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MODULAÇÃO DO METABOLISMO DA TETRAIDROBIOPTERINA EM CONDIÇÕES INFLAMATÓRIAS

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“Pain, like love, is all consuming: when you have it, not much else matters, and there is nothing you can do about it. Unlike love, however, we are actually beginning to tease apart the mystery of pain”.

Clifford J. Woolf (2010).

RESUMO

A inflamação sustentada é um componente comum da fisiopatologia de muitas doenças, seja como causa primária ou consequência secundária da deficiência energética e/ou estresse oxidativo. A inflamação crônica pode danificar tecidos e órgãos e contribuir para o desenvolvimento e progressão de várias condições médicas. O objetivo desta tese foi investigar o papel potencial do metabolismo de tetraidrobiopterina (BH4) como alvo-chave para modular a inflamação em condições em que os níveis de BH4 podem ser aumentados ou prejudicados. Como sistemas biológicos foram incluídos três desenhos experimentais: cultura de fibroblastos humanos com deficiência genética de uma proteína mitocondrial que é essencial para a tradução mitocondrial, o *TFAM*. A segunda abordagem incluiu animais submetidos a dieta rica em gordura (do inglês *high fat diet* – HFD) para induzir o acúmulo visceral de tecido adiposo e expostos ao exercício físico aeróbio visando prevenir o desenvolvimento da obesidade. O terceiro desenho experimental envolveu o uso da cafeína em um modelo animal de inflamação induzida por lipopolissacarídeo (LPS). Os resultados gerados nesta tese de doutorado evidenciaram que os fibroblastos deficientes em *TFAM* expressam o fenótipo de deficiência energética, caracterizado por redução do consumo de oxigênio basal, extravasamento de prótons e capacidade de reserva mitocondrial, bem como inibição dos complexos da cadeia respiratória, particularmente os complexos I e IV. Além disso, foi demonstrado que o estresse oxidativo também está presente nessas células como outro componente da fisiopatologia da deficiência genética, e que a atividade das enzimas antioxidantes glutationa peroxidase e superóxido dismutase foi reduzida em fibroblastos deficientes em *TFAM*. Além disso, a análise do transcriptoma dessas células mostrou que os genes responsáveis pela síntese de BH4 não foram regulados positivamente como seria esperado em condições metabólicas deficientes, denotando, portanto, que sob disfunção mitocondrial e estresse oxidativo, a ativação da produção de BH4 pode estar comprometida nos fibroblastos. Ainda, foi observado que os níveis de BH4 estavam patologicamente aumentados e o limiar nociceptivo diminuído nos animais expostos à HFD. O aumento dos níveis de BH4 ocorreu em paralelo com maior gordura corporal e acúmulo de tecido adiposo branco visceral, intolerância à glicose e maior glicemia de jejum. Esses dados mostram que o aumento do metabolismo da BH4 coincide com a redução nos escores de nocicepção, e que os níveis de BH4 urinária poderiam se tornar um possível biomarcador do desenvolvimento da hipersensibilidade à dor na obesidade. O exercício físico de intensidade

moderada preveniu o acúmulo de tecido adiposo, além de normalizar a tolerância à glicose e o comportamento nociceptivo, desta forma, induzindo efeitos positivos na saúde. A administração de cafeína demonstrou efeitos anti-inflamatórios caracterizados pela redução da expressão gênica de citocinas pró-inflamatórias *Il1b* e *Il6,* bem como dos componentes do inflamassoma Nlpr3, *Asc* e *Casp1*. Por outro lado, foi observado aumento da expressão gênica da citocina anti-inflamatória *Il10*. Juntamente com o LPS, a administração de cafeína provocou mudanças no perfil epigenético, promovendo a indução da metilação *de novo* do DNA. Em conjunto, os resultados apresentados nesta tese sugerem que o metabolismo da BH4 pode ser considerado uma faca de dois gumes, com baixos ou elevados níveis resultando em toxicidade.

Palavras-chave: Inflamação; Disfunção mitocondrial; Dor crônica; Exercício físico; Cafeína; Epigenético.

ABSTRACT

Sustained inflammation is a common component in the pathophysiology of many diseases, whether as a primary cause or secondary consequence of energy deficiency and/or oxidative stress. Tissues and organs can be damaged by chronic inflammation, and various medical conditions can be contributed to it. The potential role of tetrahydrobiopterin (BH4) metabolism as a key target for modulating inflammation in conditions where BH4 levels can be increased or impaired was investigated in this thesis. Three experimental designs were included as biological systems: culture of human fibroblasts characterized by genetic deficiency of a mitochondrial protein essential for mitochondrial translation, TFAM. The second approach involved animals that were subjected to a high-fat diet (HFD) to induce the accumulation of visceral adipose tissue and were exposed to aerobic exercise to prevent the development of obesity. The third experimental design involved the use of caffeine in an animal model of lipopolysaccharide (LPS)-induced inflammation. The results generated in this doctoral thesis revealed that the phenotype of energy deficiency is expressed by TFAM-deficient fibroblasts, which is characterized by reduced basal oxygen consumption, proton leakage, and mitochondrial reserve capacity, as well as inhibition of respiratory chain complexes, particularly complexes I and IV. Furthermore, it was demonstrated that oxidative stress is also present in these cells as another component of the pathophysiology of genetic deficiency, and the activity of the antioxidant enzymes glutathione peroxidase and superoxide dismutase was reduced in TFAM-deficient fibroblasts. Additionally, the transcriptome analysis of these cells showed that genes responsible for BH4 synthesis were not upregulated as expected under deficient metabolic conditions, indicating that activation of BH4 production may be compromised in fibroblasts under mitochondrial dysfunction and oxidative stress. It was also observed that BH4 levels were pathologically increased, and the nociceptive threshold was decreased in animals exposed to HFD. The increase in BH4 levels occurred parallel to higher body fat and accumulation of visceral white adipose tissue, glucose intolerance, and elevated fasting blood glucose. These data demonstrate that increased BH4 metabolism coincides with reduced nociception scores, and urinary BH4 levels could become a potential biomarker for the development of obesity-related hypersensitivity to pain. The accumulation of adipose tissue was prevented, glucose tolerance was normalized, and nociceptive behavior was induced positively on health by moderate-intensity physical exercise. Anti-inflammatory effects were demonstrated by caffeine administration, characterized by the

reduction of gene expression of pro-inflammatory cytokines Il1b and Il6, as well as components of the inflammasome Nlpr3, Asc, and Casp1. On the other hand, an increase in gene expression of the anti-inflammatory cytokine Il10 was observed. Alongside LPS, changes in the epigenetic profile were induced by caffeine administration, promoting de novo DNA methylation. Together, the results presented in this thesis suggest that BH4 metabolism can be considered a double-edged sword, with low or high levels resulting in toxicity.

Keywords: Inflammation; Mitochondrial dysfunction; Chronic pain; Physical exercise; Caffeine; Epigenetic.

RESUMO EXPANDIDO

Introdução

A inflamação é uma resposta protetora do organismo que visa eliminar patógenos ou moléculas causadoras de danos celulares e teciduais. Porém, a persistência dessa resposta pode ser prejudicial e desencadear em dano tecidual contribuindo para o desenvolvimento e progressão de várias condições médicas. A disfunção mitocondrial é um fator chave na manutenção da inflamação por ser o local primário de produção de espécies reativas de oxigênio (EROs) em condições fisiológicas e patológicas. No entanto, a consequência no metabolismo celular será diferenciada de acordo com o tipo e níveis de EROs produzidas. Por exemplo, baixas concentrações de EROs sinalizam para a ativação do principal sistema de defesa antioxidante, enquanto altos níveis ativarão complexos multiproteicos denominados de inflamassoma. Nas últimas duas décadas tem se demonstrado que a ativação persistente destes complexos compromete a sobrevida celular por processar citocinas pró-inflamatórias, o que contribui para a manutenção do estresse oxidativo, disfunção mitocondrial e, portanto, de inflamação. Nesse cenário, a deficiência de sistemas celulares que mantêm a fisiologia mitocondrial normal, ou que permitem que as mitocôndrias se adaptem a diferentes demandas energéticas, também é descrita como envolvida na ativação do inflamassoma. Um exemplo de uma via metabólica que pode resultar em disfunção celular induzida por inflamação é o metabolismo da tetraidrobiopterina (BH4), onde baixas ou altas concentrações podem levar à inflamação e prejudicar as funções celulares normais. A BH4 é uma pterina natural tradicionalmente conhecida por sua atividade de cofator de fenilalanina hidroxilase, tirosina hidroxilase, triptofano hidroxilase e todas as isoformas da enzima óxido nítrico sintase. Consequentemente, a BH4 é necessária para o metabolismo da fenilalanina e éteres lipídicos, e para a síntese de serotonina, dopamina e óxido nítrico. Nosso grupo de pesquisa tem demonstrado na última década que as concentrações intracelulares da BH4 também promovem outras funções que incluem a ativação do sistema antioxidante, da função mitocondrial, da geração de um sistema antioxidantes, e dos processos cognitivos, entre outros. Desta forma, níveis deficientes de BH4, não só comprometerão as reações bioquímicas onde participa como cofator enzimático, mas também poderá favorecer a disfunção mitocondrial, estresse oxidativo e inflamação. Por outro lado, também nosso grupo tem descrito que a produção exacerbada de BH4 é patogênica, causando dor, aumentando a agressividade do sistema imunológico e a progressão dos sintomas de doenças

crônicas, incluindo dor crônica, asma, esclerose múltipla, colite ulcerativa, artrite reumatoide e comprometimento cognitivo. Portanto, pode ser sugerido que a manutenção de níveis basais de BH4 é essencial para o bom funcionamento de diversas vias metabólicas e funções vitais.

Hipótese

O aumento excessivo ou a inibição do metabolismo da BH4 caracterizam doenças que cursam com inflamação crônica; assim, os níveis de BH4 podem se tornar alvos farmacológicos a fim de modular a inflamação e o curso deste tipo de doenças.

Objetivos

O objetivo geral desta tese foi identificar se o metabolismo de BH4 é modulado por doenças crônicas caracterizadas por inflamação e se o exercício e a cafeína podem provocar ambientes anti-inflamatórios que normalizariam os níveis intracelulares de BH4. Os objetivos específicos foram divididos em capítulos: ***Capítulo I:*** *a)* Determinar se culturas de fibroblastos obtidas de um indivíduo afetado por uma doença mitocondrial primária pode se tornar num modelo experimental para o estudo de novos horizontes terapêuticos, incluindo a suplementação com BH4; *b)* Elaborar uma revisão de literatura contemplando os papéis do metabolismo da BH4 na saúde e na doença. ***Capítulo II:*** *a)* Identificar se existem alterações nos limiares nociceptivos e na produção de BH4 em animais submetidos a dieta rica em gordura (do inglês *high fat diet* - HFD) e ao exercício físico; *b)* Elaborar uma revisão de literatura com as contribuições das modificações epigenéticas induzidas pela obesidade e pelo exercício físico associadas à função cognitiva. ***Capítulo III:*** *a)* Analisar os efeitos da cafeína, utilizada como um substituto do exercício físico devido às propriedades ergogênicas, sobre mediadores inflamatórios e modificações epigenéticas no músculo vasto lateral de camundongos submetidos a inflamação.

Estratégia experimental

Para atingir os objetivos mencionados acima, esta tese seguiu uma abordagem abrangente e variada que envolveu:

Capítulo I: *a)* Cultura de fibroblastos humanos deficientes em *TFAM* para análise da possível manifestação do fenótipo metabólico de doença mitocondrial. A determinação do fenótipo metabólico foi caracterizada mediante a determinação da respiração celular através do uso de um

bioanalisador metabólico, e atividade mitocondrial mediante a determinação espectrofotométrica das atividades das principais enzimas da cadeia respiratória. Também foram investigados alguns parâmetros de estresse oxidativo, como as atividades das principais enzimas antioxidantes. *b)* Elaboração de uma revisão geral que contemplou o estado da arte sobre o metabolismo da BH4 na saúde e na doença e foi proposto o uso "*off-label*" para o tratamento de doenças com disfunção mitocondrial, incluindo as de origem genético.

Capítulo II: *a)* Camundongos submetidos à HFD para posterior análise de parâmetros de composição corporal, testes comportamentais de locomoção, motivação e nocicepção, bem como os níveis urinários de BH4; além disso, camundongos alimentados com HFD foram submetidos a um protocolo de exercício físico de intensidade moderada em esteira de correr para avaliar os efeitos do exercício sob os parâmetros mencionados acima. *b)* Elaboração de uma revisão da literatura sobre modificações epigenéticas induzidas pelo exercício físico como proposta para melhorar os déficits cognitivos associados à obesidade.

Capítulo III: *a)* Camundongos submetidos a uma injeção intraperitoneal de cafeína e/ou lipopolissacarídeo (LPS) visando avaliar os efeitos da cafeína no músculo sobre a expressão genética de genes envolvidos na inflamação e sobre marcadores de epigenética.

Resultados e discussão

Os dados resultantes dessa pesquisa foram organizados em três capítulos que contemplam dois artigos de revisão de literatura e três artigos originais. No ***Capítulo I*** as descobertas do estudo revelam redução no consumo de oxigênio basal, respiração máxima, na capacidade de reserva mitocondrial, e na atividade dos complexos da cadeia respiratória mitocondrial, nomeadamente complexo I e IV, bem como aumentados níveis de ânion superóxido e reduzida capacidade antioxidante nos fibroblastos deficientes em *TFAM*. Estes resultados evidenciam que os fibroblastos deficientes em *TFAM* expressam o mesmo fenótipo de deficiência energética que se esperaria encontrar nos tecidos mais afetados pela doença, ou seja, o fígado, o cérebro e o músculo esquelético. Além disso, a análise do transcriptoma dessas células mostrou que os genes responsáveis pela síntese de BH4 não foram regulados positivamente como seria de se esperar nessas condições metabólicas, denotando, portanto, que sob disfunção mitocondrial e estresse

oxidativo, a ativação da produção de BH4 pode estar comprometida. No ***Capítulo II*** os resultados demonstram aumento no depósito de tecido adiposo branco visceral, na porcentagem de gordura corporal e na tolerância à glicose. Foi observado também uma redução dos limiares de hipersensibilidade à dor provocada por estímulos químicos e mecânicos e um aumento nos níveis de BH4 na urina de camundongos alimentados com HFD. Esses dados mostram que o aumento do metabolismo da BH4 coincide com a redução nos escores de nocicepção e que os níveis de BH4 urinária poderiam se tornar um possível biomarcador de desenvolvimento da hipersensibilidade à dor na obesidade. Também foi verificado que o exercício físico de intensidade moderada normalizou a antropometria dos animais submetidos a HFD, incluindo a redução do tecido adiposo branco, a normalização da tolerância à glicose, e a recuperação do escore basal de nocicepção. No ***Capítulo III*** os achados demonstram que a administração da cafeína provocou um efeito anti-inflamatório em um modelo de inflamação mediada por LPS em camundongos, com marcada redução na expressão gênica de citocinas pró-inflamatórias, aumento das citocinas anti-inflamatórias e diminuição dos componentes do inflamassoma, tais achados possivelmente mediados por mecanismos epigenéticos uma vez que juntamente com o LPS, a administração de cafeína provocou mudanças no perfil epigenético, promovendo a indução da metilação *de novo* do DNA.

Considerações finais

Os dados gerados nesta tese de doutorado forneceram de dados objetivos sobre o envolvimento do metabolismo da BH4 na fisiopatologia de distúrbios com componente inflamatório. Os fibroblastos deficientes em *TFAM* reproduzem as disfunções esperadas, sendo, portanto, um modelo adequado para testar potenciais tratamentos, como por exemplo a suplementação de BH4. Deve ser salientado que a deficiência em *TFAM* não conta com nenhum tipo de esperança terapêutica e os afetados tem uma sobreviva muito curta.

O aumento dos níveis de BH4 observado na urina de animais com acúmulo patológico do de tecido adiposo podem servir como um biomarcador de hipersensibilidade à dor na obesidade, podendo oferecer implicações clínicas importantes no diagnóstico e monitoramento da dor crônica nesta condição. Além disso, os resultados indicam que o exercício físico e a administração de cafeína podem ser uma estratégia alternativa para modular negativamente a inflamação. Em conjunto, estes achados podem apoiar estudos futuros na validação da suplementação da BH4 como

um promotor da saúde mitocondrial, bem como na redução dos níveis excessivos de BH4 como manejo da dor crônica.

Palavras-chave: Inflamação; Disfunção mitocondrial; Dor crônica; Exercício físico; Cafeína; Epigenética.

