UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS E TECNOLOGIA –
PRESIDENTE PRUDENTE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DO MOVIMENTO

AVALIAÇÃO DA RESPOSTA INFLAMATÓRIA EM CÉLULAS MONONUCLEARES DO SANGUE PERIFÉRICO DE JOVENS ADULTOS COM DIFERENTES COMPOSIÇÕES CORPORAIS E APTIDÕES FÍSICAS: PAPEL DA CAPSAICINA E RECEPTORES ADRENÉRGICOS

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Tiago Olean Oliveira

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Orientador: Prof. Dr. Fábio Santos Lira

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RESUMO

Níveis insuficientes de atividade física e maus hábitos alimentares relacionam-se com acúmulo excessivo de tecido adiposo visceral, alterações nas respostas do sistema nervoso simpático e inflamação crônica de baixo grau. Adoção da prática regular de exercício físico combinado com o auxílio de suplementos alimentares podem otimizar a diminuição dos estoques de gordura visceral, regular a expressão de receptores adrenérgicos β2 (AR- β_2), bem como aumentar a capacidade cardiorrespiratória, no qual em conjunto, contribuem para a redução das concentrações de citocinas pró-inflamatórias circulantes. No presente estudo, primeiramente foi analisado o impacto de diferentes status da composição corporal (eutróficos e sobrepesados/obesos) e níveis de aptidão cardiorrespiratória (sedentários e atletas) sobre o perfil metabólico e inflamatório sistêmico, posteriormente, investigamos a resposta inflamatória e metabólica frente estímulo inflamatório *ex vivo* (sangue periférico) e *in vitro* (células mononucleares do sangue periférico (PBMC)). Adicionalmente, foi avaliado o potencial efeito regulador do capsiate e o papel da via AR- β_2 em PBMC de indivíduos com diferentes composições corporais e níveis de aptidão física. Para responder a estas perguntas, foram recrutados vinte e três jovens saudáveis classificados como eutróficos, sobrepesados/obesos, sedentários e atletas. Foram realizadas avaliações de composição corporal e coleta de sangue em repouso/jejum para análises bioquímicas e ensaios *ex vivo* e *in vitro*. No primeiro artigo, as PBMCs dos indivíduos sobrepesados/obesos produziram maiores concentrações absolutas do fator de necrose tumoral – alfa (TNF-α) em paralelo com menores concentrações relativas de TNF-α, interleucina (IL) – 10 e proteína inflamatória de macrófagos – 1 alfa (MIP-1α) em comparação com indivíduos eutróficos. Adicionalmente, indivíduos sedentários apresentaram maiores concentrações de IL-6 circulantes, maiores valores relativos de TNF-α e maiores taxas de consumo de oxigênio basal em PBMC em comparação com atletas. No segundo artigo, o estímulo de capsiate, bem como a inibição seletiva do AR- β_2 não foram capazes de promover modulações nas concentrações de citocinas nas PBMCs. Em conclusão, a composição corporal e a aptidão física impactam no perfil e na resposta inflamatória das PBMCs, entretanto, o estímulo de capsiate e a inibição do AR- β_2 não promoveram a modulação da resposta inflamatória.

Palavras-Chave: Aptidão física, sistema imune, suplementação

ABSTRACT

Insufficient levels of physical activity and poor eating habits are related to excessive accumulation of visceral adipose tissue, changes in sympathetic nervous system responses, and low-grade chronic inflammation. The adoption of regular physical exercise combined with the help of food supplements can optimize the reduction of visceral fat stores, regulate the expression of β2 adrenergic receptors (AR-β2), as well as increase cardiorespiratory capacity, which together contribute for the reduction of circulating pro-inflammatory cytokine concentrations. In the present study, we first analyzed the impact of different status of body composition (eutrophic and overweight/obese) and levels of cardiorespiratory fitness (sedentary and athletes) on the systemic metabolic and inflammatory profile. ex vivo (peripheral blood) and in vitro (peripheral blood mononuclear cells (PBMC)). Additionally, the potential regulatory effect of capsiate and the role of the AR-β2 pathway in PBMC of individuals with different body compositions and levels of physical fitness were evaluated. To answer these questions, twenty-three healthy young people classified as eutrophic, overweight/obese, sedentary and athletes were recruited. Body composition assessments and blood collection at rest/fasting were performed for biochemical analysis and ex vivo and in vitro assays. In the first article, PBMCs from overweight/obese individuals produced higher absolute concentrations of tumor necrosis factor – alpha (TNF-α) in parallel with lower relative concentrations of TNF-α, interleukin (IL) – 10 and macrophage inflammatory protein – 1 alpha (MIP-1α) compared to eutrophic individuals. Additionally, sedentary individuals had higher concentrations of circulating IL-6, higher relative values of TNF-α and higher rates of basal oxygen consumption in PBMC compared to athletes. In the second article, capsiate stimulation as well as selective inhibition of AR-β2 were not able to promote modulations in cytokine concentrations in PBMCs. In conclusion, body composition and physical fitness impact the profile and inflammatory response of PBMCs, however, capsiate stimulation and AR-β2 inhibition did not promote the modulation of the inflammatory response.

Key words: Physical fitness, Immune system, supplementation

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Lista de abreviaturas e siglas

AMPK - Proteína quinase ativada por adenosina monofosfato

ANOVA – Análise de variância

AR-β2 – Receptor adrenérgico β2

ATP – Adenosina trifosfato

IMC – Índice de massa corporal

ETC – Cadeia transportadora de elétrons

ERK - Quinase regulada extracelularmente

HDL-c – Lipoproteína de alta densidade

HOMA-IR – Modelo de avaliação de homeostase de resistência à insulina

Ik-β - Inibidor do fator de transcrição nuclear *kappa* B

LDL-c – Lipoproteína de baixa densidade

LPS – Lipopolissacarideo

MCP-1 - Proteína 1 quimioatraente de monócitos-1

MIP-1α – Proteína inflamatória de macrófago - 1α

MVPA – Atividade física moderada-vigorosa

MyD88- Proteína de diferenciação mieloide

NF-κB – Nuclear factor *kappa* B

OCR – Taxa de consume de oxigênio

OXPHOS – Fosforilação oxidativa

PAMP – Padrões moleculares associados a patógenos

PBMC – Células mononuclear do sangue periférico

PGC-1α - Coativador 1 alfa do Receptor ativado por proliferador de peroxissoma gama

PI3K - Fosfatidilinositol-3-quinase

PMA - Forbol 12-Miristato 13-Acetato

RNS – Espécies reativas de nitrogênio

PPAR-γ - Receptor ativado por proliferador de peroxissoma *gama*

PRR – Receptores de reconhecimento de padrão

ROS – Espécies reativas de oxigênio

SAT – Tecido adiposo subcutâneo

SD – Desvio Padrão

TAG - Triacilglicerol

TC – Colesterol total

TLR – Receptor do tipo *toll*

TNF-α – Fator de necrose tumoral-*alfa*

VAT- Tecido adiposo visceral

VLDL – Lipoproteína de densidade muito baixa

$\dot{V}O_{2max}$ – Consumo máximo de oxigênio

SUMÁRIO

Apresentação

Esta dissertação está apresentada em concordância com as normas do modelo alternativo de dissertação do programa de pós-graduação em Ciências do Movimento da Faculdade de Ciências e Tecnologia da Universidade Estadual Paulista "Júlio de Mesquita Filho".

O presente material está dividido nas sessões:

– Projeto de pesquisa (Introdução, Objetivos e Métodos);

– Artigo científico 1 "Cytokines production and mitochondrial respiration in peripheral blood mononuclear cells: Impact of body fat and physical fitness levels" submetido e está atualmente sob revisão no periódico "*Exercise Immunology Review"*;

– Artigo científico 2 "Capsiate stimulus does not modulate cytokine production in peripheral blood mononuclear cells of healthy men with different body compositions and physical fitness" que será submetido no periódico "*Nutrients"*.

– Conclusões finais.

É importante ressaltar que ambos os artigos estão de acordo com as normas dos respectivos periódicos.

INTRODUÇÃO

A inatividade física é definida como um nível de atividade insuficiente para atender às recomendações atuais; de acordo com Organização Mundial da Saúde (OMS) são recomendados no mínimo 150 minutos semanais de atividade aeróbia de intensidade moderada, ou no mínimo 75 minutos semanais de intensidade vigorosa e/ou uma combinação de ambas as intensidades (WHO, 2020). Atualmente, a inatividade física é conhecida como um problema pandêmico, culminando em consequências econômicas e sociais afetando a saúde da população (KOHL et al., 2012). Esse hábito pode contribuir diretamente para a instalação e progressão de diversos fatores de risco associados com doenças crônicas não transmissíveis e comunicáveis (DCNT/DCNC), tais como doenças coronarianas e diabetes *mellitus* tipo 2, e alguns tipos de canceres (mama, cólon, entre outros). Estima-se que a inatividade física é responsável por 6 – 10% das principais DCNT/DCNC, além disso, 9% das mortes prematuras são atribuídas a este hábito (LEE et al., 2012).

O estilo de vida inativo está implicado em processos metabólicos alterados, reduzindo sensibilidade à ação da insulina, atenuando o metabolismo lipídico, e reduzindo a massa muscular (PEDERSEN; FEBBRAIO, 2012) e, quando associado a uma dieta rica em lipídios podem favorecer balanço energético positivo, com gasto calórico diário menor do que a ingestão calórica, contribuindo para aumento da gordura corporal, em especial, acúmulo de lipídeos no tecido adiposo, principalmente na região androide (BELAVÝ et al., 2014). Este quadro contribui para o aumento do risco de desenvolvimento de DCNT/DCNC, no qual apresentam característica em comum a inflamação crônica de baixo grau (MINIHANE et al., 2015) - marcada pelo aumento de duas a três vezes nas concentrações de citocinas pró-inflamatórias, tal como fator de necrose tumoral – alfa (TNF-α), interleucina – 6 (IL-6) e interleucina – 1 beta (IL-1β) e reduções das anti-inflamatórias tais como interleucina – 10 (IL-10), antagonista do receptor de IL-1 (IL-1ra) e adiponectina, bem como aumento da infiltração de células imunes para os tecidos metabólicos, favorecendo a polarização destas células para um fenótipo pró-inflamatório (GREGOR; HOTAMISLIGIL, 2011). Adicionalmente, indivíduos obesos apresentam consideráveis alterações nos fenótipos das células imunológicas, como o aumento na proporção de células com caráter pró-inflamatório/citotóxicas em relação as células anti-inflamatórias (ex. células T reguladoras (Treg)) (VON AH MORANO et al., 2020), bem como alteração do metabolismo celular das células mononucleares do sangue periférico (sigla do inglês PBMC), impactando na resposta inflamatória (DAI; RABINOVITCH; UNGVARI, 2012; SZEWCZYK et al., 2015).

De maneira interessante, células com caráter anti-inflamatório, tais como macrófagos ativados alternativamente (M2) e Tregs, apresentam predominância do metabolismo oxidativo, via maior fluxo da fosforilação oxidativa (OXPHOS), para a produção de trifosfato de adenosina (ATP) (LARTIGUE; FAUSTIN, 2013). Por outro lado, em condições pró-inflamatórias, ocorre rapidamente a alteração no metabolismo células para a predominância de vias glicolíticas, favorecendo a hiperpolarização da membrana mitocondrial interna, aumentando a produção de espécies reativas de oxigênio (sigla do inglês ROS) e promovendo a ativação de vias de sinalização inflamatórias tal como do fator nuclear – *kappa* B (NF-κB) (ZHANG et al., 2016), deste modo, mitocôndrias funcionais são imprescindíveis para a resposta inflamatória (XIE; LI; JIN, 2020). Foi demonstrado anteriormente que maiores concentrações de lipoproteína de baixa densidade (LDL-c) e pressão arterial elevada - características frequentemente encontrados em indivíduos obesos, estão associados com menores capacidades respiratórias em PBMCs (LI et al., 2015). Adicionalmente, mitocôndrias disfuncionais estão associadas ao aumento das concentrações de citocinas (WEST, 2017) e na produção de ROS (CHEN; ZHOU; MIN, 2018). Assim, um estilo de vida mais saudável e a manutenção de bons hábitos de saúde são essenciais para melhoria da composição corporal e na modulação do metabolismo, bem como da resposta imune.

Neste contexto, é crescente as estratégias de intervenção que utilizam da prática regular de exercício físico, intervenção nutricional ou combinação de ambos a fim de modular e/ou reverter os malefícios vinculados ao quadro inflamatório crônico de baixo grau promovido por hábitos alimentares inadequados e inatividade física (JOHNS et al., 2014; YAMASHITA et al., 2018). O exercício físico é uma estratégia não farmacológica de tratamento para reverter ou atenuar os efeitos deletérios à saúde promovidos pela inatividade física, atuando diretamente sobre a atividade muscular, gordura corporal, e inflamação crônica de baixo grau (WEDELL-NEERGAARD et al., 2018). Alguns dos efeitos anti-inflamatórios da prática regular do exercício físico podem ser atribuídos à redução do tecido adiposo (FISHER et al., 2011). Contudo, evidências crescentes demonstram que o exercício, mesmo na ausência de redução do tecido adiposo, pode impactar diretamente sobre o fenótipo das células imunes, reduzindo o número de monócitos pró-inflamatórios circulantes ($CD16^{+}$) e aumentando o número de linfócitos anti-inflamatório circulantes (ex: Treg) (GLEESON et al., 2011), diminuindo a expressão de receptores do tipo *toll* (TLR-4) em linfócitos e monócitos (ROBINSON et al., 2015), além de aumentar as concentrações de citocinas anti-inflamatórias circulantes (ex: IL-1ra e IL-10) após uma sessão de exercício e, cronicamente, reduzir as concentrações basais de IL-6 (PEDERSEN, 2017). Outro benefício atribuído a prática regular de exercício físico é o

aumento da aptidão cardiorrespiratória, considerada importante fator preditor de saúde, como redução da mortalidade precoce e desenvolvimento de doenças cardiovasculares e alguns tipos de cânceres (LAVIE et al., 2011). Adicionalmente, o aumento aptidão cardiorrespiratória está inversamente associado com menores valores de marcadores inflamatórios - tal como proteínas de fase aguda, em especial a proteína C reativa (PCR) (LAMONTE et al., 2002), redução no número de leucócitos circulantes - considerado outro parâmetro inflamatório (RANA et al., 2006), além de aumentar a respiração mitocondrial máxima das PBMCs (BUSQUETS-CORTÉS et al., 2017).

Adaptações desencadeadas pela sessão aguda de exercício físico, outro mecanismo discutido é a modulação do sistema imune por meio do aumento da ativação do sistema nervoso simpático e do eixo hipotálamo-pituitária-adrenal (HPA), responsável pela liberação de catecolaminas que modulam a ativação e função das células imunológicas. Entretanto, ainda não é totalmente esclarecido como a aptidão cardiorrespiratória pode modular as respostas imunes mediadas pela via adrenérgica. Em monócitos, a estimulação do receptor adrenérgico β2 (AR- β_2) foi capaz de induzir a inibição da liberação de citocinas inflamatórias, tais como TNF-α, IL-6. IL-1β, além de promover a contrarregulação da resposta inflamatória via liberação de IL-10 (VAN DER POLL et al., 1996; VAN POLL; LOWRY, 1997). Para entender o papel específico do AR- β_2 na resposta anti-inflamatória, Dimitrov et al. (2017) induziram a inibição farmacológica com antagonistas seletivos para β_2, e observaram aumento, mesmo na presença de agonistas do AR- β_2, da expressão de TNF-α induzida por LPS em monócitos, reforçando a o papel anti-inflamatório desta via. Em linfócitos, a exposição a catecolaminas promoveu a supressão da resposta inflamatória, inibindo a produção de citocinas inflamatórias pelas Th1 e aumentando a liberação de citocinas anti-inflamatórias (IL-10 e TGF-β) derivadas de linfócitos Th2 (ELENKOV; CHROUSOS, 2002). Os autores hipotetizaram que o efeito foi mediado pelo menos em parte, por prévia inibição da produção de IL-12 e estimulação de IL-4. Neste contexto, fica destacado o papel das catecolaminas na resposta inflamatória, particularmente mediada pelos AR- β_2.

No entanto, em quadros inflamatórios como na obesidade, uma das características é a alteração do funcionamento do eixo HPA e do SNS (hiperativação), favorecendo desregulações nas respostas neuroendócrinas e nos mecanismos de feedback estresse/imune e contribuindo para elevação do risco de desenvolvimento de doenças (PASQUALI et al., 2006). Em concordância com esta afirmação, monócitos de animais obesos sedentários apresentaram prejuízos na resposta fagocítica e microbicida, ficando mais susceptíveis a doenças (GÁLVEZ et al., 2020). Adicionalmente, foi demonstrado que indivíduos com obesidade central

apresentam menores expressões do AR- β_2 em PBMCs, possivelmente contribuindo para prejuízos no controle da resposta inflamatória nesta população (LEITE et al., 2017). Estes quadros apoiam a tese de que alterações no SNS e eixo HPA podem modular a resposta imunológica e, possivelmente, favorecer a instalação de doenças crônicas.

Em contrapartida, o exercício físico regular vem demonstrando ser uma ferramenta importante para promoção de saúde, podendo regular a expressão e resposta inflamatória mediadas pelo AR- β_2. Estudos recentes mostraram que o exercício físico agudo em intensidade moderada (20 minutos a 65-75% $\dot{V}O_{2pico}$) aumentou a produção de catecolaminas, bem como este aumento promoveu efeito supressor na produção de TNF-α em monócitos estimulados com LPS. Interessantemente, neste mesmo estudo, os autores investigaram a subunidade dos receptores adrenérgicos responsável por este efeito e, quando inibiram o AR- β_2 com um antagonista seletivo, observaram aumento na produção de TNF-α, confirmando a hipótese do importante papel dessa via (DIMITROV; HULTENG; HONG, 2017). Além do efeito agudo do exercício físico, foi demonstrado que a aptidão cardiorrespiratória é um fator preditivo para melhor responsividade de monócitos a ativação do AR- β_2, diminuindo a produção de TNF-α em indivíduos obesos (HONG et al., 2014).

Devido as diversas complicações associadas obesidade, novas estratégias vêm sendo estudadas para o tratamento e prevenção da doença, sendo a capsaicina postulada como um novo alvo potencial (BISHNOI et al., 2018). A capsaicina (trans-8-metil-N-faril-6-nonenamida), obtida por meio da planta do gênero *Capsicum*, pertencente à família dos capsainóides, juntamente com a capsiate, dihidrocapsaicina, nordi-hidrocapsaicina, homocapsaicina e homodi-hidrocapsaicina (REYES-ESCOGIDO; GONZALEZ-MONDRAGON; VAZQUEZ-TZOMPANTZI, 2011). O capsiate (4-hydroxy-3-methoxybenzyl (E)-8-methyl-6-nonenoate) extraído de pimentas "CH-19 Sweet" (*Capsicum annuum L.*) é um análogo da capsaicina que apresenta estrutura molecular similar, mas não apresenta pungência característica da capsaicina (IWAI; YAZAWA; WATANABE, 2003). No presente estudo foi utilizado o termo capsaicina para se referir a esta grande família de análogos, entre eles o capsiate. A capsaicina (capsiate) vêm sendo estudada pelo seu potencial efeito anti-inflamatório por meio da ativação de fatores de transcrição gênica específicos, tais como o receptor ativado por proliferador de peroxissoma gama (PPARγ), e por inibir a atividade do NF-κB (LEIHERER; MÜNDLEIN; DREXEL, 2013). Em estudo *in vitro* com macrófagos diferenciados a partir de células THP-1, tratados com 100 ng/mL de LPS para a ativação de vias inflamatórias e capsaicina (10 μM) para testar seus possíveis efeitos benéficos, observou-se regulação negativa do estado inflamatório com redução na produção de TNF-α, IL-6 e IL-1β

via inibição do NF-κB (TANG et al., 2015). Em animais submetidos a endotoxemia metabólica induzida por dieta rica em gordura, a suplementação de capsaicina (0,01g por 100g de dieta) por 12 semanas reduziu as concentrações plasmáticas de LPS, TNF-α, IL-1β e IL-6, paralelamente ao aumento de IL-10 (KANG et al., 2017).

Os achados supracitados sugerem que a suplementação com capsainóides seja um possível recurso nutricional para otimizar nas respostas anti-inflamatórias promovidas pela prática regular de exercício físico, contribuindo para a diminuição dos riscos à saúde causados pela inflamação crônica de baixo grau, associado ao estilo de vida inativo. Nesta perspectiva, ainda são incipientes na literatura estudos que investigaram as modulações na resposta inflamatória das células do sangue periférico de indivíduos altamente treinados e inativos fisicamente frente ao estímulo de capsaicina. Além disso, é importante elucidar o mecanismo responsável pelo efeito anti-inflamatório deste fitoquímico e se há uma dependência do AR-β_2.

JUSTIFICATIVA

A prática insuficiente de atividade física contribui para a hipertrofia do tecido adiposo visceral, promovendo um desequilíbrio metabólico, prejudicando o metabolismo das células imunes e favorecendo a instalação do quadro inflamatório crônico de baixo grau. O quadro inflamatório associada a obesidade aumenta o risco de desenvolvimento de doenças crônicas não transmissíveis como a diabetes *mellitus* tipo 2, doenças cardiovasculares e até alguns tipos de cânceres, aumentando os gastos públicos com saúde e reduzindo a expectativa de vida da população.

A prática regular de exercício físico é uma importante estratégia para promover o aumento da aptidão cardiorrespiratória, eficiência do metabolismo energético das células imunes, ambiente anti-inflamatório, redução da gordura corporal e proteção contra diversas doenças. Adicionalmente, a ativação do sistema nervoso simpático e eixo HPA durante as sessões de exercício, promove a liberação de catecolaminas e, que apresentam a capacidade de gerar respostas anti-inflamatória, mobilizar de substratos energéticos, aumentar da frequência cardíaca, dentre outras alterações mediadas pela sessão de exercício físico. Aliado aos efeitos benéficos do exercício físico e, devido a estudos preliminares mostrando a capacidade da capsaicina em promover funções semelhantes da via β adrenérgica, especialmente na resposta anti-inflamatória de células isoladas *in vitro*, foi hipotetizado que esse fitoquímico pode estimular resposta anti-inflamatória de células imunes circulante, bem como essa resposta pode ser mediada pela via β adrenérgica.

