncias (DUARTE *et al*., 2007; LUNKES *et al*., 2008).

Embora a sinalização purinérgica esteja intimamente ligada à diferentes aspectos do exercício físico, como inflamação, coagulação e atividade cardiovascular, a associação entre os dois é incerta. Apesar dos esforços recentes, dados da literatura indicando uma via de adaptação ao exercício físico, agudo e crônico, em diferentes populações envolvendo mecanismos purinérgicos, especialmente enzimas e/ou receptores, ainda são escassos. Os primeiros trabalhos envolvendo metabolismo extracelular e sanguíneo de purinas com o exercício físico iniciam na década de 50, mas somente em 1969 foi detectado o aumento nos níveis plasmáticos de ATP, em indivíduos adultos saudáveis do sexo masculino, após o exercício de força (FORRESTER; LIND, 1969; GREANEY; WENNER; FARQUHAR, 2015). Na década de 90 as relações entre exercício, nucleotidases e saúde cardiovascular começaram ser investigadas;

Lanfort *et al.* (1996) demonstraram que diferentes modalidades de treinamento (aeróbico e anaeróbico) aplicadas por 6 semanas aumentam a atividade da enzima E-5'-NT/CD73 no ventrículo cardíaco esquerdo de ratos, sugerindo um aumento na produção de ADO. No mesmo sentido, objetivando mostrar os benefícios cardiovasculares do exercício associados à mecanismos purinérgicos, Roque *et al.* (2011) evidenciaram que o treinamento moderado por 10 semanas em ratos, promoveu um aumento na atividade das NTPDases, NPPs e 5'-NT no soro e no sarcolema derivado do ventrículo esquerdo; além disso, foi observado um aumento na expressão da E-NTPDase1/CD39 e E-5'-NT/CD73, em conjunto com um aumento do fluxo sanguíneo coronário e capilarização miocárdica.

Previamente nosso grupo de pesquisa explorou os efeitos do exercício agudo moderado, em esteira rolante, no comportamento de nucleotidases solúveis no soro de indivíduos sedentários saudáveis do sexo masculino. Os dados demostraram que imediatamente após o protocolo de exercício, ocorreu um aumento na atividade das NTPDases (aumento na hidrólise de ATP e ADP), 5'-nucleotidase (aumento na hidrólise de AMP) e NPPs (aumento na hidrólise de *p*-nitrofenil 5'-timinidina monofosfato/*p*-Nph-5'-TMP), juntamente com níveis diminuídos de ATP e ADP, e elevação da ADO e INO. Esses resultados sugerem que as nucleotidases podem agir na tentativa de suprimir respostas inflamatórias e trombóticas desencadeadas pelo exercício, regulando os níveis de nucleotídeos e nucleosídeos (MORITZ *et al.*, 2017).

Múltiplas vias bioquímicas e fisiológicas estão envolvidas nas adaptações de curto e longo prazo desencadeadas pelo exercício físico e como efeito seus benefícios para saúde (HEINONEN *et al.*, 2014). A sinalização purinérgica, mesmo modulando importantes aspectos associados ao exercício, e sendo altamente relevante em temas como ciências cardiovasculares, neurociências e oncologia (SCHETINGER *et al.*, 2007; BURNSTOCK *et al.*, 2017), permanece preterida pelas ciências do exercício. Portanto, novos conhecimentos sobre as adaptações moleculares e bioquímicas ao exercício em diferentes populações é essencial para o aprimoramento de condutas não farmacológicas na prevenção e tratamento de doenças. Diante disso, o presente trabalho objetiva explorar os efeitos do exercício agudo de intensidade moderada, em indivíduos com diferentes características metabólicas, na funcionalidade de enzimas purinérgicas e em seus produtos metabólicos, sugerindo um possível meio de adaptação ao exercício ligado ao sistema purinérgico.

3 PROCEDIMENTOS METOLÓGICOS

3.1 PROBLEMA DE PESQUISA

Quais os efeitos do exercício aeróbico de intensidade moderada nos parâmetros funcionais de enzimas purinérgicas, no plasma sanguíneo de indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos?

3.2 OBJETIVOS

3.2.1 Objetivo geral

Avaliar os efeitos do exercício aeróbico de intensidade moderada, na funcionalidade de enzimas purinérgicas solúveis do plasma sanguíneo de indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos.

3.2.2 Objetivos específicos

Investigar os efeitos do exercício aeróbico de intensidade moderada, na atividade de nucleotidases solúveis no plasma sanguíneo de indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos.

Avaliar a influência do exercício aeróbico de intensidade moderada em todo espectro do metabolismo do ATP, no plasma sanguíneo de sujeitos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos.

Comparar as respostas purinérgicas ao exercício aeróbico moderado no plasma sanguíneo de indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos.

Determinar as relações entre os parâmetros purinérgicos supracitados e os marcadores de saúde cardiometabólica, tais como colesterol total, lipoproteína de alta densidade (HDL), lipoproteína de baixa densidade (LDL), triglicerídeos, glicose plasmática e consumo de oxigênio de pico (VO_{2ICO}).

Determinar as relações entre os parâmetros purinérgicos supracitados e marcadores pró- e anti-inflamatórios, tais como interleucina 1β (IL-1β), TNF-α, interleucina 8 (IL-8) e IL-10.

3.3 HIPÓTESES

H1: O exercício aeróbico agudo de intensidade moderada, aplicado à indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos, promoverá, pelo menos transitoriamente, um aumento na atividade das nucleotidases solúveis do plasma sanguíneo.

H2: O exercício aeróbico agudo de intensidade moderada, aplicado à indivíduos sedentários eutróficos, sedentários com sobrepeso e fisicamente ativos, promoverá, pelo menos transitoriamente, modificações no metabolismo plasmático de ATP, alterando os níveis de nucleotídeos e nucleosídeos.

H3: Os parâmetros purinérgicos avaliados apresentaram relação com marcadores de saúde cardiovascular (colesterol total, HDL, LDL, triglicerídeos e VO_{2PICO}) e inflamatórios (IL-1β, TNF-α, IL-8, IL-10) mensurados.

4 CAPÍTULO I: *Effects of physical exercise on the functionality of human nucleotidases: A systematic review* (Esse manuscrito foi submetido à revista *Purinergic Signaling* em 10/12/2020 – Anexo I).

Manuscript type: Systematic review

Manuscript title: Effects of physical exercise on the functionality of human nucleotidases: A systematic review

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ABSTRACT

Nucleotidases contribute in the regulation of inflammation, coagulation and cardiovascular activity. Exercise promotes biochemical and physiological adaptations, but its effects on nucleotidase activities and expression are unclear. The objective of this study was to review systematically the effects of exercise on nucleotidase functionality in healthy and unhealthy subjects. The MEDLINE, EMBASE, Cochrane Library and Web of Science databases were searched to identify, randomized clinical trials, non-randomized clinical trials, uncontrolled clinical trials, quasi-experimental, pre- and post-interventional studies that evaluated the acute or chronic effects of exercise on nucleotidases in humans. Two independent reviewers performed the study selection, data extraction and assessment of risk of bias. Of 203 articles identified, twelve were included in this review. Eight studies reported that acute exercise, in healthy and unhealthy subjects, elevated the activities or expression of nucleotidases. Four studies evaluated the effects of chronic training on nucleotidase activities in the platelets and lymphocytes of patients with metabolic syndrome, chronic kidney disease and hypertension and found a decrease in nucleotidase activities in these conditions. Additionally, acute and chronic exercise was able to modify the blood plasma and serum levels of nucleotides and nucleosides. Our results suggest that short- and long-term exercise modulate nucleotidase functionality. As such, purinergic signaling may represent a novel molecular adaptation in inflammatory, thrombotic and vascular responses to exercise.

Registration number: CRD42019110593.

Keywords: Exercise; Nucleotidases; NTPDase1; 5’-nucleotidase; Nucleotides.

INTRODUCTION

Physical exercise is a trigger for multiple adaptations in physiological and biochemical systems, making exercise an important tool in the prevention and treatment of many diseases [1, 2]. To elucidate the molecular and biochemical mechanisms involved in exercise, we decided to investigate purinergic signaling and its interaction with exercise physiology.

The concept of purinergic signaling is based on the existence of extracellular nucleotides and nucleosides, such as adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and adenosine (ADO), which contribute to the modulation of immune, coagulation, cardiac and vascular activity [3-5]. The source of these compounds in the extracellular environment occurs due to multiple pathways that include apoptosis, necrosis, cell injury, shear stress, exocytosis, ion channels, connexin and pannexin channels [6, 7].

The effects triggered by purines and pyrimidines in the extracellular environment are mediated by the P1 and P2 purinergic receptors [8]. The P1, or adenosine receptor, includes four subtypes: A_1, A_{2A}, A_{2B} and A_3, all of which are members of the G-protein-coupled family [9]. P2 receptors are divided into P2X and P2Y; the P2X receptors are ionotropic, while P2Y are metabotropic receptors. ATP is the major agonist of P2X receptors, which has seven subtypes P2X ($P2X_{1-7}$). The P2Y are also G-protein-coupled family receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) and are selective to different nucleotides, such as ATP, ADP, uridine 5'-triphosphate (UTP) and uridine 5'-diphospahte (UDP) [10].

The purinergic system includes the enzymatic degradation of ATP and other compounds by nucleotidases, which control the levels of extracellular purines and pyrimidines and the magnitude of purinergic responses [11]. The nucleotidase family includes nucleoside triphosphate diphosphohydrolases (NTPDases), nucleotide pyrophosphatases/phosphodiesterases (NPPs) and 5'-nucleotidase/CD73 (5'-NT) [6]. These enzymes can be found on the cell surface, attached to plasma membrane [12], in their soluble forms in extracellular fluid or the bloodstream [13], and associated with microvesicles and exosomes [6, 14].

The NTPDase family hydrolyzes extracellular nucleotide tri- and diphosphates, such as ATP and ADP, where the final product of NTPDase activity are monophosphates nucleotides. Eight members of the NTPDase family have been identified in mammalians (NTPDase1-8) and these enzymes are expressed in almost every tissue [12, 15]. The NPP family is composed of seven members (NPP1-7) that hydrolyze pyrophosphate or phosphodiester bonds and have a great range of substrate specificity. The NPP1, NPP3, NPP4 and NPP5 enzymes hydrolyze

nucleotides and/or dinucleotides [6, 16]. NTPDases and NPPs are co-expressed in many cell types; thus, the catalytic properties of these enzyme families are complementary, but unlike hydrolysis by the NTPDases, ATP hydrolysis by NPPs results in direct conversion to AMP and pyrophosphate (PPi) as product [17].

The ecto-5'-NT/CD73 enzyme (membrane-anchored isoform), amongst other actions, catalyzes the formation of extracellular ADO via AMP hydrolysis, shutting down ATP and ADP signaling pathways by P2 receptors. The formation of extracellular ADO allows P1 receptors activation, triggering their signaling pathways [12, 17]. The purinergic cascade proceeds with adenosine deamination and inosine (INO) as a product. In this context, adenosine deaminase (ADA) is an important enzyme, being mainly responsible for extracellular inosine levels and ADO metabolism. Additionally, nucleoside transporters can reuptake these nucleosides into cells to reestablish intracellular levels of ATP [6, 18, 19].

Some animal models studies have shown the relationship between exercise and nucleotidase functionality. Langfort et al. investigated the effects of 6 weeks of endurance and sprint training on ADA and 5'-NT/CD73 activities in the rat heart, demonstrating an increase in 5'-NT/CD73 basal activity after both training modalities [20]. Roque et al. evaluated the effects of swimming training for 6 weeks on rat serum and cardiac nucleotidase functionality; authors found an increase in ATP, ADP and AMP hydrolysis in the blood serum and cardiac sarcolemma. Besides that, swimming upregulated of NTPDase1/CD39 and 5'-NT/CD73 in the rat heart [21]. Cardoso et al. investigated the effects of 6 weeks of swimming training on nucleotidase activities in the platelets of hypertensive rats and demonstrated 6 weeks of this exercise protocol prevented the increase in platelet nucleotidase activities that was promoted by the hypertensive state [22].

In recent years, few studies have focused on investigating the influence of physical exercise on nucleotidase activities and expression in humans. Previous studies present a large methodological variety, small sample sizes, as well as a heterogeneity of exercise protocols and populations studied [23-26]. The aim of the present study was to review systematically the effects of acute, chronic, aerobic and anaerobic exercise on nucleotidase activities and expression in healthy and unhealthy subjects. Therefore, we aim to provide what we belive is the first systematic review of the effects of exercise on purinergic signaling to improve knowledge regarding exercise biochemistry.

METHODS

The present systematic review was conducted in accordance with the *Preferred Reporting Items for Systematic Review and Meta-Analyses* (PRISMA) statement [27] and was registered on the *International Prospective Register of Systematic Reviews* (PROSPERO) under registration number CRD42019110593.

Eligibility Criteria

For this study, we included randomized clinical trials (RCT), non-randomized clinical trials (non-RCT), uncontrolled clinical trials, quasi-experimental, and pre- and post-intervention studies that evaluated the effects of acute, chronic, aerobic or anaerobic exercise modalities on nucleotidase activities or expression (e.g. NTPDase1/CD39 and 5'-NT/CD73). Studies with healthy or unhealthy subjects, adults (≥ 20 years old) or elderly persons (≥ 65 years old), male or female gender were included.

Search Strategy

The search strategy was conducted in the following electronic databases: MEDLINE (via PubMed), EMBASE, Cochrane Library and Web of Science. Moreover, a manual search of the references cited in published studies was performed. The search was carried out in July 2020 and was not limited by language or date and comprised the following terms and corresponding synonyms: "Exercise", "Nucleotidases", "Ectonucleotidases", "NTPDases", "E-NTPDases", "NTPDase1", "E-NTPDase1", "CD39", "5'-Nucleotidase", "Ecto-5'-nucleotidase", "CD73", "E-NPP", "NPP", "Adenosine Deaminase", "ATP", "ADP", "AMP", "Adenosine", "Inosine". The complete search strategy used in PubMed is described in Supplementary Table S1.

Study Selection

Two investigators (A.F.V and D.M.M) independently evaluated titles and abstracts from studies found by the search strategy. When abstracts did not provide sufficient data about inclusion and exclusion criteria, the complete article was evaluated. In the second phase of study selection, full-texts were evaluated and selected by the reviewers independently. The selection

of studies was based on accordance with the eligibility criteria. Disagreements were settled by consensus, in cases of continuing disagreement a third investigator (C.E.J.M) was consulted.

Data Extraction

Two reviewers (A.F.V and D.M.M) performed data extraction independently, using standardized forms through Microsoft Excel® software. Methodological features of selected studies were collected, such as authors, year of publication, sample, methods, intervention and results. For data that were presented only graphically, the results were extracted using WebPlotDigitizer [28]. In cases of disagreements, a third investigator was consulted to provide a consensus opinion (C.E.J.M). The majors outcomes extracted were nucleotidase activities and/or expression in the biological sample evaluated (eg. serum, plasma, platelets or lymphocytes).

Assessment of Risk of Bias

The assessment of the methodological quality of studies included was performed by two reviewers (A.F.V and D.M.M) independently, using the Methodological Index for Non-Randomized Studies (MINORS) [29]. The MINORS is a validated scoring system used to assess the methodological quality of non-randomized studies; higher scores indicate less risk of bias, whereas lower scores indicate higher risk of bias. The maximum score for non-comparative studies is 16 and for comparative studies is 24. In cases of disagreements between the reviewers, a third investigator was consulted for a consensus (C.E.J.M).

Data Analysis

Data analysis was performed as a descriptive and qualitative analysis. Due to the methodological heterogeneity of studies included, it was impossible to perform meta-analyses. The descriptive and qualitative data analyzed are presented in the figures and tables.

RESULTS

Study Selection

The initial search located 203 studies; eight studies were excluded because they were duplicated among the databases searched. Of the remaining 195 studies, 178 were excluded based on their titles and/or abstracts. After full-text analysis, five articles were excluded; four did not address the outcome of interest and the other was an expanded abstract. After full-text evaluating, twelve studies were included in this review [23-26, 30-37] (Fig. 1).