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LIST OF ABBREVIATIONS AND ACRONYMS

AR: Adenosine receptor

AT: Adipose tissue

AUC: Area under curve

BAT: Brown adipose tissue

BH4: Tetrahydrobiopterin

BMI: Body index mass

CCR2: C chemokine receptor 2

CNS: Central nervous system

CRP: C-reactive protein

DAMPs: Damage-associated molecular patterns

DRG: Dorsal root ganglion

ETC: Electron transport chain

FDA: Food and drug administration

GCH1: GTP cyclohydrolase 1

GTPCH: Guanosine triphosphate cyclohydrolase I

GTT: Glucose tolerance test

HFD: High fat diet

HPLC: High performance liquid chromatography

IASP: International association for the study of pain

i.c.v.: Intracerebroventricular

IFN-γ: Interferon gamma

IL-1β: Interleukin-1 beta

IL-2: Interleukin-2

IL-4: Interleukin-4

IL-6: Interleukin-2

IL-10: Interleukin-10

IL-13: Interleukin-13

IL-18: Interleukin-18

i.p.: Intraperitoneal

LPS: Lipopolysaccharide

MCP-1: Monocyte chemoattractant protein-1

mtDNA: Mitochondrial DNA

NF-κB: Nuclear factor kappa B

NLRP3: NOD-like receptor family, pyrin domain-containing protein 3

OXPHOS: Oxidative phosphorylation

PAMPs: Pathogen-associated molecular patterns

PMDs: Primary mitochondrial diseases

PNS: Peripheral nervous system

PRRs: Pattern recognition receptors

PTPS: 6-pyruvoyl tetrahydrobiopterin synthase

ROS: Reactive oxygen species

SPR: Sepiapterin reductase

SPRi3: Sepiapterin reductase inhibitor 3

SSZ: Sulfasalazine

TFAM: Mitochondrial transcription factor A

TLRs: *Toll*-like receptors

TNF-α: Tumor necrosis factor alpha

WAT: White adipose tissue

WHO: World health organization

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INTRODUCTION

Inflammation is a host response initiated by the immune system to trauma, tissue damage and/or infection. It is a protective response aimed at eliminating danger and initiating the healing process (MEDZHITOV, 2010). In the context of microbial infection, the immune system is activated by exogenous signals known as pathogen-associated molecular patterns (PAMPs), e.g., lipopolysaccharide (LPS) and bacterial and viral nucleic acids, indicating the presence of pathogens.

On the other hand, damage-associated molecular pattern molecules (DAMPs), which are endogenous danger signals, e.g., extracellular ATP, cholesterol, uric acid, amyloid, reactive oxygen species (ROS), and others, can be responsive to unscheduled cell death, microbial invasion, or stress (PERREGAUX; GABEL, 1994). Inflammatory responses can be induced by DAMPs, either independently or in synergy with PAMPs, through the activation of pattern recognition receptors (PRRs). The subfamilies of PRRs, including *Toll*-like receptor (TLR) and nucleotide-binding oligomerization domain-like receptors (NLRs), contribute to tissue damage (MEDZHITOV, 2010). Once activated, NLRs can recruit and assemble proteins into complexes known as inflammasomes. This physiological process activates the innate immune system and initiates a controlled inflammatory response. However, sustained activation of the immune system and NLRs is known to be cytotoxic and has been linked to the physiopathology of acute and chronic diseases (HENEKA et al., 2013; SCHRODER; TSCHOPP, 2010; TERRILL-USERY; MOHAN; NICHOLS, 2014).

Persistent or unresolved inflammation can also be induced by mitochondrial dysfunction, as the organelle is the main source of ROS under both physiological and pathological conditions. While the aim and levels of the generated ROS have differential fate, with low levels known to signal for increased antioxidant defenses, high levels are detrimental to the cell's survival. In this scenario, the involvement of cellular systems that maintain normal mitochondrial physiology or enable mitochondria to adapt to different energy demands is also described in the activation of inflammasomes.

The metabolism of tetrahydrobiopterin (BH4) provides an example of a metabolic pathway in which the deficiency and excess of its intermediates can lead to inflammation-induced cell dysfunction. BH4, traditionally defined as an obligatory cofactor for the metabolism of phenylalanine and ether lipids and the synthesis of serotonin, dopamine and nitric oxide, is a natural

pteridine (WERNER et al., 1990). Considering the intracellular levels of BH4 regulating essential metabolisms, alterations in the cardiovascular, nerve, and immune system are likely to occur (KIM; HAN, 2020).

However, new fundamental roles have been uncovered for BH4 metabolism. The BH4 pathway has been described by our group as having antioxidant and anti-inflammatory activities, such as the inhibition of inflammasome assembly and activation. It has been also identified as a mitochondrial activator and a memory enhancer in the nervous system (DE PAULA MARTINS et al., 2018a; GHISONI et al., 2015; GHISONI; LATINI, 2015; LATINI et al., 2018). Furthermore, it has been demonstrated by us that the T-cell proliferation and tissue infiltration are impaired by the genetic or pharmacological inactivation of the BH4 pathways in animal models of diseases with chronic inflammation, and this mechanism involves iron dyshomeostasis and mitochondrial dysfunction. These finding have been confirmed in human cells as well (CRONIN et al., 2018; GHISONI; LATINI, 2015).

Furthermore, it has been reported that BH4 administration improves working memory and brain activation in the form of phenylketonuria, a hereditary catabolic defect where phenylalanine cannot be metabolized into tyrosine in some cases due to BH4 deficiency (CHRIST et al., 2013). Positive effects of enhanced BH4 metabolism have been also shown in mitochondrial physiology and dynamics and cognition in the brain and the immune system of experimental animals (CRONIN et al., 2018; KIM et al., 2019; LATINI et al., 2018). Thus, it is possible that reduced BH4 levels may coexist with mitochondrial dysfunction and oxidative stress, which are known activators of the inflammatory response. If this is proven to occur, new horizons could be proposed for the treatment of acute and chronic inflammatory disorders, such as primary mitochondrial diseases (PMDs), a group of genetic disorders for which there is currently no cure or effective therapies.

Thus, it is hypothesized that BH4 supplementation might represent an effective strategy to increase residual mitochondrial function in PMDs. Although this approach may not provide a cure for PMDs, it has the potential to significantly improve mortality and morbidity in a variety of mitochondrial diseases, independently of the primary genetic defect, for which there is currently no hope. This aligns with new policies in the United States (FDA, 2022) and in Brazil (law 14.313/2022) that have established incentive programs for identifying new indications for existing

Food and Drug Administration (FDA)-approved drug through off-label use. BH4 is commercially available as sapropterin dihydrochloride in tablet or powder for oral solution formulations. Since 2007, BH4 has been used in over 7,800 individuals accross different clinical trials, demonstrating its safety and tolerance (MUNTAU et al., 2021). The FDA has approved clinical trials for the off-label use of BH4 in a range of disorders, including those affecting phenylalanine metabolism, heart and vascular diseases, kidney, lung, liver, central nervous system (CNS), genetic disorders, rheumatologic diseases, menopause, and aging (clinicaltrials.gov). However, to reach this point, it is necessary to generate sufficient preclinical evidence demonstrating compromised BH4 metabolism. ***Therefore ,the first step is to identify appropriate cellular models of PMDs where mitochondrial dysfunction, oxidative stress, and inflammation can be observed alongside deficient BH4 metabolism.*** Furthermore, additional studies will be needed to demonstrate that enhancing the availability of BH4 would promote mitochondrial health and subsequently lead to a less prone inflammatory state.

On the other hand, the inflammatory environment, characterized by the release of pro-inflammatory mediators, i.e., interferon-gamma (IFN-γ), LPS, interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), can pathologically activate the BH4 metabolism, leading to excessively high levels of BH4. This is caused by the transcriptional upregulation of the rate-limiting enzyme, guanosine triphosphate cyclohydrolase I (GTPCH), which is involved in the *de novo* synthesis of BH4, by these inflammatory mediators (WERNER et al., 1990). In recent years, it has been described by our group that this upregulated metabolism triggers inflammatory and neuropathic pain, increases immune system aggressiveness, and induces oxidative stress in animal models and human diseases (CRONIN et al., 2018; FUJITA et al., 2020; LATREMOLIERE et al., 2015; RUSSO et al., 2020; STAATS PIRES et al., 2020b). Additionally, our studies have demonstrated that pharmacologic and genetic experimental inhibition of BH4, aimed at restoring its basal levels, results in analgesia, regulation of immune cell activity, and promotion of an antioxidant status (CRONIN et al., 2018; FUJITA et al., 2020; LATREMOLIERE et al., 2015).

Chronic pain is a debilitating and complex disease that is represented by an economic burden primarily due to medical treatment, decreased worker productivity, and reduced quality of life (TREEDE et al., 2019). The United States population is affected by chronic pain in approximately 20.4 % of cases (DAHLHAMER et al., 2018), and around 40 % of Brazilians are

affected as well (CARVALHO et al., 2018; SOUZA et al., 2017). The physiopathology of chronic pain is still not well understood, but evidence has extensively shown that certain chronic metabolic diseases may increase the risk of developing chronic pain. For example, it has been demonstrated that individuals affected by obesity, a disease characterized by chronic low-grade inflammation, are more sensitive to pain compared to lean individuals (MCKENDALL; HAIER, 1983). Additionally, there is a positive correlation between pain perception and an increase in body mass index (BMI) (HITT et al., 2007; SOMERS; WREN; KEEFE, 2011). However, chronic pain is another condition that lacks effective and safe treatments, thus emphasizing the urgent need to discover new ways of treating this disease. Therefore, the use of the BH4 inhibitors pathway to restore normal levels of BH4 can be proposed. ***Furthermore, the levels of BH4 in biological fluids could be used as the first quantitative biomarker for pain/analgesia in obesity and other chronic inflammatory diseases.***

It has been extensively shown that the regular practice of moderate physical exercise can be considered as a potent anti-inflammatory tool for the prevention and treatment of various inflammatory conditions, including obesity (for a review see SCHEFFER; LATINI, 2020). Additionally, increased mitochondrial activity and antioxidant status are stimulated by exercise, contributing to the observed beneficial effects. In agreement, the pathological increase of neopterin levels (SCHEFFER et al., 2019), a metabolite related to BH4 metabolism that has long been utilized as a sensitive biomarker of innate immune system activation (WERNER et al., 1990), in LPS-treated mice has been prevented through physical exercise. ***Therefore, this could serve as a valuable quantitative method to monitor the level of inflammation in chronic metabolic diseases, such as obesity, where exercise is the primary non-pharmacological treatment.***

Exercise has the potential to be considered a pleiotropic free-drug treatment that positively impacts health by negatively modulating the expression of several pro-oxidative and pro-inflammatory genes (SOUZA et al., 2022). The underlying molecular mechanisms involved changes in the epigenetic profile. For instance, an increase in the demethylation of the promoter of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1-a)*, which regulates cellular energy metabolism and mitochondrial function, has been observed in the skeletal muscle of animals subjected to voluntary wheel running (LOCHMANN et al., 2015). Similarly, the hypermethylation of the promoter of nuclear receptor subfamily 4 group A member 1 (*Nr4a1)*, a

transcriptional regulator positively associated with chronic low-grade inflammation induced by HFD-induced WAT accumulation in mice, was reversed by physical exercise (KASCH et al., 2018). Additionally, an acute session of 80 % VO_2 intensity exercise was shown to induce the hypomethylation of *PGC1-a* and *TFAM*, promoting enhanced energy production and mitochondrial homeostasis (BARRÈS et al., 2012).

The accumulation of specific metabolites like tetrahydrofolate and ascorbic acid can be induced by exercise, which can further alter chromatin compaction and regulate gene expression (ŚWIATOWY et al., 2021). In this context, it is known that BH4 levels have the potential to modulate one-carbon metabolism and, consequently, epigenetics. The enzyme dihydrofolate reductase (DHFR), which is involved in the synthesis of BH4 through the salvage pathway, also plays a crucial role in DNA methylation. In fact, a decrease in DHFR activity leads to reduced levels of tetrahydrofolate, thereby impairing DNA methylation (CHEN et al., 1984). This is relevant to the action of antifolate medications used to treat inflammatory diseases, as demonstrated by methotrexate, which is known to reduce BH4 levels (CRABTREE et al., 2009). ***Thus, BH4 metabolism and physical exercise may also regulate chromatin compaction, leading to changes in epigenetics and promoting an anti-inflammatory and antioxidant status with increased mitochondrial activity by modulating genomic functions such as DNA replication, damage, and repair, or the expression of inflammatory mediators.***

Caffeine, the most widely consumed drug globally, is mainly consumed due to its ergogenic effects (ASTORINO; ROHMANN; FIRTH, 2007; SPRIET et al., 1992). It has shown that caffeine exhibits anti-inflammatory properties by increasing the levels of the anti-inflammatory cytokines, e.g., IL-10 (TAULER et al., 2016), and reducing the levels of pro-inflammatory mediators, e.g., IL-6 (RODAS et al., 2020), in the blood of athletes. Additionally, there is evidence suggesting that caffeine may also inhibit the activity of histone deacetylases (HDACs), resulting in a more open chromatin structure that, in turn, increases gene expression or transcriptional activity. ***Therefore, it is plausible that caffeine may also modulate epigenetics to reduce the production of pro-inflammatory mediators and, for example, delay fatigue.***

1.1 HYPOTHESIS

Given the above-mentioned scientific premises, the overall hypothesis of this thesis was:

The excessive augmentation or the inhibition of the BH4 metabolism are characteristic of diseases with chronic inflammation, that can be pharmacologically targeted to normalize the intracellular concentrations in order to modulate inflammation and the course of the disease.

1.2 AIMS

The main aim of this doctoral work is:

To identify the potential role of BH4 metabolism as a critical target for modulating inflammation in conditions where BH4 levels can become significantly elevated or deficient and whether exercise and caffeine can elicit anti-inflammatory environments that would normalize BH4 intracellular levels.

The specific aims of this study are:

Chapter I:

- To determine the suitability of cultured fibroblasts as a cellular model for characterizing the impaired mitochondrial features of human *TFAM* deficiency, allowing for further investigations on potential treatments, including BH4 supplementation.

Chapter II:

- To induce AT accumulation, quantify the urinary levels of BH4, and determine the nociceptive thresholds in a animals submitted to high fat diet (HFD) and physical exercise.

Chapter III:

- To analyze the effects of caffeine on inflammatory mediators and epigenetic modifications in the *vastus lateralis* muscle of mice under LPS-induced inflammation.

CHAPTER I

TRADITIONAL AND NEW FUNCTIONS OF TETRAHYDROBIOPTERIN METABOLISM AND UNVEILING FIBROBLASTS AS A VALUABLE MODEL FOR INVESTIGATING POTENTIAL TREATMENTS FOR MITOCHONDRIAL DISORDERS

This chapter provides essential information on inflammation and primary mitochondrial diseases. Two manuscripts are part of this chapter: *1)* One literature review with an overview of the traditional and vital known functions of BH4 as an obligatory cofactor for various enzymatic reactions, which leads to the hydroxylation of aromatic amino acids resulting in the catabolism of phenylalanine, synthesis of the neurotransmitters dopamine and serotonin, cleavage of ether lipids and the production of nitric oxide (NO) (THONY; AUERBACH; BLAU, 2000). It also brings evidence of the crucial physiological role of basal levels of BH4, including its function as a ROS scavenger, highlighting its antioxidant and anti-inflammatory properties and its ability to enhance mitochondrial function (CHRIST et al., 2013; DE PAULA MARTINS et al., 2018b; GHISONI et al., 2015; LATINI et al., 2018). This work also highlights that excessive BH4 levels have been linked to pathogenic effects, including increased pain, increased aggressivity of the immune system aggression, and progression of chronic diseases, such as chronic pain and rheumatoid arthritis, among others (CRONIN et al., 2018; FUJITA et al., 2020; LATREMOLIERE et al., 2015; STAATS PIRES et al., 2020b). *2)* One experimental manuscript with the bioenergetic and oxidative stress characterization of *TFAM*-deficient human fibroblasts, which highlights the fibroblasts as an alternative to muscle biopsies for not just diagnosing mitochondrial *TFAM* deficiencies but mainly for offering a valuable and cost-effective tool to identify new therapeutical approaches. It is also stressed that cultured fibroblasts can generate patient-specific cell lines for modeling tissue-specific human diseases in mitochondrial diseases.

(P1) Antioxidants:
Tetrahydrobiopterin: Beyond its traditional role as a cofactor.
Eichwald, T.; Silva, L.B.; Pires, A.C.S.; Niero, L.; Schnorrenberger, E.; Filho, C.C.; Espíndola, G.; Huang, W.L.; Guillemin, G.; Abdenur, J.; Latini, A. (Published in 3 May 2023).

(P2) PLOS Medicine:

Bioenergetics and antioxidant status in human *TFAM* deficiency.

Tuany Eichwald, Wei Lin Huang, Alexander Stover, Mariella T Simon, Philip H Schwartz, Alexandra Latini, Jose E Abdenur. (Manuscript to be submitted to PLOS Medicine).

1.1 INFLAMMATION

Inflammation is an adaptive biological response triggered by tissue injury due to microbial invasion, harmful compounds, viral infection, trauma, even by endogenous signals such as cells undergoing apoptosis. The inflammatory response is directed to prevent further injury and/or removal of damaged areas, seeking to promote tissue regeneration (MEDZHITOV, 2008). The inflammatory process is a defense mechanism to protect and repair tissues against endogenous and exogenous damage (TAKEUCHI; AKIRA, 2010; TRACEY, 2002) and involves a coordinated action between the immune system and the injured tissue (NATHAN; DING, 2010).

The inflammatory processes consist of vascular responses, migration, and activation of leukocytes and systemic reactions, leading to the accumulation of fluids and leukocytes in extravascular tissues (MEDZHITOV, 2008). At the tissue level, inflammation translates as swelling, pain, redness, heat, and loss of function (CHEN et al., 2018; LIBBY, 2008; MEDZHITOV, 2021).

Although inflammatory processes depend on the precise nature of the initial stimulus and its location, the response to a noxious agent generally begins with the recognition of harmful stimuli, which is mediated by PRRs. PRRs are triggered by exogenous signals indicative of pathogen invasion, known as PAMPs, such as LPS; or by DAMPs, such as extracellular ATP, endogenous signs indicative of sterile tissue injury (without the presence of microorganisms) (TAKEUCHI; AKIRA, 2010; TANG et al., 2012). DAMPs and PAMPs trigger an inflammatory response mediated by the production and release of pro-inflammatory cytokines, contributing to tissue damage (PERREGAUX; GABEL, 1994).