Portanto, o presente estudo buscou elucidar, primeiramente, as possíveis modulações das respostas inflamatórias e metabólicas de células mononucleadas do sangue periférico de indivíduos com diferentes aptidões cardiorrespiratória (atletas e indivíduos sedentários) e entender os possíveis mecanismos subjacentes. No segundo momento, buscamos avaliar a produção de citocinas promovidas pelo estímulo de capsaicina e o papel dos receptores adrenérgicos em indivíduos com composições corporais e aptidões físicas.

OBJETIVO GERAL

Verificar as respostas inflamatórias nas PBMCs de indivíduos com diferentes composições corporais e níveis de aptidão física (atletas e indivíduos sedentários) e avaliar o papel dos AR-β_2, mediante ao estímulo de capsaicina.

OBJETIVO ESPECÍFICO

- Analisar a influência do condicionamento físico e composição corporal sobre o perfil inflamatório e metabólico sistêmico;
- Analisar se indivíduos com diferentes composições corporais e aptidão física exibem diferentes respostas na produção de citocinas *ex vivo*. Para isso, as concentrações de citocinas do sobrenadante do ensaio *ex vivo* foram analisadas;
- Analisar *in vitro* a resposta inflamatória de PBMCs de indivíduos com diferentes composições corporais e níveis de aptidão física. Para isso, as concentrações de citocinas do sobrenadante do ensaio *in vitro* foram determinadas;
- Analisar a capacidade respiratória mitocondrial das PBMCs de indivíduos com diferentes composições corporais e níveis de aptidão física, utilizando o método de respirometria de alta resolução.
- Analisar a expressão gênica de *Ampk*, *Ppar-γ* e *Nf-κb* nas PBMCs de indivíduos com diferentes composições corporais e níveis de aptidão física.
- Analisar *in vitro* a capacidade de produção de citocinas pelas PBMCs após tratamento com LPS ou PMA. As PBMC foram também incubadas na presença ou ausência do antagosnista AR- β_2 (ICI 118, 551), associada ou não com capsiate para determinar as concentrações de citocinas no sobrenadante da cultura celular.

MATERIAIS E MÉTODOS

Recrutamento da amostra

Foram recrutados 23 homens saudáveis, divididos em dois estudos. No estudo 1, classificamos os voluntários de acordo com o IMC (*estudo 1*): grupo eutróficos (n= 8, IMC <24.9 kg/m^2) e grupo sobrepesado/obeso (n= 9, IMC >25 kg/m^2); e para o estudo 2, classificamos de acordo com aptidão física (*estudo 2*): grupo atletas (n = 6, IMC = 23,40 kg/m^2, AFMV: 71,11 ± 14,21 min^{-1}.dia), composto por atletas profissionais de atletismo altamente treinados e em fase de competição (quatro atletas treinavam para realização de provas de curta distância (100 e 200 metros) e dois para provas de fundo (5.000 metros)), bem com, o grupo inativos fisicamente (n = 8, IMC = 20,83 kg/m^2, AFMV: 34,85 ± 22,46 min^{-1}. dia) de modo a comparar indivíduos com diferentes condicionamentos físicos. Os participantes do estudo eram homens saudáveis e testaram negativo para o Sars-CoV-2 (anticorpos IgG e IgM) antes de iniciar as avaliações. A triagem dos participantes foi realizada por meio de uma visita ao laboratório para assinatura do termo de consentimento e aplicação dos questionários. A segunda visita foi dedicada para avaliar a composição corporal e coletas de sangue. Não foram incluídos indivíduos fumantes e que apresentassem quaisquer implicações cardiorrespiratórias e ortopédicas, que estivessem utilizando medicamentos anti-inflamatórios ou substância psicoativas, uso de suplementação alimentar ou recurso ergogênico, abuso de álcool (mais de três vezes na semana) ou outras drogas e/ou não aceitassem assinar o termo de consentimento livre e esclarecido. Todos os procedimentos realizados no estudo foram aprovados pelo Comitê de Ética em Pesquisa da Universidade local (Universidade Estadual Paulista - Unesp - Presidente Prudente, SP / Brasil) (CAAE: 26011919.0.0000.5402) e de acordo com a Declaração de Helsinki (ASSOCIATION, 2013).

Procedimentos

Avaliação da composição corporal

O peso corporal e estatura dos participantes foram avaliados utilizando uma balança eletrônica (Filizola PL50, Filizola Ltd., Brasil) e um estadiômetro fixo com precisão de 0,1 cm foi utilizado para avaliar a estatura. A circunferência da cintura foi mensurada por meio de uma fita métrica inelástica. Além disso, a composição corporal dos participantes foram realizadas por meio do exame de absorciometria de raio-x de dupla energia (DXA; scanner Lunar DPX-NT, General Electric Healthcare) em três compartimentos, massa magra, massa gorda e conteúdo mineral ósseo. As variáveis obtidas foram massa gorda (%), massa gorda livre (%),

andróide, ginoide, massa gorda (%) e calculada a relação entre elas (relação A/G). A razão androide/ginoide foi calculada como uma medida de obesidade central, associada ao risco de resistência à insulina e dislipidemia (WIKLUND et al., 2008). Também avaliamos o tecido adiposo visceral e subcutâneo por meio da ultrassonografia (TOSHIBA-Eccocee, transdutor convexo 3,7 MHz, Tóquio, Japão) operado por um médico de instituição especializada em diagnóstico por imagem. Os parâmetros e métodos para determinar os estoques de tecido adiposo visceral foram baseados em trabalhos publicados anteriormente apresentados por (RIBEIRO-FILHO et al., 2003).

Consumo alimentar

A avaliação do consumo alimentar foi realizada por meio de protocolo de autorrelato preenchido pelos participantes em dois dias da semana e um dia do final de semana. Os participantes foram incentivados a manter uma rotina nutricional regular. Os participantes anotaram o peso exato dos alimentos consumidos. Essas informações permitiram calcular o valor absoluto e relativo ao peso corporal do consumo calórico total e macronutrientes (carboidratos, proteínas e lipídios) durante três dias.

Nível de atividade física

Determinamos o nível de atividade física (AF) por meio de um acelerômetro (GT3X, ActiGraph LLC, Pensacola, FL, EUA). Os participantes utilizaram o acelerômetro durante sete dias (mínimo de 4 dias por pelo menos 10 horas diárias para serem incluídos na análise). Definimos intervalos de tempo de não uso de pelo menos 60 minutos consecutivos de contagem zero, com uma permissão de interrupção de atividade de 0-100 contagens por minuto com uma duração máxima de dois minutos consecutivos (MATTHEWS et al., 2012). Os valores das contagens por minutos foram calculados como a soma da contagem total de atividades dividida pelo número de dias válidos. O tempo de comportamento sedentário foi delineado como valores <100 contagens por minuto e a atividade física vigorosa moderada como> 2.020 contagens por minuto (TROIANO et al., 2008). Os dados foram utilizando o software ActLife (versão 6.9.2, Pensacola, FL, EUA).

Teste incremental máximo

Os participantes do estudo 1 realizaram o teste incremental máximo em uma esteira ergométrica (Inbramed MASTER CI, Inbrasport®, Porto Alegre, Brasil) em ambiente com temperatura e umidade controladas. Os gases expirados foram coletados respiração a respiração

com máscara de silicone conectada ao analisador de gases (Quark PFT - Cosmed®, Roma, Itália). O consumo máximo de oxigênio ($\dot{V}O_{2máx}$) foi adotado como o maior média dos últimos 30 segundos do consumo de oxigênio quando a diferença de <2,1 $ml.kg^{-1}.min^{-1}$ comparando os dois últimos estágios finais do teste incremental. Os participantes realizaram um aquecimento de 5 minutos caminhando a 5 km / h antes do teste. A velocidade de teste inicial foi fixada em 6 km / h e aumentada 1 km / h a cada 2 min. A inclinação da esteira foi mantida em 1%, e o teste foi encerrado quando o participante atingiu a exaustão voluntária. Incentivos verbais foram fornecidos para garantir que cada voluntário corresse até a exaustão. Além disso, um monitor de frequência cardíaca (Polar Vantage NV, Electro Oy, Finlândia) e uma escala de esforço subjetivo (escala de Borg 6-20) foram usados em paralelo com o analisador de gases.

Coletas de sangue e análise

Para o artigo 1, aproximadamente 10 mL de sangue foram coletados por punção periférica de uma veia do antebraço em repouso e após jejum de 12 horas. O volume colhido foi fracionado em tubos contendo gel anticoagulante (para soro), ácido etilenodiaminotetracético (EDTA) para plasma e fluoreto/EDTA para análise de glicose. As amostras de sangue foram centrifugadas a 3.000 rpm por 15 min a 4 ° C, amostras do plasma e o soro foram armazenados a -80 ° C para análises bioquímicas e imunoensaio de ELISA. As concentrações de glicose foram determinadas por meio de kits colorimétricos (Labtest, Brasil) e de insulina por ensaio imunoabsorbância ligado a enzima (ELISA). O HOMA-IR foi calculado usando a equação: HOMA-IR = (glicose [mmol/L] × insulina [μIU/mL]/22,5) (DR et al., 1985). Triacilglicerol (TAG), colesterol e frações (TC e HDL-c) foram determinados por meio de kits colorimétricos comerciais (Labtest®, Brasil) e o não-HDL foi calculado pela subtração entre as concentrações de TAG e HDL-c. Além disso, a equação de Friedewald et al. (1972) foi utilizada para estimar as concentrações de LDL-c.

As concentrações de citocinas presentes no soro foram determinadas pela técnica ELISA de acordo com as instruções do fabricante usando kits de alta sensibilidade de detecção (R&D System, Quantikine ELISA, Inc., Minneapolis, EUA) com intervalos entre 15,6-1.000 pg / mL para TNF-α, 3,13-300 pg / mL para IL-6, 7,8-500 pg / mL para IL-10 e 7,8-500 pg / mL para sTNF-RI, com um coeficiente de variação intra-ensaio de 1,9%, 2,8%, 2,1 % e 1,9%, com sensibilidade de 6,23 pg / mL, 0,7 pg / mL, 3,9 pg / mL e 1,69 pg / mL, respectivamente; Para determinar as concentrações de TNF-α, IL-6, IL-10, IFN-γ, MCP-1 e MIP-1α presentes em sobrenadantes de culturas de PBMC (artigo 1 e 2) e sangue total estimulado (artigo 1) foi realizado o ensaio imunoenzimático (ELISA) com kits comerciais (Duoset R&D System,

Minneapolis, EUA) com intervalos entre 15,6-1000 pg / mL, 9,3-600 pg / mL, 31,2-2000 pg / mL, 9,3-600 pg / mL, 15,6-1000 pg / mL e 7,8-500 e um coeficiente de variação intra-ensaio de 1,5%, 1,3%, 2,1%, 3,1%, 4,1% e 2,2%, respectivamente.

Estimulação *ex vivo* do sangue total com lipopolissacarídeo

Para o artigo 1, a técnica de estimulação do sangue total foi realizada utilizando protocolo proposto por Barry e colegas (BARRY et al., 2018). Foram colhidos 3 mL de sangue venoso por punção periférica da veia do antebraço em tubo contendo K3 - EDTA, o sangue foi diluído numa proporção de 1:10 em meio de RPMI-1640 (livre de soro) com 1x penicilina/estreptomicina. O sangue total diluído (540 µL) foi distribuído em placas de cultura de 24 poços e adicionado estimulação com LPS (Escherichia coli, tipo: 0111: B4; Sigma, St. Louis, MO), com concentração final de 10 ng/mL. As amostras foram incubadas por seis horas a 37 ° C em 5% CO_2. Posteriormente, os sobrenadantes foram coletados e armazenados em tubos de plástico e refrigerados em -80°C até o momento das análises.

Isolamento das células mononucleares do sangue periférico (PBMC)

As células mononucleares do sangue periférico (PBMC) foram isoladas do sangue periférico por meio da técnica de separação por gradiente de densidade, no qual o sangue foi adicionado ao histopaque na proporção de 1:1 (Histopaque – 1077. Sigma-Aldrich Co. LLC) e centrifugado por 30 minutos a 400 x *g* em uma centrífuga sem freio, a fim de estimular a sedimentação dos eritrócitos e separação das PMBCs, conforme as instruções do fabricante. Após este procedimento, foi realizado contagem das PBMCs em um hemocitômetro (Câmara de Neubauer) e, posteriormente, as células foram plaqueadas na concentração de $1x10^6$ por poço em placas de poliestireno de 24 poços com meio de cultura de células (RPMI-1640 – Sigma-Aldrich) enriquecido com bicarbonato de sódio [24mM], HEPES [20mM], glutamina [2mM], 10% de soro bovino fetal, [10000U/mL] de penicilina e [10000U/mL] de estreptomicina em um volume final de 1 mL.

Tratamento *in vitro* das células mononucleares do sangue periférico (PBMC)

As células de cada participante foram estimuladas com capsiate (CAP) (Sigma-Aldrich), bloqueador seletivo do AR-β_2 – ICI 118, 551 ($5x10^{-6}$ M) (Sigma-Aldrich), LPS (10 ng/mL) ou PMA (*Phorbol 12-myristate 13-acetate*) (50 ng/mL) e ionomicina (1 µg/mL), conforme demonstrado na **Figura 1**. Para o bloqueio seletivo do receptor adrenérgico β2, tratamos as

células com [5x10^{-6} M] por 30 minutos antes dos demais estímulos (LPS, PMA, CAP), baseados no trabalho de (DIMITROV; HULTENG; HONG, 2017). Após 24h de incubação, o meio de cultura foi coletado e armazenados em – 80ºC para análises posteriores.

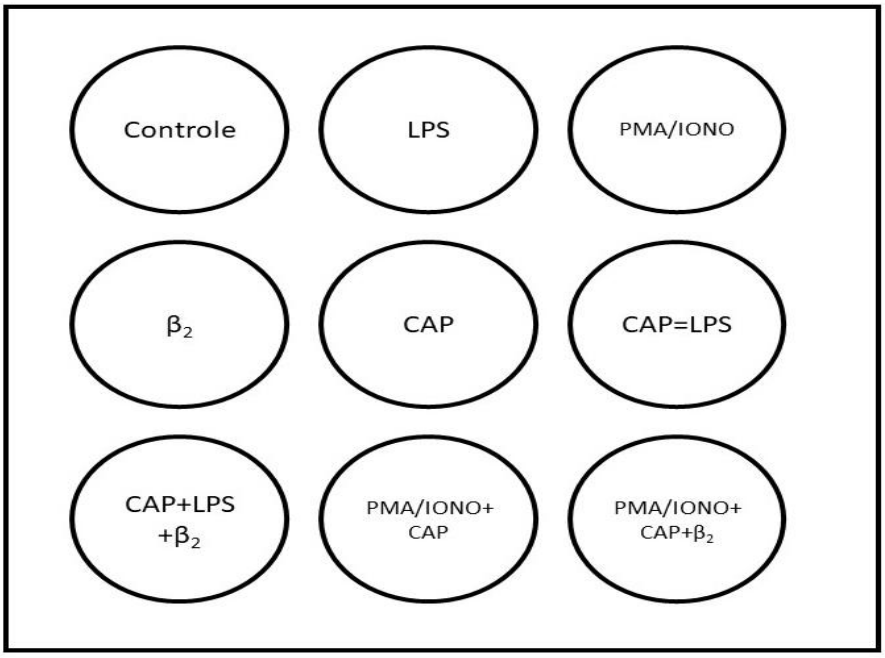

Figura 1. Desenho esquemático dos tratamentos utilizados na cultura de células.

Ensaio de MTT

Para o artigo 2, as PBMCs foram isoladas como descrito anteriormente e 2x10^5 células foram incubadas por 24 horas, a 37ºC em 5% CO_2, em placas de 96 poços com estímulos de capsiate em diferentes concentrações ([50µM] e [100µM]) em um volume final de 200 µL de meio de cultura. Após a incubação, foi adicionado 100 µL de MTT (brometo de 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2H- tetrazólio [5mg/mL] diluído em PBS 1x) em cada poço e as células foram novamente incubadas por 4 horas a 37ºC em 5% CO_2. Posteriormente, foi adicionado 100 µL de DMSO ou ácido isopropílico e realizado a leitura na absorbância de 570 nm.

Respiração mitocondrial

A respiração das células permeabilizadas foi realizada utilizando o protocolo descrito e desenvolvido por Ouroboros com modificações (PORTER et al., 2015; SILVA; OLIVEIRA, 2012). As medições de consumo de oxigênio foram realizadas incubando as células em tampão respiratório (MiR05) contendo 0,50 mmol L^{-1} de EGTA, 3,0 mmol L-1 de $MgCl_2$, 60 mmol L^{-1} de ácido lactobiônico, 20 mmol L^{-1} de taurina, 10 mmol L^{-1} KH_2PO_4, 20 mmol L^{-1} de HEPES,

0,11 mol L^{-1} de D-sacarose e 1g L^{-1} de BSA. O sensor utilizado foi desenvolvido por Olean-Oliveira e colaboradores (OLEAN-OLIVEIRA et al., 2018) e consiste em uma célula eletroquímica (câmara) contendo um eletrodo de fio de platina como um contra-eletrodo, um fio de prata como um eletrodo de pseudo-referência e um eletrodo de trabalho contendo um filme de polímero condutor com óxido de grafeno reduzido (chamado poli (azo-BBY) - rGO) sensível às mudanças de oxigênio. Em cada experimento, foi realizada a calibração utilizando uma solução MiR05 saturada com oxigênio dissolvido ($O_{2\ sat}$), uma solução em equilíbrio atmosférico ($O_{2\ atm}$) e uma solução saturada com gás N_2 (ausência de O_2) a 37°C. Antes das medições, a câmara foi preenchida com 1 ml de solução MiR05 e seu sinal foi estabilizado. As células foram suspensas em 1 ml de MiR05 e permeabilizadas pela adição de 20 µg ml^{-1} de digitonina (Sigma-Aldrich) que foi gentilmente homogeneizado por 5 minutos. A solução contendo as células foi adicionada à câmara obtendo-se um volume final de 2 ml e foram realizadas titulações. O vazamento de respiração do complexo I (CI) foi medido com adição de malato (5 mM), glutamato (10 mM) e piruvato (5 mM). A fosforilação (OXPHOS) foi obtida primeiro pela adição de ADP (2,5 mM) (OXPHOS $_{CI}$) seguida de succinato (10 mM) (OXPHOS $_{CI+CII}$) para obter a frequência respiratória máxima. Todas as medições foram registradas a 37°C.

Análise estatística

Foi realizado o teste de Shapiro-Wilk para verificar a normalidade dos dados. A estatística descritiva foi apresentada como média e desvio padrão (DP) para variáveis paramétricas e mediana e intervalo interquartil (IQR). O teste t de Student foi utilizado para comparar as características gerais dos participantes, ingestão alimentar e perfil inflamatório. Para os valores absolutos de sangue total estimulado, foi realizado a análise de variância bidirecional (ANOVA) [grupo (eutrófico versus sobrepeso/obeso) x condição (sem LPS ou com LPS)] e [grupo (sedentário versus atleta) x condição (sem LPS ou com LPS)] com ajuste pós teste de Tukey. Para testar a esfericidade da variância foi realizado o teste de Mauchly, quando violadas, foram ajustadas com correção de Greenhouse-Geisser. Para comparar a produção de citocinas nas diferentes condições propostas nos estudos 1 e 2, utilizou-se o teste de Mann-Whitney para verificar diferenças entre as médias das concentrações absolutas produzidas em cultura de sangue total e cultura de PBMC na presença ou ausência de LPS ou PMA mais ionomicina, concentrações relativas de citocinas em PBMC, sangue total estimulado e os estados de respiração mitocondrial. A significância estatística foi definida como $p < 0,05$, e os dados foram analisados usando o Statistical Package for the Social Sciences 22.0 (SPSS Inc. Chicago. IL.USA).

Artigo Científico 1

Cytokines production and mitochondrial respiration in peripheral blood mononuclear cells: Impact of body fat and physical fitness levels

Running title: *Impact on physical fitness and body fat on cytokine production*

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ABSTRACT

We aimed to investigate the effect of body fat and physical fitness levels on inflammatory and metabolic responses of peripheral blood mononuclear cells (PBMCs) in young men. We firstly investigated the influence of body mass index (BMI); eutrophic (age: 24.81 ± 4.60, BMI: 20.83 ± 2.06) versus overweight/obese (age: 29.49 ± 5.70, BMI: 29.73 ± 3.30). Secondly, we investigated the impact of physical activity levels: lower physical activity levels - LPAL (age: 24.81 ± 4.60, moderate to vigorous physical activity (MVPA): 34.85 ± 22.46 min^{-1}. day) versus higher physical activity level - HPAL (age: 22.56 ± 5.06, MVPA: 71.11 ± 14.21 min^{-1}.day). We analysed the blood concentrations of leptin, adiponectin, TNF-α, sTNF-RI, IL-6, IL-10, IFN-γ, MCP-1, and MIP-1α in the serum, and the supernatants of *in vitro* lipopolysaccharides (LPS) and phorbol 12-myristate 13-acetate (PMA)-stimulated whole blood PBMC cytokine production by ELISA assay. We also evaluated the mitochondrial respiration of the PBMCs. We observed a higher concentration of glucose (Cohen's *d* = 1.32) and lipid accumulation product index (Cohen's *d* = 1.57) in overweight/obese individuals compared to eutrophic individuals. In addition, higher concentrations of TNF-α (Cohen's *d* = 1.65) were observed in PBMCs with LPS from overweight/obese individuals compared to eutrophic individuals. Overweight/obese individuals exhibited a minor capacity for TNF-α (Cohen's *d* = 1.06), MIP-1α (Cohen's *d* = 1.19), and IL-10 (Cohen's *d* = 1.66) production in the presence of LPS, and TNF-α (Cohen's *d* = 1.33) in the presence of PMA than eutrophic individuals. Comparing physical fitness levels, LPAL individuals presented higher serum IL-6 levels (Cohen's *d* = 1.40) than HPAL. In PBMCs stimulated with LPS, LPAL individuals exhibited a greater capacity for MIP-1α (Cohen's *d* = 1.49) and IL-10 (Cohen's *d* = 1.74) production than HPAL. Interestingly, LPAL individuals exhibited a higher basal rate of O_2 consumption in PBMCs compared to HPAL (Cohen's *d* = 3.89). In conclusion, body fat composition and physical fitness levels lead to changes in energy metabolism and inflammatory responses in PBMCs.