Description of Studies

The main characteristics of the included studies are shown in Table 1. Among the twelve included studies, seven (58.33%) [23-26, 30, 33, 35] exclusively studied healthy individuals and five (41.66%) [31, 32, 34, 36, 37] reported on individuals with a disease. With the regard to the studies with healthy individuals, two studies (16.66%) [25, 26] included healthy and sedentary subjects, one (8.33%) (30) included healthy young and older adults, one (8.33%) [33] included semi-professional athletes and three (25%) [23, 24, 35] studies included healthy sedentary and trained/physically active subjects. With respect to studies that included unhealthy individuals, two studies (16.66%) [31, 32] included metabolic syndrome patients and healthy individuals, one (10%) [34] included chronic kidney disease patients and two (16,66%) [36, 37] included hypertensive patients. A total of 423 subjects were included in this systematic review, 250 (59.11%) male and 173 (40.89%) female subjects, with a mean age of 41.42 years (variation 20.56 – 67.2 years).

Descriptions of the exercise protocols applied in the selected studies are provided in Table 2. Eight (66.67%) [23-26, 30, 33, 35, 37] studies investigated the acute responses of exercise on the activity and/or expression of nucleotidases, four (33.33%) [31, 32, 34, 36] studies focused on the effects of chronic exercise on nucleotidase behavior. Among the studies that applied acute protocols of exercise, two (16.66%) (23, 24) used an incremental test, one (8.33%) [25] used the twenty-meter shuttle run test, one (8.33%) [26] employed constant intensity aerobic exercise, two (16.66%) [33, 35] used high-intensity interval exercise (HIIE), one (8.33%) [30] used resistance handgrip exercise and one (8.33%) [37] used low-intensity aerobic exercise with blood flow restriction. Additionally, of studies that employed protocols of chronic exercise, two

(16.66%) [31, 32] used concurrent training for thirty weeks and two (16.66%) [34, 36] used resistance training for eight and twenty-seven weeks, respectively.

Assessment of methodological quality

The methodological quality assessment of the studies included was performed using the MINORS tool and results are presented in Table 3. The mean score for non-comparative studies [24, 26, 33, 34, 37] was 10.6/16 (66.25% of total, variation 9–12) and for comparative studies [23, 25, 30, 31, 32, 35, 36] was 18.14/24 (75.58% of total, variation 17–18). The two major sources of bias were the unbiased assessment of the study endpoint (item 5) and loss to follow up of less than 5% (item 7).

Effects of exercise on nucleotidases

Among the selected studies, the main effects of different exercise interventions on nucleotidase activities and expression are presented in Table 2. Eight studies [23-26, 30, 33, 35, 37] investigated the outcomes of an acute exercise intervention on nucleotidases behavior in healthy individuals. Coppola et al. [23] demonstrated that acute strenuous exercise decreased NTPDase1/CD39 expression in platelets, in sedentary ($p<0.01$) and physically active ($p<0.01$) subjects; however, the expression of NTPDase1/CD39 increased in B-lymphocytes (sedentary $p<0.005$; physically active $p<0.005$).

Yegutkin et al. [24] first showed that maximal and submaximal acute exercise increased plasma ATP ($p<0.05$) and ADP ($p<0.05$) levels in trained subjects, and remained increased for 10 min after exercise, while AMP ($p<0.05$) levels increased at maximal intensity exercise ($p<0.05$). Furthermore, the activities of soluble NPP and NTPDase increased in plasma during submaximal and maximal exercise ($p<0.05$); however, soluble NTPDase activities remained increased at 10 min after exercise ($p<0.05$). The same study demonstrated that the incremental test, performed by trained individuals, increased ADP hydrolysis during ($p<0.05$) and after the test ($p<0.05$), reaching peak values after 10 min ($p<0.05$) of exercise and returning to resting levels after 30 min. The pattern of nucleotide hydrolysis was similar in arterial and venous blood. A third assay was performed in the same study with healthy sedentary subjects that performed an incremental test, showing increased levels of plasma ATP at maximal intensity ($p<0.05$). Moreover, soluble NTPDase and NPP activities transiently increased during maximal exercise

in the serum of sedentary individuals (p<0.05), returning to basal levels during the recovery period.

Karabulut et al. [25] investigated the effect of acute exercise (twenty meter shuttle run test) on ADA activity in the blood plasma of sedentary healthy men and women. This study showed an increased ADA activity in women (p=0.002) at post-exercise, but no effect of exercise in men (p=0.630). The study performed by Kirby et al. [30] assessed ATP hydrolysis in whole blood and the levels of plasma ATP during graded-intensity handgrip exercise in healthy young and older adults. The data showed a similar increase in ATP hydrolysis of young and older adults (p<0.05), combined with higher levels of plasma ATP in young adults compared to older adults (p<0.05) during acute resistance exercise.

Moritz et al. [26] analyzed the influence of a moderate acute aerobic exercise session on nucleotidase activities, and nucleotides and nucleosides levels in the blood serum of healthy sedentary male individuals. Their results showed increased activities of soluble NTPDases (p<0.05), 5'-NT/CD73 (p<0.05) and NPP (p<0.05) at post-exercise, in association with reduced serum levels of ATP (p<0.05), ADP (p<0.05) and increased serum levels of ADO (p<0.05) and INO (p<0.05).

Two studies [33, 35] evaluated the effects of acute high-intensity interval exercise protocols (HIIE), with a few differences, on nucleotidases function in the platelets and lymphocytes of healthy male subjects. Miron et al. [33] showed that an acute protocol of HIIE, when applied to semi-professional athletes did not significantly change ATP, ADP and AMP hydrolysis and NTPDase1/CD39 expression in lymphocytes at post-exercise and after 30 minutes. However, ADA activity in lymphocytes increased post-exercise (p<0.05), and remained higher after 30 minutes (p<0.05). In contrast, ATP and ADP hydrolysis in platelets decreased at post-exercise (p<0.05) and returned to basal levels after 30 minutes. On the other hand, ADA activity increased post-exercise (p<0.05) and returned to basal levels after 30 minutes.

Dorneles et al. [35] assessed the impact of HIIE on NTPDase1/CD39 and 5'-NT/CD73 expression in the $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells of low fitness and high fitness healthy male subjects. The expression of NTPDase1/CD39 increased in $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells in the low fitness (p=0.021 and p=0.03, respectively) and high fitness (p=0.025 and p=0.39, respectively) groups after the acute session of HIIE. The values increased further after 1h in the CD4+CD25- T cells of the in low fitness group (p=0.042) and in the $CD4^+CD25^+$ T cells of the high and low fitness groups (p=0.035 and p=0.033, respectively). The expression of 5'-NT/CD73 enhanced on $CD4^+CD25^+$ T cells post-exercise in both groups (p=0.01), remaining elevated at 1h after the HIIE session in low fitness (p=0.042) and high fitness group (p=0.023).

Mânica et al. [37] investigated the acute effects of low-intensity aerobic exercise, high-intensity aerobic exercise and low-intensity aerobic exercise with blood flow restriction on the nucleotidases activities in lymphocytes of hypertensive patients. The results demonstrated a similar increase in the ATP (p<0.05) and ADP (p<0.05) hydrolysis 30 min after high-intensity aerobic exercise and low-intensity aerobic with blood flow restriction. However, ADA (p<0.05) activity reduced only 30 min after high-intensity aerobic exercise. Additionally, no effect was detected after acute low-intensity aerobic exercise.

Martins et al. [31, 32] assessed the impact of 30 weeks of concurrent training on the nucleotidase activities of platelets and lymphocytes of metabolic syndrome patients. The first study [31] showed that, under pre-training conditions, platelets of metabolic syndrome patients presented an increase in NTPDase (p<0.001), 5'-NT/CD73 (p<0.001) and NPP (p<0.001) activities and a decrease in ADA (p<0.001) activity, compared to the control group. After the training period, the NTPDase (p<0.001), 5'-NT/CD73 (p<0.001) and NPP (p<0.001) activities decreased and ADA (p<0.001) activity increased, compared to the pre-training condition, resembling the control group. The second study [32] showed that, in the pre-training condition, lymphocytes of metabolic syndrome patients demonstrated an increase in ATP, ADP hydrolysis (p<0.001) and a decrease in ADA activity (p<0.001), compared to the control group. After 30 weeks of training ATP and ADP hydrolysis (p<0.05) decreased and ADA (p<0.05) activity increased, compared to the pre-training condition, demonstrating similar levels to those of the control group.

Finally, Silveira et al. [34] evaluated the effects of eight weeks of resistance training on nucleotidase activities in the platelets of chronic kidney disease patients. ATP (p<0.0001) and ADP (p<0.0001) hydrolysis decreased after eight weeks of training, while no significant differences were observed in AMP hydrolysis and ADA activity. Moreover, Lammers et al. [36] evaluated the effects of 27 weeks of resistance training on the nucleotidase activities, NTPDase1/CD39 and NTPDase2/CD39L1 expression in lymphocytes and serum levels of ATP of hypertensive and normotensive subjects. Firstly, under pre-training conditions, lymphocytes of hypertensive subjects showed an increase in ATP (p<0.05), ADP (p<0.05) hydrolysis, ADA (p<0.05) activity and serum levels of ATP (p<0.05) compared to the normotensive group. After the training period, ATP (p<0.05), ADP (p<0.05) hydrolysis and ADA (p<0.05) activity decreased, as well as the serum levels of ATP (p<0.05) in the hypertensive group compared to the pre-training condition. In contrast, the expression of NTPDase1/CD39 and NTPDase2/CD39L1 did not significantly change in lymphocytes of hypertensive or normotensive subjects after the training protocol.

DISCUSSION

This systematic review was performed to evaluate the effects of different exercise modalities on purinergic enzymes activities and expression. The findings of the current review suggest that different exercise modalities modulate the activities and expression of NTPDases, NPPs, 5'-NT/CD73 and ADA. These data indicate a novel role for purinergic signaling in linking exercise biochemistry and physiology.

As mentioned, acute and chronic exercise promotes multiple systemic adaptations [1]. Thus, it is reasonable to expect that nucleotidases responses to exercise follow some basic principles in response to the type of exercise, frequency, intensity, duration, volume and biological individuality [38]. The results of this systematic review suggest that acute exercise increases nucleotidase responses, at least in some pathways, in healthy subjects. Copolla et al. [23] and Dorneles et al. [35] observed an increase in NTPDase1/CD39 expression in B-lymphocytes, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells in healthy sedentary and physically active males after maximal exercise test and HIIE acute session. Yegutkin et al. [24], Kirby et al. [30] and Moritz et al. [26] also observed an increase in nucleotidase activities in response to different exercise protocols, applied to trained and sedentary healthy individuals. Additionally, these studies demonstrated that exercise was able to modify, at least transiently, purine levels in blood plasma and serum.

The data cited above is related to the inflammatory and coagulatory effects that are triggered by exercise. It is well known that moderate and intense acute exercise promote inflammatory and coagulatory responses, increasing levels of white blood cells (WBC), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and C-reactive protein (CRP) as well as elevating platelet count, platelet sensitivity to ADP and collagen-induced aggregation, prothrombin activation peptide 1.2 (F1.2) and thrombin/antithrombin complexes (TAT) [39-41]. These biological processes are closely related to purinergic signaling, highlighting their responses in inflammatory [42] and coagulatory [8, 43, 44] function.

Extracellular ATP has a well-established pro-inflammatory effect, promoting chemotaxis, oxygen free radical generation and IL-6 and tumor necrosis factor-α (TFN-α) production, whereas ADO has an anti-inflammatory effect, decreases superoxide anion production and pro-inflammatory cytokine release, inhibits apoptosis and increases IL-10 release [45, 46]. Additionally, ATP is an inhibitor of ADP-induced platelet aggregation [47] and ADO (produced via 5'-NT/CD73) is an important vasodilator and inhibitor of platelet aggregation [48]. The release of ATP by erythrocytes is a relevant mechanism of vasodilatation via endothelial $P2Y_{1/2/4}$

receptors in response to hypoxia, shear stress and low pH [49]. Thus, we can assume that different exercise protocols, when applied in healthy sedentary and physically active subjects, modulate the activities and expression of nucleotidases to balance nucleotides and nucleosides levels, contributing to biochemical and physiological responses to exercise.

Significant advances were made by early studies related to purine metabolism and exercise; however, these studies did not focus on nucleotidase functionality. Hellsten-Westing et al. [50] showed a reduction in the plasma levels of HX and UA after 6 weeks of HIIE training in healthy, physically active males. Kinugawa et al. [51] reported increased levels of ADO in NYHA class III chronic heart failure patients, compared to less severe patients and healthy controls at basal and post-exercise conditions. The increased levels of plasma adenosine adjusted to exercise workload may be an adaptive response in cardiac patients. Additionally, it is reasonable to assume that severe cardiac patients present higher levels of ADO, due to its cardioprotective effects such as vasodilatation, reduction of contractility and heart rate, and enhancement of O_2 and substrate delivery [52]. Recently, Zarębska et al. [53] demonstrated plasma ATP, ADP and AMP concentrations during resting, incremental test and recovery period in highly trained male athletes and physically active men.

Despite the presence of nucleotidases on hematopoietic cells, in their soluble form and attached to extracellular vesicles, endothelial nucleotidases such as NTPDase1/CD39, NTPDase2/CD39L1 and 5'-NT/CD73 are the main regulators of nucleotides and nucleosides levels in the blood [54, 55]. Furthermore, multiple pathways of nucleotide inactivation and nucleotide resynthesis are involved in the purinergic signaling. In this context, a network of enzymes may influence the extracellular levels of nucleotides and nucleosides that modulate inflammatory, thrombotic and vascular responses [6, 56].

Two other of the selected studies [31, 32] evaluated the effects of moderate concurrent training for 30 weeks on the platelets and lymphocytes of metabolic syndrome patients. Additionally, Lammers et al. [36] investigated the impacts of resistance training for 27 weeks in lymphocytes of hypertensive patients. These studies demonstrated that, under pre-training conditions, metabolic syndrome and hypertensive patients present an increase in NTPDase, NPP, 5'-NT/CD73 and ADA activity. However, ADA activity decrease under pre-training conditions in platelets and lymphocytes of metabolic syndrome patients. Such results are in agreement with previous studies that demonstrated an increase in nucleotidase activities in conditions of diabetes, hypercholesterolemia, hyperglycemia and hypertension [57-59, 61]. Martins et al. [31, 32] and Lammers et al. [36] showed that concurrent training for 30 weeks and resistance training for 27 weeks, respectively, are capable of reversing and normalizing the nucleotidase activities of

platelets and lymphocytes to activities similar to those of healthy subjects. These data can be associated with improved cardiovascular [60] and inflammatory [39] parameters promoted by chronic exercise, since long-term exercise reduces glycemia levels, blood pressure, HDL, LDL, cholesterol, triglycerides, pro-inflammatory markers and increases anti-inflammatory markers.

Correlation analysis showed a synergic function between NTPDases, NPP, 5'-NT/CD73, ADA activities and levels of glucose, HDL, triglycerides, mean platelet volume, platelet aggregation, C-reactive protein, waist circumference and blood pressure in lymphocytes and/or platelets [31, 32, 36, 59, 61]. These data suggest that, since long-term exercise is able to modify cardiometabolic parameters, the nucleotidase activities could change in relation to them.

Previous studies [26, 62-64] suggest a protective role of purinergic signaling against stressful or disease conditions. Moreover, a compensatory physiological mechanism has also been suggested, in function of the ability of nucleotidase activities to decrease ATP and ADP levels (pro-inflammatory and pro-thrombotic molecules, respectively), and increase ADO and INO levels, both cytoprotective molecules [22, 32, 44, 45, 64].