The most common PRRs are the TLRs and the NLRs (TAKEUCHI; AKIRA, 2010). Signaling through these receptors activates an intracellular signaling cascade that includes adapter molecules, kinases, and transcription factors (MARTINON; BURNS; TSCHOPP, 2002). Signal transduction pathways result in the synthesis of mediators that lead to the manifestation of inflammatory responses, resulting in the recruitment of cells to the site of injury, the production of acute phase proteins, platelet activation, fever, and vasodilation, enabling tissue repair and restoration of homeostasis (MEDZHITOV, 2021; ZHAO et al., 2020).

Among the *Toll* family, the TRL4 is activated by LPS, a molecule in the membrane of gram-negative bacteria (PARK; LEE, 2013). When TLR4 is activated, it couples the adapter protein

MyD88. It initiates several intracellular reactions, translocating the nuclear transcription kappa B (NF-κB) to the nucleus, and leading to the production of inflammatory mediators, like tumor necrosis factor-alpha (TNF-α), pro-interleukin 1 beta (pro-IL-1β), and pro-interleukin 18 (IL-18) (Figure 2) (LIU et al., 2017).

Neutrophils are one of the first types of immune system cells to reach the inflammatory site. These cells play a fundamental role in the resolution of inflammation (BISWAS; MANTOVANI, 2012) through the release of chemokines that attract monocytes and macrophages to the site of inflammation (SOEHNLEIN et al., 2008). Macrophages are immune cells with plastic characteristics; they can differentiate into specific phenotypes adopting specific functions depending on the stimulus and signals released in the microenvironment (MURRAY, 2017). Macrophages can be activated by classical (M1) and alternatively (M2) mechanisms. M1 macrophages are functionally pro-inflammatory and antimicrobial, and M2 macrophages are anti-inflammatory (MURRAY et al., 2014; WEISSER et al., 2013). M1 macrophages are activated mainly by LPS, interferon-gamma (IFN-γ), and TNF-α and induce the release of several cytokines, including IFN-γ, TNF-α, and interleukin (IL)-1β, causing positive modulation on macrophages not yet polarized (MOSSER; EDWARDS, 2008; O'SHEA; MURRAY, 2008). On the other hand, M2 macrophages are activated by cytokines such as IL-4, IL-13, and transforming growth factor beta (TGF-β) and have positive regulation of anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β. In general, M2 polarized macrophages contribute to infection prevention, tissue repair, angiogenesis, and immunomodulation (Figure 1) (BISWAS; MANTOVANI, 2012).

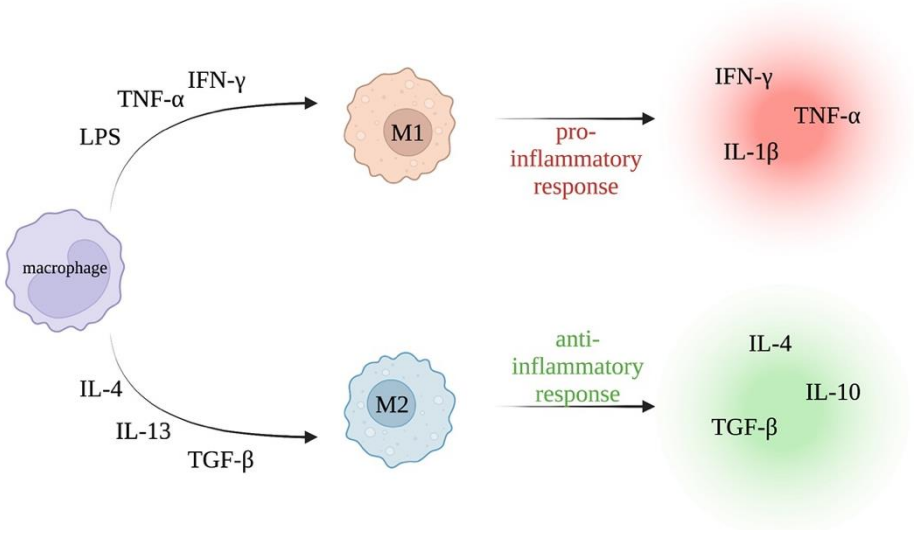

Figure 1. The plastic characteristics of macrophages.

Macrophages are activate and polarize into M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophages by stimuli such as cytokines. Upon activation, the release of pro-inflammatory lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1 beta (IL-1β) is carried out by M1 macrophages, while the anti-inflammatory cytokines IL-4, IL-10, and transforming growth factor beta (TGF-β) are released by M2 macrophages. Source: the author, 2023.

The resolution phase aims to remove cellular debris and pathogens and control the production of pro-inflammatory cytokines, thus preventing an exacerbated response and restoring homeostasis. However, if acute inflammation is not resolved, it can transition to chronic unresolved inflammation (MEDZHITOV, 2008). The chronic form of the inflammatory response has been involved in the physiopathology of a significant number of chronic diseases, such as obesity, cancer, rheumatoid arthritis, neurodegenerative diseases, inflammatory bowel diseases, and others (LIBBY, 2008; NATHAN; DING, 2010).

1.1.1 Inflammasome NLRP3

The inflammasome is a group of signaling proteins that facilitate the processing of cytokines pro-IL-1β and pro-IL-18 (MARTINON; BURNS; TSCHOPP, 2002). These cytokines have been linked to various biological responses associated with infection, inflammation, and autoimmune processes. Caspase-1, a protease that is synthesized as an inactive zymogen consisting of a caspase activation and recruitment domain (CARD) domain and two subunits, p20 and p10, is responsible for processing IL-1β and IL-18 precursors (THORNBERRY et al., 1992). The inflammasome acts as a platform that brings together two or more procaspases, allowing them to undergo proteolytic self-cleavage and form an enzymatically active heterodimer made up of p20 and p10 subunits (KOSTURA et al., 1989; MARTINON; BURNS; TSCHOPP, 2002).

There are four canonical inflammasomes that process pro-IL cleavage via caspase-1: *i)* NLRP1 (protein from the NLR family, containing pyrin 1 domain), *ii)* NLRP3 (protein from the NLR family, containing pyrin 3 domain), *iii)* IPAF (activating factor of IL-1 converting enzyme activity), and *iv)* AIM2 (absent in melanoma 2) (LAMKANFI; DIXIT, 2014). These inflammasomes differ mainly in structure, ASC accessory protein requirement, and potential

activators (FERNANDES-ALNEMRI et al., 2007). The ASC protein consists of a pyrin domain (PYD) and a CARD domain. It functions as an adapter for the interaction between PYD-containing proteins and CARD-containing proteins, such as procaspase-1 (MASUMOTO et al., 1999).

The most studied inflammasome, the NLRP3, must be primed before activation. During the priming step, various inflammatory stimuli, such as DAMPs and PAMPs, activate TLRs leading to the upregulation of *NLRP3* gene expression and synthesis of inactive NLRP3, ASC, and procaspase-1 protein. The priming step typically involves the activation of NF-κB, a transcription factor that regulates the expression of pro-inflammatory genes, including NLRP3 (LU et al., 2014). Once the NLRP3 protein is synthesized, it remains in an inactive state until the second step of activation occurs (Figure 2 left "1. PRIMING"). The activation step requires a second stimulus, the "danger signal." The danger signal can be diverse and includes many factors, such as mitochondrial ROS, potassium efflux, and cathepsin released by lysosome disruption (GUO; CALLAWAY; TING, 2015). These danger signals trigger the assembly of the NLRP3 inflammasome complex, consisting of NLRP3, the adapter protein ASC, and procaspase 1. Upon assembly, the procaspase-1 molecule undergoes autocleavage, resulting in the formation of active caspase-1. Active caspase-1 then cleaves pro-inflammatory cytokines, such as pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18), into their mature forms, which are released into the extracellular space (Figure 2 right "1. ACTIVATION") (GUO; CALLAWAY; TING, 2015). These mature cytokines contribute to the inflammatory response by recruiting immune cells and promoting cytokine production.

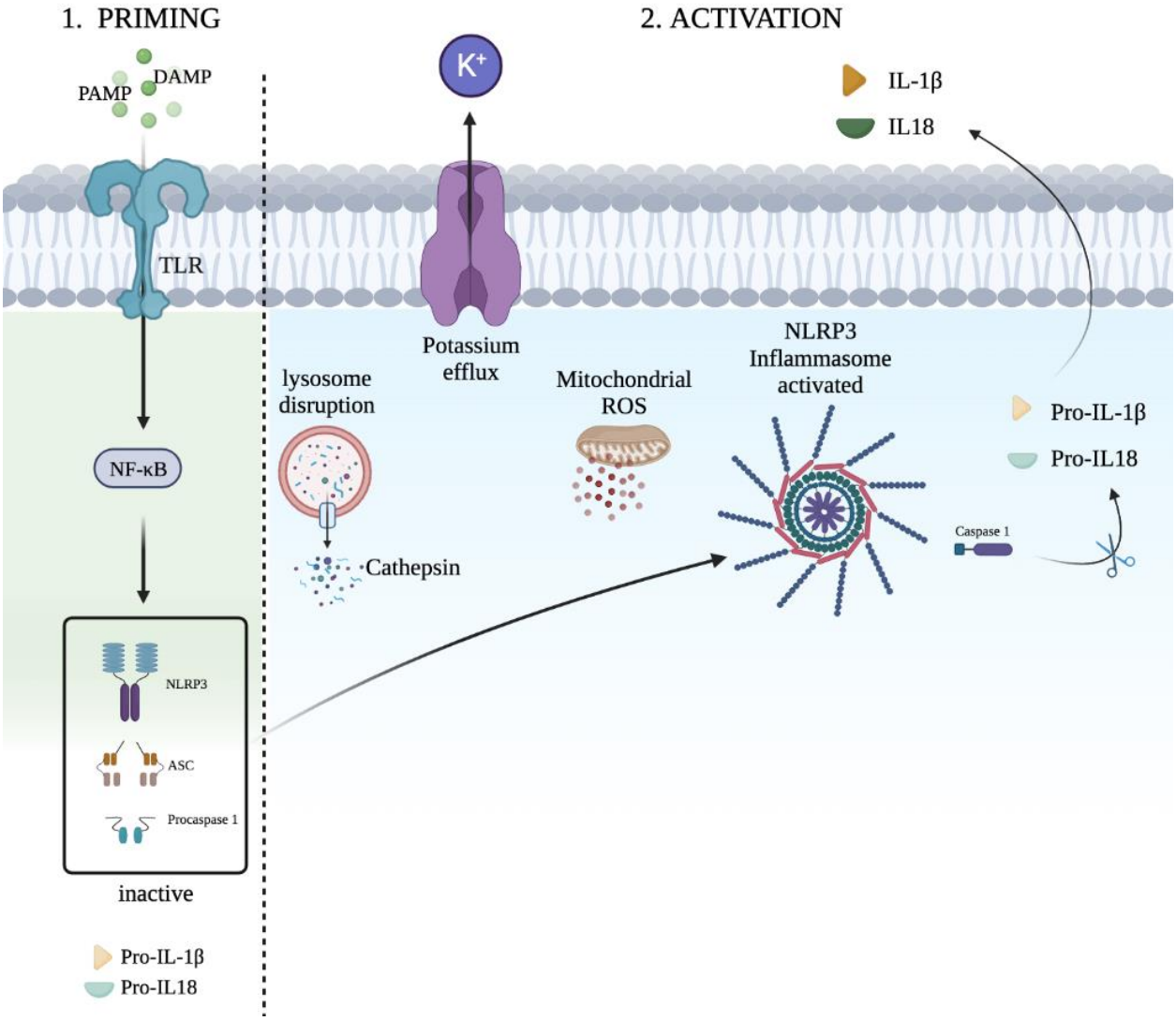

Figure 2. NLRP3 inflammasome priming and activation.

The activation of the NLRP3 inflammasome includes 2 steps, namely, priming (left) and activation (right). Left: *Toll*-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), resulting in translocation of nuclear transcription kappa B (NF-κB) to the nucleus and the promotion of the transcription of NLRP3, ASC and pro-caspase 1 (in the inactive form), as well as the pro-interleukin (IL)-1β and pro-IL-18. Right: The activation of the NLRP3 inflammasome is caused by danger signals, including the release of cathepsin due to lysosome disruption, mitochondrial ROS, and potassium efflux. This activation leads tothe activation of caspase-1, which, in turn, cleaves pro-IL-1β and pro-IL-18 into their mature interleukin forms. Pathogen-associated molecular patterns (PAMPs); damage-associated molecular patterns (DAMPs); pro-IL-1β (pro-interleukin 1 beta); pro-IL-18 (pro-interleukin 18); ROS (reactive species of oxygen); IL-1β (interleukin 1 beta); IL-18 (interleukin 18); nuclear transcription kappa B (NF-κB). Adapted from LI et al., 2021.

The NLRP3 inflammasome is critical for host immune defenses against bacterial, fungal, and viral infections (ALLEN et al., 2009; GROSS et al., 2009; LAMKANFI; DIXIT, 2014); however, the activation of the NLRP3 inflammasome has already been related to the physiopathology of several chronic diseases with an inflammatory component, i.e., in obesity (VANDANMAGSAR et al., 2011).

1.2 PRIMARY MITOCHONDRIAL DISEASES

Dysfunction of the mitochondrial respiratory chain can lead to a diverse group of disorders known as PMDs. These disorders exhibit clinical diversity and primarily impact tissues and organs that heavily depend on aerobic metabolism, including the brain and muscles (WALLACE, 1999). The underlying cause of PMDs is complex, with genetic mutations occurring in both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), resulting from pathogenic variants in the mitochondrial genome that contain 37 genes that encode 13 proteins, 22 transfer RNA (tRNA), and two ribosomal RNA (rRNA) that can affect oxidative phosphorylation (OXPHOS) function by impacting production of complex machinery needed for the electron transport chain (ETC) optimal function (CHINNERY; TURNBULL, 2001).

The ETC is organized into five complexes, including complex I (NADH: ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (CoQ-cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase). The respiratory comprises over 70 polypeptides that interact with the inner mitochondrial membrane. Thirteen of these subunits are crucial and encoded by mtDNA located within the mitochondria, along with ribosomal and transfer RNAs required for protein synthesis within the mitochondria. The nuclear genome encodes the remaining polypeptides responsible for the ETC, proteins essential for its assembly, mitochondrial structure, and the maintenance and expression of mtDNA (GORMAN et al., 2016).

The epidemiology of PMDs is approximately 1 in 5,000 live births (THORBURN, 2004). The phenotypic spectrum is broad and highly variable, ranging from isolated organs to system involvement, which often presents with prominent neurologic and myopathic features. Symptoms of PMDs vary and may include severe muscular weakness, progressive encephalopathy, liver

malfunction, chronic fatigue, cardiomyopathy, ataxia, seizures, and neurodegeneration, among others (TUPPEN et al., 2010; ZOLKIPLI-CUNNINGHAM et al., 2018).

TFAM gene is a nuclear-encoded gene that plays a crucial role in the transcription, replication, and packaging of mtDNA into nucleoids, as well as regulates nucleoid architecture, abundance, and segregation (KASASHIMA; SUMITANI; ENDO, 2011; KAUFMAN et al., 2007). Thus, *TFAM* levels are known to directly control mtDNA copy number (LARSSON et al., 1998). It is accepted to be a transcription factor regulator of mitochondrial genes that encode for the subunits of the ETC, such as NADH dehydrogenase, cytochrome c oxidase, and ATP synthase, as well as mitochondrial rRNA and RNA (CHINNERY; TURNBULL, 2001). Therefore, mutations in *TFAM* profoundly impair OXPHOS and trigger calcium-dependent stress signaling and adaptive metabolic response, resulting in mitochondrial dysfunction, impaired cellular health, and embryonic lethality (PICCA; LEZZA, 2015).

Considering the variable phenotype of PMDs, diagnosis, and management are challenging. The diagnosis has been based on demonstrating mitochondrial dysfunction in a relevant tissue biopsy (e.g., skeletal muscle or liver), with the particular pattern of a biochemical abnormality used to direct targeted molecular genetic testing of mtDNA, specific nuclear genes, or both. The management of mitochondrial diseases includes reducing morbidity and mortality, addressing organ-specific complications, and intervening when possible. However, challenges remain in understanding disease mechanisms, developing curative treatments, and preventing disease transmission.

1.2.1 PMDs and inflammation

PMDs are not typically considered inflammatory diseases. However, mitochondrial dysfunction has been implicated in human diseases with underlying inflammatory pathologies, such as diabetes *mellitus* and cardiac dysfunction. Moreover, mounting evidence shows that increased ROS generation, extracellular ATP, and mtDNA release mediated by mitochondrial dysfunction cause inflammatory responses (LÓPEZ-ARMADA et al., 2013).

Besides producing energy, mitochondria also serves as the primary location for generating ROS. Through *in vitro* studies, it has been observed that up to 2 % of electrons flowing through the ETC undergo partial reduction to produce the superoxide anion, primarily at complexes I and

complex III (CADENAS; DAVIES, 2000). Moreover, ROS production in the mitochondria may result from reverse electron transport from complex II to complex I (CADENAS; DAVIES, 2000; KUKA et al., 2014). According, it has been demonstrated higher levels of ROS in *TFAM* knockdown human fibroblasts (BALLIET et al., 2011), as well as in *Tfam* knockdown mice cardiomyocytes (ZHANG et al., 2018) and adipocytes (VERNOCHET et al., 2012). Moreover, increased levels of ROS in *TFAM*-deficient cardiac myocytes in mice can be due to a significant decrease in the expression of components of the ETC, resulting in ETC malfunctioning and greater production of ROS (VERNOCHET et al., 2012).