Keywords: Immune system, immunometabolism, physical fitness

1. INTRODUCTION

The overlap of a global inactive lifestyle and high caloric diets is strongly associated with the rise of the obesity pandemic (38, 68). Although the global population is living longer, it does not seem to be living healthier, which has a tremendous impact on public health and the economic system (53, 97). Current trends show that over four billion people will be overweight or obese by 2050 (6). Bodirsk and colleagues (2020) reported that a rising demand for food is a consequence of changes in human population, demographic structure, mass urbanisation, physical activity levels, body weight, and body mass index (BMI) (6). Insufficient levels of physical activity and obesity are critical and independent factors that increase the risk of developing several chronic diseases related to metabolic disturbances, including type 2 diabetes mellitus (75), non-alcoholic fatty liver disease (47), cardiovascular diseases (52), and metabolic syndrome (54).

The triad of the modern lifestyle (physical inactivity, obesity, and high caloric diet) is associated with a gradual and permanent positive energy balance over time, favouring an increase in body fat mass (62). Adipose tissue hypertrophy and reduced adipogenesis trigger a pro-inflammatory state, named by Gregor and Hotamisligil (2011) as the phenomenon of "metainflammation" – a vicious cycle of inflammatory states mediated by excess circulating nutrients resulting in a state of obesity. In this context, the contribution of nutrition to inflammation depends on the types of macronutrients, their quantities, and their effects on different immune cells, as well as their interaction with different organs, including skeletal muscle, brain, liver, and gut, which play a vital role in inflammation (36, 62). Our group previously reported that a higher concentration of glucose in the bloodstream is related to disturbances in both innate and adaptive immune cell profiles, including but not restricted to higher proinflammatory M1 macrophage phenotypes and monocytes, T helper cell subsets (Th1/Th17) polarisation, and lower T regulatory cells (Treg), both in frequency and function (100). Collectively, alterations in cellular immunity are directly related to increases in systemic pro-inflammatory cytokines (100), such as tumour necrosis factor-alpha (TNF-α), interleukin-6, and 1β (IL-6 and IL-1β), and diminished levels of anti-inflammatory cytokines, such as IL-10 and IL-1ra, as well as in adipokines, with higher levels of leptin and lower levels of adiponectin (67, 84). Current evidence suggests that metabolic pathways are crucial for immune cells to facilitate the energy and biosynthesis that directly regulate immune cell functions (100).

The chronic elevation in inflammatory markers induces a state of permanent leukocyte activation and senescence, as well as a sterile response against pathogen or danger signal

perturbations. In this sense, immune cells are able to identify the extracellular and intracellular pathogen-associated molecular patterns (PAMPs) and other immunogens via pattern recognition receptors (PRRs) like the toll-like receptors (TLRs) located on the cell surface or on the cytosol of the cell. Intracellular signal cascades are initiated after PAMP binding to its specific receptor, leading to an enhanced innate response and the transcription and release of proinflammatory molecules (mainly TNF-α and IL-6). Although cytokine production after immunogen stimulation is related to the enhanced immune response against the pathogen, the chronic elevation of inflammatory mediators in non-pathogenic situations hampers immunity (104). In this sense, a low but persistent inflammation depresses both innate and adaptive immune responses. Past data have highlighted that obese individuals are more prone to infections than healthy subjects (29). In line with this, obesity is related to a reduced ability to induce an inflammatory response after endotoxin challenge, such as lipopolysaccharide (LPS) (42). Thus, there is an inverse association between markers of microbial products in the peripheral blood and cardiorespiratory fitness, suggesting that increasing physical function by exercise training may reduce persistent inflammatory activation (50). Furthermore, both obesity and low physical fitness were associated with an increased expression of TLR-4, the classical PRR ligand to LPS, and higher activation of the master inflammatory transcription NF-κB in peripheral blood mononuclear cells (PBMCs) (44). Thus, chronic inflammation induced by an unhealthy lifestyle may directly impact the acute immune response by hampering the ability of PBMCs to be finely activated by pathogens (29). However, basal systemic inflammation does not reflect the immune response against pathogens, and divergent results regarding the role of lifestyle on PBMC response to immunogens have been reported in both human and rodent models (43, 73).

PBMC composed mainly by monocytes and lymphocytes are, at least in part, quiescent in the steady state, they share the ability to rapidly respond to infection, inflammation, or other perturbations (76). The transition of PBMCs between their quiescent and active states requires nutrients available to support and direct functional changes due to robust energetic demand (45, 76). The immune response in overnutrition and insufficient physical activity leads to the activation of TNF associated receptors-myeloid differentiation protein-88/inhibitor of κB/nuclear factor κB (TRAF-MyD88/Ik-β/NF-κB) cascade signalling in adipocytes, wherein the expression of monocyte chemoattractant protein-1 (MCP-1) regulates the migration and infiltration of monocytes, promoting local inflammation by increasing the invasion of leukocytes and enhancing the production of reactive oxygen and nitrogen species (ROS/RNS) (59). The PBMCs are sustained by their specific changes in cellular metabolism, in which

glucose is used to fuel this response through two different pathways, such as conversing glucose to pyruvate in the cytoplasm and to fuel oxidative phosphorylation (OXPHOS), while on metabolic pathways, PBMCs also have the flexibility to metabolise glutamine via glutaminolysis or fatty acids via β-oxidation (45, 70, 76, 95).

Thus, it is well accepted that cells such as anti-inflammatory macrophages and Tregs use oxidative metabolism to produce ATP via the Krebs cycle and electron transport chain (ETC) in the mitochondria (49). In this sense, mitochondria regulate cell development, activation, proliferation, differentiation, and the death of immune cells (102). The full oxidation of macromolecules to CO_2 generates a proton gradient and a transmembrane potential through the pumping of protons by complexes I, III, and IV of the ETC. This proton gradient is essential for ATP synthase for the conversion of ADP and phosphate into ATP (69). On the other hand, proinflammatory conditions lead to a shift from OXPHOS to glycolysis, leading to the hyperpolarisation of the inner mitochondrial membrane, the generation of ROS, mainly by mitochondrial superoxide production, and the subsequent activation of key inflammatory pathways, such as NF-κB, extracellular signal-regulated protein kinases (ERK), and phosphatidylinositol 3-kinase (PI3K)-Akt (109). Thus, mitochondrial dynamics can orchestrate immunosurveillance by modulating the metabolic and physiological states of leukocytes and inflammation, which directly affect their function (107).

Additionally, obesity and lower levels of physical activity can negatively impact the metabolism of immune cells by reducing mitochondrial respiration (14, 93). Interestingly, T cells with dysfunctional mitochondria have been shown to promote premature senescence, increase circulating TNF-α concentrations, and promote premature mortality in animals (18). In humans, a reduction of mitochondrial respiration has been shown to be associated with higher concentrations of low-density lipoprotein cholesterol (LDL-c) and high blood pressure (55). Additionally, an increased production of inflammatory cytokines (103) and ROS (12) is associated with mitochondrial dysfunction. DeConne et al. (2020) demonstrated that LDL-c concentrations are negatively associated with maximum oxygen consumption, spare respiratory capacity, and oxygen consumption rate (OCR) in healthy individuals (17). Thus, a healthier lifestyle, such as engaging in physical exercise programs and balanced diets, may be essential for maintaining mitochondrial homeostasis of circulating immune cells, reflecting an enhanced inflammatory response.

Physical activity is a well-recognized strategy to promote changes in body composition by decreasing body fat, which may reduce body mass index (BMI) and increase cardiorespiratory fitness ($\dot{V}O_{2max}$) (87). Studies have shown that high levels of cardiorespiratory

fitness are associated with a reduction in cardiometabolic risk factors, such as hypertension, hyperlipidaemia, insulin resistance, type 2 diabetes mellitus, and inflammation (7, 51). Recently, Dorneles et al. (2021) reported exciting results, demonstrating that individuals with similar BMI (<25 kg/m^2) but with different cardiorespiratory fitness presented different inflammatory responses in T lymphocytes, presenting an inflammatory status through an increase in the number of circulating T lymphocytes. In addition, the production of TNF-α in T lymphocytes was higher than that in better-conditioned individuals (21).

Engagement in physical training programs has been demonstrated to promote a negative energy balance, attenuating the harmful effects of excess body fat, even in the absence of adipose tissue reduction (5, 26, 101). Additionally, it is widely accepted that bouts of exercise promote a strong anti-inflammatory response characterized by the secretion of myokines (i.e., IL-6) and anti-inflammatory cytokines IL-4, IL-1ra, and IL-10, neutralizing inflammatory cytokines lasting for hours after the session to compensate for transient energy impairment and tissue repair. In fact, in the long-term, it is possible to establish an anti-inflammatory environment via physical exercise (33, 78, 100). The central discussion regarding physical exercise and physical fitness status mechanisms is partially supported by the activation adrenergic receptors (β2-AR) by catecholamines, and the nutrient sensor AMP-activated protein kinase (AMPK) and translocation of peroxisome proliferator-activated receptor gamma (PPAR-γ), as well as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (34, 48, 72), Concomitantly, energetic demand is augmented to supply energy to skeletal muscles for contractile activity, resulting in lower energetic substrate availability for immune cells. Emerging evidence indicates that acute exercise mobilises immunoregulatory Treg cells and haematopoietic stem cells and reduces toll-like receptor expression on monocytes and the peripheral frequency of proinflammatory terminally differentiated T cells modulating the phenotypes of circulating immune cells (24, 82). This reduces pro-inflammatory monocytes (CD14$^-$ CD16$^+$) and increases Treg cells, which have been shown to be engaged during endurance exercise training programs and are associated with higher Treg cell frequency and increased IL-10 production in response to antigen stimulation, supporting the hypothesis of the beneficial role of fitness (30, 39, 82).

Additionally, the improvement of physical fitness promotes modulation in muscle mitochondria (41) and in circulating immune cells, preventing mitochondrial damage caused by ROS (8), regulating the synthesis of mitochondrial proteins (i.e. mitochondrial uncoupling protein) (10) and increasing the membrane potential and mitochondrial metabolism of immune cells (99). Interestingly, the fatty acid oxidation capacity is linked to the anti-inflammatory

response of immune cells (102), and sedentary individuals who performed low-intensity physical exercise showed an improvement in the fatty acid oxidation capacity in PBMCs (56). This supports the idea that improving physical fitness can impact the mitochondrial metabolism functionality of PBMCs, contributing to the prevention of dysfunctions due to unhealthy lifestyles.

However, despite advances in the study of the beneficial effects of physical exercise on the immune system, studies elucidating the modulation promoted by: (1) different body composition status (eutrophic versus overweight/obese) and (2) levels of physical fitness (lower physical activity versus higher physical activity level) on the systemic inflammatory response and PBMC production are still necessary. Therefore, in the present study, we aimed to investigate the impact of BMI (study 1) and the impact of different levels of physical fitness (study 2) on the *ex vivo* and *in vitro* inflammatory responses in PBMCs.

2. METHODS

2.1. Participant Recruitment

A total of 23 healthy young men were recruited to participate in the study. Participants were included in the study if they tested negative for anti-Sars-CoV-2 (IgG and IgM antibodies), did not present any health disorders (e.g. cardiorespiratory and osteoarticular diseases), and did not use any ergogenic substance or medication for at least six months before the study. The participants of study 1 were classified as eutrophic (n = 8, BMI <24.9 kg/m^2) and overweight/obese (n = 9, BMI >25 kg/m^2). The participants of study 2 were classified as lower physical activity levels - LPAL (n = 8, MVPA: 34.85 ± 22.46 min^{-1}. day) and higher physical activity level - HPAL (n = 6, MVPA: 71.11 ± 14.21 min^{-1}. day) who trained in Presidente Prudente, São Paulo, Brazil (part of the Brazilian athletics team). Written informed consent was obtained from all the participants. This study was approved by the local research Ethics Committee of the São Paulo State University "Júlio de Mesquita Filho" and duly registered in the Brazil Platform (national electronic system created by the Federal Government to systematise the receipt of research projects involving human beings in Ethics Committees throughout the country (CAAE: 26011919.0.0000.5402). All experiments were conducted according to the 2013 Revision of the Declaration of Helsinki (3).

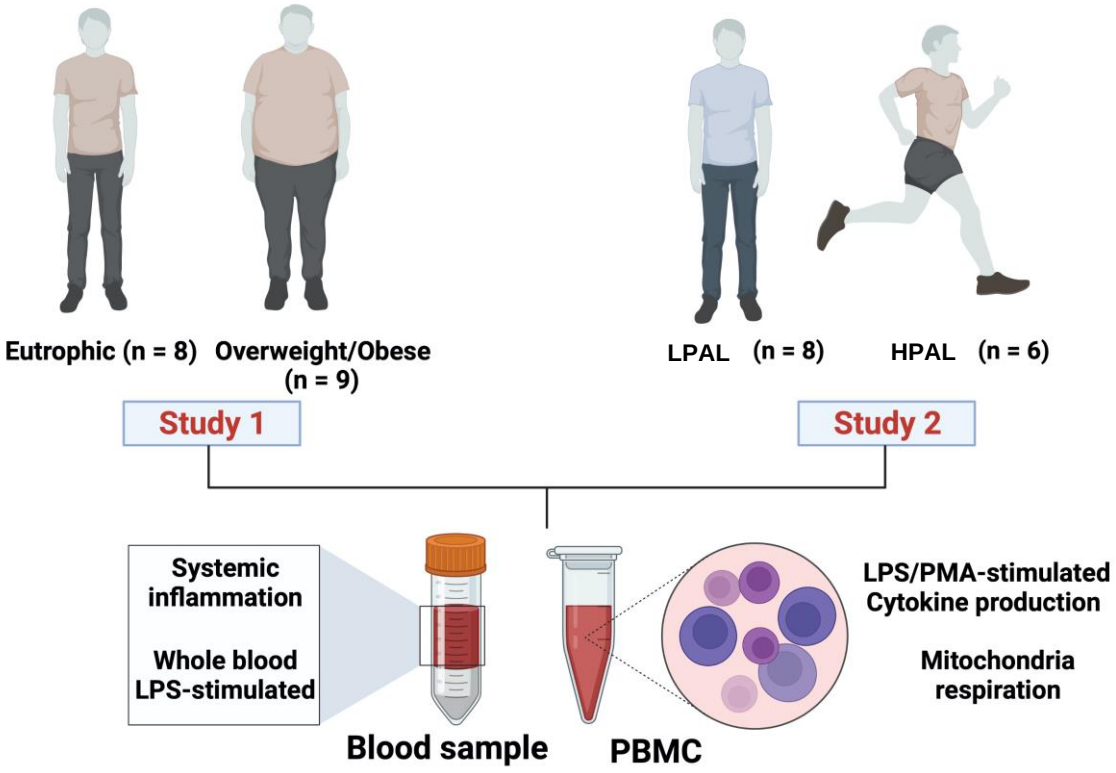

Figure 1. Experimental design of study 1 and 2.

2.2. Anthropometric and body composition assessment

The body weight was evaluated using an electronic scale (Filizola PL50 Ltd., Brazil) and the height was measured using a fixed stadiometer with an accuracy of 0.1 cm. Waist circumference was measured using a measuring tape. Additionally, the body composition in three compartments (lean mass, fat mass, and bone mineral content) was evaluated by dual-energy x-ray absorptiometry (DXA; Lunar DPX-NT scanner, General Electric Healthcare). We also obtained fat mass (%), free fat mass (%), android and gynoid fat mass (%), and the ratio between android and gynoid (A/G ratio). The android-to-gynoid ratio was calculated as a measure of central obesity, associated with the risk of insulin resistance and dyslipidemia (105). We evaluated the visceral (VAT) and subcutaneous (SAT) adipose tissue using an ultrasound device (TOSHIBA-Eccocee, convex transducer of 3.7 MHz, Tokyo, Japan) operated by a physician from an institution specialized in imaging diagnosis. The parameters and methods for determining visceral adipose tissue were based on previously published works presented by Ribeiro-Filho et al. (2003) (85). The visceral adiposity index and deep-abdominal adipose tissue (DAAT) were estimated using the mathematical formulas previously described by Amato et al. (2010) (83) and Brundavani et al. (2006) (9).

2.3. Measures of physical activity level

We measured the physical activity (PA) using an accelerometer (GT3X, ActiGraph LLC, Pensacola, FL, USA). The participants used the accelerometer during seven days (minimum of 4 days for at least 10 hours a day to be included in the analysis). We define non-use time intervals of at least 60 consecutive minutes of zero count, with an activity interruption allowance of 0-100 counts per minute with a maximum duration of two consecutive minutes (63). The values of counts per minutes were calculated as the sum of the total activity count divided by the number of valid days. Sedentary time was delineated as values <100 counts per minute and moderate-vigorous physical activity as > 2,020 counts per minute (98). Data were using ActLife software (version 6.9.2, Pensacola, FL, USA).

2.4. Maximal incremental test

Participants performed a maximal incremental test on a treadmill (Inbramed MASTER CI, Inbrasport®, Porto Alegre, Brazil) in an environment with controlled temperature and humidity. Expired gases were collected breath-by-breath with a silicon mask connected to the gas analyzer (Quark PFT - Cosmed®, Rome, Italy). The average of the last 30 seconds of the test was defined as the $\dot{V}O_{2max}$. Participants performed a warm-up of 5 min walking at 5 km/h before the test. The initial test speed was set at 6 km/h and increased by 1 km/h every 2 min. Treadmill inclination was maintained a 1%, and the test was terminated when the participant reached voluntary exhaustion. Verbal encouragements were provided to ensure that every volunteer ran to exhaustion. Additionally, a heart rate monitor (Polar Vantage NV, Electro Oy, Finland) and a subjective effort scale (Borg scale 6-20) was used in parallel with the gas analyzer.

2.5. Dietary Intake Analysis

We analyzed the participants' habitual food consumption using a self-report protocol on two weekdays and one weekend day. The participants were encouraged to maintain their regular nutritional routine. We calculated the total food intake and macronutrients such as protein, carbohydrate, and lipid consumption.

2.6. Blood sample and analysis

We collected approximately 10 mL of blood through a peripheral puncture of a forearm vein after 12-hour fasting period. The blood volume was separated into tubes containing anticoagulant gel for serum isolation, ethylenediaminetetraacetic acid (EDTA) for plasma isolation, and fluoride/EDTA for plasma isolation to glucose analysis. The blood samples were centrifuged at 3,000 rpm for 15 min at 4 °C, and the plasma and serum were stored at -80ºC until further colorimetric and enzyme-linked immunosorbent assay (ELISA) analyses. Glucose concentrations were analyzed using colorimetric kits (Labtest, Brazil) and insulin using ELISA commercial kits (Monobind Inc., USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the equation: HOMA-IR = (glucose [mmol/L] × insulin [μIU/mL]/22.5) (64). Triacylglycerol (TAG), cholesterol, and fractions (total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c)) were analyzed by commercial colorimetric kits (Labtest®, Brazil), and Non-HDL was calculated by subtraction between triacylglycerol (TAG) and HDL-c concentrations. Additionally, the Friedewald et al. (1972) (28) equation was used to estimate LDL-c. The lipid accumulation product was estimated using a mathematical formula previously described by Kahn (2005) (46).

The concentrations of cytokines present in the serum were performed by ELISA technique according to the manufacturer's instructions using high detection sensitivity kits (R&D System, a biotechne brand, Quantikine ELISA, Inc., Minneapolis, USA) with ranges between 15.6–1,000 pg/mL for TNF-α, 3.13–300 pg/mL for IL-6, 7.8–500 pg/mL for IL-10 and 7.8–500 pg/mL for sTNF-RI, with an intra- assay coefficient of variation of 1.9%, 2.8%, 2.1%, and 1.9%, respectively. The concentrations of TNF-α, IL-6, IL-10, IFN-γ, MCP-1 and MIP-1α present in supernatants from PBMC cultures and stimulated whole blood were determined by ELISA with commercial kits (Duoset R&D System, Minneapolis, USA) with ranges between 15.6–1000 pg/mL, 9.3–600 pg/mL, 31.2–2000 pg/mL, 9.3–600 pg/mL, 15.6–1000 pg/mL and 7.8–500 and an intra-assay coefficient of variation of 1.5%, 1.3%, 2.1%, 3.1%, 4.1%, and 2.2%, respectively.

2.7. Whole blood stimulated with LPS

We use a protocol similar to that described by Barry et al. (2018) (4) for the whole blood stimulated *ex vivo* assay. Approximately 3 mL of blood was collected in tubes containing K3-EDTA and diluted 1:10 in serum-free RPMI medium (Sigma) containing 5 mM glucose with 1x penicillin/streptomycin. Diluted whole blood was plated in 24-well culture plates (540 μL) and incubated in the presence or absence of lipopolysaccharide (LPS) (Escherichia coli, type:

0111: B4; Sigma, St. Louis, MO) at the final concentration of 10 ng/mL for six hours at 37°C in 5% CO_2. After this period, the supernatant was collected and stored at -80ºC for further analysis.

2.8. Peripheral blood mononuclear cell culture

The samples were added to Histopaque®-1077 (Sigma-Aldrich Co. LLC) (1:1) for PBMC isolation, centrifuged at 400 x g for 30 minutes at room temperature. The PBMC were washed with phosphate-buffered saline (PBS) and resuspended in 1 mL of enriched-medium RPMI. A total of $1x10^6$ PBMC/mL were incubated for 24 hours at 37°C and 5% CO_2 in cell culture medium (RPMI-1640 Sigma-Aldrich Co. LLC) enriched with glutamine [2mM], HEPES [20mM], 10% fetal bovine serum, and antibiotics penicillin [100U/mL] and streptomycin [0.1 mg/mL] in 24-well plates (Kasvi - PR / Brazil). PBMCs were cultured in the absence or presence of LPS [10 ng/mL] (Escherichia coli, type: 0111: B4; Sigma, St. Louis, MO), or with Phorbol 12-Myristate 13-Acetate (PMA) [50 ng/ml] (Sigma, St. Louis, MO) plus ionomycin [1 μg/ml] (Sigma, St. Louis, MO). After 24 hours, supernatants were collected and stored at -80ºC for further cytokine analysis.