STUDY STRENGTHS AND LIMITATIONS

This systematic review is the first to highlight the effects of exercise on nucleotidase functionality in healthy and unhealthy subjects using a high-quality methodological approach in accordance with PRISMA statement. One of the limitations of our work was due to the methodological diversity in the evaluation of nucleotidase activities and expression applied among the included studies, such as high-performance liquid chromatography (HPLC), flow cytometry, thin layer chromatography (TLC) and spectrometric assay. However, the pattern of nucleotidases responses to different, acute or chronic, exercise modalities was quantified as demonstrated in Table 2.

Additional limitations are present of our study and should be pointed out: (1) no randomized clinical trial was found in our search; (2) there was methodological differences in the exercise protocols used in selected studies (since there are a limited number of published studies on this research subject, it was impossible to restrict the exercise method or type); (3) due to the high heterogeneity of data and subjects included in this systematic review, it was not possible to perform a meta-analysis; (4) taking into account the quality assessment, some studies were considered to be methodologically limited; (5) we did not include gray literature in the search strategy.

CONCLUSIONS

Our results suggest that acute and chronic protocols of exercise induce at least transitory adaptations in NTPDase, NPP, 5'-NT/CD73 and ADA activities and expression in different biological samples. These nucleotidases adaptations modified nucleotides and nucleosides levels, possibly modulating coagulation, inflammation and vascular activity associated with the exercise response. Moreover, since long-term exercise induces improvements of cardiometabolic and inflammatory parameters, purinergic enzymes functionality may be related to these markers. The data from this systematic review suggest novel biochemistry and physiological adaptations induced by exercise, based on purinergic signaling. However, further studies are necessary to determine the effects of exercise on different purinergic components and their physiological and clinical impact.

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Compliance with ethical standards

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

This work does not contain any studies with human participants or animals performed by any of the authors.

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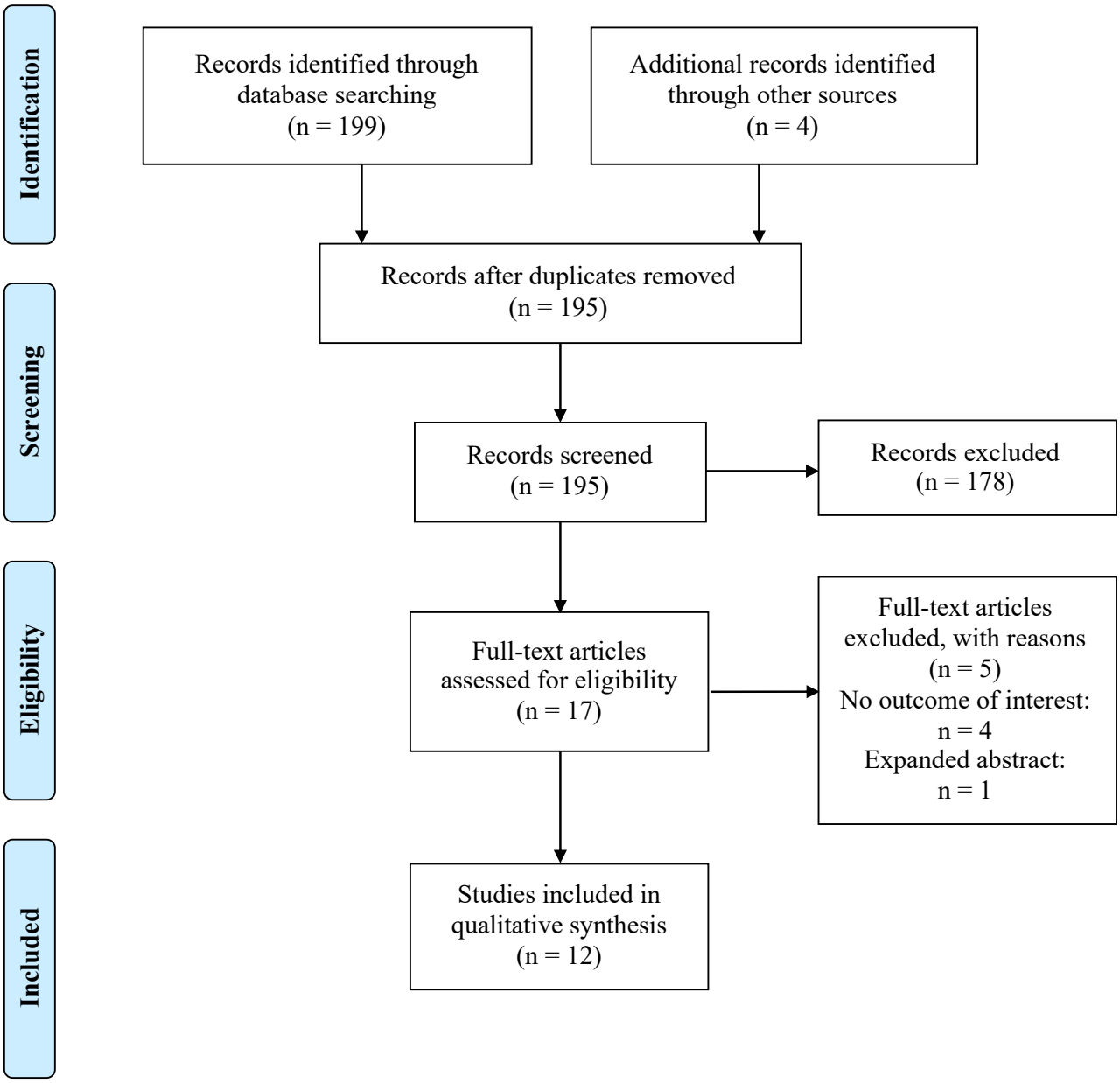

Figure 1. Flowchart of the included studies.

Supplementary Table 1. Search strategy used in PubMed

Step	Search terms
#1	"Exercise"[Mesh] OR "Exercises" OR "Physical Activity" OR “Activities, Physical” OR “Activity, Physical” OR “Physical Activities” OR “Exercise, Physical” OR “Exercises, Physical” OR “Physical Exercise” OR “Physical Exercises” OR “Acute Exercise” OR “Acute Exercises” OR “Exercise, Acute” OR “Exercises, Acute” OR “Exercise, Isometric” OR “Exercises, Isometric” OR “Isometric Exercises” OR “Isometric Exercise” OR “Exercise, Aerobic” OR “Aerobic Exercise” OR “Aerobic Exercises” OR “Exercises, Aerobic” OR “Exercise Training” OR “Exercise Trainings” OR “Training, Exercise” OR “Trainings, Exercise”
#2	“Nucleotidases”[Mesh] OR “Apyrase” OR “Phosphohydrolase, ADP” OR “ADPase” OR “ATP-ADPase” OR “ATP ADPase” OR “ATP-Diphosphatase” OR “ATP Diphosphatase” OR “ATP Diphosphohydrolase” OR “Diphosphohydrolase, ATP” OR “Adenosine Diphosphatase” OR “Diphosphatase, Adenosine” OR “ENTPD1 protein, human” OR “ATPDase, human” OR “CD39 antigen, human” OR “ectonucleoside triphosphate diphosphohydrolase 1, human” OR “NTPDase-1, human” OR “ectoATPase” OR “ecto-adenosine triphosphatase” OR “ecto-atpase” OR “ATPase, ecto” OR “NTPDase2” OR “CD39L1 protein, human” OR “ENTPD2 protein, human” OR “NTPDase-3, human” OR “ectonucleoside triphosphate diphosphohydrolase 3, human” OR “CD39-like 3 protein, human” OR “CD39L3 protein, human” OR “ecto-Mg-ATPase” OR “Myoglein” OR “ecto-nucleotidase” OR “CD39 antigen” OR “ecto-apyrase” OR “NTPDase1” OR “ectoADPase” OR “ectonucleoside triphosphate diphosphohydrolase 1” OR “NTPDase-1” OR “ecto-ADPase” OR “ecto-ATP diphosphohydrolases” OR "CD39" OR “5'-Nucleotidase” OR “5' Nucleotidase” OR “5'-Nucleotidase Phosphoribolase” OR “5' Nucleotidase Phosphoribolase” OR “Adenylate Phosphatase” OR “AMP Phosphatase” OR “Cytidylate Phosphatase” OR “Ecto-5'-Nucleotidase” OR “Ecto 5' Nucleotidase” OR “IMP Nucleotidase” OR “CD73 Antigens” OR “IMPase” OR “Inosinate Phosphatase” OR “Pyrimidine 5'-Nucleotidase” OR “Pyrimidine 5' Nucleotidase” OR “Thymidine Phosphatase” OR “Uridylate 5'-Nucleotidase” OR “Uridylate 5' Nucleotidase” OR “Antigen, CD73” OR “CD73 Antigen” OR “Antigens, CD73” OR “5'-AMP Nucleotidase” OR “5' AMP Nucleotidase” OR “IMP Phosphatase” OR “ectonucleotide pyrophosphatase phosphodiesterase 1” OR “nucleotide pyrophosphatase-alkaline phosphodiesterase I” OR “ecto-nucleotide pyrophosphatase phosphodiesterase 1” OR "CD73" OR “glycoprotein PC-1” OR “plasma cell membrane glycoprotein PC-1” OR “Enpp1 protein, zebrafish” OR “MAPF protein, Bos taurus" OR “major acidic fibroblast growth factor-stimulated phosphoprotein, Bos taurus” OR “major aFGF-stimulated phosphoprotein, Bos taurus” OR “ENPP1 protein, rat” OR “ENPP1 protein, human” OR “NPP1 protein, human” OR "alkaline phosphodiesterase 1, human" OR "PC-1 glycoprotein, human" OR "plasma-cell membrane glycoprotein 1, human" OR "ectonucleotide pyrophosphatase-phosphodiesterase 1, human" OR "ENPP1 protein, mouse" OR "nucleotide pyrophosphatase - phosphodiesterase I" OR "NPP-PDE" OR “Adenosine Deaminase” OR “Deaminase, Adenosine” OR “Adenosine Aminohydrolase” OR “Aminohydrolase, Adenosine” OR "ADA"
#3	“Adenosine Triphosphate”[Mesh] OR “ATP” OR “Adenylpyrophosphate” OR “Adenosine Triphosphate, Magnesium Salt” OR “Magnesium Adenosine Triphosphate” OR “MgATP” OR “Adenosine Triphosphate, Manganese Salt” OR “MnATP” OR “Manganese Adenosine Triphosphate” OR “Atriphos” OR “Adenosine Triphosphate, Chromium Salt” OR “CrATP” OR “Cr(H2O)4 ATP” OR “Chromium Adenosine Triphosphate” OR “Adenosine Triphosphate, Calcium Salt” OR “CaATP” OR “Adenosine Triphosphate, Chromium Ammonium Salt” OR “Adenosine Triphosphate, Magnesium Chloride” OR “ATP-MgCl2” OR “ATP MgCl2” OR “Striadyne” OR “Adenosine Diphosphate”[Mesh] OR “Diphosphate, Adenosine” OR “ADP” OR “Adenosine Pyrophosphate” OR “Pyrophosphate, Adenosine” OR “Adenosine 5'-Pyrophosphate” OR “5'-Pyrophosphate, Adenosine” OR “Adenosine 5' Pyrophosphate” OR “Magnesium ADP” OR “ADP, Magnesium” OR “MgADP” OR “Adenosine Monophosphate”[Mesh] OR “AMP” OR “Adenosine 5'-Phosphate” OR “5'-Phosphate, Adenosine” OR “Adenosine 5' Phosphate” OR “Adenosine Phosphate Dipotassium” OR “Dipotassium, Adenosine Phosphate” OR “Phosphate Dipotassium, Adenosine” OR “Disodium, Adenosine Phosphate”) OR “Phosphate Disodium, Adenosine” OR “Adenosine 2'-Phosphate” OR “Adenosine 2' Phosphate” OR “2'-Adenylic Acid” OR “2' Adenylic Acid” OR “Acid, 2'-Adenylic” OR “2'-Adenosine Monophosphate” OR “2' Adenosine Monophosphate” OR “Monophosphate, 2'-Adenosine” OR “Adenylic Acid” OR “2'-AMP” OR “5'-Adenylic Acid” OR “5' Adenylic Acid” OR “Acid, 5'-Adenylic” OR “Adenosine 3'-Phosphate” OR “Adenosine 3' Phosphate” OR “Phosphaden” OR “Adenosine”[Mesh] OR “Adenocard” OR “Adenoscan” OR “Inosine”
#4	#1 AND #2 AND #3

Table 1. Sample population characteristics of included studies

Study, Year	Characteristics of subjects	Sample Size (M/F)	Age (years)	Body Mass (Kg)	BMI (Kg/m^2)
Coppola et al., 2005	G1: Healthy, sedentary G2: Healthy, physically active	G1: 08 (M) G2: 08 (M)	G1: 34 ± 7 G2: 34 ± 6	Not informed	G1: 26.3 ± 3 G2: 26.7 ± 3
Yegutkin et al., 2007[†]	Healthy, endurece-trainded	20 (M)	29 ± 3	78.9 ± 8.2	24.4 ± 2.5
Yegutkin et al., 2007[†]	Healthy, sedentary	7 (3 M / 04 F)	30 ± 5	80.6 ± 23.1	27.9 ± 8
Karabulut et al., 2011	G1: Healthy, sedentary males G2: Healthy, sedentary females	G1: 20 (M) G2: 20 (F)	G1: 21.67 ± 0.69 G2: 20.56 ± 0.75	G1: 76.42 ± 8.94 G2: 61.32 ± 6.76	G1: 23.6 ± 2.8 G2: 21.1 ± 2.3
Kirby et al., 2012	G1: Healthy, young adults G2: Healthy, older adults	G1: 38 (33 M / 05 F) G2: 26 (24 M / 02 F)	G1: 23 ± 6.16 G2: 64 ± 5	Not informed	G1: 23.7 ± 1.84 G2: 26.5 ± 3.56
Martins et al., 2016[a]	G1: Metabolic syndrome patients G2: Healthy individuals	G1: 38 (15 M / 23 F) G2: 30 (13 M / 17 F)	G1: 59.4 ± 3.0 G2: 58.3 ± 2.8	Not informed	G1: 35.13 ± 5.13 G2: 23.2 ± 3.3
Martins et al., 2016[b]	G1: Metabolic syndrome patients G2: Healthy individuals	G1: 20 (15 M / 05 F) G2: 20 (14 M / 06 F)	G1: 57.95 ± 3.30 G2: 59.40 ± 3.95	G1: 95.25 ± 9.91 G2: 74.30 ± 11.41	G1: 38.24 ± 8.69 G2: 23.93 ± 2.88
Moritz et al., 2017	Healthy, sedentary	10 (M)	25.30 ± 2.94	81.51 ± 13.43	25.59 ± 3.44
Miron et al., 2018	Semi-professional athletes	19 (M)	27 ± 2.2	75 ± 8.6	21.4 ± 9.7
Silveira et al., 2018	Chronic kidney disease hemodialysis patients	34 (18 M / 16 F)	50.95 ± 18.4	Not informed	Not informed
Dorneles et al., 2019	G1: Low fitness, healthy G2: High fitness, healthy	G1: 15 (M) G2: 15 (M)	G1: 25.3 ± 1.4 G2: 26.1 ± 1.9	G1: 70.8 ± 3.68 G2: 71.3 ± 2.20	G1: 23.5 ± 1.5 G2: 23.7 ± 1.3
Lammers et al., 2020	G1: Hypertensive patients, sedentary G2: Normotensive individuals, sedentary	G1: 31 (F) G2: 28 (F)	G1: 56.17 ± 4.3 G2: 53.64 ± 3.6	G1: 71.21 ± 3.5 G2: 66.61 ± 2.6	G1: 29.85 ± 4.9 G2: 25.22 ± 4.5
Mânica et al., 2020	Hypertensive patients, sedentary	16 (F)	67.2 ± 3.7	70.7 ± 8.1	29.2 ± 3.7

Data presented as mean ± standard deviation (SD).
G: group; M: male; F: female; Kg: kilogram; BMI: body mass index; NYHA: New York Heart Association. [†]Two studies presented in the same article.