In this way, it has been demonstrated that higher ROS production also leads to inflammasome NLRP3 activation (BULUA et al., 2011; ZHOU et al., 2011), as well as cause the activation of MAPKs, which in turn may aid in the production of IL-6 and TNF-α (BULUA et al., 2011). Corroborating, the NLRP3 deletion abolishes ROS elevation-induced IL-1β expression (ZHOU et al., 2011). Finally, ROS can also activate NF-κB, which is involved not just in the NLRP3 inflammasome activation but also in the higher expression of the IL-2 gene (MANNA et al., 1998; SCHRECK; RIEBER; BAEUERLE, 1991).

The ATP, a well-known DAMP, can also lead to an inflammatory response. The mechanisms of ATP release to cytosol include diffusion through membrane pores or damaged membranes, and active transport, which was demonstrated to occur in a various cells, including neurons, fibroblasts, macrophages, and neutrophils (FITZ, 2007). The increased release of ATP serves as a danger signal associated with the development of inflammation, mainly through NLRP3 inflammasome activation and consequent IL-1β maturation (RITEAU et al., 2010).

Mitochondrial dysfunction driving inflammatory responses is also due to the mtDNA, also considered a DAMP. Overall, mtDNA is released from mitochondria to the cytosol via BCL-2-associated X, apoptosis regulator (BAX), and BCL-2 antagonist/killer 1 (BAK1) pores or the permeability transition pore complex (PTPC). Outside mitochondria, mtDNA accumulation is a potent activator of cyclic GMP–AMP synthase (cGAS), resulting in stimulator of interferon response cGAMP interactor 1 (STING1) signaling and consequent synthesis of cytokines such as interferon-β1 (IFNβ1), IL-6 and TNF (RODRÍGUEZ-NUEVO et al., 2018)

In addition, it was demonstrated that oxidized mtDNA released into the cytosol due to mitochondrial dysfunction acts as a potent activator of the NLRP3 inflammasome. A feedback loop

has been described in which inflammasome activation promotes the release of mtDNA through a mechanism dependent on ROS, thereby establishing a connection between heightened NLRP3 activation downstream and enhanced mitochondrial permeability transition pore (mPTP) opening upstream (NAKAHIRA et al., 2011; SHIMADA et al., 2012). These observations delineate a close association between mitochondrial dysfunction and NLRP3 inflammasome activation (Figure 3).

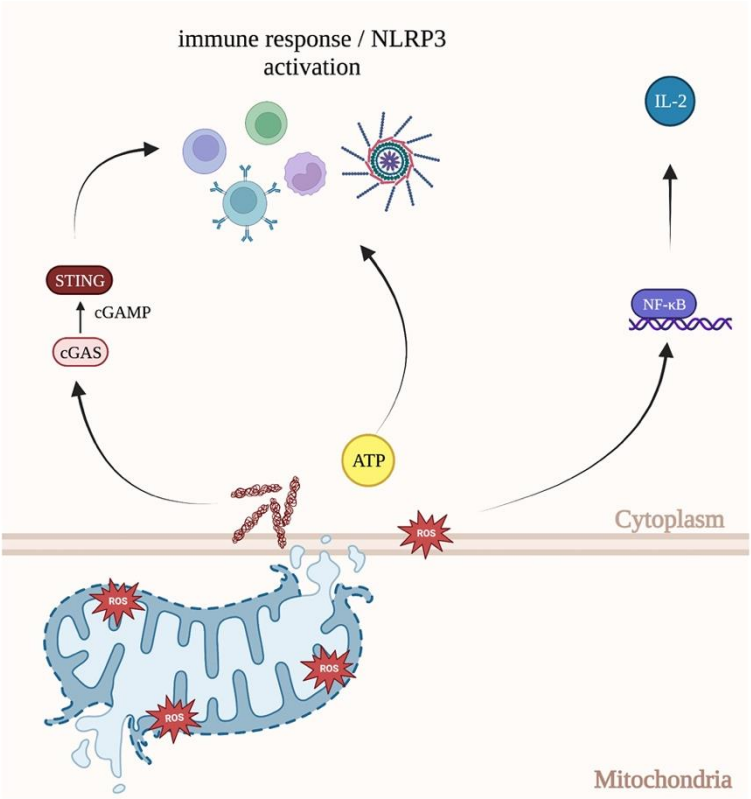

Figure 3. Mitochondrial dysfunction leads to immune response activation.

Higher ROS production is elicited by the dysfunctional mitochondrial in primary mitochondrial diseases, leading to NF-κB activation and IL-2 production. Furthermore, the release of mtDNA and ATP to the cytosol results in the release of pro-inflammatory cytokine and activation of the inflammasome. ROS (reactive oxygen species); IL-2 (interleukin-2); NF-κB (nuclear factor kappa B); ATP (adenosine triphosphate). Source: the author, 2023.

LITERATURE REVIEW ARTICLE: TETRAHYDROBIOPTERIN: BEYOND ITS TRADITIONAL ROLE AS A COFACTOR

(P1) Antioxidants (impact factor 7.675):
Tetrahydrobiopterin: Beyond its traditional role as a cofactor.
Eichwald, T.; Silva, L.B.; Pires, A.C.S.; Niero, L.; Schnorrenberger, E.; Filho, C.C.; Espíndola, G.; Huang, W.L.; Guillemin, G.; Abdenur, J.; Latini, A. (Published in 3 May 2023).

BH4 is traditionally known as an obligatory cofactor for the production of biogenic amines, lipid metabolism via alkylglycerol monooxygenase, and all isoforms of nitric oxide synthase (NOS) enzymes (WERNER et al., 1990; WERNER; BLAU; THÖNY, 2013). Thus, BH4 plays a critical role in the hydroxylation process of aromatic amino acids, leading to the catabolism of phenylalanine and the production of neurotransmitters dopamine and serotonin, as well as the cleavage of ether lipids and the biosynthesis of NO (THONY; AUERBACH; BLAU, 2000).

Genetic BH4 deficiency is associated with a group of rare disorders that can cause intellectual disability, movement disorders, and other neurological symptoms (BLAU et al., 2001; BLAU; VAN SPRONSEN, 2014; WERNER; BLAU; THÖNY, 2013). However, non-genetic disorders have also been linked to impaired BH4 metabolism, such as neurodegenerative and neurological disorders, including Parkinson's disease (DE PAULA MARTINS et al., 2018c), depression (PAN et al., 2011) and schizophrenia (RICHARDSON et al., 2006).

Moreover, our research group and others have uncovered additional biological properties for BH4 metabolism, including its positive functions on the antioxidant system, mitochondrial activity, inflammatory status, and cognition (CHRIST et al., 2013; DE PAULA MARTINS et al., 2018a; GHISONI et al., 2015; GHISONI; LATINI, 2015; LATINI et al., 2018; PATEL et al., 2002). Furthermore, it was demonstrated that the excessive production of BH4 has pathological effects, leading to symptoms such as pain, heightened immune system aggression, and the progression of chronic diseases like pain, asthma, multiple sclerosis, ulcerative colitis, rheumatoid arthritis, and cognitive impairment (CRONIN et al., 2018; FUJITA et al., 2020; LATREMOLIERE et al., 2015; STAATS PIRES et al., 2020b).

Given that, it was conducted an extensive review of the state-of-the-art of BH4 metabolism that integrates both the traditional and newly emerging roles of BH4. This literature review may assist in identifying knowledge gaps and research opportunities, serving as a valuable resource for future investigations. A deeper comprehension of the significance of proper BH4 synthesis may pave the way for novel therapeutic approaches in various diseases, e.g., mitochondrial diseases, and contribute to research implications, including the advancements in clinical trials.

Key Results & Implications

It is presented here state-of-the-art of BH4 metabolism, its function as a cofactor in enzymatic conversions involved in the metabolism of neurotransmitters, phenylalanine, NO, and lipid esters, and its newly described functions on cytoprotection. It is also given the interplay between BH4 and mitochondrial activity, its role in enhancing cytoprotective pathways, and its potential as a therapeutic target for various conditions associated with mitochondrial dysfunction, such as metabolic disorders, neurodegenerative diseases, and primary mitochondriopathies. Finally, the off-label use of BH4 supplementation for hereditary metabolic disorders associated with impaired energy production is proposed.

antioxidants

MDPI

Review

Tetrahydrobiopterin: Beyond Its Traditional Role as a Cofactor

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Abstract: Tetrahydrobiopterin (BH4) is an endogenous cofactor for some enzymatic conversions of essential biomolecules, including nitric oxide, and monoamine neurotransmitters, and for the metabolism of phenylalanine and lipid esters. Over the last decade, BH4 metabolism has emerged as a promising metabolic target for negatively modulating toxic pathways that may result in cell death. Strong preclinical evidence has shown that BH4 metabolism has multiple biological roles beyond its traditional cofactor activity. We have shown that BH4 supports essential pathways, e.g., to generate energy, to enhance the antioxidant resistance of cells against stressful conditions, and to protect from sustained inflammation, among others. Therefore, BH4 should not be understood solely as an enzyme cofactor, but should instead be depicted as a cytoprotective pathway that is finely regulated by the interaction of three different metabolic pathways, thus assuring specific intracellular concentrations. Here, we bring state-of-the-art information about the dependency of mitochondrial activity upon the availability of BH4, as well as the cytoprotective pathways that are enhanced after BH4 exposure. We also bring evidence about the potential use of BH4 as a new pharmacological option for diseases in which mitochondrial disfunction has been implicated, including chronic metabolic disorders, neurodegenerative diseases, and primary mitochondriopathies.

Keywords: antioxidant; neopterin; sepiapterin; mitochondrial enhancer; memory; inflammation; oxidative stress

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1. Tetrahydrobiopterin Biosynthesis

6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4) is an enzyme cofactor that is essential for the synthesis of monoamine neurotransmitters, for the metabolism of phenylalanine (Phe) and lipid esters, and for the production of nitric oxide (NO) [1]. The intracellular concentration of BH4 is strictly maintained at low levels by three finely tuned biosynthetic pathways: the de novo, recycling, and salvage pathways (Figure 1).

The synthesis of BH4 is a complex metabolic pathway and involves several metabolites, including 7,8-dihydrobiopterin (BH2), which is the oxidized form of BH4, neopterin that has been associated with inflammatory conditions for decades, sepiapterin that has recently been identified as another precursor of BH4, and BH4 itself. The de novo BH4 pathway reduces guanosine triphosphate (GTP) into BH4 by the successive action of three enzymes: GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydrobiopterin (PTPS), and sepiapterin reductase (SPR). In the absence of SPR, non-specific reductases (aldose reductase (AR) and carbonyl reductase (CR)) can also generate BH4. SPR, AR, and CR also participate in the

salvage pathway, which utilizes a metabolic intermediate previously formed in the de novo pathway, 6-pyruvoyl-tetrahydrobiopterin, to generate a non-stable intermediate that non-enzymatically forms sepiapterin. Sepiapterin is then metabolized by SPR or CR into BH2, which is further transformed into BH4 by the action of dihydrofolate reductase (DHFR). Finally, in the recycling pathway, after BH4 is used as a mandatory enzyme cofactor and transformed into quinonoid dihydrobiopterin (qBH2) by pterin 4α-carbinolamine dehydratase (PCD), dihydropteridine reductase (DHPR) acts on the qBH2 intermediate to regenerate BH4 [1,2] (Figure 1).

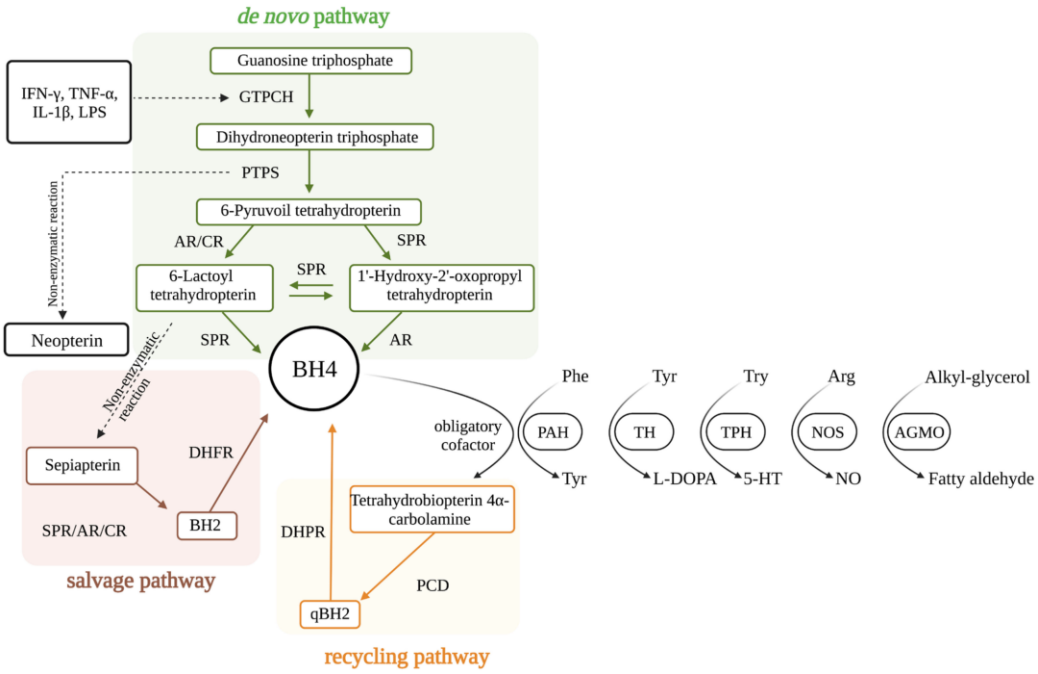

Figure 1. Metabolic pathways involved in the biosynthesis of tetrahydrobiopterin (BH4). ***De novo* pathway:** guanosine triphosphate cyclohydrolase (GTPCH), 6-pyruvolyl tetrahydropterin synthase (PTPS), and sepiapterin reductase (SPR) transform guanosine triphosphate into BH4. The last enzymatic step catalyzed by SPR can be overcome by the unspecific reductases aldose reductase (AR) and carbonyl reductase (CR). This is possible due to an active interaction between the de novo and the salvage pathways, where AR, CR, and/or SPR utilize intermediates of the de novo pathway to generate the key intermediate of the salvage pathway, sepiapterin. **Salvage pathway:** sepiapterin is transformed into dihydrobiopterin (BH2), then reduced to BH4 by dihydrofolate reductase (DHFR). **Recycling pathway:** Pterin-4-alpha-carbinolamine dehydratase (PCD) transforms BH2 in dihydrobiopterin quinoid (qBH2), which is reduced back to BH4 by dihydropteridine reductase (DHPR). BH4 is an obligatory cofactor for the activity of the aromatic amino acid hydroxylases, phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), and tryptophan hydroxylase (TPH), for all isoforms of nitric oxide synthase (NOS), and for alkylglycerol monooxygenase (AGMO). Abbreviations: IL-1β: interleukin-1 beta; TNF-α: tumor necrosis factor-alpha; INF-γ: interferon-gamma; IL-6: interleukin-6; LPS: lipopolysaccharide.

Under physiological conditions, GTPCH is the rate-controlling enzyme in the BH4 pathway [1]. GTPCH is encoded by *GCH1*, which is positively regulated by a variety of inflammatory and oxidant mediators, including lipopolysaccharide (LPS), interferon

gamma (IFN-γ), interleukin 1 beta (IL-1β) [3], tumor necrosis factor alpha (TNF-α) [4], hydrogen peroxide [5], and others (Figure 1). During inflammation *GCH1* is markedly upregulated, however, levels of other constitutive downstream enzymes that mediate the de novo pathway (PTPS and SPR) are only slightly increased, leading to a *pseudo* metabolic blockage and a consequent accumulation of neopterin [6]. Indeed, neopterin has long been used as a sensitive biomarker for innate immune system activation for multiple acute and chronic conditions [7,8].

GTPCH is also regulated by allosteric feedback via BH4 and Phe, which interact with the GTPCH feedback regulatory protein (GFRP) [9]. GFRP acts as both a positive and negative regulator of GTPCH [10]. High Phe levels are known to stimulate GTPCH activity, resulting in activation of the de novo pathway and BH4 production. When intracellular levels of BH4 are sufficient for proper metabolism, GTPCH activity is negatively modulated [11].

2. Levels of BH4 and Related Metabolites in Biological Fluids

Pediatric and adolescent reference values for the biological fluid levels of neopterin, biopterin (fully oxidized BH4), and BH4 are shown in Table 1 [12]. The table shows that the levels of pterins in cerebrospinal fluid (CSF) and urine decrease with age (Table 1), but no correlation with age is seen for neopterin and biopterin levels in serum and dried blood spot samples. These trends contrast with those observed in adults, where serum/plasma and urine neopterin levels correlate with age, but not sex [13] (Table 2). Neopterin levels are highest in the elderly, probably due to chronic inflammation [14]. The renal clearance of neopterin is similar to that of creatinine, therefore levels cannot be measured in patients with impaired renal function [15,16].

Table 1. Reference values for tetrahydrobiopterin (BH4) and related metabolites.