2.9. Measurement of high-resolution respirometry in permeabilized PBMC

A total of $0.5x10^6$ PBMCs of three volunteers of each group were utilized to measure mitochondrial respiration. Respiration of permeabilized cells was performed using the protocol describe and developed by Ouroboros (OROBOROS INSTRUMENTS, Innsbruck, Austria) with modifications (81, 92). Oxygen consumption measurements were made by incubating the cells in respiratory buffer (MiR05) containing 0.50 mmol L^{-1} EGTA, 3.0 mmol L^{-1} $MgCl_2$, 60 mmol L^{-1} lactobionic acid, 20 mmol L^{-1} taurine, 10 mmol L^{-1} KH_2PO_4, 20 mmol L^{-1} HEPES, 0.11 mol L^{-1} D-sucrose and 1 g L^{-1} BSA. The sensor used was developed by Olean-Oliveira at al. (2018) (71), which consists of an electrochemical cell (chamber) holding a platinum wire electrode as a counter electrode, a silver wire as a pseudo-reference electrode and a working electrode containing a conductive polymer film with reduced graphene oxide (called poly(azo-BBY)-rGO) sensitive to oxygen changes. On the morning of each experiment, a background calibration was performed using a MiR05 solution saturated with dissolved oxygen ($O_{2\ sat}$), a solution in atmospheric equilibrium ($O_{2\ atm}$) and a solution saturated with N_2 gas (absence of O_2) at 37°C. Prior to the measurements, the chamber was filled with 1 ml of MiR05 solution, and its signal was stabilized. The cells were suspended in 1 ml of MiR05 and permeabilized by adding 20 μg ml^{-1} of digitonin (Sigma, St. Louis, MO) which was kindly homogenized for 5 minutes. The solution containing the cells was added to the chamber obtaining a final volume

of 2 ml and titrations were performed. ROUTINE respiration which represents the consumption of mitochondrial O_2 under endogenous conditions was measured from the addition of cells to the respiration medium (MiR05). The complex I (CI) LEAK respiration was measured with addition of malate (5 mM), glutamate (10 mM) and pyruvate (5 mM), which represents mitochondrial O_2 consumption after inhibition of ATP synthesis compensating for proton leak. The oxidative phosphorylation (OXPHOS) was obtained first by ADP (2.5 mM) addition (OXPHOS $_{CI}$) followed by succinate (10 mM) (OXPHOS $_{CI+CII}$) to obtain the maximal respiratory rate, reflecting the maximum capacity of mitochondrial producing ATP from oxidative phosphorylation (80). All measurements were recorded at 37 °C.

3.0. RNA isolation and RT-PCR assays

Total PBMC RNA was extracted with Brazol reagent (LGC Biotechnology Ltda. – Cotia – SP) according to the manufacturer's recommendations and 11 volunteers were used for the RT-PCR analyzes. Reverse transcription to complementary DNA (cDNA) was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems—Thermo Fisher Scientific, Foster, CA). The cDNA was stored at -80 ºC for subsequent analysis (AMPK, PPAR-γ and NF-kB) by RT-PCR with Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are shown in the **Table 1**. Quantification of gene expression was carried out using the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) as an internal control. Relative quantification of genes of interest was calculated using the $2^{-\Delta\Delta CT}$ formula, in which cycle threshold (CT) is the difference between the CT value for the gene of interest and CT value for the housekeeping gene (2).

Table 1. Prime sequences of RT-PCR analysis

Gene	Primer forward	Prime reverse
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
AMPK	GGCACGCATACCCTTGAT	TCTTCCTTCGTACACGCAAATAA
PPAR-γ	CAGCCTTTAACGAAATGACCA	TGTGGAGTAGAAATGCTGGA
NF-κB	GAAGCACGAATGACAGAGGC	GCTTGGCGGATTAGCTCTTTT

3.1. Statistical Analysis

We performed the Shapiro-Wilk test to verify the normality. Descriptive statistics were presented as mean and standard deviation (SD). Student's t test was used to compare the general

characteristics of the participants, dietary intake, and inflammatory profile. For the absolute values of whole blood stimulated, a two-way analysis of variance (ANOVA) [group (eutrophic versus overweight/obese) x condition (without LPS or with LPS)] and [group (LPAL versus HPAL) x condition (without LPS or with LPS)] was conducted followed by Tukey's post hoc test. To test the sphericity of the variance, a Mauchly's test was performed, in which case the analyzes were adjusted with a Greenhouse-Geisser correction. To compare cytokine production under the different conditions proposed in studies 1 and 2, the Mann-Whitney test was used to verify differences in absolute concentrations produced in whole blood culture and PBMC culture in the presence or absence of LPS or PMA plus ionomycin, relative concentrations of cytokines in PBMC, whole blood stimulated and the states of mitochondrial respiration. Statistical significance was set at p <0.05, and the data were analyzed using the Statistical Package for Social Sciences 22.0 (SPSS Inc. Chicago. IL.USA).

3. RESULTS

3.1 *Impact of body composition on metabolic parameters, whole blood-stimulated blood, and cultured PBMCs*

The general characteristics of eutrophic and overweight/obese individuals are shown in Table 2. As expected, overweight/obese individuals had a higher BMI (Cohen's d = 3.24, p < 0.001), body weight (Cohen's d = 3.46, p < 0.001), body fat (%) (Cohen's d = 1.60, p = 0.006), gynoid and android body fat (gynoid: Cohen's d = 1.68, p = 0.004; android: Cohen's d = 1.78, p = 0.003), android/gynoid fat ratio (Cohen's d = 1.27, p = 0.018), visceral and subcutaneous adipose tissue (visceral: Cohen's d = 1.63, p = 0.006; subcutaneous: Cohen's d = 1.25, p = 0.015), waist circumference (Cohen's d = 2.28, p < 0.001), visceral adiposity index (Cohen's d = 1.35, p = 0.0004), and DAAT (Cohen's d = 2.39, p = 0.0002) compared with eutrophic individuals. Furthermore, overweight/obese individuals presented a lower lean body mass (Cohen's d = 1.65, p = 0.005) and $\dot{V}O_{2max}$ (Cohen's d = 1.69, p = 0.002) than eutrophic individuals. In the metabolic profile, we observed a higher concentration of glucose (Cohen's d = 1.73, p = 0.003) in overweight/obese individuals than in eutrophic individuals **(Table 2)**. On the other hand, we did not observe significant differences in sedentary time, moderate to vigorous physical activity, total intake, carbohydrate, protein, and lipid intake in overweight/obese individuals compared to eutrophic individuals. In this sense, the body composition distribution, regardless of physical activity, dietary intake, and metabolic parameters, appears to be prominent in eutrophic and overweight/obese individuals.

Table 2. General characteristics in eutrophic and overweight/obese individuals in study 1.

Variables	Study 1		P-value
	Eutrophic	Overweight/obese	
General characteristics			
Age (y)	24.81 ± 4.60	29.49 ± 5.70	0.078
Weight (kg)	62.51 ± 6.33	97.04 ± 12.61	<0.001*
BMI (kg.m^2)	20.83 ± 2.06	29.73 ± 3.30	<0.001*
$\dot{V}O_{2max}$ (mL.min^{-1}.kg)	51.51 ± 5.30	40.25 ± 7.76	0.002*
MVPA time (min.d^{-1})	34.85 ± 22.46	26.17 ± 24.11	0.492
Sedentary time (min.d^{-1})	697.30 ± 260.60	590.60 ± 52.80	0.466
Body composition			
Fat mass (%)	21.35 ± 2.21	30.49 ± 7.75	0.006*
Lean Mass (%)	75.20 ± 2.19	66.83 ± 6.84	0.005*
Fat Gynoid (%)	21.19 ± 3.63	30.58 ± 7.03	0.004*
Fat Android (%)	18.79 ± 4.35	35.98 ± 12.91	0.003*
A/G ratio	0.91 ± 0.16	1.14 ± 0.20	0.018*
Waist Circumference (cm)	74.94 ± 4.93	97.67 ± 13.18	<0.001*
Visceral Adipose Tissue (cm)	3.09 ± 1.01	5.20 ± 1.53	0.006*
Visceral Adiposity index (log)	1.87 ± 0.12	2.41 ± 0.31	0.0004*
Deep-Abdominal Adipose-Tissue Index (DAAT, cm^2)	90.15 ± 31.15	244.70 ± 85.31	0.0002*
Metabolic profile			
Total Cholesterol (mg.dL^{-1})	157.50 ± 27.21	162.00 ± 27.93	0.423
TAG (mg.dL^{-1})	113.80 ± 7.74	116.90 ± 19.21	0.670
LDL-c (mg.dL^{-1})	95.86 ± 23.31	100.40 ± 25.41	0.708
Non-HDL (mg.dL^{-1})	118.70 ± 23.55	123.80 ± 26.74	0.682
HDL-c (mg.dL^{-1})	38.88 ± 9.35	38.20 ± 6.82	0.869
Glucose (mg.dL^{-1})	76.91 ± 2.81	85.74 ± 6.66	0.003*
Insulin (µUI.mL)	10.66 ± 8.46	15.11 ± 11.08	0.372
HOMA-IR	2.05 ± 1.65	3.16 ± 2.28	0.146
Lipid Accumulation Product Index	13.02 ± 6.77	43.92 ± 19.50	0.0007*
Inflammatory profile			
Leptin (ng/mL)	4.08 ± 4.01	17.90 ± 11.60	0.006*
Adiponectin (µg/mL)	6.49 ± 2.67	5.80 ± 2.43	0.587
Adipo/Lep ratio	5.20 ± 7.51	2.38 ± 4.31	0.089
Leptin/VAT (cm) ratio	1.22 ± 0.96	3.17 ± 2.07	0.028*
Adiponectin/VAT (cm) ratio	2.32 ± 1.17	1.31 ± 0.99	0.114
Leptin/SAT (cm) ratio	5.14 ± 5.47	10.04 ± 6.13	0.104
Adiponectin /SAT (cm) ratio	8.13 ± 5.12	4.19 ± 2.66	0.061
TNF-α (pg/mL)	9.03 ± 2.03	8.72 ± 1.61	0.737
sTNF-RI (pg/mL)	65.05 ± 9.85	60.82 ± 16.53	0.539
IL-6 (pg/mL)	3.65 ± 1.39	3.86 ± 1.50	0.774
IL-10 (pg/mL)	10.95 ± 4.30	8.12 ± 3.29	0.146
IL-10/TNF-α ratio	1.25 ± 0.48	0.94 ± 0.41	0.176
Dietary profile			
Total intake (kcal.kg $^{-1}$)	28.86 ± 7.79	25.18 ± 5.82	0.351
Protein (g.kg $^{-1}$)	1.16 ± 0.27	1.11 ± 0.33	0.722
Carbohydrate (g.kg $^{-1}$)	3.01 ± 1.13	2.75 ± 1.04	0.657
Lipids (g.kg $^{-1}$)	1.13 ± 0.64	1.17 ± 0.48	0.912

Note: BMI, body mass index; MVPA, moderate-to-vigorous physical activity; A/G ratio, android, and gynoid ratio; TAG, triacylglycerol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; Adiponectin/Leptin ratio, adiponectin and leptin ratio; IL, interleukin; TNF-α, tumor necrosis factor – α; sTNF-RI, soluble tumor necrosis factor receptor type I; Data are presented as mean ± SD. *P<0.05.

With regards to the overall metabolic and pro- and anti-inflammatory profile, overweight/obese individuals presented higher concentrations of glucose (Cohen's d = 1.32, p = 0.003) and lipid accumulation product index (Cohen's d = 1.57, p = 0.0007), as well as leptin (Cohen's d = 1.59, p = 0.006) and leptin/VAT ratio (Cohen's d = 1.20, p = 0.028) compared with eutrophic individuals, but not for adiponectin, adiponectin/leptin ratio, leptin/SAT ratio, adiponectin/VAT ratio, adiponectin/SAT ratio, or circulating cytokines TNF-α**,** sTNF-RI, IL-6, IL-10, and IL-10/TNF-α ratio **(Table 2)**. Evaluating the pro- and anti-inflammatory response in whole blood-stimulated blood in the presence and absence of LPS, we observed a higher production of IL-6 (Cohen's d = 1.88, p = 0.020) in overweight/obese individuals after stimulation with LPS **(Figure 2**, panel B**)**. On the other hand, the pro- and anti-inflammatory response of cultured PBMCs in the presence and absence of LPS, there was a higher production of LPS-induced TNF-α (Cohen's d = 1.65, p = 0.006) in overweight/obese individuals than in eutrophic individuals **(Figure 3**, panel A**)** and a lower production of TNF-α (Cohen's d = 1.06, p = 0.046), IL-10 (Cohen's d = 1.66, p = < 0.001) **(Figure 3,** panel F and H**)**, and MIP-1 (Cohen's d = 1.19, p = 0.026) relative to non-LPS stimulation in overweight/obese individuals compared with eutrophic individuals **(Figure 3,** panel J**)**. Cultured PBMCs of overweight/obese individuals showed a lower production of TNF-α (Cohen's d = 1.33, p < 0.001) relative to PMA plus ionomycin compared with eutrophic individuals **(Figure 4**, panel C). In this context, we observed a higher response to pro-inflammatory challenge and lower anti-inflammatory response in overweight/obese individuals than in eutrophic individuals. Therefore, the results of our studies address body composition as a pivotal mediator of the pro-inflammatory response of cultured PBMCs in overweight/obese individuals.

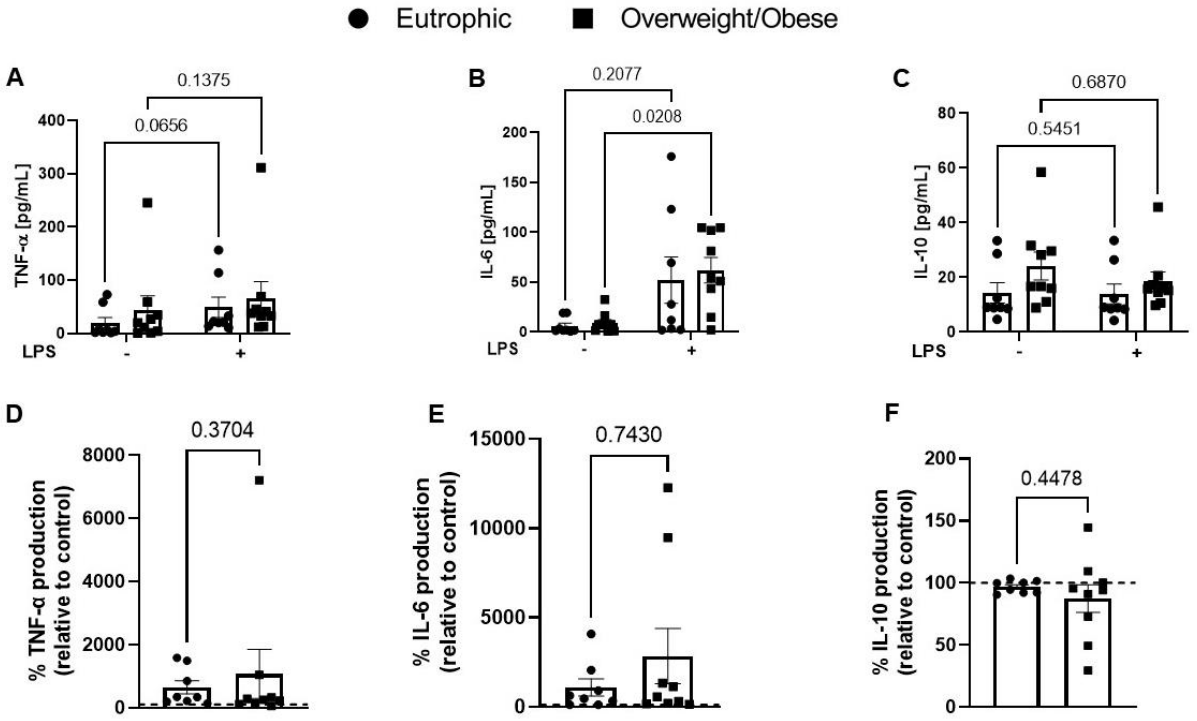

Figure 2. Whole blood stimulated in absence or presence of LPS [10 ng/mL]. Production of TNF-α (A), IL-6 (B), IL-10 (C), TNF-α relative to LPS (D), IL-6 relative to LPS (E) and IL-10 relative to LPS (F) in eutrophic and overweight/obese individuals from study 1. Data are expressed as mean ± SD.

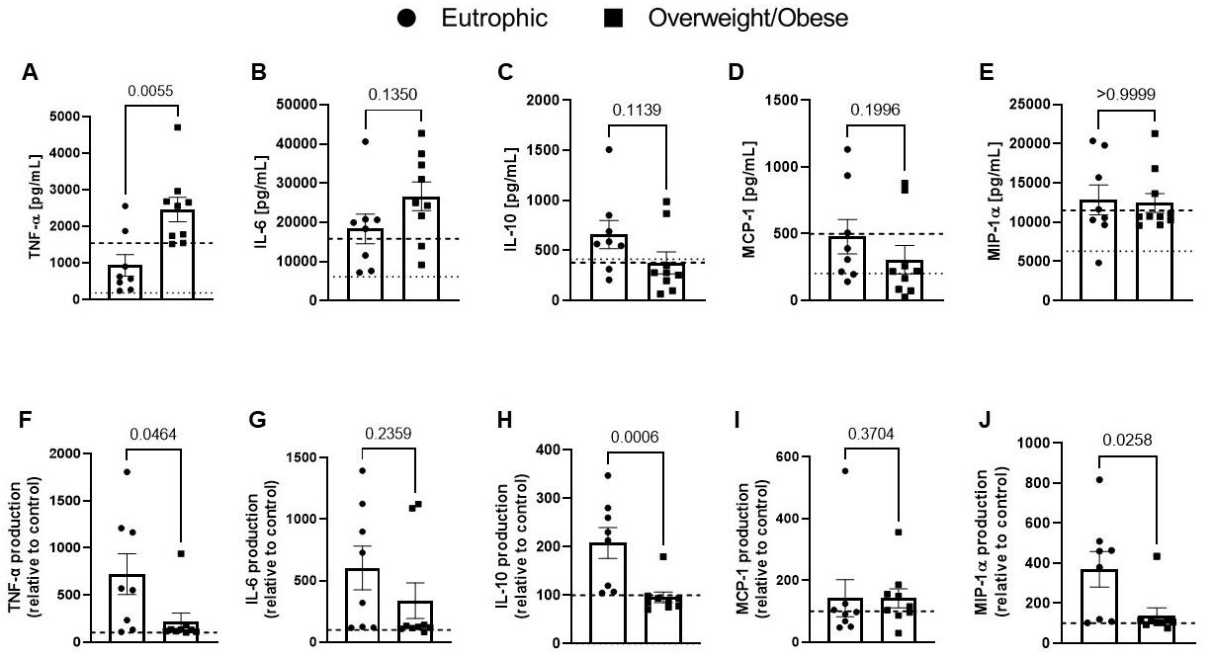

Figure 3. Cultured PBMCs in absence or presence of LPS [10 ng/mL]. Production of TNF-α (A), IL-6 (B), IL-10 (C), MCP-1 (D), MIP-1α (E), TNF-α relative to LPS (F), IL-6 relative to LPS (G), IL-10 relative to LPS (H), MCP-1 relative to LPS (I) and MIP-1α relative to LPS (J) in eutrophic and overweight/obese individuals from study 1; Data are expressed as mean ± SD.

The mitochondrial respiration profiles of PBMCs in study 1 are shown in **Figure 5**. We did not observe any differences in terms of the rates of O_2 consumption in the ROUTINE, LEAK, $OXPHOS_{(CI)}$, and $OXPHOS_{(CI+CII)}$ states in the PBMCs of overweight/obese individuals

compared to eutrophic individuals. Thus, body composition appears to have no direct impact on the mitochondrial respiration of PBMCs.

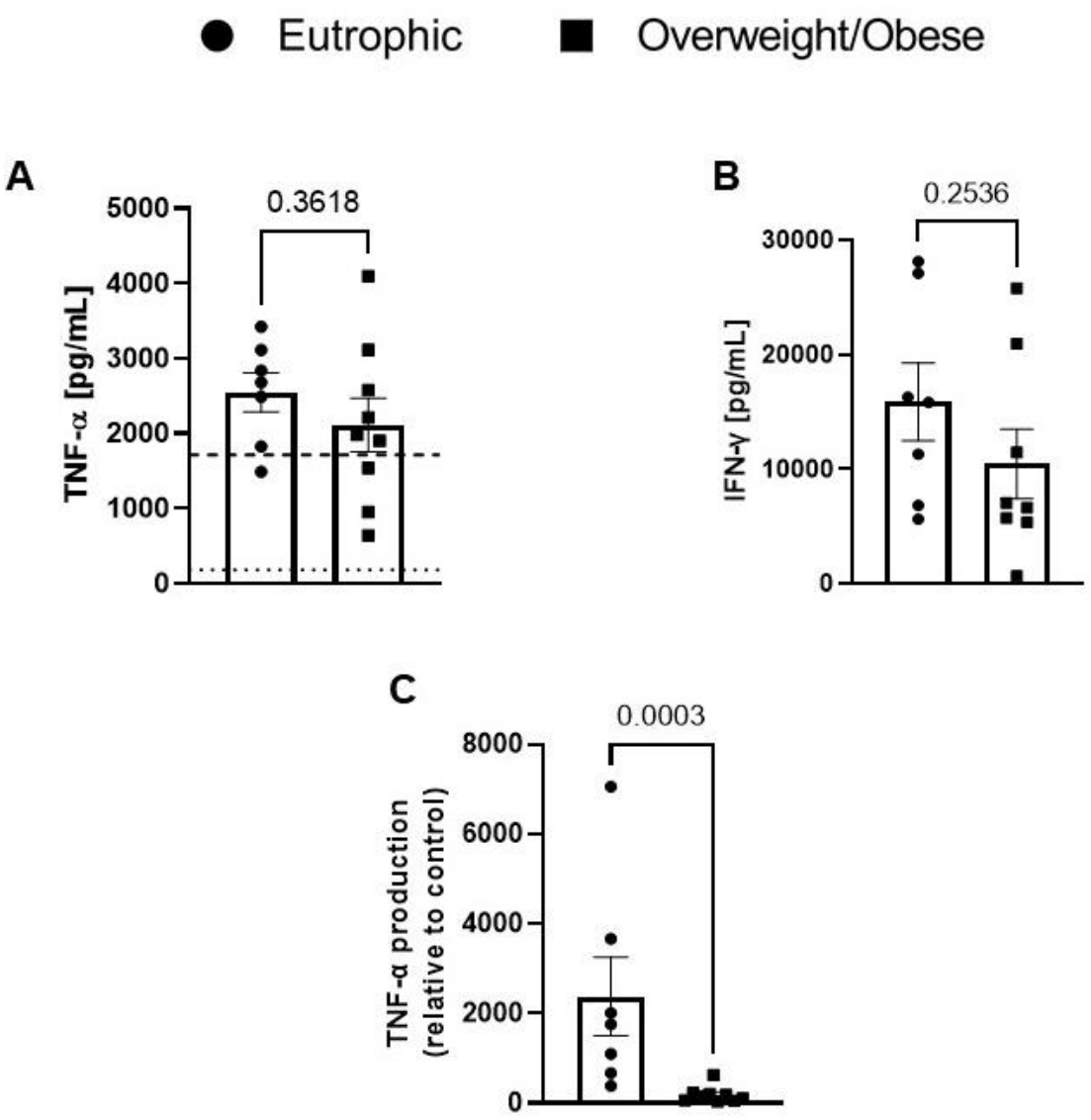

Figure 4. Cultured PBMCs in absence or presence of PMA [50 ng/mL] plus Ionomycin [1 µg/mL]. Production of TNF-α (A), IFN-γ (B), TNF-α relative to PMA (E), in eutrophic and overweight/obese individuals from study 1. Data are expressed as mean ± SD.