Table 2. Main results summary of included studies

Study, Year	Exercise protocol	Sample Analyzed	Results
Coppola et al., 2005	Incremental test: the test started with workload of 30 W and load increments of 10 W/min. The test was finished at exhaustion.	Platelet B-Lymphocytes T-Lymphocytes	↓ CD39 expression in platelets post-exercise (G1, 2-fold; G2, 1.71-fold). ↑ CD39 expression B-Lymphocytes post-exercise (G1, 2-fold; G2, 1.28-fold). ↔ in CD39 expression in T-Lymphocytes post-exercise.
Yegutkin et al., 2007[†]	Protocol 1: 15 min of submaximal exercise on a cycle ergometer, followed by 3 min rest and a constant maximal load exercise bout. Protocol 2: Incremental test in cycle ergometer with workload of 25, 50, 75, 90 and 100% of peak power until exhaustion.	Blood plasma Blood serum	Protocol 1: ↑ ATP and ADP levels at submaximal (ATP, 1.68-fold; ADP, 1.82-fold) and maximal (ATP, 2.43-fold; ADP, 2.37-fold) exercise. ↑ AMP levels during maximal exercise (2.-fold). ↑ NPP and NTPDase activities during submaximal (NPP, 1.29-fold; NTPDase, 1.31-fold) and maximal (NPP, 1.18-fold; NTPDase 1.69-fold) exercise. Protocol 2: ↑ NTPDase activity during exercise in venous (1.31-fold) and arterial (1.71-fold) blood.
Yegutkin et al., 2007[†]	Incremental cycle-ergometer exercise to exhaustion.	Blood plasma	↑ ATP levels during maximal exercise (1.45-fold). ↑ NTPDase (1.31-fold) and NPP (1.21-fold) activities during maximal exercise.
Karabulut et al., 2011	Twenty-meter shuttle run.	Blood plasma	↔ in ADA activity post-exercise (G1). ↑ activity of ADA post-exercise (G2, 13.53-fold).
Kirby et al., 2012	Fifteen min of graded-intensity handgrip exercise: 5 min period each of 5, 15 and 25% of MVC workload.	Whole blood Blood plasma	↑ ATP hydrolysis at 5 (2.10-fold), 15 (2.15-fold) and 25% (2.21-fold) of MVC (G1 and G2). ↑ levels of ATP at 5 (1.54-fold), 15 (1.69-fold) and 25% (2.03-fold) of MVC (G1)
Martins et al., 2016[a]	Concurrent and moderate training with aerobic and resistance exercises, during 30 weeks / 3 times per week.	Platelets	↑ NTPDase (ATP, 2.41-fold; ADP, 1.55-fold), E-5'-nucleotidase (2.19-fold) and NPP (6.97-fold) activities pre-training (G1). ↓ NTPDase (ATP, 1.86-fold; ADP, 1.44-fold), E-5'-nucleotidase (1.59-fold) and NPP (4.66-fold) activities post-training (G1). ↓ ADA (2.1-fold) activity pre-training (G1). ↑ ADA (1.92-fold) activity post-training (G1).
Martins et al., 2016[b]	Concurrent and moderate training with aerobic and resistance exercises, during 30 weeks / 3 times per week.	Lymphocytes	↑ NTPDase (ATP, 1.67-fold; ADP, 2.11-fold) activity pre-training (G1). ↓ NTPDase (ATP, 1.54-fold; ADP, 2.07-fold) activity post-training (G1). ↓ ADA (1.53-fold) activity pre-training (G1). ↑ ADA (1.64-fold) activity post-training (G1).
Moritz et al., 2017	Thirty min of aerobic exercise on treadmill at 70% of MHR.	Blood serum	↑ ATP (2.62-fold), ADP (2.59-fold), AMP (1.53-fold) and *p*-Nph-5'-TMP (1.48-fold) hydrolysis post-exercise. ↓ levels of ATP (1.27-fold) and ADP (1.22-fold) post-exercise; ↑ levels of ADO (1.23-fold), INO (1.24-fold) and UA (1.24-fold) post-exercise.
Miron et al., 2018	HIIE: 0 to 10 min at 55% of MHR; 10 to 20 min at > 90% of MHR; 20 to 30 min at 50 – 70% of MHR; 30 to 40 min at > 90% of MHR.	Platelets Lymphocytes	↔ ATP, ADP and AMP hydrolysis post-exercise (Lymphocytes). ↔ NTPDase1 expression post-exercise (Lymphocytes). ↑ ADA (2.28-fold) activity post-exercise (Lymphocytes). ↓ ATP (1.28-fold) and ADP (1.44-fold) hydrolysis post-exercise (Platelets).

			↑ ADA (1.83-fold) activity post-exercise (Platelets).
Silveira et al., 2018	Resistance training for 8 weeks / 3 times per week. Subjects performed 3 sets of 12 to 15 repetitions, with 1 to 2 Kg.	Platelets	↓ ATP (2.11) and AMP (1.61-fold) hydrolysis post-training. ↔ ADP hydrolysis and ADO deamination post-training.
Dorneles et al., 2019	High-intensity interval exercise, consisted of 10 bounts of 60 sec at 85 – 90% of MHR, alternated with 75 sec at 50 MHR in treadmill.	PBMC	↑ CD39 Expression post-exercise on $CD4^+CD25^+$ (G1, immediately post 1.34-fold, post-1h 1.3-fold; G2, immediately post 1.2-fold, post-1h 1.33-fold) and $CD4^+CD25^-$ (G1, immediately post 2.03-fold, post 1h 1.58-fold; G2, immediately post 1.38-fold) T cells. ↑ CD73 expression post-exercise on $CD4^+CD25^+$ T cells (G1, immediately post 1.6-fold, post-1h 1.42-fold; G2, immediately post 1.3-fold, post-1h 1.36-fold). ↔ CD73 expression post-exercise on $CD4^+CD25^-$ T cells.
Lammers et al., 2020	Resistance training for 27 weeks / 2 times per week / 45 – 60 min of continuous exercise per day, moderate intensity.	Lymphocytes Blood serum	↓ ATP (G1, 1.25-fold) and ADP (G1, 1.33-fold) hydrolysis post-training. ↓ ADA (G1, 1.28-fold) activity post-training. ↔ NTPDase1 and 2 expression post-training. ↓ levels of ATP (G1, 1.17-fold) post-training.
Mânica et al., 2020	Protocol 1: high-intensity aerobic exercise, 50% of VO_{2max} for 10 min. Protocol 2: low-intensity aerobic exercise, 30% of VO_{2max} for 10 min. Protocol 3: low-intensity aerobic exercise with blood flow restriction, 30% of VO_{2max} and occlusion pressure to 130% of systolic blood pressure at rest.	Lymphocytes	Protocol 1: ↑ ATP (1.30- fold), ADP (1.18-fold) hydrolysis and ↓ADA (1.84-fold) activity 30 min after exercise. Protocol 2: ↔ ATP, ADP hydrolysis and ADA activity post-exercise. Protocol 3: ↑ ATP (1.44-fold), ADP (1.16-fold) hydrolysis and ↔ ADA activity 30 min after exercise.

Data presented as mean ± standard deviation (SD).

↑: significant increase; ↓: significant decrease; ↔: no change; G: group; HIIE: high-intensity intermittent training; W: watt; BP: blood pressure; RPM: rotations per minute; MVC: maximum voluntary contraction; MHR: maximum heart rate; PBMC: peripheral blood mononuclear cells; HX: hypoxanthine; UA: uric acid; ADO: adenosine; ATP: adenosine 5'-triphosphate; ADP: adenosine 5'-diphophate; AMP: adenosine 5'-monophosphate; NPP: nucleotide pyrophosphatase/phosphodiesterase; NTPDase: nucleoside triphosphate diphosphohydrolases; ADA: adenosine deaminase; *p*-Nph-5'-TMP: *p*-nitrophenyl 5'-thymidine monophosphate; INO: inosine; VO_{2max}: maximum oxygen uptake. [†]Two studies presented in the same article.

Table 3. Methodological quality of included studies, assessed by MINORS

Study, Year	Score												
	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7	Item 8	Item 9	Item 10	Item 11	Item 12	Score
Coppola et al., 2005	2	2	2	2	0	2	0	1	2	2	2	1	18/24
Yegutkin et al., 2007	2	1	2	2	0	2	0	1	-	-	-	-	10/16
Karabulut et al., 2011	2	2	2	2	0	2	0	1	2	2	2	1	18/24
Kirby et al., 2012	2	2	2	2	0	2	0	1	2	2	2	1	18/24
Martins et al., 2016[a]	2	1	2	2	0	2	0	1	2	2	2	1	17/24
Martins et al., 2016[b]	2	2	2	2	0	2	0	1	2	2	2	1	18/24
Moritz et al., 2017	2	2	2	2	0	2	0	1	-	-	-	-	11/16
Miron et al., 2018	2	2	2	2	0	2	0	1	-	-	-	-	11/16
Silveira et al., 2018	1	1	2	2	0	2	0	1	-	-	-	-	9/16
Dorneles et al., 2019	2	2	2	2	0	2	0	1	2	2	2	1	18/24
Lammers et al., 2020	2	2	2	2	0	2	1	2	2	2	2	1	20/24
Mânica et al., 2020	2	2	2	2	0	2	0	2	-	-	-	-	12/16

MINORS: Methodological index for non-randomized studies.
The items are scored 0 (nor reported), 1 (reported but inadequate) or 2 (reported and adequate). The ideal score for non-comparative studies is 16 and 24 for comparative studies. Item 1: a clearly stated aim; item 2: inclusion of consecutive patients; item 3: prospective collection of data; item 4: endpoints appropriate to the aim of the study; item 5: unbiased assessment of the study endpoint; item 6: follow-up period appropriate to the aim of the study; item 7: loss to follow up less than 5%; item 8: prospective calculation of the study size; item 9: an adequate control group; item 10: contemporary groups; item 11: baseline equivalence groups; item 12: adequate statistical analyses.

5 CAPÍTULO II: *Acute moderate-intensity aerobic exercise promotes purinergic and inflammatory responses in sedentary, overweight and physically active subjects* (Esse manuscrito aceito para publicação na revista *Experimental Physiology* em 17/02/2021 – Anexo II).

Manuscript title: Acute moderate-intensity aerobic exercise promotes purinergic and inflammatory responses in sedentary, overweight and physically active subjects.

Running title: Exercise induces purinergic and inflammatory responses

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New Findings

What is the central question of this study?

How does moderate-intensity aerobic exercise affect the behavior of purinergic enzymes in sedentary, overweight and physically active subjects? What is the relationship between purinergic and inflammatory responses triggered by exercise?

What is the main finding and its importance?

Our data demonstrate that moderate-intensity aerobic exercise modifies the activity of purinergic enzymes and the levels of nucleotides and nucleosides. These results are similar in subjects with different biological characteristics. 5'-nucleotidase activity and adenosine levels are associated with inflammatory responses. This study suggests that a purinergic pathway is related to the inflammatory responses triggered by exercise.

Abstract

Purinergic signaling is a mechanism of extracellular communication that modulate events related to exercise, such as inflammation and coagulation. Herein, we evaluated the effects of acute moderate-intensity exercise on the activities of purinergic enzymes and plasma levels of adenine nucleotides in individuals with distinct metabolic characteristics. We analyzed the relationship between purinergic parameters, inflammatory responses and cardiometabolic markers. Twenty-four healthy males were assigned to three groups: normal weight sedentary (n=8), overweight sedentary (n=8) and normal weight physically active (n=8). The volunteers performed an acute session of moderate-intensity aerobic exercise on a treadmill at 70% of V̇O2peak; blood samples were drawn at baseline, immediately post-exercise and at 1h post-exercise. Immediately post-exercise, all subjects showed increases in ATP, ADP, AMP and p-Nph-5'-TMP hydrolysis, while AMP hydrolysis remained increased at 1h after exercise. High-performance liquid chromatography analysis demonstrated lower levels of ATP and ADP at post- and 1h post-exercise in all groups. Conversely, adenosine and inosine levels increased at post-exercise, but only adenosine remained augmented at 1h after exercise in all groups. With regard to inflammatory responses, the exercise protocol increased TNF-α and IL-8 concentrations in all subjects, but only TNF-α remained elevated at 1h after exercise. Significant correlations were found between the activity of 5'-NT, adenosine levels, V̇O2peak, triglyceride, TNF-α and IL-8 levels. Our findings suggest a purinergic signaling pathway that participates, at least partially, in the inflammatory responses triggered by acute moderate-intensity exercise. The response of soluble nucleotidases to acute moderate exercise appears to be similar between subjects of different biological profiles.

Keywords: Exercise; Nucleotidases; NTPDases; 5'-nucleotidase; Inflammation.

Introduction

Physical exercise is a well-known trigger for short and long-term molecular and tissue adaptations, and is a non-pharmacological tool for the prevention and treatment of many diseases (Heinonen et al., 2014; Pedersen et al., 2015). In turn, purinergic signaling is an elegant system of extracellular communication that has been poorly explored in exercise science, whereas nucleotides and nucleosides are known to modulate immune, thrombotic, vascular and cardiac responses (Faas et al., 2017; Atkinson et al., 2006; Burnstock & Ralevic, 2014).

Nucleotides and nucleosides such as adenosine 5'-triphosphate (ATP) and adenosine (ADO) are released to the extracellular environment through several pathways (cell injury, shear stress, exocytosis, connexin and pannexin channels) (Yegutkin, 2014; Lohman & Isakson, 2014). Once released, nucleotides and nucleosides may directly bind to purinergic receptors (P1 or P2) or be enzymatically degraded by purinergic enzymes, named nucleotidases. These enzymes are found attached to the cell membrane, in their soluble form in extracellular fluid or bloodstream and associated with extracellular vesicles (Yegutkin, 2008; Jiang et al., 2014). The main action of nucleotidases is to control the extracellular levels of purines and pyrimidines, regulating the magnitude of purinergic responses (Bagatini et al., 2018). The nucleoside triphosphate diphosphohydrolase family (NTPDase1-8) hydrolyzes tri- and diphosphate nucleotides into monophosphates nucleotides (Robson et al., 2006). The nucleotide pyrophosphatases/phosphodiesterases (NPP) family has seven members (NPP1-7), but only NPP1, 3, 4 and 5 hydrolyze nucleotides and/or dinucleotides (Zimmermann et al., 2012; Lopez et al., 2020). Finally, the major role of 5'-nucleotidase (5'-NT) is the formation of ADO, which can bind to P1 receptors (adenosine receptors), suffer deamination via adenosine deaminase (ADA) or be transported into the cell by nucleoside transporters (Yegutkin, 2014).

Currently, the significance of purinergic signaling in exercise biochemistry and physiology is still unclear, despite recent studies. Previously, our research group demonstrated that sedentary individuals that were submitted to acute moderate exercise presented increased nucleotidase activities in their blood serum post-exercise. Moreover, these results were associated with reduced serum levels of ATP, adenosine 5'-diphosphate (ADP) and increased levels of ADO and inosine (INO) at post-exercise (Moritz et al., 2017). Yegutkin et al. (2007) showed that maximal and submaximal acute exercise increased NTPDase and NPP activities in the blood serum of sedentary and trained individuals. Coppola et al. (2005) observed that acute strenuous exercise, also performed by sedentary and trained subjects, modified the expression of E-NTPDase1/CD39 in platelets and B-lymphocytes. Dorneles et al. (2019) demonstrated that

an acute session of high-intensity interval exercise (HIIE) increased E-NTPDase1/CD39 and E-5'-NT/CD73 expression on $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells post-exercise, in low and high physical fitness male subjects.

The purinergic signaling, which regulates important aspects related to exercise, such as coagulation and inflammation, appears to have been somewhat neglected in the field of exercise science, allowing relevant gaps concerning short- and long-term exercise-induced responses. Taken together with the data above, physical inactivity and overweight promote a pro-inflammatory state and a higher risk of cardiovascular diseases, both conditions linked to the purinergic pathway (Bagatini et al., 2018; Lavie et al., 2019). In the present study, we hypothesize that, at least transiently, exercise may play a modulatory role in the activities of NTPDases, NPPs and 5'-NT in the blood plasma of subjects with different metabolic profiles. Therefore, the objective of this work was to evaluate the effects of acute moderate-intensity aerobic exercise on the activity of soluble purinergic enzymes, quantify the whole spectrum of ATP metabolism in the blood plasma of sedentary, overweight and physically active male individuals, and correlate these purinergic parameters with the inflammatory responses triggered by our exercise protocol and cardiometabolic markers.