	CSF			Urine	
Age	**BH4 (nmol/L)**	**Biopterin (nmol/L)**	**Neopterin (nmol/L)**	**Biopterin (mmol/mol creatinine)**	**Neopterin (mmol/mol creatinine)**
Newborns	25–121	20–70	15–35	0.5–3.0	1.1–4.0
0–1 year	24–59	15–40	12–30	0.5–3.0	1.1–4.0
2–4 year	20–61	10–30	9–20	0.5–3.0	1.1–4.0
5–10 years	20–49	10–30	9–20	0.5–3.0	1.1–4.0
11–16 years	20–49	10–30	9–20	0.5–2.7	0.2–1.7
		Serum		**Dried blood spot**	
		Biopterin (nmol/L)	**Neopterin** (nmol/L)	**Biopterin** (nmol/L Hb)	**Neopterin** (nmol/g Hb)
All ages		4–18	3–11	0.08–1.20	0.19–2.93

Cerebrospinal fluid (CSF), serum, urine, and dried blood spot BH4 levels, by age group. Adapted from [12].

Neopterin concentrations in CSF are somewhat lower than those in serum or plasma in adults [15] (Table 2). Similarly, neopterin concentrations are elevated in the blood, but not the CSF of individuals with neurological neuroinflammatory chronic conditions who also have normal blood brain barrier (BBB) function [16]. Neopterin present in the CSF is likely to have a central origin, since it can cross the BBB only at a very low quotient (1/40) [7,15,16]. Additionally, our group recently demonstrated that neopterin is secreted by human brain cells, neurons, astrocytes, and microglia after LPS or IFN-g challenge [17].

Unfortunately, there are no defined reference levels for BH4 in the adult population, probably due to the methodological difficulties in measuring the metabolites in the pathway. Moreover, sample processing for BH4 quantification requires acidic treatment and/or the addition of antioxidants, since the pterin is extremely sensitive to pH, and it oxidizes into BH2 at a pH higher than 4. In addition, processed samples need to be frozen immediately and must always be protected from direct light [18,19]. In contrast, neopterin is a stable molecule that does not require excessive processing and it is stable in urine samples left at room temperature or 4 °C for days, or frozen for months. Because of the lack of defined

reference levels for BH4, it is appropriate to have the controls measured in parallel when measuring BH4 in biological samples, like serum/plasma and urine from different cohorts.

Table 2. Neopterin levels in biological fluid from adults.

Neopterin Levels	
Age	**CSF (nmol/L)**
19–75	4.2 ± 1.0
Age	**Urine (μmol/mol creatinine)**
19–25	125 ± 32
26–35	112 ± 33
36–45	124 ± 33
46–55	126 ± 34
56–65	137 ± 37
>65	142 ± 39
Age	**Serum (nmol/L)**
19–75	5.3 ± 2.7
>75	9.7 ± 5.0

Adapted from [13].

3. BH4 as an Enzyme Cofactor

BH4 is known widely to act as an enzyme cofactor for a select few enzymatic reactions, which involve Phe hydroxylase (PAH), Tyr 3-hydroxylase (TH), and tryptophan-5 hydroxylase (TPH) (Figure 2a), all isoforms of NO synthases (NOS I, II and III) (Figure 2b), and alkylglycerol monooxygenase (AGMO).

3.1. PAH

The transformation of Phe into the semi-essential amino acid L-tyrosine (Tyr) is catalyzed by PAH (EC: 1.14.16.1) [20]. PAH was the first enzyme to be discovered to have BH4 as a mandatory cofactor for the hydroxylation of Phe. PAH contains a non-heme non-iron–sulfur iron (Fe^{2+}) in the active site, where BH4 and molecular oxygen bind to promote hydroxylation of the amino acid. As a result, Tyr is formed, and BH4 is transformed into qBH2, which will be used by PCD and DHPR to regenerate BH4 in the recycling pathway [1]. According to the human protein atlas (humanproteinatlas.org, accessed on 15 March 2023), PAH distribution is tissue specific, with highest levels in liver, in agreement with its participation in the hepatic synthesis of Tyr [21].

PAH genetic deficiency leads to hyperphenylalaninemia (HPA) and to the most common inborn error of amino acid metabolism, phenylketonuria (PKU) [22]. Both conditions, HPA and PKA, can be identified by measuring the Phe and Phe/Tyr ratio during routine neonatal screening [23]. Mild PAH deficiencies result in modest elevations of Phe (below 360 umol/L) and do not require any treatment. However, severe PAH deficiency leads to PKU, characterized by the persistent accumulation of Phe greater than 360 mmol/L, which becomes toxic to the brain and impairs cognitive development [21]. The clinical symptoms of untreated PKU include intellectual disabilities, microcephaly, seizures, tremors, ataxia behavioral abnormalities, psychiatric symptoms, and decreased skin and hair pigmentation. Affected individuals also show decreased production of myelin, as well as dopamine, norepinephrine, and serotonin [21]. Treatment consists of a Phe-restricted diet and should be initiated as soon as possible after birth in order to normalize blood concentrations of Phe and Tyr, and to prevent cognitive deficits [21]. Some patients respond to additional treatment with the PAH cofactor BH4, which allows for an increase in protein intake or even liberalization of the diet [24]. More recently, treatment with an alternative enzyme,

phenylalanine ammonia lyase (PAL), that deaminates Phe to cinnamic acid and ammonia has shown very promising results [25].

Another form of HPA was identified in a group of patients with increased Phe levels, who showed developmental regression, abnormal movements, and seizures after a few months of normal early development. This unusual presentation was called "atypical or malignant HPA" [26]. It was later established that the elevation in Phe in these patients was caused by deficient BH4 concentrations, establishing the fact that pterin can act as an enzyme cofactor donating electrons (Figure 2a), and as a pharmacological chaperone. In fact, it was shown that BH4-mediated structural stabilization restored the dysfunctional PAH monomer binding to the PAH active site, leading to enhanced PAH activity [27]. Currently, children identified as having increased Phe levels in their newborn screening sample undergo additional testing, including the measurement of BH4 and BH4-related metabolites concentrations, enzymatic activity for enzymes involved in BH4 synthesis, and/or molecular testing [28]. In some centers, a BH4-loading test is also performed measuring Phe levels at different times after a single dose of sapropterin dihydrochloride (commercial BH4 analogue) (see Section 4 below) [28].

3.2. TH

The first enzymatic step for dopamine synthesis is catalyzed by TH (EC: 1.14.16.2), which transforms Tyr into L-3,4-dihydroxyphenylalanine (L-DOPA) [29] (Figure 2a). Similar to PAH, TH uses molecular oxygen to hydroxylate its substrate in a reaction that requires finely tuned intracellular BH4 levels [30]. Electrons transferred from BH4 interact with a non-heme, non-iron–sulfur iron (Fe^{2+}) in the active site during hydroxylation. TH can be regulated by phosphorylation at a serine residue (e.g., Ser^{8}, Ser^{19}, Ser^{31}, and Ser^{40}) present in the active site, which rapidly increases activity levels (for a review see [31]). TH is mostly expressed in brain tissue, primarily in the cytosol, but is also associated with neurotransmitter-containing vesicles in the plasma membrane (humanproteinatlas.org, accessed on 15 March 2023).

Deficient TH activity results in impaired dopamine synthesis and lower concentrations of its catabolite homovanillic acid (HVA) [32]. Any change in TH expression or activity directly impacts dopamine neurotransmission, and a TH deficiency may lead to the development of idiopathic Parkinson's disease (PD). Indeed, postmortem human striatum from patients with PD shows reduced TH levels [33].

Hereditary TH deficiency causes L-DOPA–responsive dystonia (DRD), an autosomal recessive movement disorder that can present in childhood or adolescence, with different degrees of severity, and is characterized by progressive dystonia with diurnal variation, and response to treatment with L-DOPA [34,35]. Pharmacological treatment with L-DOPA and/or dopamine agonists is effective, but prognosis depends on symptom severity and how early treatment is initiated, ranging from completely resolved symptoms to persistent dystonia and rigidity [36]. Supplementation with BH4 has recently been shown to restore dopamine content and TH activity in a rodent model of PD [37].

It is important to note, that there are other genetic conditions that can mimic DRD but are not caused by defects in TH, therefore molecular confirmatory testing is important to confirm the diagnosis for TH deficiency [38].

3.3. TPH

Tryptophan (Try) is transformed into 5-hydroxytryptophan by the biological activity of TPH (EC: 1.14.16.4) in the presence of molecular oxygen and BH4. Similar to the other two BH4-dependent hydroxylases, TPH hydroxylates its substrate through the interaction of molecular oxygen with a non-heme, non-iron–sulfur iron (Fe^{2+}) in the active site. BH4 facilitates the activation of molecular oxygen and leaves the reaction as the intermediate pterin 4a-carbinolamine, which is reduced back to BH4 by PCD and DHPR activities in the BH4 recycling pathway [39] (Figure 2a). The activity of THP can be enhanced by the presence of Try and BH4, or by phosphorylation. For example, THP1 is a target of

protein kinase A, while THP2 can be phosphorylated by calmodulin-dependent protein kinase II [40]. Increased TH activity may increase the availability of serotonin, which has antidepressant effects [41]. On the contrary, impaired TH activity, e.g., following exposure to a selective and irreversible inhibitor of THP, such as *p*-chlorophenylalanine, may precipitate depression in animals.

TPH has two isoforms. The TPH1 isoform is mainly expressed in peripheral tissues, where it regulates vasoconstriction and the control of immune responses. Previous research has shown that 90% of circulating serotonin is produced by the gut [42]; however, THP1 is also expressed in the skin, pineal gland, and the central nervous system. TPH2 is present exclusively in the brain and is involved in the central control of food intake, sleep, and mood [43].

No clear association between genetic defects in TPH and human disease has been found to date.

3.4. NOS

All NOS isoforms (NOSI, II, and III, formerly called neuronal, inducible, and endothelial NOS) (EC: 1.14.13.39) catalyze the conversion of $_{L}$-arginine (Arg) into $_{L}$-citrulline plus nitric oxide [44]. All NOS isoforms have similar homodimeric structures, where each monomer has an N-terminal oxygenase domain with binding sites for Arg, heme, and BH4. Additional cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) bind to the reductase domain, which donates electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the oxygenase domain [45]. BH4 is not involved in activation of the oxygen as it is in the BH4-dependent hydroxylases, but supplies one electron to the reaction, yielding a protonated trihydropterin radical [46]. This radical is then regenerated to BH4, while bound to the enzyme by a process that involves the transferring of an electron from NADPH via the FAD and FMN sites of the reductase domain (Figure 2b). BH4 presumably also supplies a proton to the NOS active site via a proton bridge [1].

BH4 also enhances the structural functions of NOS during the synthesis of NO. BH4 allows dimer stabilization and dimer formation, protects against proteolysis, and increases Arg binding [47]. Under these physiological conditions, NOS is coupled, and NO is formed. However, when BH4 is consumed, e.g., during oxidative stress, and the BH4/BH2 ratio is compromised, NOS is uncoupled, and in addition to NO production, the anion superoxide radical is formed. As a result, a short-lived and reactive biological oxidant called peroxynitrite ($ONOO^{-}$) is generated from the diffusion-controlled reaction of the free radicals, superoxide and NO [48].

NOSI and NOSIII are constitutively expressed, and their activity is regulated by calcium and calmodulin. NOSI is present in brain tissue; however, the protein has also been identified by immunohistochemistry in the spinal cord, the sympathetic ganglia and adrenal glands, the peripheral nitrergic nerves, the epithelial cells of various organs, kidney macula densa cells, pancreatic islet cells, and the vascular smooth muscle [49]. The largest source of NOSI (in terms of tissue mass) in mammals is skeletal muscle [49]. NOSI has been implicated in modulating learning, memory, and neurogenesis due to the ability to generate the gas retrograde neurotransmitter NO [50]. In this scenario, BH4 has been shown to facilitate learning and memory by supporting NO production in rodents, through the activity of NOSI [18].

NOSIII is mostly expressed in endothelial cells, but has also been detected in the heart, platelets, brain, placenta, and kidney [49]. NOSIII dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing the levels of cyclic GMP in smooth muscle cells [51].

NOSII is the only inducible NOS whose expression can be induced by bacterial LPS, cytokines, and other agents. Although identified primarily in macrophages, the expression of the enzyme can be stimulated in virtually any cell or tissue. Once expressed, NOSII is constantly active and is not regulated by calcium, as are NOSI and NOSIII [49]. When present

in immune cells, NOSII produces large quantities of NO, with de novo synthesis of BH4 (mandatory NOS cofactor) required for sustained enzyme activity (Figure 1). High levels of NO produced by activated macrophages may not only be toxic to undesired microbes, parasites, or tumor cells, but also compromise cell viability and homeostasis, due to the high reactivity of NO towards protein-bound iron. NO can inhibit key enzymes that contain iron in their catalytic centers, including complexes of the respiratory chain, as well as enzymes involved in the Krebs cycle and in DNA, leading to DNA oxidation and fragmentation [52].

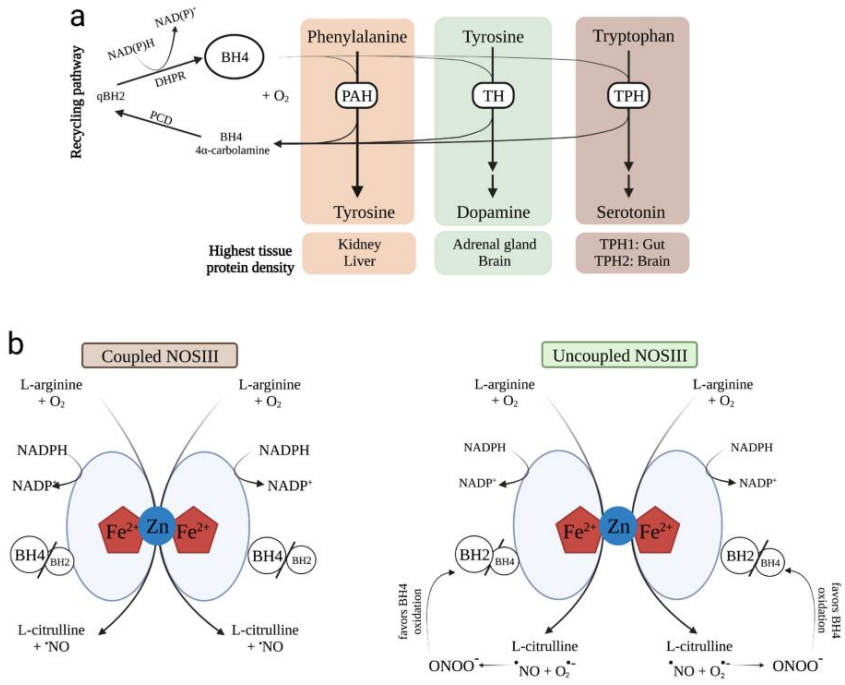

Figure 2. (**a**) Phenylalanine (PAH), tyrosine (TH), and tryptophan (TPH) hydroxylases are dependent on tetrahydrobiopterin (BH4) for their biological activities. Due to its cofactor function, BH4 is required to synthesize tyrosine from phenylalanine, dopamine from tyrosine, and serotonin from tryptophan. After functioning as a cofactor, BH4 is regenerated via the BH4 recycling pathway. Dihydrobiopterin quinoid (qBH2) is then formed by pterin 4α-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR). Protein levels are highest in kidney and liver for PAH; in the adrenal gland and brain for TH; and in the gut and brain for TPH1 and TPH2, respectively. (**b**) Tetrahydrobiopterin (BH4) availability is compromised by the uncoupling of nitric oxide synthase III (NOSIII). *Coupled NOSIII*: L-arginine and molecular oxygen (O_2) are the substrates for NOS III to produce $_L$-citrulline and nitric oxide (NO). The reaction requires as cofactors, the mandatory presence of O_2 and appropriate levels of BH4, as well as reduced nicotinamide adenine dinucleotide phosphate (NADPH). Coupled NOSIII is presented as a heme-containing dimer stabilized by zinc (Zn). Zn is responsible for connecting two NOSIII monomers at the heme group site. BH4 exerts structural and biochemical functions, helping to stabilize the dimer and controlling the coupling of O_2 to $_L$-arginine oxidation. When BH4 levels become deficient and the levels of its oxidized product dihydrobiopterin (BH2) increase (reduced BH4/BH2 ratio), the NOSIII-catalyzed reaction results in the formation of $_L$-citrulline, NO, and superoxide anion (O_2^-). O_2^- is a very reactive radical species that produces peroxynitrite ($ONOO^-$), a highly reactive oxidant that favors BH4 oxidation. Abbreviations: Fe^{2+}: iron; $NADP^+$: nicotinamide adenine dinucleotide phosphate. Adapted from Kim and Han, 2020 [53]. The symbol "•" is used to indicate the presence of an unpaired electron in •NO (nitric oxide), $O_2^\bullet$ (superoxide anion), and $ONOO^-$ (peroxynitrite).

3.5. AGMO

The cleavage of an ether bond of free alkylglycerols and lyso-alkylglycerophosphocholines or lyso-alkylglycerophosphoethanolamines is catalyzed by AGMO (EC: 1.14.16.5) in the presence of molecular oxygen and BH4. AGMO is localized in the membrane of the endoplasmic reticulum (www.proteinatlas.org, accessed on 15 March 2023). Although the highest expression occurs in the liver and white adipose tissue, there is some brain regional specificity of the enzyme, which seems to be essential for proper brain function.