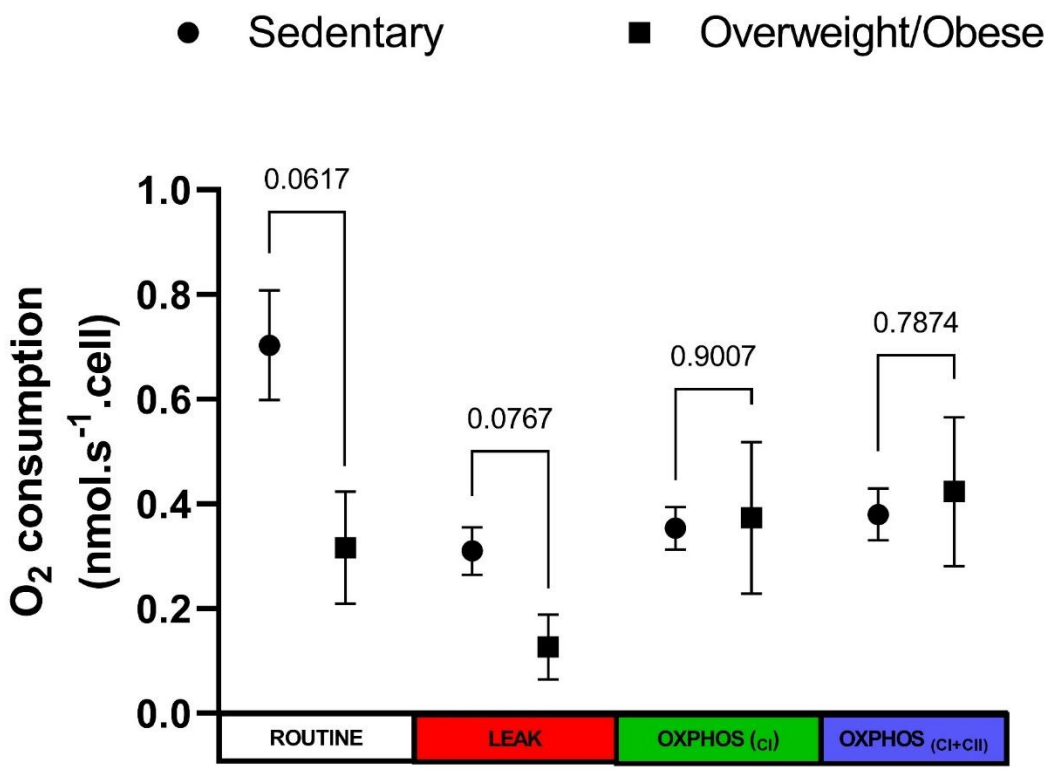

Figure 5. Oxygen consumption rate in different status of mitochondrial respiration in eutrophic and overweight/obese individuals from study 1. Data are expressed as mean ± SD.

We analysed the expression of genes related to inflammatory and energy pathways in PBMCs (**Figure 6 and 7**) and found that the eutrophic and overweight/obese groups showed increases in NF-κB expression (Cohen's d = 2.88; p = 0.0011 and Cohen's d = 5.42; p = 0.0036, respectively) after PMA stimulation compared to untreated cells (**Figure 7**, panel C). For the relative and absolute expression of AMPK and PPAR-γ, NF-κB in PBMCs treated in the presence or absence of LPS, and the absolute and relative expression of AMPK and PPAR-γ stimulated with PMA, no significant differences were observed between groups and stimuli. This suggest that body composition does not modulate the expression of inflammatory and energy genes in PBMCs.

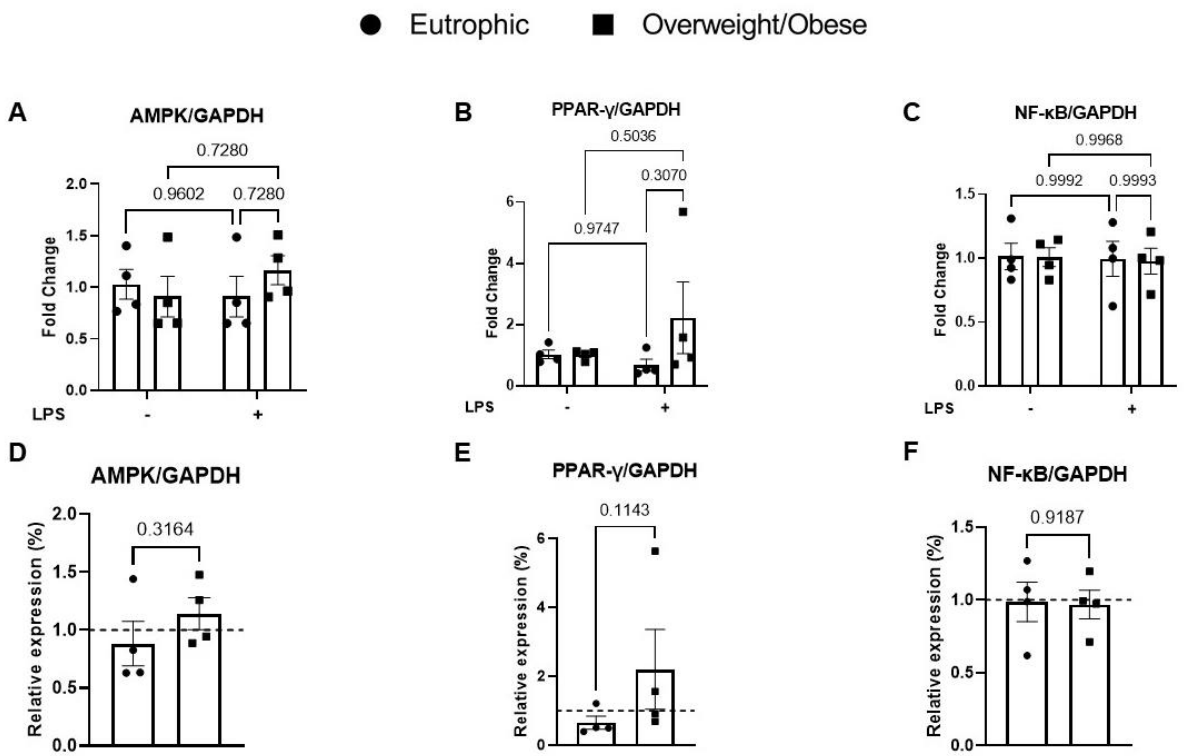

Figure 6. Expression of AMPK (A), PPAR-γ (B) and NF-κB (C) and relative gene expression of AMPK (D), PPAR-γ (E) and (F) in PBMC stimulated in absence or presence of LPS [10 ng/mL] of eutrophic and overweight/obese individuals from study 1.

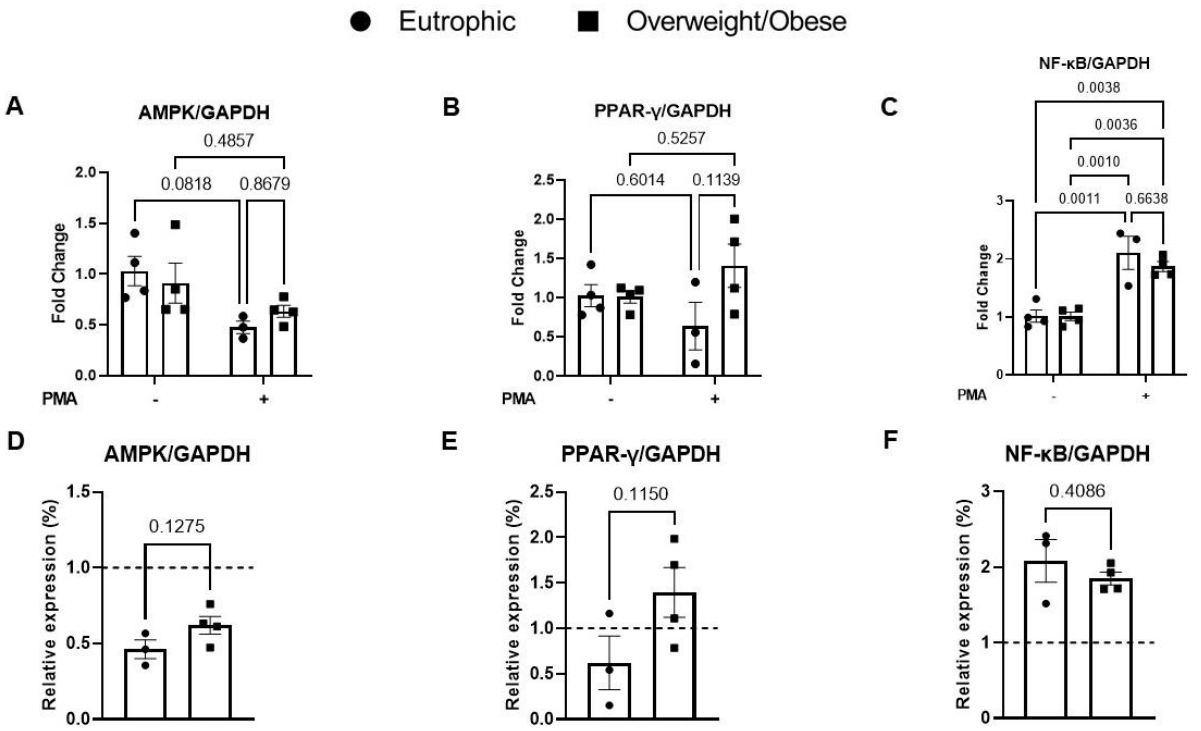

Figure 7. Expression of AMPK (A), PPAR-γ (B) and NF-κB (C) and relative gene expression of AMPK (D), PPAR-γ (E) and (F) in PBMC n absence or presence of PMA [50 ng/mL] plus Ionomycin [1 µg/mL] of eutrophic and overweight/obese individuals from study 1. Data are expressed as mean ± SD.

***3.2** Levels of physical fitness regardless of body composition on metabolic parameters, whole blood-stimulated blood, and cultured PBMCs*

The general characteristics of LPAL individuals and HPAL are shown in **Table 3**. Regarding the levels of physical activity, HPAL presented higher times in MVPA (Cohen's d = 1.93, p = 0.008) than LPAL individuals. With regards to body composition, HPAL presented higher BMI (Cohen's d = 1.93, p = 0.008) and lean body mass (Cohen's d = 3.94, p = 0.001) than LPAL individuals. In contrast, HPAL presented lower body fat (%) (Cohen's d = 3.98, p = <0.001), gynoid and android fat (Cohen's d = 3.11, p = < 0.001; Cohen's d = 3.37, p = < 0.001), android/gynoid ratio (Cohen's d = 1.31, p = 0.033), and SAT (Cohen's d = 2.76, p = 0.001) than LPAL individuals. Moreover, HPAL presented higher relative protein consumption (Cohen's d = 1.52, p = 0.023) and carbohydrates (Cohen's d = 1.49, p = 0.021) than LPAL individuals. We did not observe differences in sedentary time, body weight, waist circumference, VAT, total intake, and lipid intake between HPAL and LPAL individuals. Regarding the metabolic profile, we observed lower circulating concentrations of TAG (Cohen's d = 2.06, p = 0.003) in HPAL than in LPAL individuals. Thus, we observed a higher impact of MVPA on body composition, food intake, and metabolic parameters between LPAL individuals and HPAL.

Table 3. General characteristics in LPAL and HPAL individuals in study 2.

Variable	Study 2		P-value
	LPAL	HPAL	
General characteristics			
Age (y)	24.81 ± 4.60	22.56 ± 5.06	0.401
Weight (kg)	62.51 ± 6.33	71.00 ± 8.69	0.055
BMI (kg.m^2)	20.83 ± 2.06	23.40 ± 1.69	0.029*
$\dot{V}O_{2max}$ (mL.min^{-1}.kg)	-	-	-
MVPA time (min.d^{-1})	34.85 ± 22.46	71.11 ± 14.21	0.008*
Sedentary time (min.d^{-1})	697.30 ± 260.60	693.40 ± 356.00	0.594
Body composition			
Fat mass (%)	21.35 ± 2.21	11.65 ± 2.64	<0.001*
Lean Mass (%)	75.20 ± 2.19	84.22 ± 2.38	<0.001*
Fat Gynoid (%)	21.19 ± 3.63	10.03 ± 3.55	<0.001*
Fat Android (%)	18.79 ± 4.35	7.18 ± 2.18	<0.001*
A/G ratio	0.91 ± 0.16	0.73 ± 0.11	0.033*
Waist Circumference (cm)	74.94 ± 4.93	78.28 ± 6.07	0.277
Visceral Adipose Tissue (cm)	3.09 ± 1.01	3.46 ± 1.04	0.535
Subcutaneous Adipose Tissue (cm)	0.94 ± 0.29	0.32 ± 0.13	0.001*
Visceral Adiposity index (log)	1.87 ± 0.12	1.96 ± 0.15	0.241
Deep-Abdominal Adipose-Tissue Index (DAAT, cm^2)	90.15 ± 31.15	113.4 ± 43.58	0.265
Metabolic profile			
Total Cholesterol (mg.dL^{-1})	157.50 ± 27.21	146.80 ± 22.37	0.448
TAG (mg.dL^{-1})	113.80 ± 7.74	101.00 ± 4.14	0.003*
LDL-c (mg.dL^{-1})	95.86 ± 23.31	83.25 ± 19.94	0.309
Non-HDL (mg.dL^{-1})	118.70 ± 23.55	103.40 ± 19.78	0.226
HDL-c (mg.dL^{-1})	38.88 ± 9.35	43.35 ± 4.44	0.228
Glucose (mg.dL^{-1})	76.91 ± 2.81	80.43 ± 5.36	0.136
Insulin (µUI.mL)	10.66 ± 8.46	8.14 ± 5.37	0.536
HOMA-IR	2.05 ± 1.65	1.66 ± 1.18	0.629
Lipid Accumulation Product Index	13.02 ± 6.77	15.45 ± 7.45	0.534
Inflammatory profile			
Leptin (ng/mL)	4.08 ± 4.01	2.89 ± 2.70	0.573
Adiponectin (µg/mL)	6.49 ± 2.67	7.15 ± 3.98	0.717
Adipo/Lep ratio	5.20 ± 7.50	8.17 ± 10.62	0.662
Leptin/VAT (cm) ratio	1.22 ± 0.96	0.90 ± 0.89	0.542
Adiponectin/VAT (cm) ratio	2.32 ± 1.17	2.36 ± 1.88	0.954
Leptin/SAT (cm) ratio	5.14 ± 5.47	10.66 ± 12.38	0.279
Adiponectin /SAT (cm) ratio	8.13 ± 5.12	21.47 ± 4.83	<0.001*
TNF-α (pg/mL)	9.03 ± 2.03	9.23 ± 2.03	0.808
sTNF-RI (pg/mL)	65.05 ± 9.85	57.20 ± 12.18	0.206
IL-6 (pg/mL)	3.65 ± 1.39	2.08 ± 0.76	0.019*
IL-10 (pg/mL)	10.95 ± 4.30	7.63 ± 2.17	0.111
IL-10/TNF-α ratio	1.25 ± 0.48	0.87 ± 0.38	0.132
Dietary profile			
Total intake (kcal.kg $^{-1}$)	28.86 ± 7.80	31.04 ± 12.49	0.132
Protein (g.kg $^{-1}$)	1.16 ± 0.27	1.78 ± 0.51	0.023*
Carbohydrate (g.kg $^{-1}$)	3.02 ± 1.14	5.74 ± 2.31	0.021*
Lipids (g.kg $^{-1}$)	1.13 ± 0.64	3.00 ± 3.94	0.202

Note: BMI, body mass index; MVPA, moderate-to-vigorous physical activity; A/G ratio, android, and gynoid ratio; TAG, triacylglycerol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; Adiponectin/Leptin ratio, adiponectin and leptin ratio; IL, interleukin; TNF-α, tumor necrosis factor – α; sTNF-RI, soluble tumor necrosis factor receptor type I; Data are presented as mean ± SD. *P<0.05.

The pro- and anti-inflammatory profiles of HPAL and LPAL individuals are shown in **Table 3**. HPAL presented lower IL-6 (Cohen's d = 1.40, p = 0.019) and adiponectin/SAT ratio (Cohen's d = 2.68, p < 0.001) compared to LPAP individuals. However, no differences were observed in leptin, adiponectin, adiponectin/leptin ratio, leptin/VAT ratio, leptin/SAT ratio, adiponectin/VAT ratio, TNF-α, sTNF-RI, IL-10, and IL-10/TNF-α ratio. Regarding the inflammatory response induced by LPS-stimulated whole blood, there was no difference in the production of TNF-α, IL-6, and IL-10 in HPAL and LPAL individuals. In the PBMC culture, HPAL presented lower levels of production of IL-10 (Cohen's d = 1.74, p = 0.019) and MIP-1α (Cohen's d = 1.49, p = 0.041) compared to non-LPS stimulation compared to LPAL individuals **(Figure 9**, panels H and J). Regarding the culture of PBMCs stimulated in the absence or presence of PMA plus ionomycin, there was no difference in the production of TNF-α and IFN-γ between the LPAL individuals and HPAL. Thus, the different levels of physical conditioning appear to impact the production of cytokines in PBMCs, in which LPAL individuals have a greater release of anti-inflammatory cytokines and chemokines than HPAL.

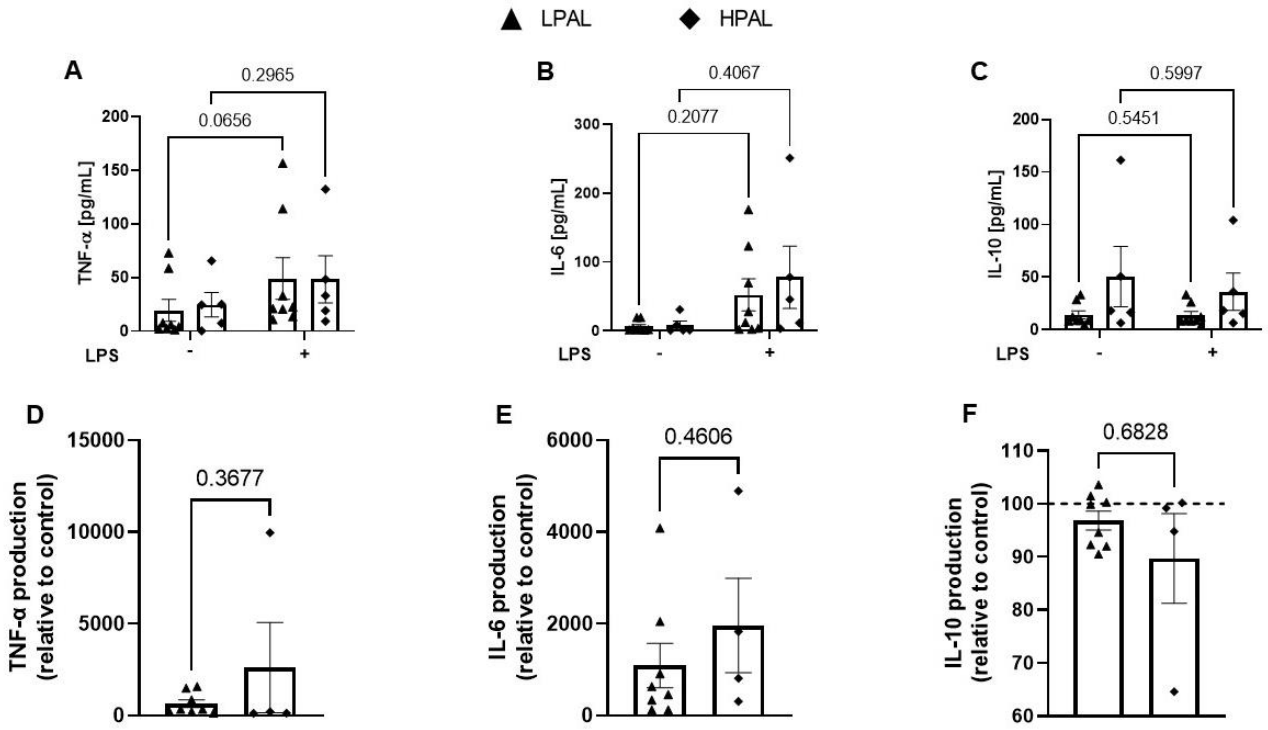

Figure 8. Whole blood stimulated in absence or presence of LPS [10 ng/mL]. Production of TNF-α (A), IL-6 (B), IL-10 (C), TNF-α relative to LPS (D), IL-6 relative to LPS (E) and IL-10 relative to LPS (F) in LPAL and HPAL individuals from study 2. Data are expressed as mean ± SD. Data are expressed as mean ± SD.