Materials and Methods

Ethical approval

The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul (protocol number 79422417.2.0000.5347) and was conducted according to the declaration of Helsinki, except for registration in a database. All volunteers were informed about experimental procedures before giving their written consent and were informed that they could terminate their participation at any time.

Chemicals

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophophate (AMP), adenosine (ADO), inosine (INO), hypoxanthine (HX), xanthine (XT), uric acid (UA), *p*-nitrophenyl thymidine 5'-monophosphate (*p*-Nph-5'-TMP), Coomassie brilliant blue G, TRIS, methanol, tetrabutylammonium hydroxide and potassium phosphate

monobasic were obtained from Sigma-Aldrich CO (ST. Louis, MO, USA). All the other reagents were of analytical grade.

Participants

Prior to the recruitment, a sample size calculation was performed based on our previous study (Moritz et al., 2017) for the outcome of nucleotidases activities. The calculus was carried out using the WinPEPI program version 11.65, in which a α=0.05 and power of 80%. The total sample size obtained was 24 individuals.

Twenty-four healthy male adults were recruited from the community using flyers and advertisements on the radio/internet and in newspapers/magazines. The volunteers were divided into three groups: normal weight sedentary (NWSED, n=8, 26.38±2.97 years), overweight sedentary (OWSED, n=8, 25.75±2.92 years) and normal weight physically active (PHACT, n=8, 23.13±3.18 years). Participants were included according to following inclusion criteria: 1) NWSED: body mass index (BMI) 18.6-24.9 Kg/m^2, had not been engaged in any exercise program for at least 6 months and peak oxygen uptake ($\dot{V}O_{2peak}$) 40-45 mL/Kg/min; 2) OWSED: BMI 25-29.9 Kg/m^2, had not been engaged in any exercise program for at least 6 months and $\dot{V}O_{2peak} < 40$ mL/Kg/min; 3) PHACT: BMI 18.6-24.9 Kg/m^2, had been performing at least 3 hours of exercise per week for a minimum of 6 months and $\dot{V}O_{2peak} > 45$ mL/Kg/min.

The volunteers had no previous metabolic, cardiovascular and orthopedic disorders, had not received pharmacological treatment for at least 30 days and were non-smokers. Those individuals with a history of alcohol abuse (2 ≥ doses per day) were excluded. The volunteers were instructed to abstain from caffeine for ≥ 12h before the test and informed consent was obtained from all subjects included in the study.

Experimental design

The experimental protocol was performed on two different days in the Exercise Research Laboratory of the Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). On the first day, all volunteers were clinically assessed, and data regarding history disease as respiratory, cardiac, metabolic, inflammatory and orthopedic diseases, medical history of the first-degree family, pharmacological treatment, diet and exercise routine were collected. Subjects with a previous or familial condition that could influence our results were excluded from the study. The participants answered the Physical Activity Readiness Questionnaire

(PAR-Q) (Shephard, 1988), which was applied to exclude individuals with any improper health condition. The body composition was evaluated using a five-components method, based on the anatomical site markings and the technique of measuring skinfolds, following the standards of the International Society for the Advancement of Kinanthropometry (ISAK) (Marfell-Jones et al., 2006).

An incremental cardiopulmonary exercise test was performed on a treadmill to determine the workload of our acute exercise protocol. $\dot{V}O_{2peak}$ was determined by the breath-by-breath method, using an open-circuit spirometry system (Quark CPET, COSMED, Rome, Italy). Heart rate (HR) was continuously measured by a telemetric band (Polar Electro Oy, Kempele, Finland). The test started with a warm-up period, consisting of 3 min of walking at 5 km/h followed by increments of 1 km/h every minute until exhaustion, and the subjects were verbally stimulated to perform at maximum effort during the test. The recovery period consisted of 3 min of walking at 5 km/h. $\dot{V}O_{2peak}$ was identified as the highest value in a line of tendency plotted against the time (Wasserman et al., 1964; Dekerle et al., 2003).

Seven days after the preliminary assessment, on the second day of the experimental protocol, subjects arrived fasted at the laboratory for a basal blood sample collection (pre-exercise condition). Subsequently, they received a standard meal composed of 0.5 g/Kg carbohydrates (NWSED, total calories 304.69±11.98 Kcal, carbohydrates 38.71±1.83 g, proteins 19.31±0.83 g, fats 8.07±0.14 g; OWSED, total calories 329.15±26.25 Kcal, carbohydrates 42.46±4.02 g, proteins 21.02±1.83 g, fats 8.37±0.31 g; PHACT, total calories 299.8±29.35 Kcal, carbohydrates 37.96±4.5 g, proteins 18.97±2.05 g, fats 8.02±0.35 g) and remained at rest for 30 min. The individuals were then submitted to 30 min of aerobic exercise on a treadmill at 70% of $\dot{V}O_{2peak}$, which was continuously monitored by the open-circuit spirometry system. Blood samples were collected again immediately and at 1h after the exercise session.

Blood sample collection

Blood samples (10 mL) were collected from the antecubital vein in the pre-exercise condition after at least 8h of fasting, immediately post-exercise and at 1h after the exercise protocol. The blood was collected into heparin anticoagulant tubes and centrifuged at 4ºC, 1,500 x *g* for 10 min. The plasma sample was then collected and stored at -80°C for posterior analysis.

Protein determination

Protein was measured by the Commassie blue method, using serum albumin as standard (Bradford, 1976).

NTPDase and 5'-nucleotidase activity assays

To assay NTPDase and 5'-nucleotidase activities in the blood plasma, the samples were incubated with 112.75 mM (final concentration) Tris-HCl buffer, pH 7.4. The samples were pre-incubated for 10 min at 37°C, and to start the reaction, ATP, ADP and AMP were added to the reaction medium to a final concentration of 3 mM. After 50 min, the incubation was stopped with 5% trichloroacetic acid (TCA; final concentration) and subsequently chilled on ice. The samples were centrifuged at 4°C, 16,000 x *g* for 10 min and the amount of inorganic phosphate (Pi) released was determined by the malachite green colorimetric method with minor modifications (Chan et al., 1986). Controls were performed to correct the non-enzymatic nucleotide hydrolysis. Incubation times, sample dilutions and protein concentrations were chosen to guarantee the linearity of the reaction as previously established (Moritz et al., 2017). All samples were processed in triplicate and enzyme activities were expressed as nmol of Pi released per minute per milligram of protein (nmol/min/mg).

NPP activity assay

NPP activity was evaluated using *p*-Nph-5'-TMP (an artificial substrate used for the in vitro assay of this enzyme), as described by Sakura et al (1998). Briefly, the reaction was performed in a medium containing Tris-HCl at a final concentration of 112 mM, pH 8.9, and the samples with 1 mg of plasma protein were pre-incubated for 10 min at 37°C. The enzyme reaction was started by the addition of 0.5 mM (final concentration) of *p*-Nph-5'-TMP. After 60 min, 200 µL of NaOH 0.2 M were added to the medium to stop the reaction. The amount of *p*-nitrophenol released from the hydrolysis of the substrate was measured at 410 nm using a molar extinction coefficient of 18.8 x 10^{-3}/M/cm. Controls were performed to correct the non-enzymatic substrate hydrolysis. Incubation times, sample dilutions and protein concentrations were chosen to guarantee the linearity of the reaction as previously established (Moritz et al., 2017). All samples were processed in triplicate and enzyme activities were expressed as nmol of *p*-nitrophenol released per minute per milligram of protein (nmol/min/mg).

High-performance liquid chromatography (HPLC) analysis

Purine levels and metabolic residues of ATP hydrolysis in plasma samples pre-, post-exercise and post-1h of exercise were evaluated by HPLC. Firstly, sample proteins were denatured by 0.6 M of perchloric acid and centrifuged at 4°C, 16,000 x *g* for 20 min. After that, the supernatants were neutralized with 4 M KOH and centrifuged again at 4°C, 16,000 x *g* for 20 min. After the second centrifugation, supernatants were collected and filtered with 0.22 µm syringe filter (Cobetter Filtration, Hangzhou, China). Aliquots of 20 µL were applied to a reverse-phase HPLC (Shimadzu, Kyoto, Japan) using a C_{18} column (Gemini C_{18}, 25 cm, 4.6 mm, 5 µm, Phenomenex, CA, USA). The elution was carried out by applying a linear gradient from 100% solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium chloride, pH 6.0) to 100% solvent B (solvent A + 30% methanol) over a 30-min period (flow rate 1.2 mL/min), according previously described (Voelter et al., 1980). The amounts of purine were measured by absorption at 254 nm. The retention times of standards were used to parameters for identification and quantification. Purine concentration are expressed in micromolar (µM).

Inflammatory markers

Plasma levels of interleukin-1β (IL-1β), interleukin-10 (IL-10) and tumor necrosis factor α (TNF-α) were evaluated by enzyme-linked immunosorbent (ELISA), according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Furthermore, plasma levels of interleukin-8 (IL-8) were determined by cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

General biochemistry analysis

Glucose, total cholesterol, triglycerides and high density-lipoprotein cholesterol (HDL-C) were analyzed using an automated enzymatic colorimetric method (Cobas C111, Roche Diagnostics, Basel, Switzerland). Low-density lipoprotein cholesterol (LDL-C) was estimated by the Friedewald equation (Friedewald et al., 1972).

Statistical analysis

Data were analyzed using IBM SPSS (Statistical Package for Social Science, 20.0, IBM, USA). Firstly, a Shapiro-Wilk test was realized to evaluate the normality of the data. The analysis of the homoscedasticity of the variances was performed using the Levene test and sphericity using the Mauchly test. Participants' baseline characteristics were compared by one-way ANOVA followed by a Bonferroni post hoc. Biochemical outcomes were analyzed using two-way ANOVA with repeated measures (three groups vs three-time points), followed by Bonferroni post hoc. Correlation analysis was performed using the Pearson correlation factor. Results are expressed as mean ± standard deviation (SD) and differences were considered to be significant when $p \leq 0.05$.

Results

Participants' characteristics

The baseline characteristics of the participants of the study are shown in Table 1. No significant differences were found among the groups with regard to age, height, muscle mass or maximum heart rate (MHR). As expected, body mass and body mass index (BMI) were higher in the OWSED group compared to the NWSED (p=0.032 and p<0.001, respectively) and PHACT (p=0.049 and p<0.001, respectively) groups, according to the inclusion criteria. Additionally, fat mass was also higher in the OWSED group, compared to the NWSED (p=0.006) and PHACT groups (p=0.001). Of note, the mean body fat percentage was higher in the OWSED group, compared to the PHACT group (p=0.027), but not compared to the NWSED individuals (p=0.143). The $\dot{V}O_{2peak}$ values were significantly different among the groups (OWSED=38.58±1.94; NWSED=43.84±0.82; PHACT=50.31±2.34; p<0.001), highlighting the differences in cardiorespiratory fitness between the groups.

Nucleotidases activities

Levels of ATP, ADP, AMP and *p*-Nph-5'-TMP hydrolysis in the blood plasma of NWSED, OWSED and PHACT subjects at pre-exercise (PRE), post-exercise (POST) and at 1h post-exercise (POST1H) are shown in Figure 1 (Fig. 1A, B, C and D, respectively). ATP

hydrolysis (Fig. 1A) increased in response to exercise in all groups (POST, $p<0.001$), remaining increased in the PHACT group at 1h post-exercise (POST1H, $p=0.031$). Thus, a time effect was observed ($p<0.001$) however, there was no interaction group x time ($p=0.307$) and no group effect ($p=0.657$). Similarly, ADP hydrolysis (Fig. 2B) showed a time effect ($p<0.001$), with an increase at the post-exercise time in all groups ($p<0.001$) that was maintained at 1h post-exercise (POST1H) in the OWSED group ($p=0.025$). No interaction of group x time ($p=0.719$) and no group effect ($p=0.327$) were observed, although ADP hydrolysis remained increased at POST1H in the OWSED ($p=0.001$) and PHACT ($p=0.003$) groups. Moreover, AMP hydrolysis (Fig. 1C) increased at post-exercise in all groups (POST, $p<0.001$) and remained increased at 1h post-exercise (POST1H; NWSED, $p=0.047$; OWSED, $p<0.001$; PHACT, $p=0.002$). Our analysis revealed time and group effects ($p<0.001$ and $p<0.011$, respectively), with significant differences between the OWSED and PHACT groups at pre- ($p=0.027$) and post-exercise ($p=0.021$). Finally, the activity of NPP, measured by hydrolysis of *p*-Nph-5'-TMP, also increased in response to exercise in all groups (POST, $p<0.001$), reflecting a time effect ($p<0.001$). No interactions between time x group ($p=0.813$) nor any group effect ($p=0.581$) were observed.

Nucleotide and nucleoside levels

The plasma levels of nucleotides and nucleosides in the NWSED, OWSED and PHACT subjects, in response to the protocol of acute moderate exercise, are presented in Figure 2 (Fig. 2A, B, C, D, E, F, G and H, respectively). Levels of ATP and ADP (Fig. 2A and B, respectively) were reduced post-exercise and remained decreased at 1h post-exercise in the NWSED (ATP, $p<0.001$, $p=0.001$; ADP, $p<0.001$, $p=0.009$; respectively), OWSED (ATP, $p=0.006$, $p=0.047$; ADP, $p=0.002$, $p=0.027$; respectively) and PHACT (ATP, $p<0.001$, $p=0.004$; respectively) groups. As such, the analysis demonstrated a time effect ($p<0.001$) on nucleotide levels, although no interaction between time x group were observed for ATP ($p=0.386$) and ADP ($p=0.856$), nor any group effect ($p=0.900$ and $p=0.347$, respectively). Additionally, no differences were found for AMP levels in any of the times or conditions evaluated (Fig. 2C).

In contrast, plasma levels of adenosine (NWSED, $p<0.001$; OWSED, $p=0.034$; PHACT, $p=0.002$) and inosine ($p<0.001$ for all groups), increased post-exercise. However, ADO levels remained enhanced in all groups at 1h post-exercise (POST1H; NWSED, $p=0.002$; OWSED, $p<0.001$; PHACT $p<0.001$) and INO levels remained increased only in the OWSED group ($p=0.002$). The analysis of ADO and INO levels showed a time effect (both $p<0.001$), although

no interaction for time x group (p=0.161 and p=0.069, respectively) or group effect (p=0.699 and p=0.901, respectively) were detected. In contrast, no time x group interaction (p=0.564), or time effect (p=0.166), was detected in relation to XT levels, however a group effect was found (p=0.002); significantly higher levels of XT were observed in the NWSED group, compared to the OWSED and PHATC groups at post exercise (POST; p=0.009 and p=0.006, respectively) and at 1h post-exercise (POST1H; p=0.019 and p=0.006, respectively). Similarly, time effects were found for HX (p<0.001) and UA (p<0.001) following the protocol, where exercise increased both HX (NWSED, p=0.002; OWSED, p<0.001; PHACT, p=0.002) and UA (NWSED, p=0.001; OWSED, p=0.002; PHACT, p=0.038) levels. However, levels of UA remained enhanced at 1h post-exercise (POST1H) for NWSED (p=0.048) and OWSED (p=0.002). In this regard, a time effect was detected (p<0.001, both), but no time x group interaction (XT, p=0.613; UA, p=0.277) or group effect (XT, p=0.766; UA, p=0.115) were found.