AGMO was first described in 1964 [54] and it remained for several decades as an orphan enzyme, until the encoding gene, *TMEM195*, was discovered [55]. *TMEM195* is thought to play a role in neurodevelopmental disorders, such as autism spectrum disorders (ASD) [56–58]. The relationship of *TMEM195* with the BH4 metabolic pathway is unknown; however, reduced levels of BH4 have been found in the urine and CSF of children with ASD [59]. Moreover, a clinical trial demonstrated that treatment with 20 mg/kg/day sapropterin dihydrochloride significantly improved social awareness, mannerisms, hyperactivity, and inappropriate speech, compared to a placebo in ASD affected individuals [60].

4. Hereditary Deficiencies of BH4

BH4 deficiencies are a group of rare inherited neurological disorders caused by mutations in genes encoding enzymes involved in BH4 synthesis (Figure 1). The deficiencies are mainly characterized by neurotransmitter dysfunction, with or without HPA, which nowadays is typically identified at the newborn screening [61]. Neurological alterations become clear and evident at the median age of four months, and symptoms can include poor suction, impaired tone, brain atrophy, and microcephaly [62]. Early diagnosis is essential for the initiation of therapies aimed at preventing physical and mental damage.

The overall frequency of BH4 deficiencies remains to be established. The average incidence of all types of HPA in Europe has been estimated at 1:10,000 [63], with BH4 deficiencies responsible for 1 to 2% of these cases. PTPS deficiency accounts for 54% of these cases, while defects in DHPR, GTPCH, and PCD account for 33%, 4%, and 5%, respectively [64].

4.1. GTPCH Deficiency

GTPCH deficiency is a rare form of HPA, and 37 cases have been described in the International Database of Tetrahydrobiopterin Deficiencies (BIODEF) (biopku.org, accessed on 15 February 2023). Mutations in *GCH1* can occur in recessive (AR-GTPCH; OMIM #233910) or dominant forms (AD-GTPCH; OMIM #600225). Both, the AR-GTPCH and AD-GTPCH forms show monoamine neurotransmitter abnormalities, but only the former is accompanied by HPA [65]. In AR-GTPCH, neopterin values are usually very low, ranging from 0.09 to 0.20 mmol/mol creatinine in the urine and from 0.05 to 3.0 nmol/L in the CSF. The reduced levels of neopterin are proportional to the compromise of GTPCH activity and BH4 synthesis. Indeed, HPA is markedly reduced after a BH4 loading test (BH4 20 mg/kg) [28]. Clinical presentation of GTPCH deficiency (AD or AR forms) is characterized by diurnal fluctuation of the symptoms, with aggravation in the evening. The most frequent symptoms include hypertonia, dystonia, gait difficulties, and hyperreflexia [66]. AR-GTPCH-affected individuals present more prominent developmental delay, and global dystonia. Treatment involves a Phe-restricted diet accompanied by oral administration of sapropterin dihydrochloride (BH4 supplementation), L-DOPA/decarboxylase inhibitor, 5-hydroxytryptophan, and folinic acid (Table 3) [67]. Treatment for the AD form of GTPCH deficiency includes the restitution of BH4, via oral administration of sapropterin dihydrochloride, and a L-DOPA/decarboxylase inhibitor (Table 4) [67].

4.2. PTPS Deficiency

The deficiency of PTPS (OMIM #261640) is the most frequent and heterogeneous of all BH4 hereditary defects. At the time of submission of this manuscript, 735 cases had been reported in the BIODEF: 168 with typical severe form, 35 with atypical mild peripheral

form, and only 2 with transient presentation (biopku.org, accessed on 15 March 2023). Almost two hundred genotypic variants have been described for the *PTPS* gene. Neopterin values in PTPS deficiency are very high when compared to controls (Table 1), ranging from 4.95 to 51.16 mmol/mol creatinine in the urine and 11 to 449 nmol/L in the CSF. The BH4 loading test shows a reduction in plasma Phe concentrations after the administration of 20 mg/kg BH4 [28].

Individuals affected by the typical form of the disease represent 80% of all cases and have more pronounced symptoms, with marked impairment in neurophysiological development, truncal hypotonia, increased appendicular tone, global dystonia, loss of head control, bradykinesia, swallowing difficulties, somnolence and hyperthermia, among others [62]. The atypical form is also known as the peripheral form, due to the presence of normal levels of neopterin and neopterin/BH4 ratio in the CSF, less significant HPA, and minor changes in the neurotransmitter levels, which predict excellent prognosis with normal neurological development [68].

The treatment of PTPS deficiency can include a Phe-restricted diet, oral sapropterin dihydrochloride (BH4 supplementation) combined with neurotransmitter precursors (L-DOPA/decarboxylase inhibitor), 5-hydroxytryptophan, and folinic acid (Table 3).

4.3. DHPR Deficiency

The deficiency of DHPR (OMIM #261630) is an autosomal recessive genetic defect in the *QDPR* gene that affects the BH4 recycling pathway. Out of the 303 cases listed in the BIODEF so far, 267 presented the severe form, 12 the atypical mild form, and 21 with an unknown subtype (biopku.org, accessed on 15 March 2023).

DHPR deficient individuals present normal or slightly increased neopterin values, ranging from 0.48 to 23.23 mmol/mol creatinine in the urine and from 11 to 70 nmol/L in the CSF (See Tables 1 and 2 for comparison with normal levels). The BH4 loading test shows a reduction in plasma Phe concentrations after the administration of 20 mg/kg BH4 [28].

Clinical presentation of DHPR deficiency is variable, but the severe forms are among the most devastating of all the BH4 deficiencies. Symptoms include progressive mental retardation secondary to extensive neuronal loss, abnormal vascular proliferation in the brain and basal ganglia calcification, and sudden death [69–71]. Treatment includes the administration of neurotransmitter precursors L-DOPA/decarboxylase inhibitor, 5-hydroxytryptophan, and folinic acid combined with a Phe-restricted diet (Table 3). Mildly affected individuals may not require any treatment [72].

4.4. PCD Deficiency

The deficiency of PCD (OMIM #264070) causes a mild form of HPA in newborns [73]. So far, 30 patients with PCD are listed in the BIODEF (biopku.org, accessed on 15 March 2023), and a total of 32 gene variants have been found to be responsible for PCD deficiency [74]. Neopterin values are increased in the disease, ranging from 4.07 to 22.48 mmol/mol creatinine in the urine and from 46 to 117 nmol/L in the CSF. The BH4 loading test shows a reduction in plasma Phe concentrations after the administration of 20 mg/kg of BH4 [28].

Usually, patients with PCD deficiency show no major alterations in Phe and neurotransmitter levels and no significant clinical abnormalities other than transient alterations in tone, having an excellent prognosis [75]. Indeed, it has been proposed that unspecific enzymes might compensate for the deficiency later in life [76]. When needed, treatment includes a Phe-restricted diet, oral sapropterin dihydrochloride, neurotransmitter precursors (L-dopa/decarboxylase inhibitor), 5-hydroxytryptophan, and folinic acid (Table 3).

Table 3. Tetrahydrobiopterin (BH4) biosynthesis defects with hyperphenylalaninemia (HPA).

Pterin Metabolism Defects with HPA						
Disease Name	**OMIM**	**Gene**	**Affected Enzyme**	**Symptoms**	**Diagnostic Based on Metabolites in CSF**	**First Line Treatment**
Autosomal recessive GTP cyclohydrolase I deficiency	233910	*GCH1*	GTPCH I	Developmental delay, hypotonia, hypertonia, dystonia, hypersalivation	↓ HVA ↓ 5-HIAA ↓ /N 5-MTHF ↓ Biopterin ↓ Neopterin N Sepiapterin	Phe-reduced diet Sapropterin dihydrochloride L-Dopa/DC inhibitor 5-Hydroxytryptophan Folinic acid
6-pyruvoyl-tetrahydropterin synthase deficiency	261640	*PTS*	PTPS	Developmental delay, hypotonia, hypertonia, epilepsy, cognitive impairment, low birth weight	↓ HVA ↓ 5-HIAA ↓ /N 5-MTHF ↓ Biopterin ↑ Neopterin N Sepiapterin	Phe-reduced diet Sapropterin dihydrochloride L-Dopa/DC inhibitor 5-Hydroxytryptophan Folinic acid
Q-dihydropteridine reductase deficiency	261630	*QDPR*	DHPR	Developmental delay, hypotonia, hypertonia, epilepsy, microcephaly	↓ HVA ↓ 5-HIAA ↓ /N 5-MTHF ↑ Biopterin ↓/N Neopterin N Sepiapterin	Phe-reduced diet L-Dopa/DC inhibitor 5-Hydroxytryptophan Folinic acid
Pterin-4-alpha-carbinolamine dehydratase deficiency	264070	*PCBD1*	PCD	Developmental delay, hypotonia, hypomagnesemia, MODY3-like diabetes	NR HVA NR 5-HIAA NR 5-MTHF N Biopterin N Neopterin N Sepiapterin	Phe-reduced diet Sapropterin dihydrochloride L-Dopa/DC inhibitor 5-Hydroxytryptophan Folinic acid

BH4 deficiencies with HPA are described in the table. Main clinical and biochemical alterations, as well as the available palliative treatment are indicated for each disease. Abbreviations: Phe: phenylalanine, DC: decarboxylase; HVA: homovanillic acid; 5-HIAA: 5-Hydroxyindoleacetic acid; 5-MTHF: 5-Methyltetrahydrofolate; ↓: decrease; ↑: increase; N: normal; NR: not reported. Adapted from [77].

4.5. SPR Deficiency

The deficiency of SPR (OMIM #182125) was the last BH4 inherited metabolic disorder to be identified [78]. So far, 55 patients with SPR are listed in the BIODEF (biopku.org, accessed on 15 March 2023), 4 of them presenting the mild/peripheral subtype and 47 presenting as severe. Individuals with SPR deficiency do not have HPA; therefore, they are not detected by newborn screening, and the pterin levels in urine can be normal [78,79]. The absence of HPA is believed to occur due to the presence of alternative non-specific enzymes (such as CRs and/or ARs) that compensate for the deficient activity of SPR [28]. Normal to slightly increased levels of neopterin can be found in the CSF (Table 4) [28].

SPR deficiency is a genetic disease with autosomal recessive inheritance [78]. Patients with SPR deficiency can present in infancy only with severe developmental delay, which is followed by typical symptoms of a neurotransmitter defect: inconsolable crying, hypotonia, tremor, and ataxia, with diurnal variations. Psychiatric manifestations are common [80,81]. So far, 104 reported pathogenic variants distributed over the entire SPR gene have been reported [74]. Treatment includes the administration of oral sapropterin dihydrochloride combined with L-DOPA/decarboxylase inhibitor, and 5-hydroxytryptophan (Table 4).

Table 4. Tetrahydrobiopterin (BH4) biosynthesis defects without hyperphenylalaninemia (HPA).

Pterin Metabolism Defects without HPA						
Disease Name	**OMIM**	**Gene**	**Affected Enzyme**	**Symptoms**	**Diagnostic Based on Metabolites in CSF**	**First Line Treatment**
Autosomal dominant GTP cyclohydrolase I deficiency	600225	*GCH1*	GTPCH I	Hypertonia, diurnal fluctuation of symptoms, dystonia, gait difficulties, hyperreflexia	↓ /N HVA ↓ /N 5-HIAA NR 5-MTHF ↓ /N Biopterin ↓ /N Neopterin N Sepiapterin	Sapropterin dihydrochloride L-Dopa/DC inhibitor
Sepiapterin reductase deficiency	182125	*SPR*	SPR	Developmental delay, hypotonia, hypertonia, cognitive impairment, impaired speech development, dysarthria, diurnal fluctuation of symptoms, dystonia, oculogyric crises, dyskinesia/other involuntary movements, hypokinesia, hypersalivation, psychiatric and sleep problems	↓ HVA ↓ 5-HIAA N 5-MTHF ↑ Biopterin N Neopterin ↑ Sepiapterin	Sapropterin dihydrochloride L-Dopa/DC inhibitor 5-Hydroxytryptophan

BH4 deficiencies without HPA are described in the table. Main clinical and biochemical alterations, as well as the available palliative treatment are indicated for each disease. Abbreviations: DC: decarboxylase; HVA: homovanillic acid; 5-HIAA: 5-Hydroxyindoleacetic acid; 5-MTHF: 5-Methyltetrahydrofolate; ↓: decrease; ↑: increase; N: normal; NR: not reported. Adapted from [77].

5. New Fundamental Roles of BH4 Metabolism Denoting Its Multifaceted Biological Functions

New fundamental roles for BH4 metabolism have recently been uncovered. Our group has described the physiological properties for the BH4 pathway as having antioxidant and anti-inflammatory activities (inhibits the assembly and activation of the inflammasome) and being a mitochondrial activator, as well as a memory enhancer in the nervous system [7,17,18,82,83]. In addition, we have demonstrated that the genetic or pharmacological inactivation of *Gch1* impairs T cell proliferation and tissue infiltration, by a mechanism that involves iron homeostasis and mitochondrial activity in animal models of diseases with chronic inflammation, which was further confirmed in human cells [83].

Conversely, we have also demonstrated that exacerbated activation of the BH4 pathway is pathogenic, causing mitochondrial dysfunction, increasing the aggressiveness of the immune system, inducing pain hypersensitivity, and the progression of symptoms of chronic diseases, including chronic pain, asthma, multiple sclerosis, ulcerative colitis, rheumatoid arthritis, cognitive impairment, and others [83–87].

Our group has also shown that pain clinical scores can be attenuated or normalized if the physiological metabolic flux of the BH4 pathway is reestablished. This effect was observed in various animal models for chronic pain [84]. The discovery of this mechanism opened new and innovative therapeutic horizons to treat chronic inflammatory conditions safely and efficiently, based on the inhibition of SPR [83–85]. The use of specific inhibitors for BH4 synthesis called SPRi3 and QM385, reduced the BH4 levels in sensory neurons in active pain, eliciting analgesia [83–85]. Also, the identification of increased levels of sepiapterin in biological fluids as a marker of pharmacological BH4 metabolism engagement, offered for the first time, a quantitative and independent measure of analgesia [88].

6. Biological Effects of Neopterin

There is vast literature exploring the potential mechanistic toxic roles of neopterin, due to its association with acute and chronic inflammation (more than 3500 publications in the last five decades). In general, these studies have shown increased translocation of the nuclear factor kappa B (NF-kB), increased intracellular calcium, reactive oxygen species (ROS) production, oxidative stress, mitochondrial dysfunction, increased proto-oncogene expression, apoptosis, and reduced cell viability in a variety of human and rodent cells exposed to neopterin [89–94]. All these mechanisms have already been implicated in the physiopathology of multiple inflammatory states, including obesity and obesity-related comorbidities, such as cardiovascular diseases, type 2 diabetes mellitus, atherosclerosis, hypertension, and musculoskeletal and neurodegenerative diseases. In fact, our group has also demonstrated increased neopterin in individuals affected by diabetic neuropathic pain [86] and in acute-on-chronic liver failure patients [95]. However, information regarding the functional physiological activities of neopterin is scarce.

Neopterin has been reported to be a silent and inert by-product of BH4 metabolism, acting as a marker for activation of the innate immune response. However, our group has described new activities for neopterin under non-inflamed physiological conditions. For example, we demonstrated increased resistance to induced oxidative stress in the mouse brain after a single intracerebroventricular (i.c.v.) administration of neopterin (4 pmol), which generated slightly increased neopterin levels in the CSF [17,82]. The experimental treatment increased glutathione, glutathione-metabolizing enzyme activity, respiratory chain complex I/IV activity, and basal oxygen consumption, but reduced glycolysis, through activation of the nuclear factor-erythroid factor 2-related factor 2 (Nrf2)/antioxidant response element (ARE) pathway [17,82]. All these positive effects of neopterin might have contributed to the mnemonic effect previously demonstrated [96]. Neopterin administration (i.c.v.) enhanced learning and memory by facilitating long-term potentiation (LTP) [96], which represents the acquisition and maintenance of memories at the synaptic level [97].

Similar antioxidant functions were induced in cell-based systems, including primary rat astrocytes, C6 astroglial cells, and human nerve cells, when exposed to very low concentrations of neopterin [17,82]. In addition, an increased mitochondrial number was observed in neopterin-treated primary dorsal root ganglion (DRG) neurons (sensory neurons), indicating that mitochondrial fusion–fission processes or biogenesis are induced or maintained by physiological levels of neopterin. Figure 3a–d shows the positive effects of boosting neopterin levels on mitochondrial content and the expression of Nrf-2/ARE-related genes in the DRG neurons and mouse brain. Figure 3 shows that the single exposure (24 h) of sensory neurons to neopterin increased the number of mitochondria (Figure 3b) when compared to the controls (Figure 3a), suggesting a boost in energy metabolism. In agreement, the i.c.v. administration of neopterin (4 pmol) increased the expression of Nrf-2 (Figure 3c) and Tfam (Figure 3d), which suggest increased antioxidant defenses and mitochondrial health. In this scenario, Nrf-2 is a master transcription factor that finely regulates the cellular antioxidant response [98,99]. The Nrf2/ARE system regulates the transcription of approximately 250 genes, including antioxidants crucial for cellular redox control in the brain and skeletal muscle, tissues highly dependent on mitochondrial activity for energy synthesis. Some of the modulated genes encode for the antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase, hemeoxygenase type 1 (HO-1), peroxiredoxin, thioredoxin reductase, thioredoxin, and metallothionein. The increase in the protein content of these enzymes has been correlated with several beneficial effects in tissues with high mitochondrial content (for a review see [100]). Furthermore, Tfam is a protein encoded by TFAM that performs multiple functions of transcriptional activation and organization of mitochondrial DNA (mtDNA) [101,102]. Tfam is also necessary for the regulation of enzymes involved in oxidative phosphorylation, as well as for the maintenance of mtDNA, thus playing a role in the organization of the mitochondrial genome [102]. These novel data suggest that neopterin at physiological concentrations might serve as a key metabolite-activating antioxidant and energy pathways that can

promote cell survival. Crosstalk among metabolic routes to promote cellular homeostasis has already been demonstrated for other metabolic intermediates, e.g., NAD+ [103]. It has been extensively demonstrated that the NAD+ pathway functions as a critical regulator to maintain physiologic processes, enabling cells to adapt to environmental changes, including nutrient perturbation, genotoxic factors, circadian disorder, infection, inflammation, and xenobiotics [103].