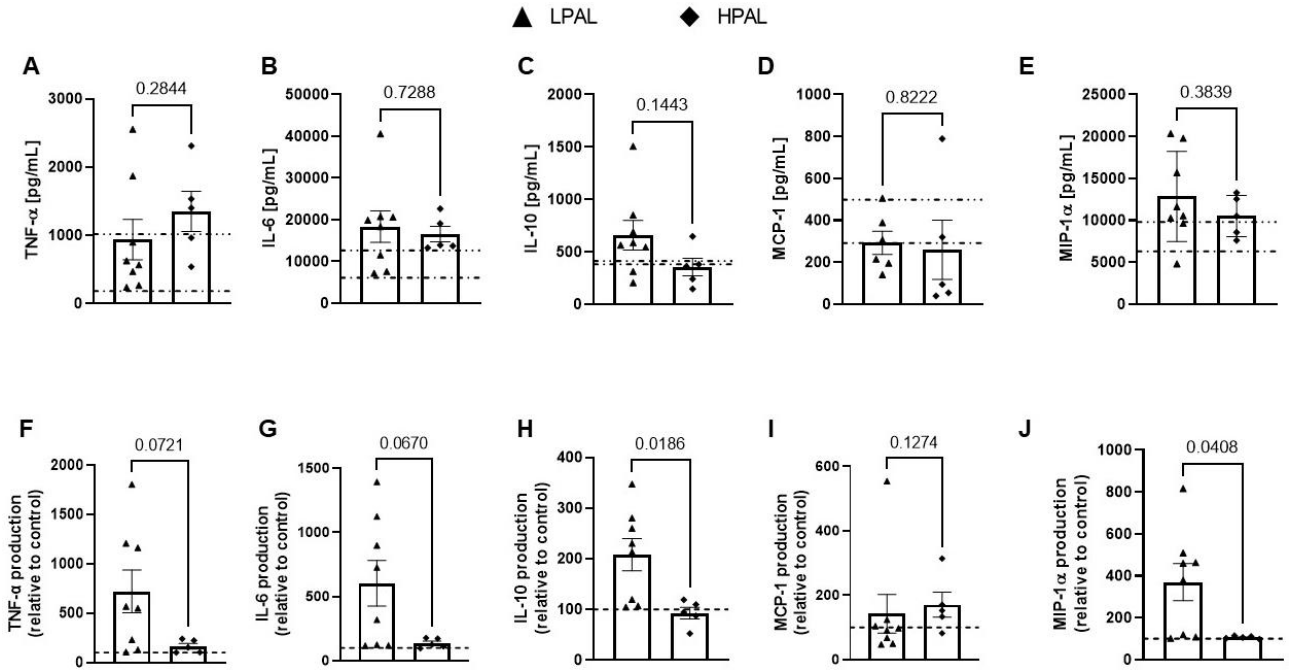

Figure 9. Cultured PBMCs in absence or presence of LPS [10 ng/mL]. Production of TNF-α (A), IL-6 (B), IL-10 (C), MCP-1 (D), MIP-1α (E), TNF-α relative to LPS (F), IL-6 relative to LPS (G), IL-10 relative to LPS (H), MCP-1 relative to LPS (I) and MIP-1α relative to LPS (J) in LPAL and HPAL individuals from study 2; Data are expressed as mean ± SD.

The mitochondrial respiration of permeabilized PBMCs from LPAL individuals and HPAL is shown in **Figure 11**. The ROUTINE rate was higher in LPAL individuals than in HPAL (Cohen's d = 3.89, p = 0.009). However, there were no differences in the LEAK, OXPHOS $_{(CI)}$, and OXPHOS $_{(CII)}$ between the groups studied in Study 2. Therefore, we observed that physical conditioning was able to modulate the state of O_2 consumption in PBMCs, in which a low level of physical fitness impacts the ROUTINE state.

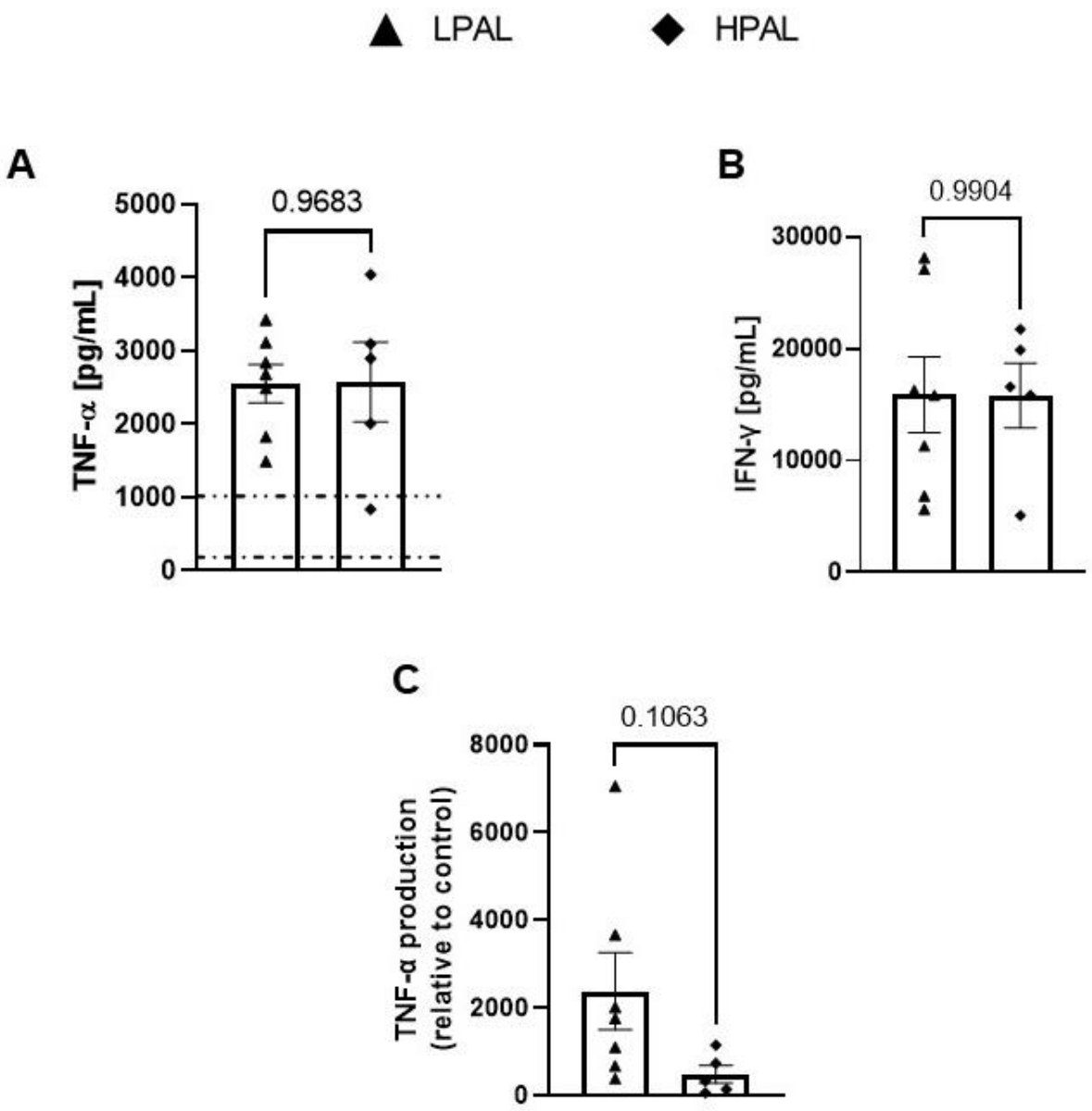

Figure 10. Cultured PBMCs in absence or presence of PMA [50 ng/mL] plus Ionomycin [1 µg/mL]. Production of TNF-α (A), IFN-γ (B), TNF-α relative to PMA (C), in LPAL and HPAL individuals from study 2. Data are expressed as mean ± SD.

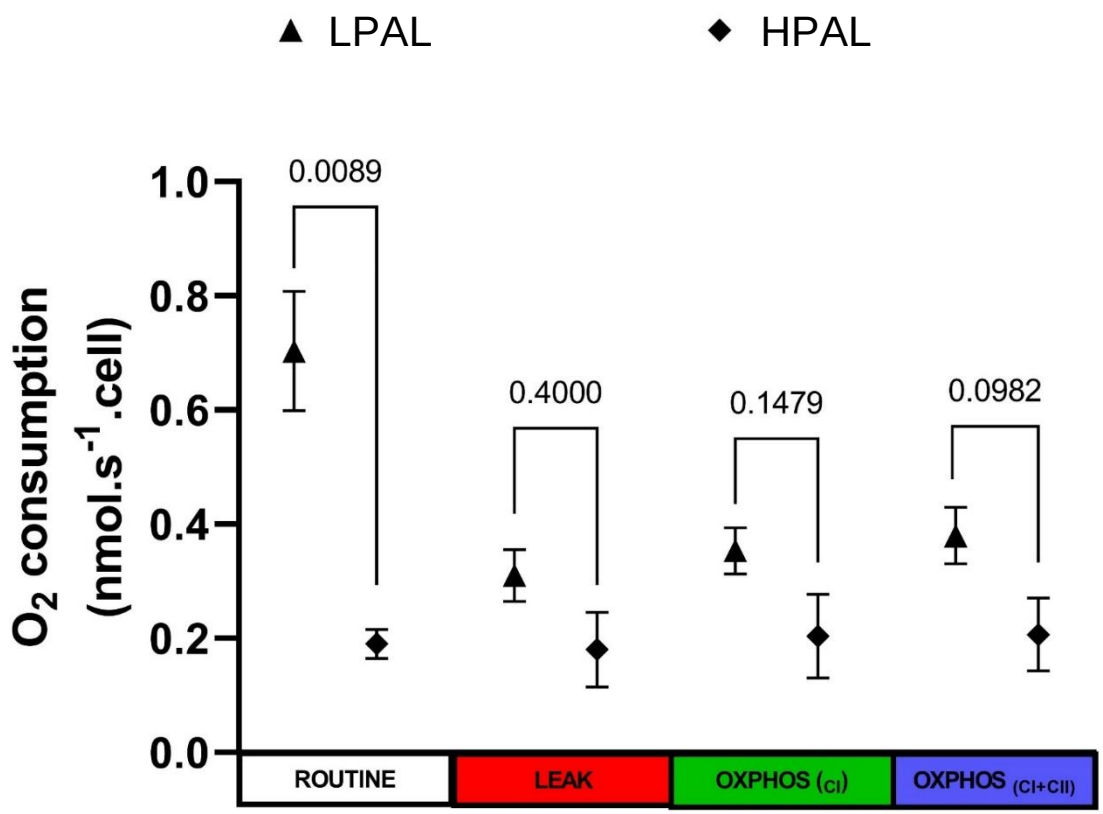

Figure 11. Oxygen consumption rate in different status of mitochondrial respiration in LPAL and HPAL individuals from study 2. Data are expressed as mean ± SD.

When we evaluated gene expression in PBMCs from individuals with different physical fitness levels (**Figure 12 and 13**), both LPAL individuals and HPAL showed increases in NF-κB expression (Cohen's $d = 2.88$; $p = 0.0042$ and Cohen's $d = 9.56$; $p = 0.0001$, respectively) after PMA stimulation compared to untreated cells (**Figure 13**, panel C). However, when comparing PBMCs after inflammatory stimulation with PMA, HPAL showed higher levels of NF-κB expression (Cohen's $d = 2.07$; $p = 0.0342$) compared to the LPAL group. No differences were observed between groups and conditions in terms of the absolute and relative expression of AMPK, PPAR-γ, and NF-κB after LPS stimulation and the absolute and relative expression of AMPK and PPAR-γ after PMA stimulation. These results indicate that individuals with higher physical fitness had higher levels of inflammatory gene expression than LPAL individuals.

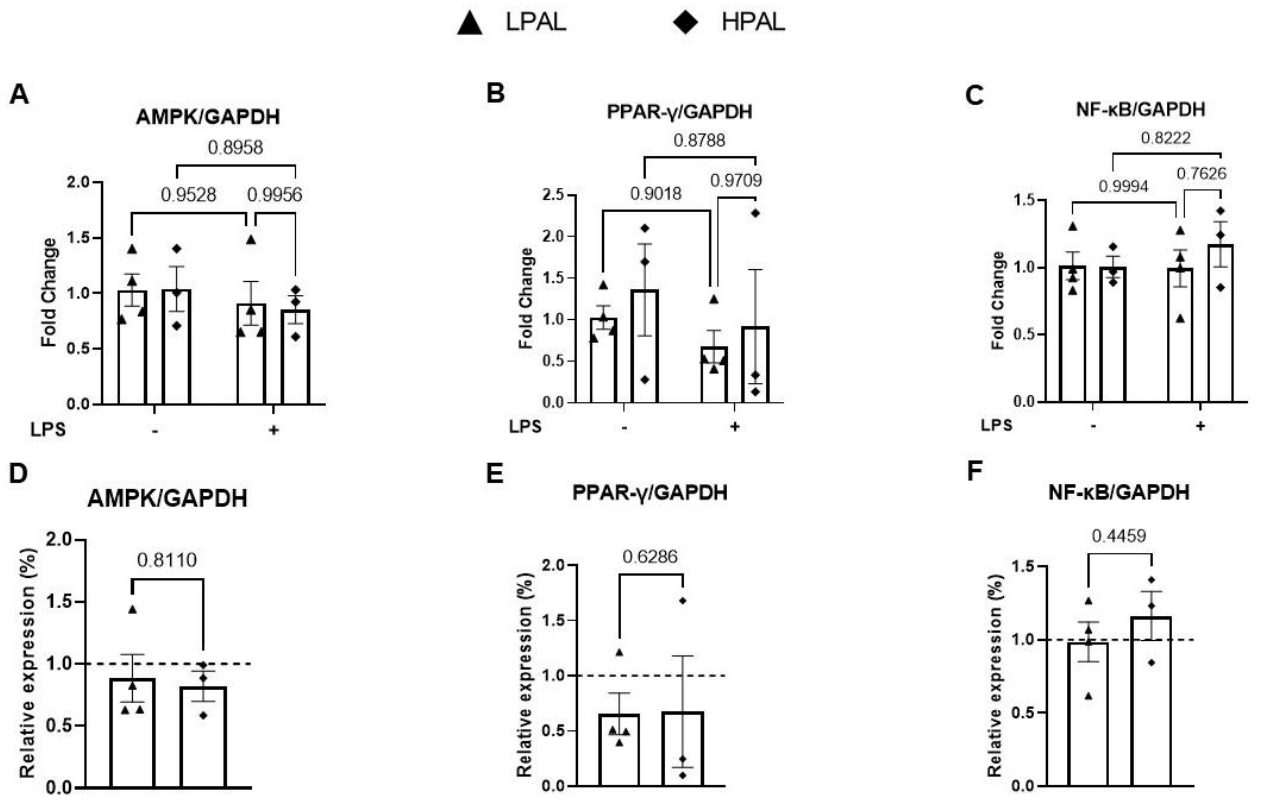

Figure 12. Expression of AMPK (A), PPAR-γ (B) and NF-κB (C) and relative gene expression of AMPK (D), PPAR-γ (E) and (F) in PBMC stimulated in absence or presence of LPS [10 ng/mL] of LPAL and HPAL individuals from study 2. Data are expressed as mean ± SD.

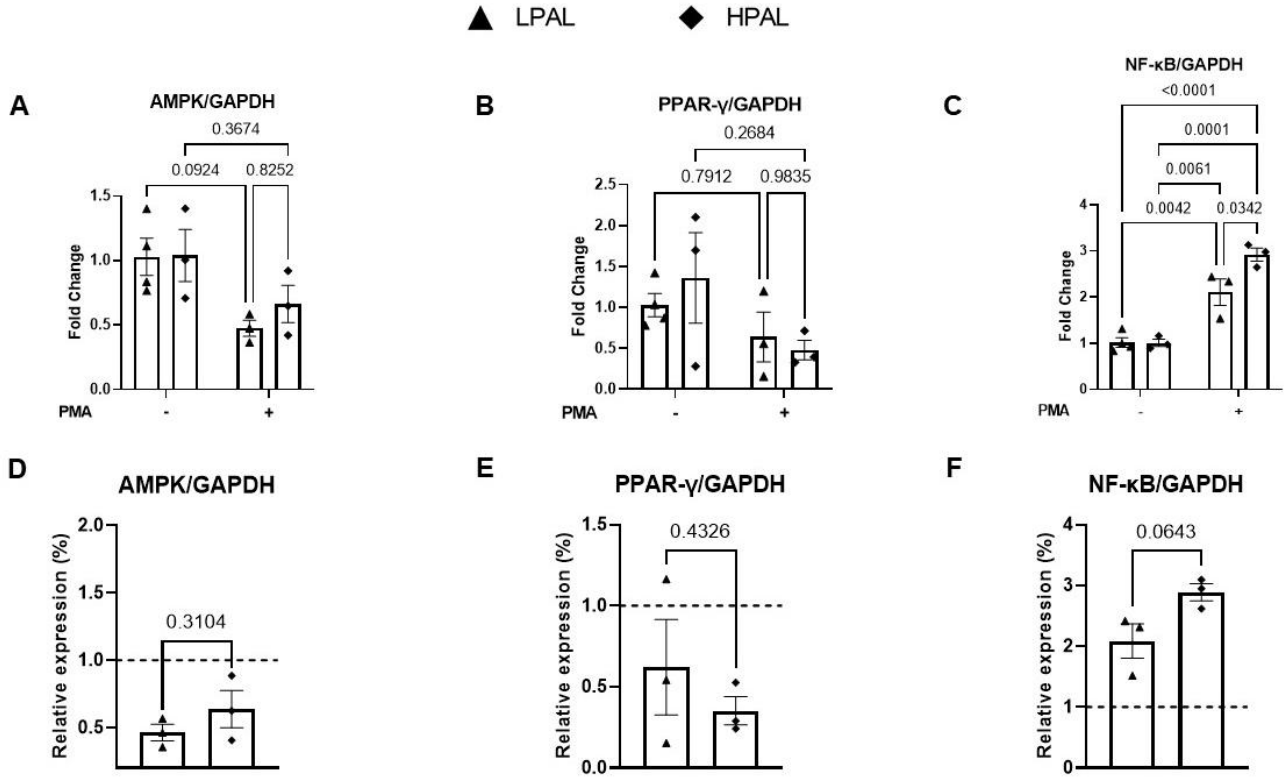

Figure 13. Expression of AMPK (A), PPAR-γ (B) and NF-κB (C) and relative gene expression of AMPK (D), PPAR-γ (E) and (F) in PBMC n absence or presence of PMA [50 ng/mL] plus Ionomycin [1 µg/mL] of LPAL and HPAL individuals from study 2. Data are expressed as mean ± SD.

4. DISCUSSION

We aimed to investigate the separate effects of different body composition and physical fitness status on systemic and cellular inflammatory responses. Our main findings were that the LPS-stimulated PBMCs of overweight/obese individuals produced higher concentrations of TNF-α than those of eutrophic individuals. However, the relative inflammatory response to different challenges in the presence of LPS and PMA promoted a significantly lower production of TNF-α, IL-10, and MIP-1α compared with eutrophic individuals. Regarding the differences promoted by different levels of physical fitness, we demonstrated that LPAL individuals have systematically higher concentrations of IL-6 and higher levels of IL-10 and MIP-1α production in LPS-stimulated PBMCs, as well as higher ROUTINE rates, than HPAL. Furthermore, PBMCs from eutrophic individuals tended to present a higher routine and leak O_2 mitochondrial consumption compared to the overweight/obese group. Finally, ROUTINE rate was reduced in the PBMCs of the HPAL group than in the LPAL group. Therefore, these findings corroborate in part with our initial hypothesis that body composition and physical fitness can affect energy metabolism and inflammatory responses.

4.1 Impact of body composition

Our results are in agreement with previous studies (19, 20), in which overweight/obese individuals were found to produce higher resting concentrations of TNF-α in LPS-stimulated PBMC cultures than eutrophic individuals, validating the hypothesis that an excessive increase in body fat stimulates a sustained activated state in immune cells as a result of chronic stimulation by inflammatory environment/metabolic dysfunction (32). Interestingly, evidence has shown that the increase in TNF-α production may be due to increased NF-κB transcription activity and p65 subunit expression, in parallel with reduced IκB-β activity (32) and increased expression of TLR-4 in circulating monocytes (15). Furthermore, increases in TNF-α production are facilitated by histone H4 hyperacetylation status in LPS-stimulated PBMCs of obese men, indicating an epigenetic mechanism that perpetuates the inflammatory response (20). Thus, it is possible that there is an alteration in the transcription of upstream genes related to inflammatory pathways, since in the present study there were no alterations in the expression of NF-κB.

Poor nutrition factors may explain another proposed mechanism for the increase in the inflammatory status of immune cells in overweight/obese individuals. Chronic exposure to high concentrations of glucose may have been associated with a pro-inflammatory response mediated by NF-κB activation (108), promoting metabolic dysregulation in cells and inhibiting the critical enzyme related to the control of homeostasis cell energy, mainly by AMP-activated protein kinase (AMPK)

(16, 35). AMPK plays an essential role in the immune response, going beyond its essential role in periods of low energy, increasing glucose uptake, fatty acid oxidation, and mitochondrial biogenesis, among other metabolic activities (58). In immune cells, AMPK activation is related to macrophage polarisation to the anti-inflammatory phenotype (M2), activating anti-inflammatory pathways that promote the activation of inflammatory pathways, such as NF-κB (88). Additionally, the excess nutrient status can promote a reduction in AMPK phosphorylation, decreasing its activation, consequently allowing for the activation of NF-κB and increased cytokine production (108).