Inflammatory markers

Table 2 depicts levels of markers of acute inflammatory responses to the exercise protocol for the NWSED, OWSED and PHACT groups. No differences were detected in IL-1β and IL-10 levels for any of the parameters analyzed (time, group, or time x group interaction). However, plasma levels of TNF-α increased at post-exercise (NWSED, p=0.002; OWSED, p<0.001; PHACT, p=0,028) and remained increased at 1h post-exercise for all groups (NWSED, p=0.018; OWSED, p=0.006; PHACT, p=0.047); there was a time effect (p<0.001), but no group effect (p=0.748) or time x group interaction (p=0.626) were found. Additionally, plasma IL-8 also increased post-exercise in all groups (NWSED, p=0.003; OWSED, p=0.002; PHACT, p<0.001), and remained increased at 1h post-exercise in the PHACT group (p=0.005); our data revealed a time effect (p<0.001), but no group effect (p=0.256) nor time x group interaction were detected (p=0.175).

Blood biochemistry

General blood plasma biochemical metabolic variables at baseline are shown in Table 3. No differences were found between the groups with regard to total cholesterol, HDL-C and LDL-C. In contrast, glucose and triglyceride levels were higher in the OWSED group,

compared to the NWSED (p=0.003 and p<0.001, respectively) and PHACT (p=0.035 and p=0.001) groups.

Correlation analysis

Correlation analyses were performed to evaluate potential connections between NTPDase, NPP and 5'-NT activities with cardiorespiratory parameters, blood biochemistry and inflammatory markers. A significant positive correlation was found between 5'-NT activity and $\dot{V}O_{2peak}$ (PRE, r=0.514, p=0.010; POST, r=0.502, p=0.012; POST1H, r=0.593, p=0.002; Fig. 3A, 3B and 3C, respectively). In addition, a positive correlation was also found between plasma ADO levels and $\dot{V}O_{2peak}$ (PRE, r=0.423, p=0.039; POST, r=0.452, p=0.027; POST1H, r=0.431, p=0.036; Fig. 3D, 3E and 3F, respectively). With regard to blood biochemistry parameters, a positive correlation was observed between 5'-NT activity and triglyceride levels (PRE, r=0.481, p=0.017; POST, r=0.409, p=0.047; POST1H, r=0.428, p=0.037). Likewise, NPP activity and triglyceride levels were positively correlated (PRE, r=0.429, p=0.037; POST, r=0.490, p=0.015; POST1H, r=0.436, p=0.033).

With respect to the inflammatory markers, the activity of 5'-NT was negatively correlated with plasma IL-8 levels (PRE, r=–0.442, p=0.030; POST, r=–0.623, p<0.001; POST1H, r=–0.501, p=0.013; Fig. 4A, 4B and 4C, respectively), and positively correlated with TNF-α levels (PRE, r=0.498, p=0.017, POST, r=0.478, p=0.018; POST1H, r=0.558 e p=0.005; Fig. 4D, 4E and 4F, respectively). In contrast, a negative correlation was observed between NPP activity and plasma TNF-α levels (PRE, r=–0.468, p=0.021; POST, r=–0.574, p=0.003; POST1H, r=–0.477, p=0.018). Additionally, a positive correlation was found between plasma ADP levels and plasma IL-8 (PRE, r=0.537, p=0.007; POST, r=0.546, p=0.006, POST1H, r=0.553, p=0,005); however, a negative correlation was observed between plasma ADO levels and plasma IL-8 (PRE, r=–0.486, p=0.016; POST, r=–0.551, p=0.005, POST1H, r=–0.452, p=0.026).

Discussion

This is the first study to investigate and compare the responses of nucleotidases in the plasma of normal weight sedentary, overweight sedentary and physically active young male adults in response to acute moderate-intensity aerobic exercise. Furthermore, we evaluated the

levels of nucleotides and nucleosides in the plasma of these individuals and their relations with and blood biochemical parameters, cardiorespiratory fitness and inflammatory markers.

Primarily, our results indicate a significant increase in NTPDases, NPPs and 5'-NT activities, as demonstrated by the evaluation of ATP, ADP, *p*-Nph-5'-TMP and AMP hydrolysis, immediately after the exercise protocol in sedentary, overweight and physically active subjects (Fig. 1A, B, C and D, respectively). At 1h after exercise, ATP and ADP catabolism remained increased only in the PHACT and OWSED groups, respectively, while AMP hydrolysis remained increased in all three groups. Consistent with these data, plasma levels of ATP, ADP, ADO, INO, HX and UA were altered post-exercise and at 1h post-exercise in NWSED, OWSED and PHACT subjects (Fig. 2A, B, D, E, G and H, respectively).

Although sedentary and overweight subjects demonstrate a pro-inflammatory basal state, lower cardiorespiratory fitness, and a higher risk of cardiovascular disease (Wei et al., 1999; Lavie et al. 2019), the purinergic responses triggered by moderate-intensity exercise were analogous to physically active individuals. Previously, was demonstrated that treadmill exercise at intensities from 83% to 94% of maximum oxygen uptake ($\dot{V}O_{2max}$) increased the plasma levels of ATP in highly trained and physically active male subjects (Zarębska et al., 2018). Thus, our results suggest that moderate-intensity aerobic exercise may promote the release of ATP by erythrocytes, lymphocytes, endothelial and muscle cells, however, at this exercise intensity, the activity of purinergic enzymes is able to counteract the enhance of nucleotides in the blood plasma. Moreover, since several mechanisms contribute to the release of nucleotides, such as injury cells, shear stress and hypoxia, a possible common purinergic pathway is shared among individuals of different metabolic characteristics in response to acute exercise.

The modulatory effects of acute and long-term exercise models on purinergic enzymes have been reported in previous studies. To the best of our knowledge, Langfort *et* al. were the first to show that six weeks of endurance or sprint training increased E-5'-NT/CD73 activity in the rat heart (Langfort et al., 1996). In this context, the functionality of human nucleotidases seems responsive to different exercise stimuli. Incremental exercise until exhaustion increased E-NTPDase1/CD39 expression in B-lymphocytes in sedentary and trained individuals; in contrast, the expression in platelets decreased (Coppola et al., 2005). Similarly, incremental exercise, constant-load submaximal and maximal-load exercise, increased NTPDase and NPP activities in the serum of trained and sedentary individuals post-exercise and remained increased up to thirty minutes (Yegutkin et al., 2007). Furthermore, an acute session of HIIE increased E-NTPDase1/CD39 and E-5'-NT/CD73 expression in $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells of low- and high-physical fitness men, and this expression remained increased for 1h

after the session (Dorneles et al., 2019). Additionally, the performance of an HIIE session by semi-professional athletes also increased ADA activity in lymphocytes and platelets, while E-NTPDase activity decreased in platelets post-exercise (Miron et al., 2019). Previously, we demonstrated that a moderate-intensity aerobic exercise session elevated ATP, ADP, AMP and *p*-Nph-5'-TMP hydrolysis post-exercise in the serum of sedentary male individuals, combined with reduced levels of ATP and ADP, and higher levels of ADO, INO and UA (Moritz et al., 2017). Taken together with the data above, we hypothesize that different models of acute exercise lead to a transitory modulation of NTPDse, 5'-NT and NPP functionality in trained and untrained subjects.

NTPDase1 and 5'-NT are key regulators of immunological and coagulation processes (Faas et al., 2017; Atkinson et al., 2006). Over the last few years, it has been suggested that these enzymes have a protective role in cardiac and vascular tissues due, at least in part, to the control of extracellular levels of ATP, ADP and ADO (Schetinger et al., 2007; Marcus et al., 2005; Eckle et al., 2007). Extracellular ATP and ADP have well-established pro-inflammatory and pro-thrombotic effects, respectively, promoting oxygen free radical generation, chemotaxis, IL-6 and TNF-α production, and platelet aggregation (Faas et al., 2017; Kanthi et al., 2014). In contrast, extracellular ADO and INO have anti-inflammatory, antithrombotic and vasodilatory roles, decreasing superoxide anion and pro-inflammatory cytokine production, regulating neutrophil function, inducing angiogenesis, and inhibiting apoptosis and platelet aggregation (Faas et al., 2017; Schetinger et al., 2007; Haskó et al., 2000; Hsiao et al., 2005). HPLC analysis indicated that, in response to our protocol of acute moderate-intensity aerobic exercise, ATP and ADP plasma levels decreased and remained at decreased at 1h after exercise in the NWSED, OWSED and PHACT groups. Moreover, plasma levels of ADO and INO increased post-exercise in all groups, but only ADO levels remained higher after 1h. Like ADO and INO, plasma levels of UA were augmented post-exercise; UA is a product of ATP metabolism and has important antioxidant properties in blood plasma, coupled with health and disease aspects (Yu et al., 2019).

Our results may reflect the nucleotidase behavior at different time points post-exercise, taking into account that moderate and intense acute exercise promote transitory pro-inflammatory and pro-thrombotic responses, such as increasing the plasma levels of lymphocytes, natural killer (NK) cells, IL-6, IL-8, C-reactive protein (CRP), platelet count, β-Thromboglobulin, thrombin/antithrombin complexes (TAT) and sensitivity to collagen- and ADP-induced aggregation (Cerqueira et al., 2020; Lippi & Maffulli, 2009; Posthuma et al., 2015). With regard to the immune- and thromboregulatory roles of NTPDases, NPPs and 5'-

NT, it is plausible that a purinergic signaling pathway may be triggered in an attempt to control the pro-thrombotic and pro-inflammatory effects of acute exercise through modulation of nucleotides and nucleosides levels (Bagatini et al., 2018; Yegutkin, 2020).

Although we observed similarities in the parameters studied in NWSED, OWSED and PHACT individuals, some peculiarities should be noted. We cannot exclude the participation of other soluble nucleotidases, such as alkaline phosphatases and acid phosphatases, that may contribute to extracellular ATP metabolism at pre-, post-, and 1h post-exercise of exercise (Yegutkin, 2014; Rudberg et al., 2000; Fragala et al., 2017). Furthermore, E-NTPDase1/CD39, E-NTPDase2/CD39L1 and E-5'-NT/CD73 are highly expressed at the membranes of platelets and endothelial cells, and participate in the metabolism of nucleotides in the bloodstream (Atkinson et al., 2006; Kanthi et al., 2014; Heber & Volf, 2015). The magnitude and duration of purinergic signaling includes ATP-generating and ATP-consuming or inactivation pathways, in addition to ADO-generating, ADO-consuming or inactivation and coexisting reuptake (Yegutkin, 2008; Yegutkin et al., 2002; Pastor-Anglada et al., 2018). Therefore, multiple purinergic signaling pathways may partially contribute to the findings of this study.

We also showed that our protocol of acute moderate-intensity aerobic exercise induced inflammatory responses, increasing IL-8 and TNF-α plasma levels post-exercise and at 1h after exercise in NWSED, OWSED and PHACT individuals. However, no differences were detected among the groups in IL-1β and IL-10 plasma levels (Table 2). Firstly, cytokine plasma levels are associated with exercise intensity, duration, type, and clinical condition of the individuals (Cerqueira et al., 2020). Our data are in agreement with previous studies that also demonstrated increased levels of IL-8 and TNF-α at different time points after moderate and high-intensity acute exercise, in healthy trained and untrained subjects (Landers-Ramos et al., 2014; Christiansen et al., 2013; Liu & Timmons, 2016). Moreover, Brenner et al. (1999), Kim et al. (2015) and Dorneles et al. (2016) showed that moderate-intensity exercises, applied in healthy individuals, did not significantly modify the plasma levels of IL-1β and IL-10. In this context, we found a significant negative correlation between 5'-NT activity and IL-8, and a significant positive correlation between 5'-NT activity and TNF-α plasma levels pre-, post- and 1h post-exercise (Fig. 4A, B, C, D, E and F, respectively). Further studies are necessary to fully explain these opposite relationships between 5'-NT activity and the IL-8 and TNF-α plasma levels, both classic pro-inflammatory cytokines. The analysis also revealed significant correlations between NPP activity and TNF-α, as well as between ADP, ADO and IL-8 plasma levels. Taken together, these data reinforce the hypothesis that a purinergic pathway is involved in the inflammatory responses that are triggered by acute exercise.

Some cardiometabolic parameters, such as cholesterol, triglycerides and glucose levels, have been associated with the behavior of platelet nucleotidases in different conditions (Duarte et al., 2007; Lunkes et al., 2008; Martins et al., 2016). As our results indicate significantly higher resting plasma levels of glucose and triglycerides in the OWSED group (Table 3), the correlation analysis found a positive correlation between the activities of NPP and 5'-NT, and triglyceride levels. Interestingly, we found a significant positive correlation between 5'-NT activity, ADO plasma levels and $\dot{V}O_{2peak}$; this correlation was observed at pre-, post- and 1h post-exercise (Fig. 3A, B, C, D, E and F, respectively). These new findings further support a role for 5'-NT and ADO in cardiovascular health. The functionalities of 5'-NT and concentrations of ADO are closely linked to coronary blood flow, vasodilatory effect in vascular beds, blood pressure, cardiac activity and regulation of cardiorespiratory responses to hypoxia and inhibition of cardiac inflammation (Eckle et al., 2014; Guieu et al., 2020; Quast et al., 2017; Holmes et al., 2018). Additionally, a greater cardiorespiratory fitness ($\dot{V}O_{2peak}$) is associated with a lower risk of cardiovascular disease and all-causes of mortality in healthy and unhealthy subjects (Blair et al., 1996; Wei et al., 1999). Further studies are necessary to explore the meaning of these results since products of ATP metabolism have previously been applied in a model of performance prediction in highly trained athletes, and plasma hypoxanthine was indicated as a strong predictor of performance (Zieliński et al., 2013).

In summary, the present work demonstrates that acute moderate-intensity aerobic exercise induces transitory increments in the activities of soluble NTPDases, NPPs and 5'-NT, controlling the plasma levels of nucleotides and nucleosides. These responses may remain altered for 1h after exercise and are similar between subjects of different biological profiles and levels of cardiorespiratory fitness. Furthermore, our protocol of exercise was able to promote inflammatory responses that demonstrated significant correlation with the activites of purinergic enzymes, and nucleotide and nucleoside levels. Our results support the hypothesis that a purinergic signaling pathway modulates the inflammatory and thrombotic responses triggered by acute moderate- and high-intensity exercise; this may be a protective mechanism that presents similarities in healthy individuals of distinct characteristics. Finally, we show a significant positive correlation between 5'-NT, ADO and $\dot{V}O_{2peak}$, all markers associated with cardiovascular health.

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Competing interests

The authors declare no conflict of interest.

Author contributions

C.E.J.M., F.F., A.M.O.B. and A.R.-O. contributed to the conception and design of the work. C.E.J.M., F.P.B., A.F.V. and S.V.M. enrolled subjects and conducted data collection. C.E.J.M., F.P.B., J.N.S., A.F.D. and P.R.P. performed the experimental procedures and data analysis. C.E.J.M. wrote the manuscript. A.M.O.B., F.F. and A.R.-O. edited and revised the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Baseline characteristics of participants

	NWSED (n=8)	OWSED (n=8)	PHACT (n=8)
Age (years)	26.37±2.97	25.75±2.91	23.12±3.18
Body mass (Kg)	74.16±9.74	85.02±5.95*#	75.01±7.63
Height (m)	1.78±0.06	1.75±0.05	1.78±0.07
BMI (Kg/m^2)	23.40±2.01	27.75±1.26*#	23.65±1.20
Muscle mass (Kg)	33.20±5.73	37.87±2.20	35.50±6.24
Fat mass (Kg)	19.65±5.30	27.41±3.98*#	18.21±4.03
Body fat (%)	27.08±5.55	31.92±2.93#	25.01±5.79
HRrest (beats/min)	66.87±6.08	73.75±8.71#	64.5±7.03
MHR (beats/min)	187.5±11.01	183.5±11.96	187.87±10.37
$\dot{V}O_{2peak}$ (mL/Kg/min)	43.96±1.09	38.82±2.48*#	50.30±2.34*

Values are presented as means ± SD. NWSED, normal weight sedentary; OWSED, overweight sedentary; PHACT, physically active; BMI, body mass index; HRrest, resting heart rate; MHR, maximum heart rate; $\dot{V}O_{2peak}$, peak oxygen uptake. *Indicates difference from NWSED group ($p<0.05$). #Indicates difference from PHACT group ($p<0.05$).