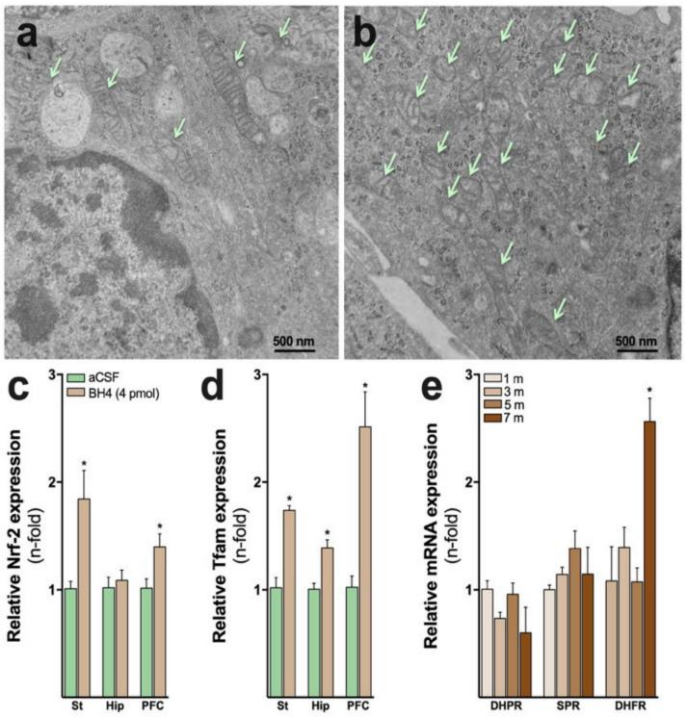

Figure 3. Tetrahydrobiopterin (BH4) pathway in the brain of naïve mice and the effect of its metabolites on mitochondrial parameters in sensory neurons. (**a**,**b**) Increased mitochondrial number in dorsal root ganglia neurons (sensory neurons) exposed to 50 nM neopterin for 24 h. (**b**) Arrows denote increased organelle number. Bars indicate 500 nm. Electron microscopy micrographs: 10,000× magnification. (**c**,**d**) Increased Nrf-2 and Tfam expression in different brain regions after 24 h of a single intracerebroventricular injection of 4 pmol BH4 (1 µL) in C57Bl6 mice. St: striatum; Hip: hippocampus; PFC: prefrontal cortex; aCSF: artificial cerebrospinal fluid. (**e**) Expression of the genes coding dihydropteridine reductase (DHPR; BH4 recycling pathway), dihydrofolate reductase (DHFR; BH4 salvage pathway), and sepiapterin reductase (SPR; BH4 de novo and salvage pathways) in the hippocampus of naïve C57Bl6 mice. 1, 3, 5, and 7 m: 1, 3, 5 or 7-month-old mice. * $p < 0.05$.

7. Biological Effects of BH4

Considering that neopterin is a byproduct of the de novo BH4 pathway and that its levels reflect the magnitude of GTPCH activity, similar intracellular effects should be expected for BH4 under non-inflammatory conditions. Indeed, positive effects on the antioxidant system, mitochondrial physiology, and dynamics and cognition were observed in the brain and the immune system of animals with enhanced BH4 metabolism [18,83].

As previously shown for neopterin, a single i.c.v. administration of BH4 enhanced the hippocampal cognition via a mechanism linked to the activation of glutamatergic neurotransmission and cell threshold reduction, which facilitated LTP triggering in various

rodent strains [18]. In agreement, it has been reported that BH4 administration in PKU-affected patients (a hereditary inability to transform Phe into Tyr) improved working memory and brain activation [104].

The antioxidant activities of BH4 might have also contributed to the observed mnemonic effect. In fact, it has been demonstrated that BH4 is a more reactive scavenger of superoxide, hydroxyl and thiyl radicals, and peroxynitrite than ascorbate at the physiological blood pH [105,106]. It has also been proposed that BH4 would enhance mitochondrial activity when thiol compounds, such as glutathione are reduced [107], suggesting the existence of a mitochondrial BH4 pool. Moreover, it was shown experimentally that *Gch1* (gene encoding the rate-limiting enzyme of BH4 biosynthesis) is a direct target of Nrf-2 in skin cells submitted to radiation (Nrf-2 has a binding site in the proximal promoter of the *Gch1* gene), and the resultant increased intracellular BH4 levels promoted cytoprotection by neutralizing ROS [108]. This is in agreement with previous results showing neopterin-induced Nrf-2 nuclear translocation with increased content of key cytoprotective downstream proteins, including the antioxidant HO-I (it metabolizes heme into biliverdin, a potent antioxidant) in rodent and human cells [17]. It has also been shown that immune cells lacking BH4 production showed increased content of iron-related proteins, including mitoferrin, ferritin, and frataxin, all mitochondrial proteins [83], as a potential compensatory effect.

It is also known that BH4 autooxidation can be induced by a variety of biochemical reactions, including the interaction with ferricytochrome *c* with the production of BH2 via the formation of an intermediate radical [109,110]. In this scenario, it was shown that BH4 efficiently reduces ferricytochrome *c* to ferrocytochrome *c* (transition between complexes III and IV of the respiratory chain) at physiological BH4 levels in activated T cells. Thus, ablated mouse *Gch1* T cells impaired mitochondrial respiration, which was improved by the replenishment of BH4 with sepiapterin, or by delivering ferrocytochrome *c* directly into the mitochondria [83]. The data presented in Figure 3d also shows that BH4 enhanced the expression of Tfam (a mitochondrial transcription factor involved in the genesis of new mitochondria) in the mouse brain. Increased Tfam was also demonstrated to be induced by BH4 in the heart, in addition to peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) and estrogen-related receptor alpha, which are nuclear transcription factors that cooperate with Tfam for mitochondrial biogenesis [111].

The oxidation of BH4 will compromise the BH4/BH2 ratio, which is determinant for proper NOS activity and, therefore, the production of NO. BH2 serves no cofactor actions and binds to NOS with approximately the same affinity as its reduced form. Under these conditions, NOS activity becomes uncoupled resulting in the production of the superoxide radical and $ONOO^-$ [48], toxic reactive compounds that rapidly oxidize BH4 favoring the establishment of oxidative stress [105,106] (Figure 2b). Uncoupled NOS has already been described in a variety of experimental and clinical chronic conditions; namely type 2 diabetes mellitus, hypertension, cigarette smoke-induced lung dysfunction, heart failure, atherogenesis, chronic pain, and neurodegenerative diseases, which are all comorbidities of chronic diseases. Given the intrinsic BH4 deficiency/NOS uncoupling relationship, many clinical studies have focused on pharmacological interventions to enhance BH4 bioavailability to attenuate vascular dysfunction in chronic conditions [112–115].

Due to the positive intracellular effects of BH4, its deficiency may be considered a risk factor for the development of chronic diseases, including obesity, aging, vascular disorders, neurodegenerative diseases, and others, characterized by inflammation, oxidative stress, and mitochondrial dysfunction. In agreement, Figure 3e depicts increased DHFR expression in the hippocampi of naïve 7-month-old mice, phenomenon that might represent a compensatory mechanism to maintain BH4 at physiological levels during natural aging. In agreement, reduced BH4 levels have been reported in the CSF, blood and postmortem brain of individuals affected by common neurodegenerative diseases [116,117].

BH4 levels have also been proposed as being required for normal brain maturation. For example, data generated from experimental studies showed the association of reduced BH4 levels and brain maturation delay in a mouse model for prenatal hypoxia in congenital

heart disease [118]. In agreement with various other studies, exogenous BH4 administration resulted in enhanced sensorimotor coordination, normalizing the delay in myelination due to hypoxia, and decreasing white matter apoptosis during brain development. In addition, rabbits exposed to hypoxia-ischemia showed reduced neuronal death in the cortex, basal ganglia, and thalamus, suggesting that BH4 deprivation in premature brains may be a risk factor for survival [119].

8. GTPCH and SPR Deficiencies Affect Energy Metabolism

Emerging evidence obtained from multiple animal models depicting energy depletion in tissues with high mitochondria content has shown that BH4 deficiency compromises mtDNA transcription, mitochondrial biogenesis, and respiration. In agreement, it was shown that T cell physiology is also dependent on BH4 appropriate concentrations [83]; thus, it seems feasible to propose that the dependency of mitochondrial health on BH4 synthesis is a physiological path of tissues with high energy demands. For example, low levels of BH4 have been associated with a broad range of cardiovascular and metabolic diseases, including hypertension, hypertrophy, ischemic heart disease, and diabetes mellitus, in animal models and human patients [120–122]. Indeed, it has been shown that several metabolic pathways and proteins are modulated in a mouse heart when the synthesis of BH4 is compromised. A systems-based integrative data analysis to investigate systematic changes in the cardiac mitochondrial proteome of SPR-null mice demonstrated specific nodes in a pathway–pathway network that involved compromised energy production, and lipid, carbohydrate, and amino acid metabolisms. This analysis also showed a specific protein–protein network with compromised content of oxidative phosphorylation proteins. In agreement, reduced oxygen consumption (a measure of mitochondrial physiology), impaired ATP synthesis with loss of mitochondrial membrane potential, and increased ROS formation were confirmed in the hearts of SPR-null mice [111]. As expected, the remodeled bioenergetics and the oxidant environment reduced cardiac function and decreased life expectancy. However, the restitution of BH4 to normal levels (20 mg/kg/day, i.p.; 2 weeks) rescued the mitochondrial activity, attenuated ROS production, and normalized cardiac systolic function, along with body size and weight [111]. These effects were mostly linked to the positive regulation of PGC-1α [111], a coactivator of transcription needed for mitochondrial biogenesis [123]. In fact, BH4 deficiency impaired the expression of the PGC-1α-dependent genes that regulate mitochondrial biogenesis, mtDNA transcription, and mRNA translation [111], suggesting a prominent role of BH4 metabolism in mitochondrial biogenesis.

It has also been shown that BH4 deficiency, due to the lack of SPR activity, resulted in impaired mitophagy, a process required to maintain the quality and quantity of healthy mitochondria [124]. Dysregulation of mitochondrial quality control was evident in the brain, liver, muscle, and lung of SPR-null mice, and it was counterbalanced when BH4 intracellular concentrations were normalized [125]. The underlying molecular mechanisms compromising mitophagy were proposed as being linked to limited availability of Tyr, a semi-essential amino acid, whose synthesis is dependent on BH4 production. In agreement, human PKU-derived lymphocytes showed a high Phe/Tyr ratio with insufficient mitophagy [125], revealing an intimate relationship between BH4 deficiency and impaired autophagy.

The effects induced by the deficiency of BH4 have also been characterized in cells that are fully dependent on mitochondrial activity to generate energy. The pharmacological inhibition of GTPCH by using the selective and direct-acting inhibitor 2,4-diamino-6-hydroxypyrimidine resulted in oxidative stress, mitochondrial depolarization, ATP depletion, inhibition of complex IV, and necrosis in cortical neurons submitted to hypoxia [126]. All these effects were counteracted when neuronal BH4 levels were restored, by incubating cells with exogenous BH4 during the hypoxic period. Similarly, hypoxia-induced damage in BH4-deficient neurons was prevented when a NOS inhibitor, hemoglobin, or superoxide dismutase plus catalase were present during the hypoxic period [126], suggesting that NOS

uncoupling might be involved in deficient energy metabolism. From the data presented, it can be concluded that energy metabolism requires appropriate intracellular levels of BH4 to support bioenergetic processes that are independent of those catalyzed by BH4 as an enzyme cofactor.

9. Non-BH4-Linked Genetic Deficiencies of BH4 Metabolism

9.1. ASD

ASD is a disorder that describes individuals who have persistent deficits in social communication and social interaction with restricted, repetitive patterns of behavior, interest, or activities. ASD represents a large spectrum of classifications and presentations, from mild to severe impairment. ASD's physiopathology is not very well understood; however, evidence from the literature suggests a specific modulation of the BH4 pathway affecting the activity of the monoaminergic neurons that might be downregulated in the disease. Indeed, reduced levels of BH4 have been demonstrated in the blood, urine, and CSF of ASD-affected children [59,127]. Additionally, several reports have demonstrated a therapeutic effect of BH4 supplementation in children with ASD. BH4 daily doses of 1 to 3 mg/kg for 4 to up to 105 weeks elicited marked improvements in social responsiveness, communication, and cognitive abilities in over 300 mildly to severely affected ASD children [128–134]. These improvements were marked in children with higher intelligence and younger than 5 years old [128,132]. Interestingly, BH4 daily supplementation with 20 mg/kg induced similar effects than lower doses [60,114,128].

The low levels of BH4 in young children with ASD has been proposed to be related to high NO production in response to excessive inflammation and overactivation of the immune system [135]; folate deficiency, which will impact negatively on the BH4 salvage pathway activity through the participation of DHFR [136,137]; and excessive oxidative stress that will consume the antioxidant BH4 [18,138].

The involvement of the BH4 pathway was also suggested in a study that investigated 247 patients presenting autism, who were referred from pediatric-psychiatric to pediatric- metabolic outpatient clinics. Six patients from this group were affected by different metabolic disorders, and one of them by PKU [139]. In addition, genome-wide studies showed a significant nominal association in the *PTPS* gene with ASD [140]. Furthermore, a spontaneous copy number variation in the *AGMO* gene, leading to a deletion within exons 2–8, was identified in a patient with ASD [56]. Another study demonstrated that de novo mutations within the *AGMO* gene were involved in ASD [57].

9.2. Human Rabies

Reduced levels of BH4 and BH4-dependent metabolites have been demonstrated in human rabies [141]. In agreement, oral BH4 supplementation has been shown to be responsible for a rapid increase in the CSF concentrations of BH4 and the neurotransmitters dopamine and serotonin, resulting in increased survival chances for patients affected by rabies [142]. These findings strongly suggest that the neurochemical dysfunction observed in patients infected with rabies virus is, at least in part, due to impaired BH4 intracellular concentrations.

9.3. Cerebral Malaria

Impaired BH4 metabolism has also been identified in cerebral malaria, having the lowest CSF levels of BH4 when the affected individuals were in a deep coma. In addition, elevated CSF neopterin, its levels were positively associated with the duration of fever before coma, indicating that inflammation and the consequent induction of oxidative stress may be responsible for reduced BH4 levels [143]. In turn, low BH4 concentrations compromise effector immune function, contributing to coma [144]. Again, this agrees with previous studies showing that appropriate BH4 levels are needed for immune cell physiology [83].

9.4. PD

It has been extensively proposed that low levels of BH4 might be linked to the impaired dopaminergic neurotransmission in this neurodegenerative disease. High gene expression of *SPR* has been identified in the postmortem brains of patients affected by PD [116], pointing to a compensatory and protective mechanism due to the reduction in BH4 levels. In agreement, SPR has been proposed as a protective factor in neurons against methyl-4-phenylpyridinium-induced toxicity [145]. On the other hand, the expression of AR and PTS genes and CSF BH4 concentrations were decreased in brains affected by PD, indicating compromised BH4 metabolism and, consequently, impaired dopaminergic metabolism and neurotransmission [116,146,147]. In agreement, we have shown that the administration of levodopa to rodents increases the availability of BH4 in the striatum [148]. Furthermore, when levodopa was supplemented in mice submitted to an experimental model of PD, the levels of BH4 were rescued, suggesting that part of the effects mediated by this pharmacological treatment involves the restauration of the levels of BH4 metabolism. Indeed, this approach stimulated the BH4 salvage pathway by upregulating DHFR expression [148].

9.5. Alzheimer's Disease (AD)

AD is the most common form of neurodegenerative disease, estimated to contribute 60–70% of all the cases of dementia worldwide [149]. According to the prevailing amyloid cascade hypothesis, amyloid-β deposition in the brain is the initiating event in AD, although evidence is accumulating that this hypothesis is insufficient to explain many aspects of AD pathogenesis. The interplay among mitochondrial dysfunction, inflammation, and oxidative stress has extensively been proposed as key factors of its physiopathology. Therefore, it could be expected that the levels of BH4 under this scenario are compromised. In fact, BH4 metabolism (enzymes and metabolites) was demonstrated to be reduced in several brain regions of postmortem histologically confirmed AD cases [117,150]. Additionally, the administration of BH4 rescued memory impairment in 13-month-old 3×Tg-AD mice, without having any impact on the neuropathology [151], suggesting that the memory deficit may be linked to BH4 metabolism. The evidence demonstrating reduced BH4 availability in AD is the basis for the ongoing clinical trial based on the off-label (drug repurposing) use of BH4 (ClinicalTrials.gov, accessed on 15 March 2023).