Another characteristic of inflammatory upregulation is an increased resting concentration of leptin, which is indicative of adipose tissue dysfunction. Leptin is secreted mainly by adipocytes, acting in the central nervous system and peripheral tissues, regulating energy homeostasis, neuroendocrine functions, and metabolism, and circulating leptin concentrations are associated with body fat depots (79). This adipokine has been implicated in the immune response of monocytes, as well as the activation, proliferation, and induction of the release of pro-inflammatory cytokines (TNF-α, IL-6, IL-1) (13), and T lymphocytes, favouring cytokine production via the JAK-STAT signalling pathway (89). In this context, our findings show that overweight/obese individuals had higher concentrations of glucose, leptin, and leptin/VAT ratio than eutrophic individuals, which implies an increase in TNF-α concentrations found in overweight/obese individuals. On the other hand, under a pro-inflammatory stimulus, PBMCs cultured in overweight/obese individuals showed an impaired inflammatory response, producing lower concentrations of TNF-α, IL-10, and MIP-1α relative, which are critical for bacterial and viral immunoregulatory resistance, as well as chemotactic activities (1, 65, 90). One plausible mechanism is premature cellular senescence induced by obesity, which is marked by changes in cell function during aging Therefore, the decline in the function of PBMCs in overweight/obese individuals may be related to the inability to respond, and these results are corroborated by a previous study (43). However, more studies are needed to confirm this hypothesis by analysing senescence markers, such as telomeres, protein expression.

In the present study, we found no differences in the mitochondrial respiration rates of PBMCs between eutrophic and overweight/obese individuals. Although, there are studies that demonstrate a possibility that excessive nutrient intake (57), as well as obesity (110) on the development of mitochondrial dysfunction, impacting the energy production capacity, in addition to increasing the production of ROS and activation of inflammatory pathways (109). However, further studies are needed to better understand the ways in which obesity can impact the metabolism of circulating cells.

4.2 Impact of levels of physical fitness

Even in the absence of differences in circulating concentrations of TNF-α or IL-10, higher circulating IL-6 levels were found in LPAL individuals than in HPAL, corroborating previous study (74). Increased efficiency in the sensitivity of IL-6 is acquired after successive exercise sessions, contributing to a reduction in resting concentrations in trained individuals (25). Another potential mechanism for differences in IL-6 concentrations is through the role of adipose tissue in the production of cytokines, such as IL-6 (37). In the present study, LPAL individuals had higher amounts of body fat than HPAL. In addition, HPAL had a higher adiponectin/SAT ratio than LPAL individuals, demonstrating more efficient adipose tissue in producing molecules that can modulate the inflammatory response (61). Adiponectin has been involved in some inflammatory response processes, modulating macrophage differentiation, favouring the differentiation of monocytes to M2 macrophages, which showed an anti-inflammatory profile (60) and reduced pro-inflammatory cytokines, such as IFN-γ, TNF-α, and IL-6 (106).

Furthermore, it is essential to highlight the pleiotropic effects of IL-6, presenting pro- and anti-inflammatory functions (40), in which the skeletal muscles during contraction during physical exercise can generate acute increases to promote an anti-inflammatory response, stimulating the release of IL-10 and IL-1ra by skeletal muscle and immune cells, such as monocytes (77). However, the chronic increase in IL-6 rest concentrations and other factors, such as physical inactivity, may increase the risk for the onset of chronic diseases, such as type 2 diabetes mellitus and cardiovascular diseases (77).

In cultured LPS-stimulated PBMCs, a higher concentration of IL-10 may reflect a compensatory mechanism (11), in which untrained individuals may have a lower expression of IL-10 receptors or upstream proteins that stimulate the release of IL-10 compared to well-trained (2). Our research group has investigated the differences between individuals with different cardiorespiratory fitness and showed that individuals with high cardiorespiratory fitness have higher expression of peroxisome proliferator-activated gamma receptor (PPAR-γ) in monocytes compared to individuals with low cardiorespiratory fitness (2). PPAR-γ is a transcription factor that binds and activates transcription cofactors, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), stimulating anti-inflammatory genes and inhibiting inflammatory pathways, such as NF-κB (23). Our results do not comment on differences in AMPK and PPAR-γ gene expression between LPAL and HPAL. However, it is possible that HPAL have a higher protein expression of transcription factors, such as PPAR-γ and PGC-1α, requiring lower concentrations of IL-10 to immunomodulate the inflammatory response induced by IL-10. Decreased cytokine production by PBMCs in HPAL after LPS stimulation may indicate an immunotolerance phenotype and the downregulation of innate receptors. Physical activity influences the systemic inflammatory response by inducing diminished

expression of TLR-4, the main LPS sensor, and the co-receptor CD14 in the monocytes of elderly individuals (27, 66, 86). Moreover, habitual strength training lowers TNF-α production by LPS-stimulated monocytes, concomitant with lower TLR-4 expression in monocytes and diminished $CD14^{+}CD16^{+}$ non-classical monocytes in previously untrained subjects (96).

Other studies reinforce our findings of an adaptation to the anti-inflammatory profile of athletes. Higher physical fitness promoted by regular training promotes improvement in cardiorespiratory fitness and can generate changes in the phenotypes of circulating immune cells. Dorneles et al. (2019) demonstrated that well-trained individuals have a lower frequency of monocytes with more pro-inflammatory characteristics (intermediate and non-classical) in parallel with higher frequencies of regulatory T cells (Treg) when compared to their peers with low cardiorespiratory fitness, with a similar inflammatory profile (22).

Interestingly, in the present study, HPAL were found to have higher levels of NF-κB expression after stimulation with PMA compared to LPAL individuals. In this sense, it is possible that individuals with higher levels of physical fitness have a higher capacity for immune responses. Similar to previous results, our group demonstrated that individuals with higher levels of cardiorespiratory fitness have augmented expression of genes upstream of the NF-κB pathway (TLR4 and IKKα) (2). Additionally, it is possible that changes in the protein expression of this pathway have changed, as six weeks of eccentric training promoted reductions in NF-κB phosphorylation (31). Thus, it is possible that, through the improvement in physical fitness as a result of exercise, there is a refinement of the pathways responsible for promoting responses to pathogens. However, studies investigating the protein expression of inflammatory pathways, as well as the phenotype of circulating cells in individuals with different physical fitness, are needed.

Interestingly, we found statistically diminished routine ATP production by mitochondrial respiration in the PBMCs of HPAL. Some studies have demonstrated that higher maximal respiration in PBMCs is associated with greater knee extensor strength and grip strength, indicating that a better respirometric profile in peripheral immune cells is related to physical function (10). However, we cannot exclude the possibility that the dietary habits of HPAL are directly related to the respiratory capacity of immune cells. In this regard, caloric restriction decreased the transcriptome and functional mitochondrial bioenergetic pathways in female athletes (91). Here, HPAL reported a higher daily consumption of carbohydrates than LPAL individuals, which may favour a more glycolytic pathway in PBMCs. In fact, exercise increases systemic nutrient sensing by blood leukocytes and changes mitochondrial oxidative capacity (94), and might favor augmented in the process of the mitochondrial biogenesis, exhibiting increased number and density of mitochondrial. Thus, future studies should

focus on identifying the role of diet and other lifestyle habits in the leukocyte mitochondrial function of HPAL.

Considering our findings, some limitations should be mentioned to strengthen the results, including the absence of assessments to distinguish the phenotypes of circulating cells. Thus, within the mixed PBMC cell types, it is possible that the differential composition of the subpopulations in the subject groups may have an effect on metabolic measurements. Additionally, molecular evaluations to evaluate the protein expression of pathways involved in inflammatory responses in different populations would be of great value to clarify the differences and importance of body composition and physical fitness. However, we emphasise that the participants in this study were healthy men without any accentuated chronic metabolic diseases. Future studies should be carried out to understand the role of body composition and physical fitness in elderly individuals with metabolic diseases, such as diabetes mellitus type 2, among other factors that may alter the inflammatory response.

CONCLUSION

We conclude that overweight/obese individuals exhibit a higher inflammatory profile without changes in mitochondrial respiration parameters than lean individuals. On the other hand, HPAL with higher physical activity levels exhibited a fine regulation of anti-inflammatory cytokine production and more efficient mitochondrial respiration with alterations in gene expression. Therefore, the maintenance of lower body fat and higher physical activity levels leads to better inflammatory and metabolic responses in peripheral blood mononuclear cells.

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Artigo Científico 2

Capsiate does not modulate cytokine production in peripheral blood mononuclear cells of healthy men with different body compositions and physical fitness

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Abstract: Low levels of physical activity and obesity can promote disturbances in the sympathetic nervous system (SNS), reflecting on the host's immune response. On the other hand, engaging in exercise programs, along with the aid of nutritional supplements such as capsiate, can help control the detriments promoted by reductions in physical activity and poor eating habits, modulating the immune response. The present study aimed to investigate the modulations promoted by capsiate stimulation in the circulating cells of healthy young people and to understand the role of β2 adrenergic receptors (AR-β2) in this response. Twenty-three young people were recruited to participate in the study. They were classified as eutrophic (n=8, BMI <24.9 kg/m2), overweight/obese (n=9, BMI ≥25 kg/m2), sedentary (n=8, MVPA: 34.85 ± 22.46 min-1.day) and athletes (n= 6, MVPA: 71.11 ± 14.21 min-1.day). Peripheral blood mononuclear cells (PBMC) were cultured, stimulated in the presence or absence of capsiate (CAP), selective AR-β2 blocker, LPS or PMA (Phorbol 12-myristate 13-acetate). Capsiate and the AR-β2 antagonist were not able to promote modulations in the inflammatory responses of PBMC from individuals with different body compositions and physical fitness. In conclusion, capsiate stimulation as well as the AR-β2 antagonist did not promote modulations in inflammatory responses in either group.

Keywords: immune regulation; supplementation; physical activity

1. Introduction

Insufficient levels of physical activity and obesity have been worryingly discussed worldwide [1], being associated with an increased risk of developing chronic non-communicable (NCD), such as insulin resistance, diabetes mellitus type 2, cardiovascular diseases and cancer, impacting health systems and, ultimately, generating high economic costs [2]. One of the mechanisms related to this set of diseases is the installed inflammatory process, called low-grade chronic inflammation [3]. This process is characterized by a two to three-fold increase in concentrations of inflammatory cytokines such as TNF-α, IL-6, IL-1β and a reduction in anti-inflammatory cytokines such as IL-10, IL-1ra, adiponectin [4]. Individuals who have low daily energy expenditure due to reduced levels of physical activity, associated with unbalanced diets, favors the accumulation and hypertrophy of visceral adipose tissue, as in obesity [4]. In this scenario, there is an imbalance in tissue homeostasis, causing this tissue to activate inflammatory signaling pathways, due to mechanical and metabolic stresses, promoting the infiltration of immune cells to the site [5]. The infiltration of immune cells into adipose tissue, associated with increased concentrations of inflammatory cytokines, promote the polarization of these cells towards a more inflammatory profile, generating a vicious cycle [4,5].

Another impairment caused by inflammation associated with low levels of physical fitness and obesity is changes in the functioning of the sympathetic nervous system (SNS) and of the hypothalamic-pituitary-adrenal (HPA) axis [6,7]. In this scenario, there is a hyperactivation of these axes, favoring a deregulation in neuroendocrine responses and neuro/immune feedback mechanisms, increasing the risk of developing NCD [8]. It has been shown that the SNS is associated with immune responses, in which both lymphoid organs and immune cells have adrenergic receptors, which are divided into α (α1 and α2) and β (β1, β2 and β3) [9], being the β2 adrenergic receptor (AR-β2) implicated in some inflammatory responses of peripheral blood mononuclear cells (PBMC) [10]. The AR-β2 is a G protein-coupled receptor, which after binding catecholamines, promotes the activation of a downstream signaling cascade, reducing the synthesis of inflammatory cytokines synthesis [11,12]. Thus, the need to maintain healthy habits for the maintenance and refined control of adrenergic responses in parallel with immune responses is remarkable.

On the other hand, it is well established that engaging in physical exercise programs can help to reduce the health detriments promoted by low physical fitness and obesity, due to the risk of developing NCD [13]. Physical exercise, in addition to changing body composition [14], can also reduce the release of inflammatory cytokines, regardless of the change in body composition, changing phenotypes of circulating cells [15], and reducing the expression of toll-like receptor 4 (TLR4) [16]. Additionally, it was demonstrated that acute exercise at moderate intensity (20 minutes at 65-75%

V̇O2peak) increases the release of catecholamines, generating an anti-inflammatory response via AR-β2 receptors, production of TNF-α [17]. Interestingly, this effect was nullified when they selectively blocked AR-β2 in monocytes from volunteers, demonstrating the important anti-inflammatory role of this pathway [17].

The adoption of nutritional strategies in parallel with a more active lifestyle can promote positive modulations in the immune response [18]. Capsaicinoids and capsinoids, such as capsaicin and capsiate, activate the TRPV1 receptor, promoting, among other effects that have been studied, improvement in physical performance and energy expenditure [19]. Recently, our group investigated the effects of capsiate on the performance of healthy young people in the 10 km event and it was hypothesized that the effects of this supplement could be dependent on physical fitness [20]. In addition to the effects on performance, the activation of this receptor seems to promote modulations in inflammatory responses [21]. It was demonstrated in THP-1 macrophages in the presence of TLR-4 activator (lipopolysaccharide (LPS)) and capsaicin there was a reduction in the production of inflammatory cytokines [22]. Furthermore, studies with mouse macrophages that do not express the TRPV1 receptor, capsaicin stimulation promoted a reduction in the release of TNF-α [23], raising the hypothesis that the anti-inflammatory effects of this supplementation may be independent of this receptor.

Given the ability of physical exercise to modulate the adrenergic and immune response, associated with the anti-inflammatory potential promoted by capsiate supplementation, we hypothesized that individuals with different body compositions and physical fitness may present different inflammatory responses and, additionally, we seek to understand whether adrenergic receptors participate in the anti-inflammatory response promoted by capsiate. Thus, the aim of this work was to investigate the anti-inflammatory responses in peripheral blood mononuclear cells (PBMC) of healthy young people with different body compositions (study 1) and different physical fitness (study 2) if this response is dependent on the AR-β2.

2. Materials and Methods

Twenty-three healthy men were recruited, divided into two studies, according to BMI (study 1): classified as normal weight (n= 8, BMI <24.9 kg/m^2) and overweight/obese (n= 9, BMI >25 kg/m2) m^2); and according to physical fitness (study 2): in which six professional track and field athletes (highly trained - athletes group. Four athletes train for short distance events (100 and 200 meters) and two for long distance events (5,000 meters) and all were in the competition phase), in order to compare the differences in physical fitness with their peers with low levels of physical activity (sedentary group = 8). Study participants were healthy men, who, before starting the

evaluations, tested negative for Sars-CoV-2 (IgG and IgM antibodies), without any health disorder. Participants were screened through a visit to the laboratory to sign the consent form and apply the questionnaires. The second visit was dedicated to assessing body composition and blood collections. Smokers who had any cardiorespiratory and orthopedic implications, who were using anti-inflammatory drugs or psychoactive substances, use of food supplementation or ergogenic aid, alcohol abuse (more than three times a week) or other drugs and/or did not accept to sign the free and informed consent form. All procedures performed in the study were approved by the Research Ethics Committee of the local University (Universidade Estadual Paulista - Unesp - Presidente Prudente, SP / Brazil) (CAAE: 26011919.0.0000.5402) and in accordance with the Declaration of Helsinki [24].

2.1 Blood collections and analysis

For studies 1 and 2, approximately 10 mL of blood was collected by peripheral puncture of a forearm vein. Collections were performed at rest and after fasting for 12 hours. This volume was fractionated in tubes containing anticoagulant gel (for serum), ethylenediaminetetraacetic acid (EDTA) for plasma and fluoride/EDTA for glucose analysis. Blood samples were centrifuged at 3,000 rpm for 15 min at 4 °C, and plasma and serum were stored at -80 °C until further biochemical analysis and ELISA. Glucose concentrations were analyzed in colorimetric kits (Labtest, Brazil) and insulin in commercial kits (Monobind Inc., USA) by enzyme-linked immunosorbent assay (ELISA). The HOMA-IR was calculated using the equation: HOMA-IR = (glucose [mmol/L] × insulin [μIU/mL]/22.5) [25]. Triacylglycerol (TAG), cholesterol and fractions (TC and HDL-c) were analyzed by commercial colorimetric kits (Labtest®, Brazil), and non-HDL was calculated by subtracting TAG and HDL-c concentrations. In addition, the Friedewald et al. (1972) [26] was used to estimate LDL-c.

The concentrations of cytokines present in serum were performed by ELISA technique according to the manufacturer's instructions using high sensitivity detection kits (R&D System, a biotechne brand, Quantikine ELISA, Inc., Minneapolis, USA) with intervals between 15.6 -1,000 pg / mL for TNF-α, 3.13-300 pg / mL for IL-6, 7.8-500 pg / mL for IL-10 and 7.8-500 pg / mL, with a coefficient of variation intra-assay 1.9%, 2.8%, 2.1% and 1.9%, respectively; To measure the concentrations of TNF-α, IL-6, IL-10, IFN-γ, MCP-1 and MIP-1α present in PBMC culture supernatants and stimulated whole blood, they were determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (Duoset R&D System, Minneapolis, USA) with ranges between 15.6-1000 pg / mL, 9.3-600 pg / mL, 31.2-2000 pg / mL, 9.3-600 pg / mL, 15.6-1000 pg / mL and 7.8-500 and an intra-assay coefficient of variation of 1.5%, 1.3%, 2.1%, 3.1%, 4.1% and 2. 2%, respectively.

2.2 Isolation and stimulation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from the blood using the density gradient separation technique, which was added above the histopaque in a 1:1 ratio (Histopaque – 1077. Sigma-Aldrich Co. LLC) and after 30 minutes of centrifugation at 400 xg in a centrifuge without brake, erythrocytes sedimentation and peripheral blood mononuclear cells separation, according to the manufacturer's instructions. After this procedure, the PBMCs were counted in a hemocytometer (Neubauer Chamber) and then placed at a concentration of 1x106 per well in 24-well polystyrene plates with cell culture medium (RPMI-1640 – Sigma-Aldrich) enriched with sodium bicarbonate [24mM], HEPES 20 [mM], glutamine ([2mM]), 10% fetal bovine serum and antibiotics [10000U/mL] penicillin and [10000U/mL] streptomycin in a final volume of 1 mL.

Cells from each participant were stimulated with capsiate (CAP) (Sigma-Aldrich), selective AR-β2 blocker – ICI 118, 551 (5x10-6 M) (Sigma-Aldrich), LPS (10 ng/mL) or PMA (Phorbol 12-myristate 13-acetate) (50 ng/mL) and ionomycin (1 µg/mL), as shown in Figure 1. For selective β2 adrenergic receptor blockade, we treated cells with [5x10-6 M] for 30 minutes before the other stimuli (LPS, PMA, CAP), based on the study of [17]. After 24 hours of incubation, the culture medium was collected and stored at -80ºC for further analysis.

2.3 MTT assay

Peripheral blood mononuclear cells were isolated as described above and incubated 2x105 cells for 24 hours at 37°C in 5% CO2 in 96-well plates with capsiate stimuli at different concentrations, [50µM] and [100µM] in a final volume of 200 µl of culture medium. After incubation, we inserted 100 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide [5mg/mL] diluted in PBS 1x) into each well and incubated again incubated again for 4 hours at 37ºC in 5% CO2. Subsequently, we added 100 µL of DMSO or isopropyl acid and the reading was taken at the absorbance of 570 nm.

2.4 Statistical analysis

The Shapiro-Wilk test was used to verify normality and descriptive statistics were presented as mean and standard deviation. For absolute and relative values in the *in vitro* assay, a two-way analysis of variance (ANOVA) [group (eutrophic versus overweight/obesity) versus condition (stimulus)] and [group (sedentary versus athlete) versus condition (stimulus)] with post Tukey hockey. To test the variance sphericity, the Mauchly test was performed, in which case the analyzes were adjusted by the Greenhouse-Geisser correction. Statistical significance was set at $p<0.05$ and data were analyzed using the Statistical Package for Social Sciences 22.0 (SPSS Inc. Chicago. IL.USA).

3. Results

3.1 Determination of dose, response and cytotoxicity of capsiate stimulus

To determine the dose of capsiate used in the study, five healthy individuals were recruited to participate in the trial. PBMCs were isolated and stimulated at capsiate concentrations in a range of 25 µM to 200 µM in the absence or presence of LPS and after 24 hours we analyzed the concentrations of TNF-α (Figure 1). At concentrations from 50 µM to 200 µM, reductions in TNF-α concentrations ($p < 0.05$) were observed compared to cells stimulated with LPS alone. In this way, we determined the concentration for the present work at 50 µM. Later, to verify whether capsiate stimulation alone was promoting modulations in TNF-α concentrations, capsiate alone was not able to promote an increase in PBMC inflammatory responses and when associated with LPS, there was a reduction in TNF-α concentrations (**Figure 2, panel A**). We also verified the cytotoxicity of the capsiate stimulus by means of the MTT assay (Figure 2, panel B). The concentration of 100 µM promoted a reduction in the viability of PBMC cells ($p<0.05$) compared to control cells. However, concentrations of 50 µM did not promote significant reductions in cell viability. Thus, we determined concentrations of 50 µM capsiate to stimulate peripheral blood mononuclear cells, as this concentration promoted reductions in TNF-α concentrations, maintaining cell viability.

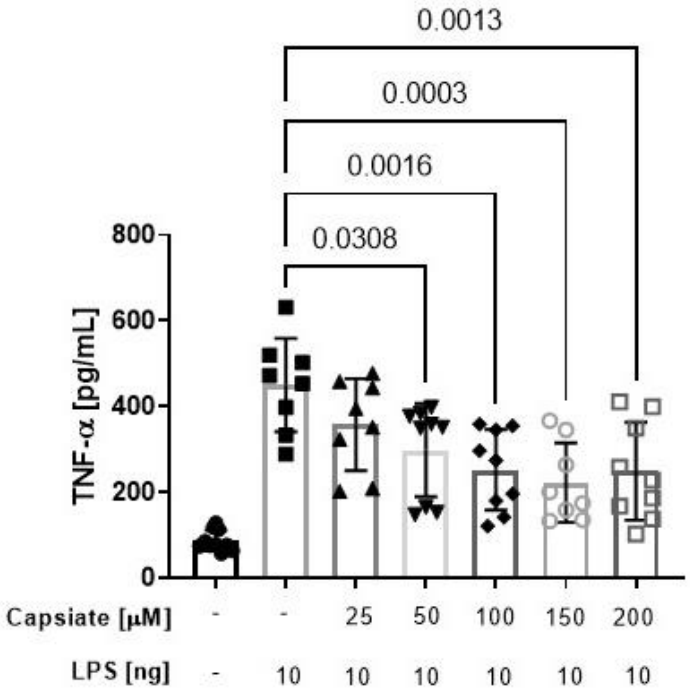

Figure 1. (B) TNF-α production in peripheral blood mononuclear cells in the presence or absence of LPS [10 ng/mL] and capsiate [25 µM - 200 µM]. Data presented as mean ± standard deviation.