Table 2. Plasma inflammatory markers in response to acute aerobic moderate exercise

	NWSED (n=8)			OWSED (n=8)			PHACT (n=8)		
	PRE	POST	POST1H	PRE	POST	POST1H	PRE	POST	POST1H
IL-1β (pg/mL)	5.41±1.86	6.03±2.87	5.71±2.71	6.21±1.60	7.61±2.14	7.32±3.2	6.1±2.6	7.2±2.94	6.34±3.58
TNF-α (pg/mL)	1.80±0.78	3.46±1.86*	3.22±1.72*	2.26±0.88	4.50±1.78*	3.89±1.24*	2.79±1.41	3.95±1.88*	3.98±2.02*
IL-8 (pg/mL)	2.5±1.05	3.58±1.22*	3.18±1.22	3.64±1.22	4.76±1.46*$^{\#}$	3.75±1.25	2.57±1.11	4.04±1.29*	3.82±1.43*
IL-10 (pg/mL)	3.60±1.65	4.16±1.88	3.44±1.76	3.42±1.85	4.23±1.84	4.29±1.72	4.13±1.69	5.06±1.81	5.40±1.80

Values are presented as means ± SD. NWSED, normal weight sedentary; OWSED, overweight sedentary; PHACT, physically active. PRE, pre-exercise; POST, post-exercise; POST1H, 1h post-exercise. IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IL-8, interleukin-8; IL-10, interleukin-10. *Indicate difference form PRE ($p<0.05$). $^{\#}$Indicate difference from POST1H ($p<0.05$).

Table 3. Biochemical characteristics of participants

	NWSED (n=8)	OWSED (n=8)	PHACT (n=8)
Glucose (mg/dL)	89.62±6.63	101.23±6.05*#	92.73±5.72
Cholesterol (mg/dL)	171.65±33.62	172±24.83	153.64±32.73
Triglycerides (mg/dL)	67.11±12.44	108±17.87*#	71.31±18.67
HDL-C (mg/dL)	53.05±13.62	50.34±6.44	50.93±8.99
LDL-C (mg/dL)	103.94±30.75	98.64±25.50	89.85±26.33

Values are presented as means ± SD. NWSED, normal weight sedentary; OWSED, overweight sedentary; PHACT, physically active; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol. *Indicates difference from NWSED group ($p<0.05$). #Indicates difference from PHACT group ($p<0.05$).

Figure legends

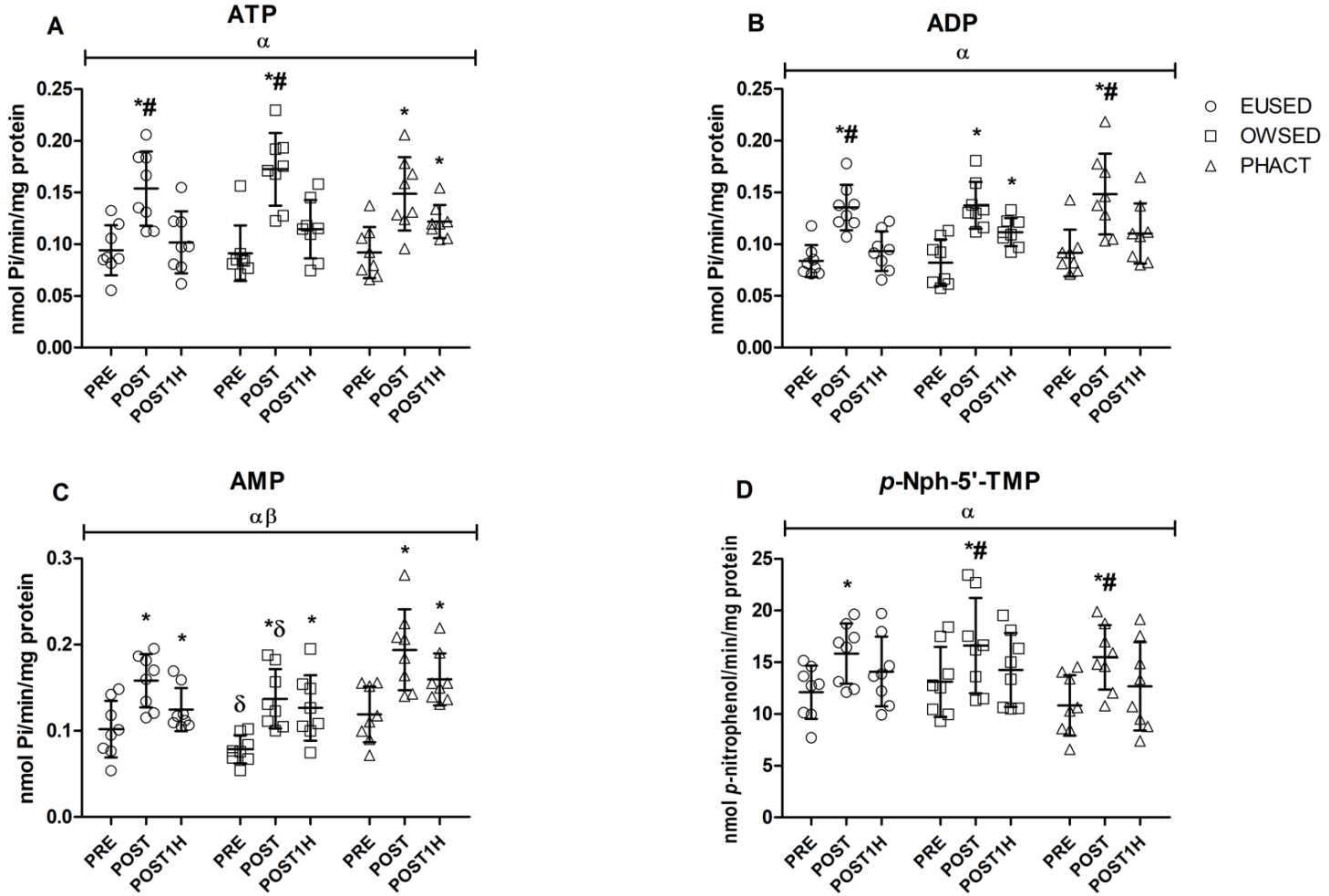
Figure 1. (A) ATP, (B) ADP, (C) AMP and (D) *p*-Nph-5'-TMP hydrolysis in the plasma of normal weight sedentary (NWSED, ○, n=8), overweight sedentary (OWSED, □, n=8) and physically active (PHACT, △, n=8) individuals at pre-exercise (PRE), post-exercise (POST) and 1h post-exercise (POST1H). Values are presented as means ± SD. $^{\alpha}$Indicates time effect (p<0.05) $^{\beta}$Indicates group effect (p<0.05). *Indicates difference from PRE (p<0.05). $^{\#}$Indicates difference from POST1H (p<0.05). $^{\delta}$Indicates difference from PHACT group (p<0.05).

Figure 2. Plasma levels of (A) ATP, (B) ADP, (C) AMP, (D) ADO, (E) INO, (F) XT, (G) HX and (H) AU in normal weight sedentary (NWSED, ○, n=8), overweight sedentary (OWSED, □, n=8) and physically active (PHACT, △, n=8) individuals at pre-exercise (PRE), post-exercise (POST) and 1h post-exercise (POST1H). Values are presented as means ± SD. $^{\alpha}$Indicates time effect (p<0.05). $^{\beta}$Indicates group effect (p<0.05). *Indicates difference from PRE (p<0.05). $^{\delta}$Indicates difference from PHACT group (p<0.05). $^{\theta}$Indicates difference from OWSED group (p<0.05).

Figure 3. Correlation analysis between 5'-nucleotidase (5'-NT) activity and $\dot{V}O_{2peak}$ values at (A) pre-exercise, (B) post-exercise and (C) 1h post-exercise (n=24); correlation analysis between plasma levels of adenosine and $\dot{V}O_{2peak}$ values at (D) pre-exercise, (E) post-exercise and (F) 1h post-exercise (n=24). Correlation analyses were performed using the Pearson correlation factor (p<0.05).

Figure 4. Correlation analysis between 5'-nucleotidase (5'-NT) activity and plasma IL-8 levels at (A) pre-exercise, (B) post-exercise and (C) 1h post-exercise (n=24); correlation analysis between TNF-α plasma levels at (D) pre-exercise, (E) post-exercise and (F)1h post-exercise (n=24). Correlation analyses were performed using the Pearson correlation factor (p<0.05).

Figure 1


A
ATP
α
nmol Pi/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
PRE
POST
POST1H
B
ADP
α
EUSED
OWSED
PHACT
C
AMP
αβ
0.3
0.2
0.1
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D
p-Nph-5'-TMP
nmol p-nitrophenol/min/mg protein
25
20
15
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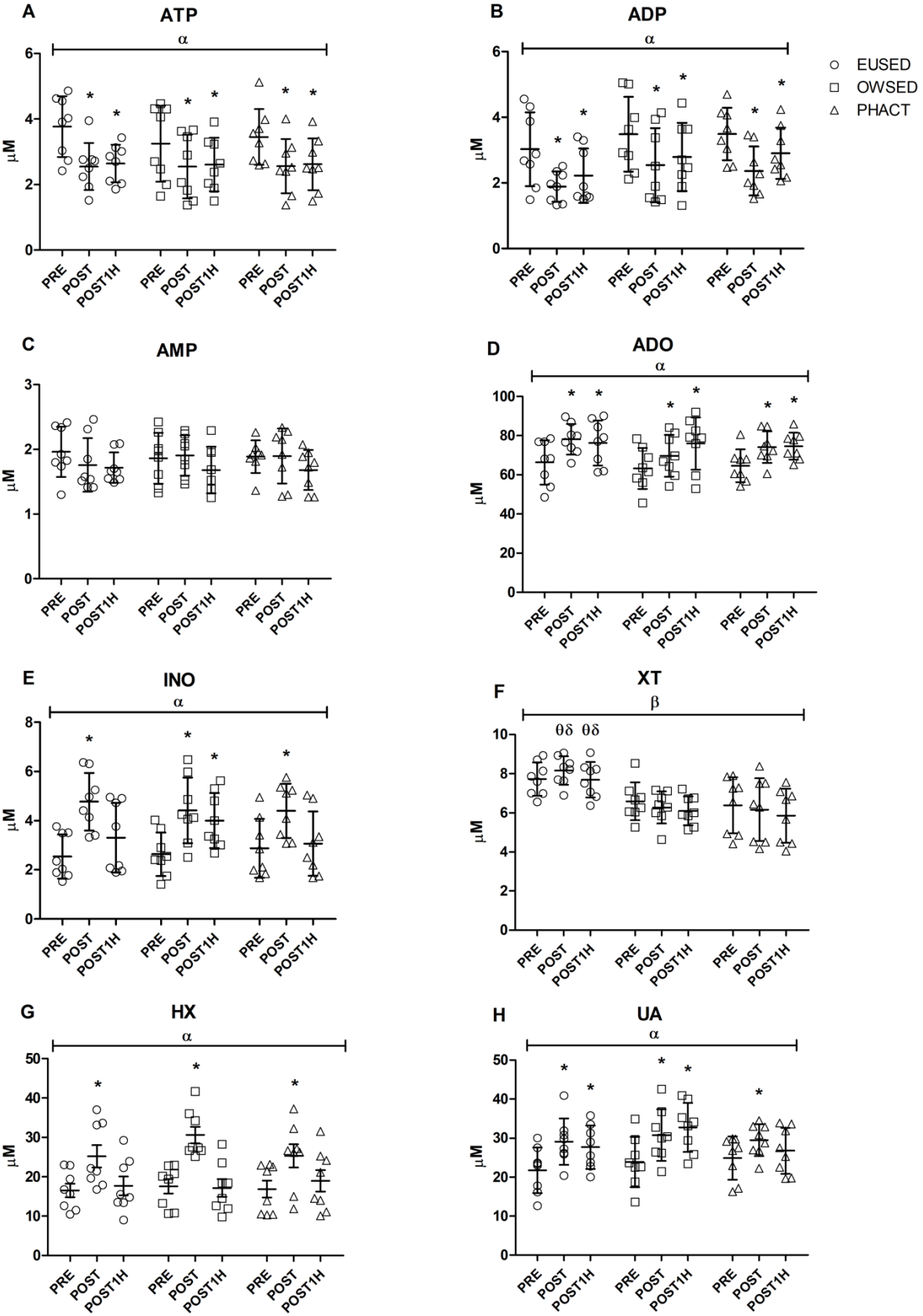
Figure 2