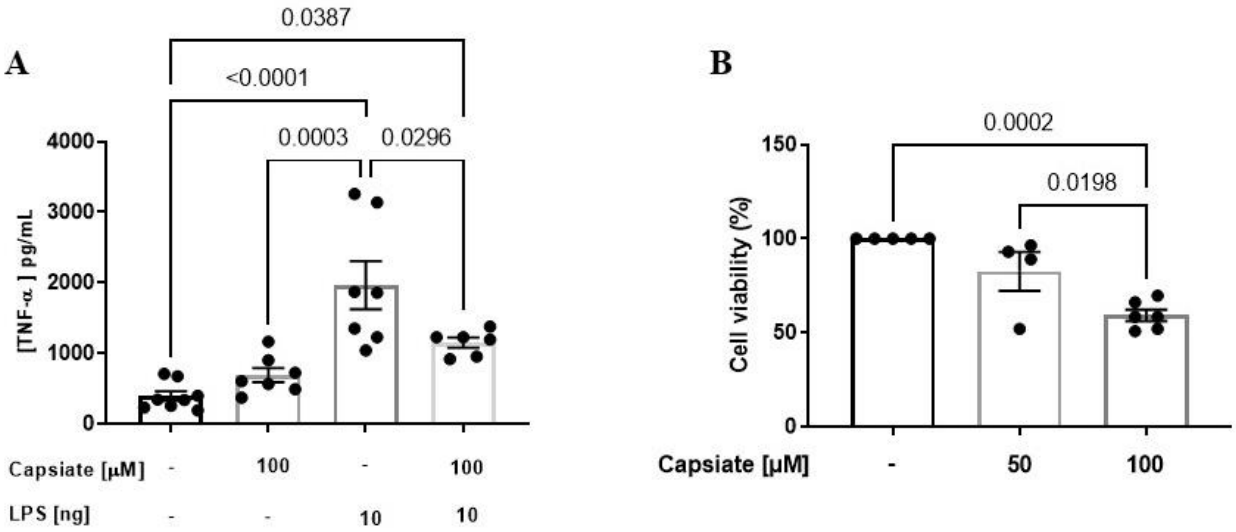

Figure 2. (A) Evaluation of the effect of capsiate alone or conjugated with LPS on the production of TNF-α in peripheral blood mononuclear cells. (B) Peripheral blood mononuclear cell (PBMC) cytotoxicity (MTT) assay; Data presented as mean ± standard deviation (one way ANOVA).

3.2 Impact of different body compositions and physical fitness on the modulations of inflammatory responses of PBMCs stimulated with capsiate and β2 adrenergic receptor inhibitor

Initially, the contribution of the body composition of volunteers in the inflammatory response of PBMCs treated in the presence or absence of LPS, capsiata, as well as the selective inhibitor of the β2 adrenergic receptor was investigated. Capsiate and the β2 adrenergic receptor antagonist were not able to promote reductions in absolute and relative concentrations of TNF-α, IL-6 and IL-10 in overweight/obese individuals compared to eutrophic individuals (Figure 3). Similarly, when we analyzed the contribution of physical fitness in the response to capsiate and the role of the β2 adrenergic receptor, no difference was shown between the sedentary and athlete groups in the absolute and relative concentrations of TNF-α, IL-6 and IL10.

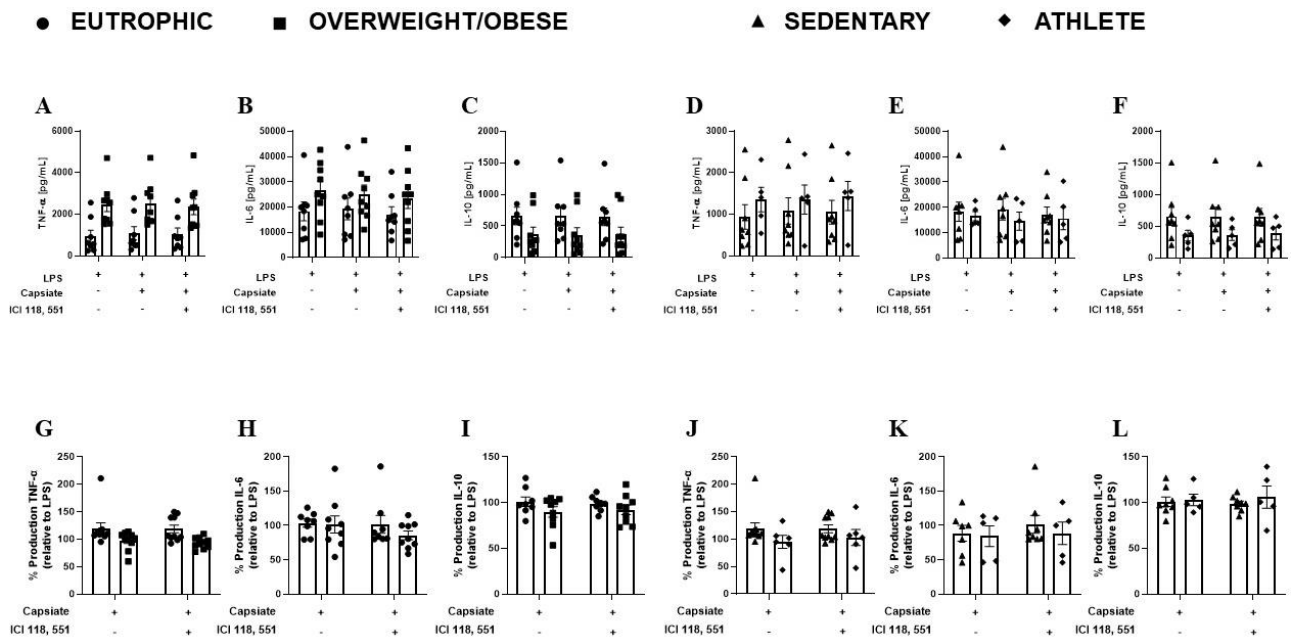

Figure 3. PBMCs cultured in the absence or presence of LPS [10 ng/mL], capsiate [50μM] and β2 receptor antagonist (ICI 118,551) [10^{-6}M]. Production of TNF-α (A), IL-6 (B), IL-10 (C), TNF-α in relation to LPS (G), IL-6 in relation to LPS (H) and IL-10 in relation to LPS (I) in eutrophic and overweight/obese subjects from study 1; TNF-α (D), IL-6 (E), IL-10 (F), TNF-α in relation to LPS (J), IL-6 in relation to LPS (K) and IL-10 in relation to LPS (L) in sedentary subjects and athletes from study 2. Data are expressed as mean ± standard deviation.

Furthermore, to verify the possible modulations of capsiate and β_2 adrenergic receptor in the inflammatory response of lymphocytes, we stimulated PBMCs with PMA + ionomycin (**Figure 4**). Regarding body composition, similarly to stimulation with LPS, capsiate and the β2 adrenergic receptor were not able to promote modulations in the relative and absolute concentrations of TNF-α and IFN-γ comparing eutrophic and overweight/obese individuals. Assessing physical fitness, capsiate and the selective β_2 adrenergic receptor antagonist was not able to promote a reduction in the production of TNF-α and IFN-γ in both groups. Thus, we demonstrate that capsiate was not able to promote an anti-inflammatory response in peripheral blood mononuclear cells stimulated in the presence and absence of inflammatory stimuli and that blockade of the β_2 adrenergic receptor does not seem to modulate the inflammatory response in cells from individuals with different body compositions and physical skills.

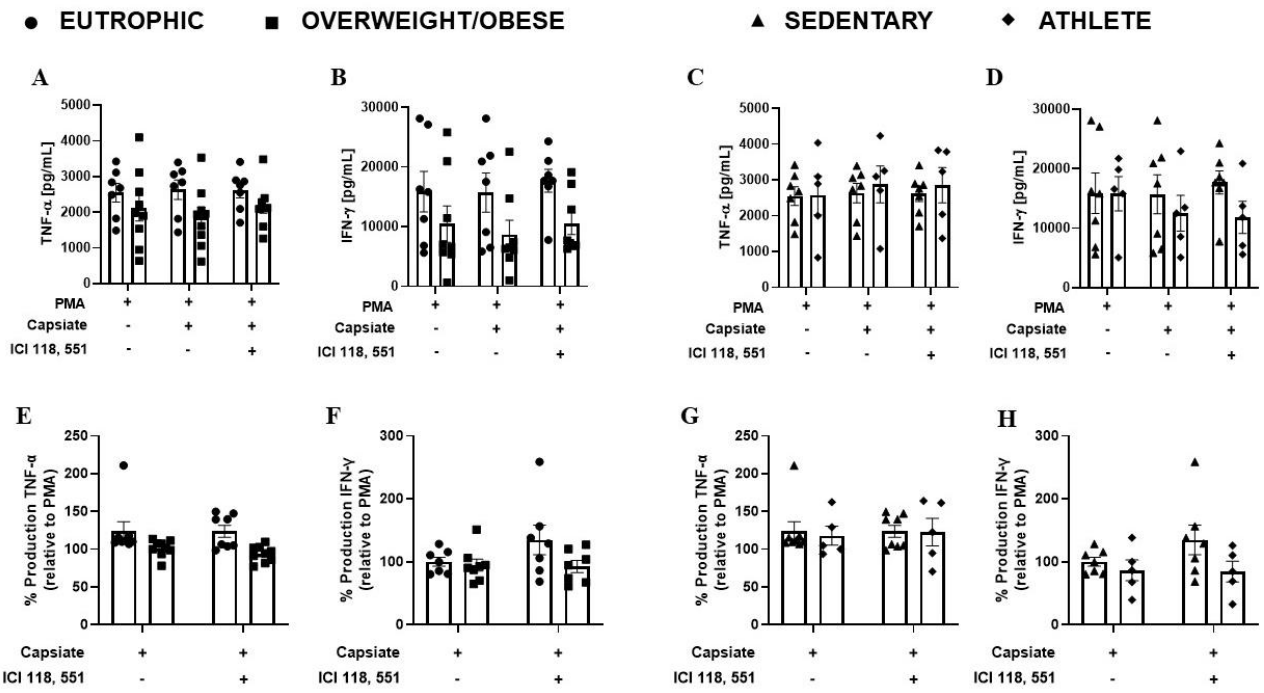

Figure 4. PBMCs cultured in the absence or presence of PMA [50 ng / mL] plus ionomycin [1 µg/mL], capsiate [50 µM] and β2 receptor antagonist (ICI 118,551) [10^{-6}M]. Production of TNF-α (A), IFN-γ (B), TNF-α in relation to PMA (E) and IFN-γ in relation to PMA (F), in eutrophic and overweight/obese subjects from study 1; TNF-α (C), IFN-γ (D), TNF-α relative to PMA (G) and IFN-γ relative to PMA (G), in sedentary subjects and athletes in study 2. Data are expressed as mean ± standard deviation.

The production of chemoattractant cytokines are shown in **Figure 5**. When comparing body composition, there was no reduction or difference in absolute and relative production of MCP-1 and MIP-1α. Regarding physical fitness, we also did not find reductions or differences in absolute and relative concentrations of MCP-1 and MIP-1α when stimulated with capsiate and β_2 adrenergic receptor inhibitors comparing sedentary individuals and athletes. In this sense, we verified that both capsiate and the inhibition of the β_2 adrenergic receptor were not able to promote modulations in the chemoattractant responses of the peripheral blood mononuclear cells of individuals with different bodies compositions and physical aptitudes.

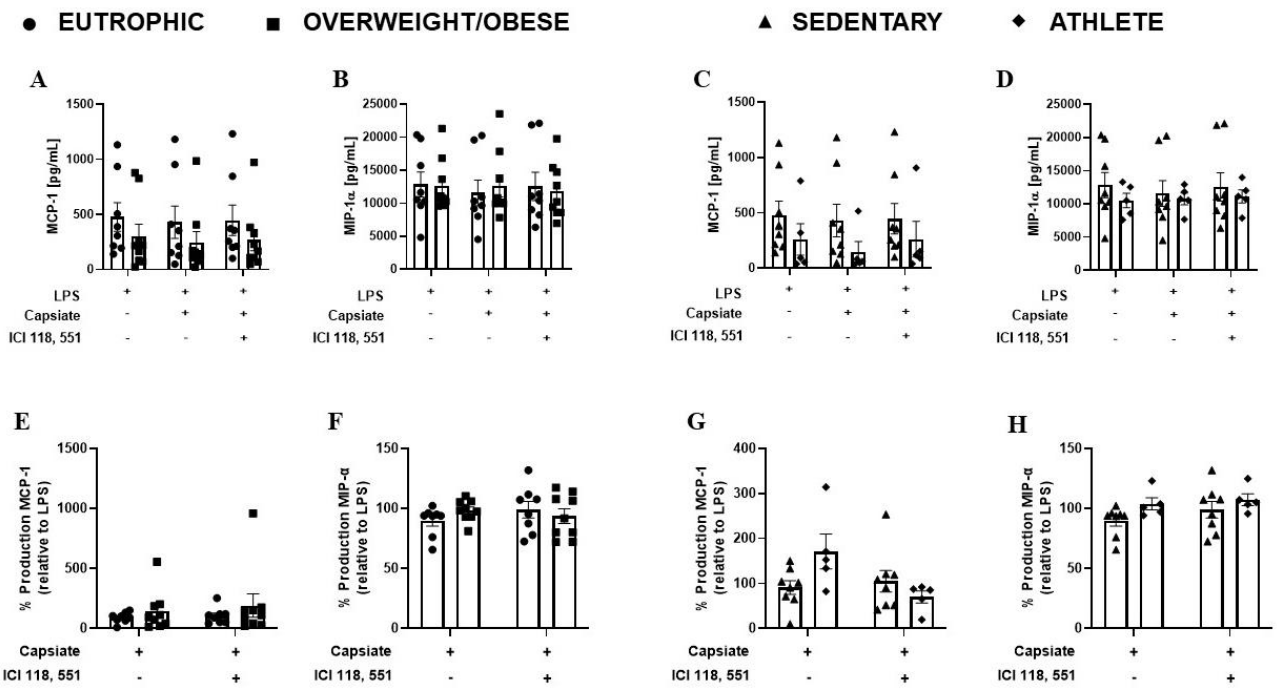

Figure 5. PBMCs cultured in the absence or presence of LPS [10 ng / mL], capsiate [50µM] and β2 receptor antagonist (ICI 118,551) [10^{-6}M]. MCP-1 (A), MIP-1α (B), MCP-1 versus LPS (E), MIP-1α versus LPS (F) production in eutrophic and overweight/obese subjects from study 1; MCP-1 (C), MIP-1α (D), MCP-1 versus LPS (G), MIP-1α versus LPS (H) in sedentary subjects and athletes from study 2. Data are expressed as mean ± standard deviation.

4. Discussion

In the present study, we investigated possible modulations in the anti-inflammatory response of PBMCs from individuals with different body compositions and physical fitness and to understand whether this possible response is dependent on AR-β_2. The results presented demonstrate that capsiate is not capable of promoting reductions in the production of cytokines, being independent of body composition and physical fitness. Additionally, we found that this inflammatory response is not modulated by AR-β_2.

In the present study, the in vitro assay to determine the concentration for capsiate stimulation promoted a reduction in the release of TNF-α induced by LPS at concentrations from 50 µM, demonstrating the anti-inflammatory potential of this phytochemical. Currently, in the literature, few studies have been performed with PBMCs, making direct comparison difficult, however, in THP-1 monocyte lineage, pretreatment with capsaicin (90 minutes, at a concentration of 125 µM), promoted modulations in the mediated inflammatory response by PHA after 24 hours [27]. Thus, it is possible that the reduction in the release of TNF-α in PBMCs in the present study is mediated by the inhibition of the degradation of the IκBα protein, which stabilizes the NF-κB complex in the cytoplasm, preventing its binding to DNA and, consequently, reducing the synthesis of inflammatory proteins.

Interestingly, findings in the literature demonstrated that capsainoids could exert their anti-inflammatory effect independently of the TRPV1 receptor, in which peritoneal macrophages from

rats stimulated in the presence of LPS and treated in parallel with capsaicin (10 - 50µM) demonstrated a reduction in the production of inflammatory cytokines, even in the presence of the TRPV1 receptor antagonist (capsazepine) [28]. Reinforcing these findings, Park et al. (2004), used RAW 264.7 macrophages that do not express the TRPV1 receptor and, after treatment with capsaicin, there was a reduction in the production of TNF-α induced by LPS, highlighting an alternative mechanism to the TRPV1 receptor [23]. Thus, the understanding of the adjacent mechanisms by which the anti-inflammatory effects of capsainoids occur are still unknown, in this sense, after previous publications by our group [20], assuming that the effects of capsainoids could be dependent body composition and physical fitness of volunteers. Additionally, preliminary studies showing the ability of capsaicin to promote similar functions of the β-adrenergic pathway, especially in the anti-inflammatory response of isolated cells in vitro, we hypothesized that this phytochemical may stimulate the anti-inflammatory response of circulating immune cells, as well as this response. be mediated by the β adrenergic pathway, being also influenced by different physical conditioning.

In the present study, capsiate was not able to promote anti-inflammatory effects and the blockade of the β2 adrenergic pathway did not modulate the inflammatory response in the present study. A possible explanation for the responses of PBMCs when stimulated in the presence of capsiate is due to the low consumption of spicy foods by the participants, reflecting a possible lack of sensitivity to capsiate, in which they did not have a habit of consuming peppers (data not shown), possibly due to our gastronomic culture, unlike Asian cultures, among others, which present a higher frequency of consumption of spicy foods [29]. Thus, it is possible that individuals who have a daily consumption of spicy foods may have a more pronounced inflammatory response compared to individuals who do not have this habit. However, further work investigating the role of chronic consumption, as well as the doses needed for this adaptation are needed.

While we did not find differences in the modulation of cytokine production, Kunde et al. (2018) investigated the modulations of inflammatory responses of THP-1 monocytes, stimulated with 5 µg/mL of LPS associated with the activation of TRPV1 receptors through capsaicin stimulation ([1.25 – 125 µM]), and found an increase at the concentrations of TNF-α, MCP-1, IL-6 when compared to wells stimulated with LPS alone [32]. Another interesting point regarding the results should be highlighted is the fact that it is one of the only works available in the literature that used primary human cells (PBMC), in which most of the currently available publications used animal lines or immortalized cells, which could explain the possible differences in the results found in relation to inflammatory responses. It is important to point out that the model used (PBMC) is of great relevance, given the proper proportions, it is the closest to a physiological environment. However, future studies

that seek to understand the modulations promoted by capsiate in different types of immune cells present in PBMCs are extremely important.

Physical conditioning has been shown to be essential for the modulation of the immune system, through the modulation of adrenergic receptors, in which it was shown that cardiorespiratory fitness is a predictive factor for better monocyte responsiveness to β2 adrenergic receptor activation, decreasing production of TNF-α in obese individuals [30]. In the present study, we hypothesized that the anti-inflammatory effects of capsainoids, which have been discussed in the literature, are independent of TRPV1 receptors, present interactions with the adrenergic receptor pathway, as the anti-inflammatory is similar. However, selective blockade of β2 adrenergic receptors did not promote changes in inflammatory responses in PBMCs stimulated in the presence of capsiate. Future work is needed to seek to understand the possible pathways by which they are activated by capsinoids and how body composition and physical fitness modulate the immune response, in addition to investigating possible benefits of capsainoids supplementation in distinct metabolic conditions, such as type 2 diabetes mellitus, dyslipidemia, among other metabolic disorders.

Therefore, future studies are needed to investigate the effects of capsainoids and the differences between them, since it is possible that the anti-inflammatory effects found in the literature promoted by capsainoids may present a molecular specificity, since the compounds are analogous**,** being the variation between the compounds in the linkage of the vanillic group with the fatty acid, being linked via the amide linkage in capsaicin and the ester linkage in capsiate [31]. Additionally, further studies are needed to investigate the specific effects of capsaicin, as well as the possible contribution of different physical abilities to the inflammatory responses found in the current project.

Some limitations in the work need to be mentioned, such as the absence of flow cytometry to trace the profile of circulating cells in each individual and how this profile could respond to the stimulus. Specific separation methods to understand whether there are differences in responses to stimuli between lymphocytes and monocytes from humans. The results presented should be applied to healthy individuals without established metabolic diseases, however, further work on individuals with established metabolic and inflammatory diseases and to understand the modulations promoted by capsiate should be developed.

5. Conclusions

Thus, we conclude that capsiate does not promote a reduction in the production of inflammatory cytokines in peripheral blood mononuclear cells of individuals with different body compositions and physical fitness.

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CONCLUSÃO

Em conclusão, nossos achados sugerem que indivíduos sobrepesados/obesos apresentam exaustiva resposta inflamatória crônica, direcionando para menor eficiência na resposta inflamatória, tanto sistêmica quanto celular. Por outro lado, indivíduos com alto nível de atividade física apresentam menores concentrações circulantes de IL-6 e maior eficiência metabólica celular, refletidas pelo menores taxas de consumo de oxigênio basal de PBMCs quando comparados com sedentários. Desta forma, o engajamento em programa de exercícios físicos e manutenção da composição corporal são essenciais para uma resposta imune adequada.

Por outro lado, a partir do segundo artigo, nós podemos observar que o estímulo de capsiate e o bloqueio seletivo do AR-β2 não foram capazes de promover modulações nas respostas inflamatórias das PBMCs em indivíduos com diferentes status da composição corporal e aptidão cardiorrespiratória.

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