A
ATP
B
ADP
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POST1H

Figure 3

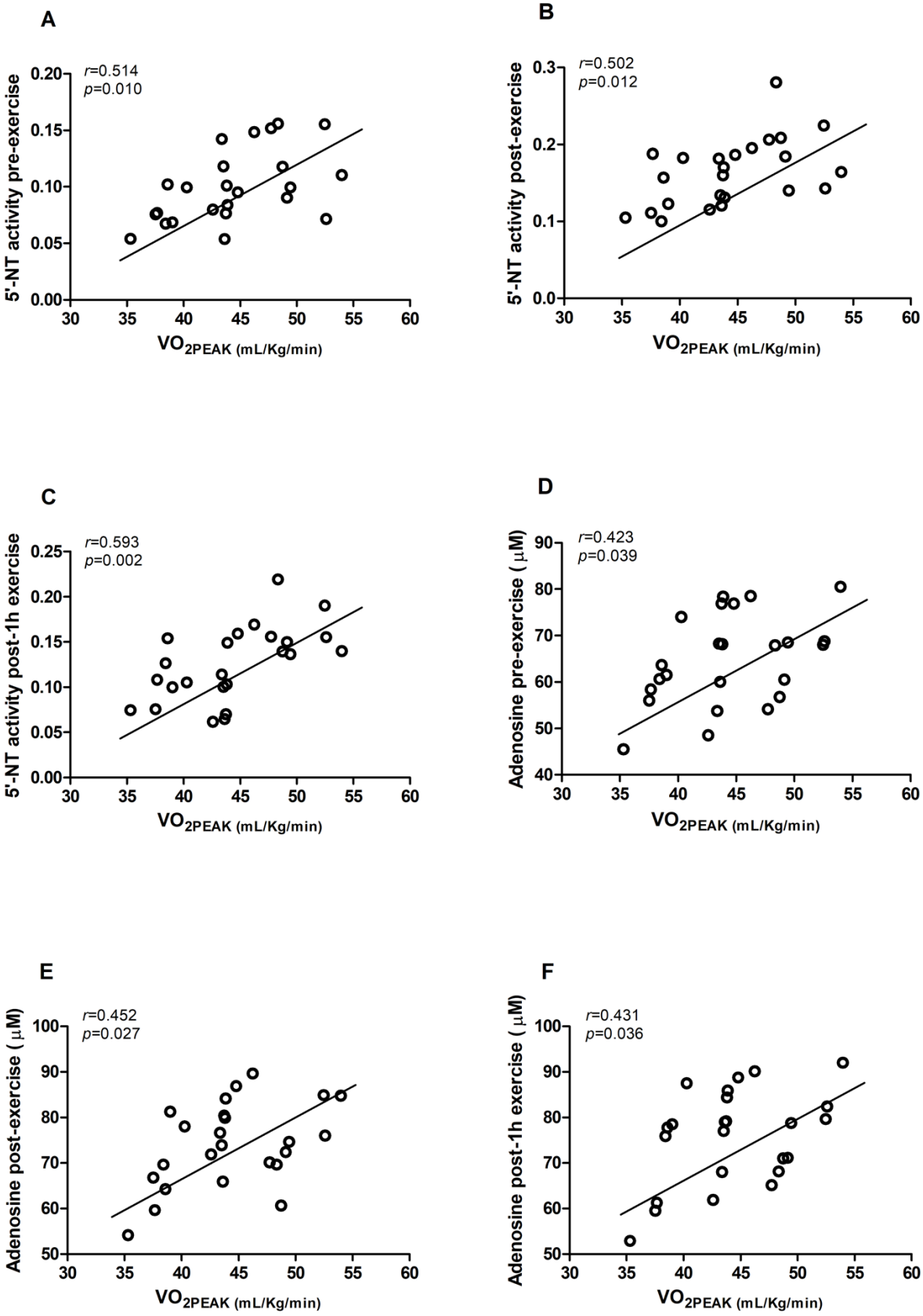
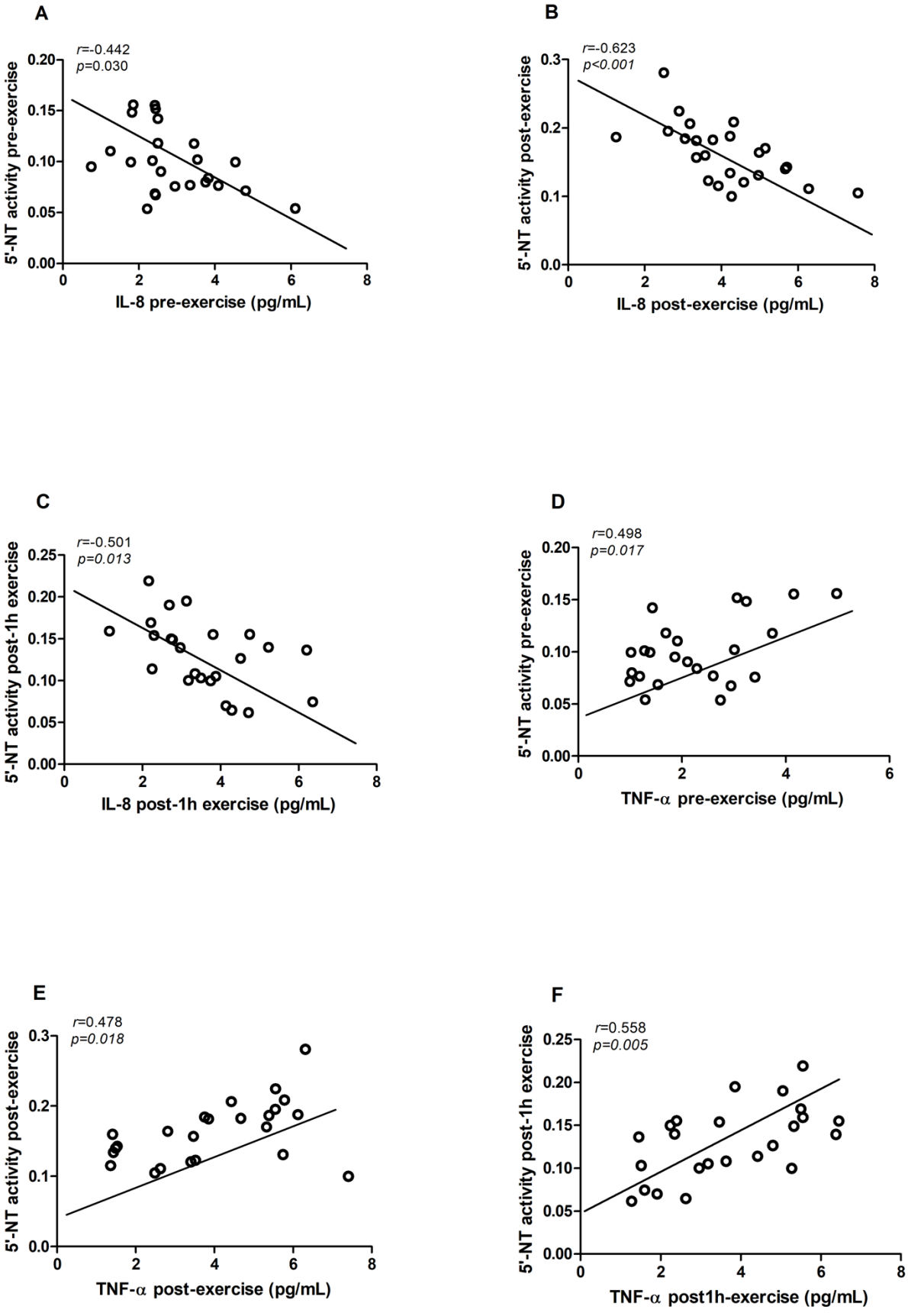

A
r=0.514
p=0.010
5'-NT activity pre-exercise
VO2PEAK (mL/Kg/min)
B
r=0.502
p=0.012
5'-NT activity post-exercise
VO2PEAK (mL/Kg/min)
C
r=0.593
p=0.002
5'-NT activity post-1h exercise
VO2PEAK (mL/Kg/min)
D
r=0.423
p=0.039
Adenosine pre-exercise (μM)
VO2PEAK (mL/Kg/min)
E
r=0.452
p=0.027
Adenosine post-exercise (μM)
VO2PEAK (mL/Kg/min)
F
r=0.431
p=0.036
Adenosine post-1h exercise (μM)
VO2PEAK (mL/Kg/min)

Figure 4


A
r=-0.442
p=0.030
5'-NT activity pre-exercise
IL-8 pre-exercise (pg/mL)
B
r=-0.623
p<0.001
5'-NT activity post-exercise
IL-8 post-exercise (pg/mL)
C
r=-0.501
p=0.013
5'-NT activity post-1h exercise
IL-8 post-1h exercise (pg/mL)
D
r=0.498
p=0.017
5'-NT activity pre-exercise
TNF-α pre-exercise (pg/mL)
E
r=0.478
p=0.018
5'-NT activity post-exercise
TNF-α post-exercise (pg/mL)
F
r=0.558
p=0.005
5'-NT activity post-1h exercise
TNF-α post1h-exercise (pg/mL)

6 CONSIDERAÇÕES FINAIS

A presente tese de doutorado aborda um tema raro no campo da bioquímica e fisiologia do exercício, buscando descrever, dentro de suas claras limitações, a participação da sinalização purinérgica nas adaptações agudas e crônicas ao exercício físico. Os dados apresentados que compõem esta tese (Capítulos I e II), sugerem consistentemente uma via de resposta purinérgica desencadeada por exercícios de intensidade moderada e alta, assim como suas relações com parâmetros cardiometabólicos vinculados ao exercício.

Primeiramente, a revisão sistemática (Capítulo I), até onde sabemos, é o primeiro trabalho deste tipo com alto rigor metodológico a sumarizar todos os diferentes tipos de estudos em humanos, demonstrando os efeitos do exercício físico na funcionalidade de enzimas purinérgicas. Assim, nós buscamos verificar de maneira acurada a capacidade que diferentes modalidades de exercícios agudo ou crônico, de moderada ou alta intensidade, possuem em modular a funcionalidade das nucleotidases, alterando sua atividade e/ou expressão em diferentes amostras biológicas de indivíduos saudáveis ou não-saudáveis. Destacamos que em nossas buscas, encontramos um baixo número de estudos relacionados ao tema, indicando a lacuna presente na literatura. Posto isto, em indivíduos saudáveis o exercício agudo, de intensidade moderada ou alta, parece modular positivamente a atividade e/ou expressão dessas enzimas, no entanto essa resposta não é equivalente ou regular em diferentes tipos de amostras biológicas, como plaquetas, linfócitos e plasma ou soro sanguíneo. Nesse sentido, os efeitos do exercício crônico nas nucleotidases aparentemente está ligado aos níveis de marcadores de saúde cardiovascular, como a pressão arterial, colesterol total, triglicerídeos e glicose plasmática. Ou seja, na medida que o exercício promove uma melhora dos valores pressóricos, perfil lipídico e glicemia, a funcionalidade dessas enzimas e seus produtos metabólicos retornam aos níveis basais, assemelhando-se à grupos controles saudáveis.

Posteriormente, o Capítulo II da tese refere-se ao manuscrito do artigo original intitulado: *Acute moderate-intensity aerobic exercise promotes purinergic and inflammatory responses in sedentary, overweight and physically active subjects*. Nossa investigação envolvendo indivíduos sedentários eutróficos, sedentários com sobrepeso e eutróficos fisicamente ativos, com diferentes perfis metabólicos, demonstra uma resposta purinérgica similar entre esses grupos. Portanto, embora indivíduos sedentários eutróficos ou com sobrepeso apresentem um perfil pró-inflamatório de baixa intensidade, maior risco doença cardiovascular e menor capacidade cardiorrespiratória, uma via comum de resposta purinérgica ao exercício aeróbico de intensidade moderada é compartilhada nos grupos estudados. Além

disso, nossos dados indicam correlações significativas principalmente entre a atividade da enzima 5'-NT, níveis de ADO e os marcadores inflamatórios IL-8 e TNF-α. Conjuntamente, nossos dados permitem sugerir o envolvimento, ao menos transitoriamente, das nucleotidases nas respostas inflamatórias desencadeadas pelo exercício. Um dos achados mais interessantes revelados por este trabalho é a correlação positiva entre a atividade da 5'-NT, os níveis plasmáticos de ADO e a capacidade cardiorrespiratória, fortalecendo a compreensão da importância desses parâmetros na saúde cardiovascular.

Em última análise, os resultados dessa tese apontam para uma via de sinalização envolvendo nucleotídeos e nucleosídeos extracelulares, contribuindo parcialmente na modulação das respostas desencadeadas por exercícios de curto- e longo-prazo com altas e médias intensidades. Por conseguinte, podemos sugerir a sinalização purinérgica como um possível mecanismo de proteção tecidual, participando da regulação de estímulos inflamatórios, trombóticos e atividade cardiovascular provocados pelo exercício físico (Figura 1).

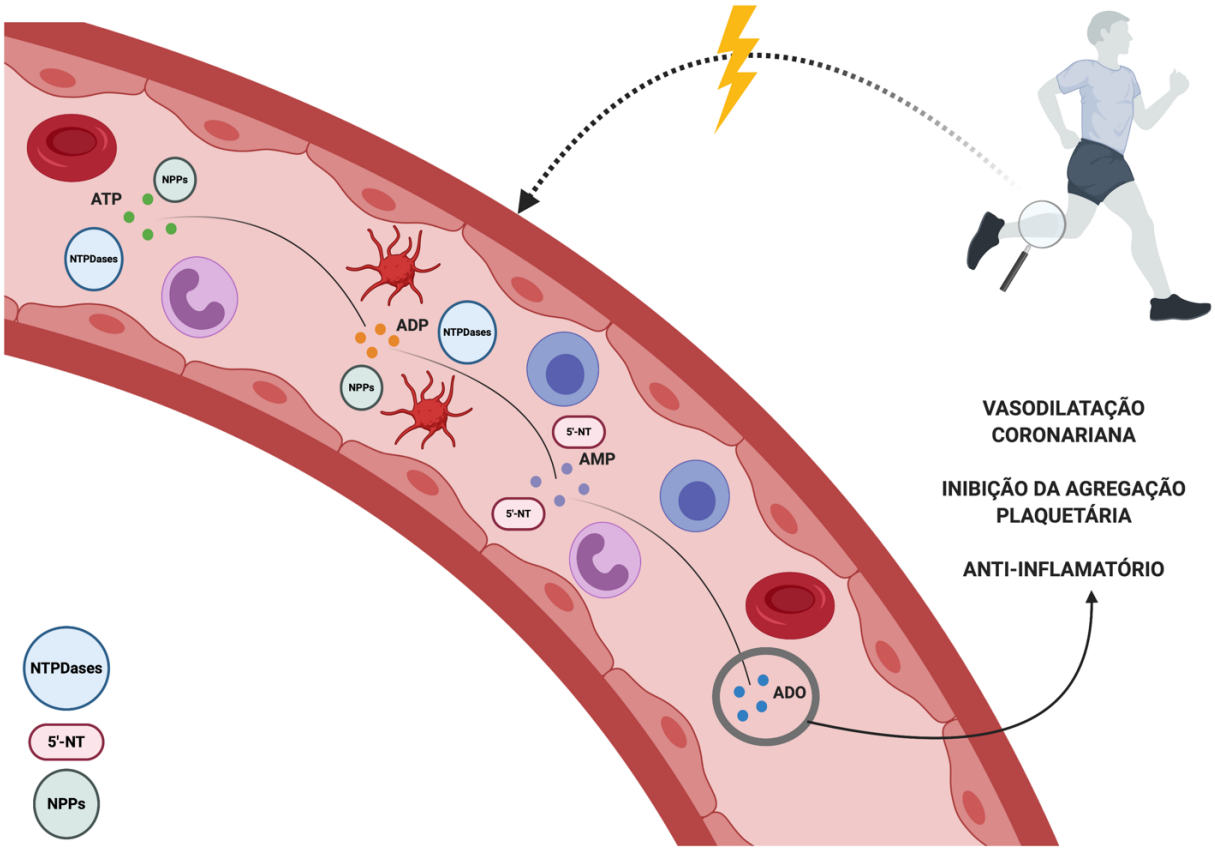


Figura 1. Resposta purinérgica ao exercício físico de alta e moderada intensidade. O exercício estimula a liberação de ATP por acarretar dano tecidual, hipóxia transitória e aumento de força de cisalhamento. Devido ao aumento do ATP, as nucleotidases acopladas às membranas celulares e solúveis na corrente sanguínea, hidrolisam ATP e ADP, participando da modulação da inflamação e coagulação, consequentemente pela atividade da 5'-NT ocorre o aumento da concentração de ADO. Assim, os efeitos protetivos estão associados ao metabolismo de ATP e ADP, evitando respostas pró-inflamatórias e pró-coagulatórias demasiadas, e ao aumento da ADO e sua ligação em receptores adenosinérgicos nas células alvo.

7 PERSPECTIVAS

Incialmente, enfatizamos a necessidade de futuros estudos relacionados ao tema apresentarem um tamanho amostral mais contundente e desenhos experimentais mais sofisticados, principalmente ensaios clínicos randomizados. Semelhantemente, a inclusão de indivíduos com perfis pró-inflamatórios mais exacerbados, como obesos de grau I, pré-diabéticos e com síndrome metabólica, possivelmente propiciassem respostas purinérgicas com uma correlação mais forte aos marcadores inflamatórios. Nesse sentido, podemos sugerir próximos trabalhos com a investigação de um painel inflamatório mais amplo e estudos de associação entre as respostas inflamatórias e purinérgicas desencadeadas pelo exercício.

Além disso, o projeto de pesquisa que originou esta tese de doutorado, tem como um dos seus objetivos investigar o papel do exercício físico na liberação de vesículas extracelulares (VEs), e a expressão e atividade de enzimas purinérgicas associadas a elas, como a E-NTPDase1/CD39 e E-5'-NT/CD73. Nesse sentido, nosso trabalho, apesar de muito esforço de todos os colaboradores, sofreu com diferentes intercorrências para realizar a padronização da técnica de isolamento de vesículas derivadas do plasma sanguínea e, respectivamente, a citometria dessas amostras biológicas. Esse fato é justificado principalmente por tratar-se de uma técnica nova, em um contexto nacional e internacional, sem a existência de uma técnica previamente estabelecida para as nossas condições. Consequentemente, estas análises ainda estão em andamento e em breve serão concluídas e os dados estarão disponíveis.

Outra lacuna presente na literatura, é sobre a atuação de diferentes modelos e intensidades de exercício nos receptores purinérgicos. Os efeitos do exercício na expressão dos receptores do tipo P2X, P2Y e P1 no endotélio, plaquetas, hemácias, linfócitos, neutrófilos, monócitos, macrófagos, adipócitos e células cardíacas. Dessa forma, será possível indicar o impacto completo da sinalização purinérgica nas respostas bioquímicas e fisiológicas desencadeadas pelo exercício. Adicionalmente, outra estratégia para implementação em futuros trabalhos é a utilização de inibidores das NTPDases e 5'-NT, possibilitando, entre outros, a compreensão do impacto dessas enzimas no contexto do exercício.

Por fim, a investigação sobre a funcionalidade da 5'-NT, os níveis plasmáticos de ADO e a relação com a capacidade cardiorrespiratória de indivíduos saudáveis e não-saudáveis é de proeminente importância. Considerando que dados prévios demonstram os fortes vínculos desses parâmetros na saúde cardiovascular, as relações supracitadas devem ser exploradas em trabalhos futuros como possíveis preditores de performance.

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9 ANEXOS

9.1 SUBMISSÃO NA REVISTA *PURINERGIC SIGNALLING*

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