9.6. Fabry Disease

The inborn error of metabolism known as Fabry disease is caused by the deficient activity of α-galactosidase-A and the, subsequent, accumulation of glycosphingolipids (mainly globotriaosylceramide, Gb3) [152]. The disease is characterized by a variety of clinical manifestations, including acroparesthesias, angiokeratomas, stroke, hypertrophic cardiomyopathy, and progressive renal impairment [153]. Enzyme replacement therapy is currently the standard of care for symptomatic Fabry patients, but its physiopathology is not well understood. It was recently demonstrated that BH4 is decreased in the plasma of female Fabry patients, which was not corrected by enzyme replacement therapy. When the metabolism was investigated in the animal model of the disease, the Fabry mouse, BH4 was confirmed to be decreased in the heart and kidney, but not in the liver and aorta. Moreover, Gb3 levels were inversely correlated with BH4 levels in animal tissues and cultured patient cells, pointing out that the accumulation of the metabolite is favored by the oxidation of BH4. In agreement, the administration substrate reduction therapy restored the levels of BH4 and the clinical phenotype. Additionally, the intervention rescued markers of oxidative stress present in the experimental model, e.g., the levels of glutathione were recovered, increasing the antioxidant defenses of the cell.

10. BH4 Administration as a New Therapeutic Horizon for Mitochondrial Diseases

Mitochondriopathies are disorders characterized by defects in the mitochondrial structure and function due to mutations, depletion, or deletions of nuclear DNA and/or mtDNA

(mtDNA) [154]. Changes in the mitochondrial structure and function can compromise the functioning of the mitochondrial respiratory chain, reduce energy production, alter the cellular redox state, increase the production of ROS, deregulate calcium homeostasis, and induce apoptosis, eliciting mtDNA destabilization (for a review see [155]). At present, there is no cure and only supportive and symptomatic therapies are available. Obstacles to finding effective treatments include the rarity of the disease, clinical diversity, genetic heterogeneity, difficult clinical trials, and poorly understood pathophysiology.

Based on our knowledge about the non-canonical biological roles of BH4 metabolism and preclinical studies from the last decade, we hypothesize that BH4 supplementation might be an innovative and safe way to treat disorders whose physiopathology involves the interplay of mitochondrial dysfunction, oxidative stress, and inflammation. Given that BH4 seems to be essential for proper mitochondrial activity and antioxidant activation in tissues with high energy demands, it is plausible that BH4 supplementation might represent an effective strategy to increase residual mitochondrial function in genetic mitochondrial diseases.

There is evidence in the literature, generated from clinical and basic studies, demonstrating that impaired mitochondrial function, and increased oxidation and inflammation are also common pathological mechanisms in hereditary metabolic disorders [156–162]. Thus, by increasing BH4 intracellular levels, it could be expected that key antioxidant, anti-inflammatory, and mitochondrial-related functions would be enhanced, for example, in the brain [7,17,18,96,163]. Although this approach might not represent the cure for primary mitochondrial disorders, it has the potential to alleviate disease burden and improve the quality of life for affected individuals and their families.

BH4 is sold commercially, as sapropterin dihydrochloride, in tablets or powder for oral solution formulations, and it is available in 63 countries around the world. Sapropterin dihydrochloride was approved on 13 December 2007 by the U.S. Food and Drug Administration (FDA) in 2007 to be used in conjunction with a Phe-restrictive diet to reduce blood Phe levels in patients with HPA due to BH4-responsive PKU (www.accessdata.fda.gov, accessed on 15 March 2023; application number ND 022181). Since then, more than 7800 patients (including 1560 children under 4 years of age) have been treated with sapropterin dihydrochloride in the United States. Several clinical trials have shown the compound is safe and well tolerated [164]. The recommended doses to reduce HPA or to increased Phe tolerance have ranged between 1 to 20 mg/kg [67].

According to the FDA, the use of an approved drug for an unapproved use, often called "off-label" use, is justified if it is judged to be medically appropriate to treat a particular condition. In this scenario, the off-label use of sapropterin dihydrochloride has grown during the last two decades, supporting clinical trials for cardiac, pulmonary, rheumatologic, dermic, and psychiatric diseases, dementia, menopause, aging, and inherited disorders (Figure 4a).

All these clinical trials are published on ClinicalTrials.gov and were based on the safety of sapropterin dihydrochloride administration [164], and the large body of information generated from preclinical studies supporting the potential benefits induced by BH4 supplementation. Detailed information about the clinical trials aimed at identifying the beneficial effects of BH4 administration on a variety of diseases can be found at www.ClinicalTrials.gov.

The physiopathology of the abovementioned disorders is still not fully defined; however, there is a clear consensus that mitochondrial dysfunction, sustained inflammatory responses, and increased oxidative stress, play a role in their onset and development. Additionally, it has been proven that the supplementation with antioxidants (e.g., ascorbic acid, vitamin E), mitochondrial activators or energy substrates (e.g., creatine, lipoic acid, coenzyme Q), and anti-inflammatory drugs (e.g., sulfasalazine, biologicals) may decrease the associated mortality and morbidity. Therefore, it seems plausible to propose the off-label use of sapropterin dihydrochloride supplementation for hereditary metabolic disorders linked to energy production.

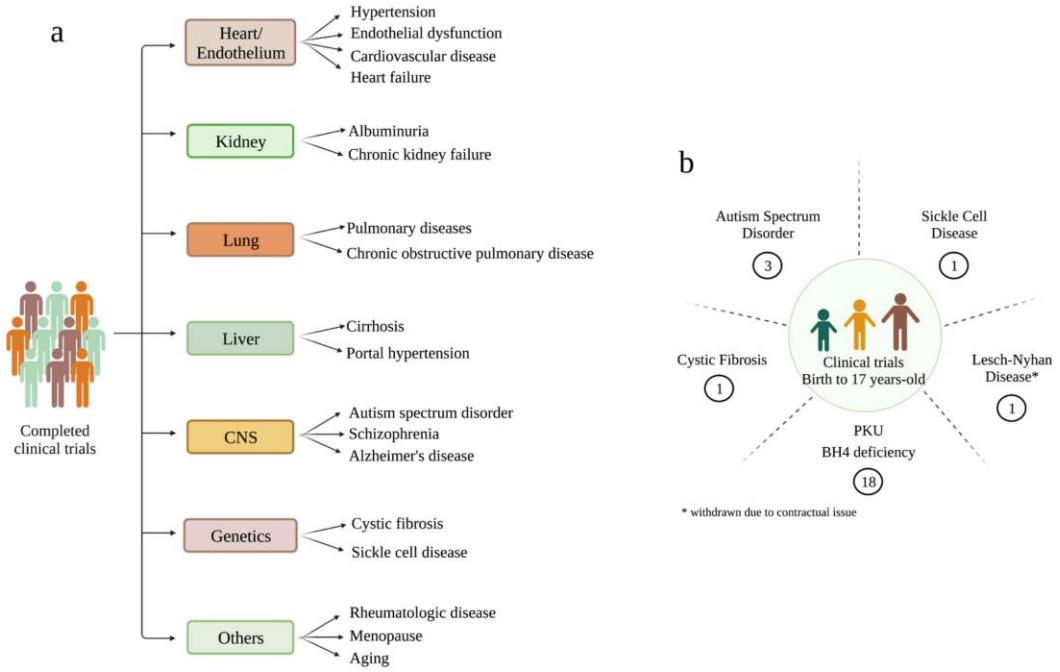

Figure 4. Food and Drug Administration (FDA)-approved clinical trials for the off-label use of tetrahydrobiopterin (BH4) for a variety of disorders not primarily affecting phenylalanine (Phe) metabolism. (**a**) Completed clinical trials where BH4 was administered to individuals affected by heart and vascular diseases, kidney, lung, liver, central nervous system (CNS), and genetic disorders. Other miscellaneous conditions were also included in the clinical trials, such as rheumatologic diseases, menopause, and aging. (**b**) Clinical trials involving the administration of BH4 in children affected by genetic disorders. Circled numbers correspond to the number of clinical trials based on BH4 administration. * The study was withdrawn before enrolling its first participant, due to contractual issues.

11. Conclusions

BH4 metabolism has emerged in the last decade as a promising metabolic target to modulate toxic pathways that may accelerate cell death. Strong evidence generated by our group and others has shown that BH4 metabolism has multiple biological roles, supporting essential pathways that generate energy, enhance antioxidant resistance, and protect against sustained inflammation. Therefore, BH4 should not be understood solely as an enzyme cofactor, and neopterin should not be considered only as an inert byproduct of BH4 metabolism.

BH4 metabolism can instead be depicted as a cytoprotective pathway that is finely regulated by the concerted action of de novo, salvage, and recycling pathways to regulate intracellular concentrations of BH4 and BH4-related metabolites. If this finely tuned balance in BH4 concentration is perturbed, several biological systems are compromised, resulting in impaired neurotransmission, immune responses, metabolism, and vascular activity (Figure 5). Correction of the imbalance resulted in improved cell homeostasis and survival. All these imbalances can potentially be attenuated by BH4 supplementation and could be beneficial in n mitochondrial disorders. However, it should be stressed that abnormally high intracellular levels of BH4 do not promote cytoprotection. On the contrary, we have shown, in human and experimental chronic diseases, that clinical presentation is worsened

when levels of BH4 surpass therapeutic or physiological levels (Figure 5). Indeed, when an inhibitor of SPR was used to normalize BH4 levels, symptoms were attenuated. Thus, BH4 metabolism can be considered a double-edge sword, with too little or too much resulting in toxicity.

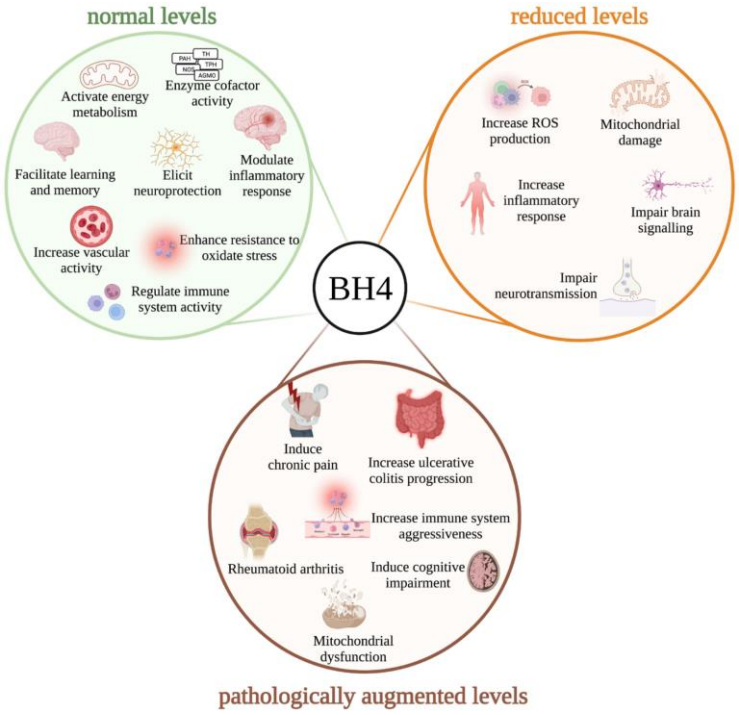

Figure 5. Tetrahydrobiopterin (BH4) metabolism as a central hub regulating physiological and toxic pathways. **Normal BH4 levels** (green circle): Physiological levels of BH4 sustain the traditional coenzyme activity of the pathway, favoring the correct metabolism of aromatic amino acids and ether lipids, and the biosynthesis of nitric oxide. Under these conditions, appropriate BH4 levels activate energy metabolism, enhance cellular resistance to oxidative stress, modulate the inflammatory response, facilitate learning and memory, regulate immune system activity, increase vascular activity, and exert neuroprotective effects. **Reduced BH4 levels** (orange circle): When BH4 levels are perturbed, ATP synthesis and brain lipid signaling are impaired, an oxidant status is induced, neurotransmission is compromised, and inflammation is favored. **Pathologically augmented BH4 levels** (brown circle): Excessive intracellular BH4 levels induce mitochondrial dysfunction, compromise memory and learning, increase the aggressivity of the immune system, promote the progression of inflammatory and autoimmune diseases, and elicit chronic pain. Thus, BH4 metabolism can be considered a double-edged sword: too little or too much results in cytotoxicity.

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ORIGINAL RESEARCH ARTICLE: BIOENERGETICS AND ANTIOXIDANT STATUS IN HUMAN *TFAM* DEFICIENCY.

(P2) PLOS Medicine (impact factor 11.613):
Bioenergetics and antioxidant status in human *TFAM* deficiency.
Tuany Eichwald, Wei Lin Huang, Alexander Stover, Mariella T Simon, Philip H Schwartz, Alexandra Latini, Jose E Abdenur (Manuscript to be submitted to PLOS Medicine).

PMDs are characterized by abnormalities in the structure and function of mitochondria, which may result from mutations, depletion, or deletions of mtDNA or mutations of nDNA (CHINNERY; TURNBULL, 2001). Currently, there is no cure, and only supportive and symptomatic therapies are available for PMDs. Obstacles finding effective treatments include the rarity of the disease, clinical diversity, genetic heterogeneity, difficult clinical trials, and, mainly, the ill-defined physiopathology.

This group of rare diseases commonly affect high energy demand tissues, such as the brain, skeletal muscle, liver, and heart. Muscle biopsy is the most common way to diagnose PMDs (WALLACE, 1999). However, muscle biopsy is an invasive procedure, and given that multiple biopsies may be required for diagnosis and as a source for future research, alternative non-invasive methods should be warranted. In this scenario, a skin biopsy is considered a minor surgical procedure performed under local anesthesia. Compared to muscle biopsy, skin biopsy is less invasive and has a lower risk of complications. Moreover, fibroblasts can be cultured through a skin biopsy become a renewable source of cells *in vitro* due to their highly proliferative characteristic.

Although high energy demand tissues are known to have a more significant number of mitochondria, and fibroblasts are generally considered to be cells with low mitochondrial content, here it is demonstrated the advantage of using fibroblasts in characterizing the mitochondrial impairment in *TFAM*-deficient fibroblasts. The deficient metabolic phenotype was confirmed *in vitro*, highlighting the significance of this finding in providing a valuable and cost-effective tool for identifying future potential treatments.

Key Results & Implications

Here, the characterization of bioenergetic and antioxidant activity was performed in fibroblasts obtained from an individual affected by *TFAM* deficiency. In *TFAM*-deficient fibroblasts, functional investigations revealed the following: *i)* impaired energy metabolism; *ii)* inhibited activity of respiratory chain complexes; *iii)* hypometabolism characterized by a failure to upregulate the glycolytic pathway; *iv)* higher production of superoxide anion; *v)* reduced activity of the main antioxidant enzymes. Altogether, it was demonstrated that *TFAM* depletion was characterized by reduced bioenergetics and antioxidant status.

Bioenergetics and antioxidant status in human *TFAM* deficiency

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Running title: *TFAM bioenergetics in fibroblasts*

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Keywords: mitochondrial DNA depletion, oxygen consumption, electron chain transport, *TFAM* deficiency, reactive oxygen species.

Abstract

Defects in the mitochondrial transcription factor A (*TFAM*) gene have been associated with mitochondrial DNA (mtDNA) depletion syndrome, genetic disorder characterized by reduced mtDNA content in affected tissues. *TFAM* deficiency can cause defects in transcription, replication, and organization of mtDNA into nucleoids, leading to a range of clinical symptoms. Here, we characterize the energy metabolism and redox status of fibroblasts obtained from a *TFAM* depletion syndrome-affected patient. Functional investigations in *TFAM* deficient fibroblasts showed impaired energy metabolism characterized by reduced oxygen consumption, proton leak, and mitochondrial spare respiratory capacity. In agreement, we also observed inhibition of the complexes of the respiratory chain, in particular complexes I and IV. *TFAM* deficient cells failed to upregulate the glycolytic pathway, a phenomenon that is expected under this type of mitochondrial deficit. These alterations indicate hypometabolism which possibly promoted a pro-oxidant status with higher production of superoxide anion, favoring oxidative stress and damage to cellular biomolecules. Finally, this scenario of oxidative stress and dysfunctional mitochondria in *TFAM* depleted fibroblasts led to an imbalance of the activities of the main antioxidant enzymes. Altogether, we show here that *TFAM* depletion was characterized by reduced bioenergetics and antioxidant status. The identification of these metabolic and phenotypic alterations in *TFAM* deficient fibroblasts offers a valuable and affordable tool for the better understanding of the physiopathology of the disease and therefore for the development of new pharmacological treatments. This is relevant since these cells can be isolated by minimally invasive methods, present specific cumulative cellular damage that characterize the mitochondrial disease and can be successfully reprogrammed to become induced pluripotent stem cells offering the opportunity to generate tissue-specific cell lines that can help model tissue-specific human diseases.

1. Introduction

Primary mitochondrial disorders (PMD) are an heterogeneous group of inherited genetic disorders, occurring in approximately 1 in 5,000 live births (Thorburn, 2004). PMD are characterized by abnormalities in the structure and function of mitochondria, which may result from mutations, depletion, or deletions of mitochondrial DNA (mtDNA) or mutations of nuclear DNA (nDNA) (Chinnery and Turnbull, 2001). The phenotypic spectrum of PMD is broad and highly variable, ranging from isolated organ system involvement in adults to multi-systemic, lethal disease in infants. Thus, the variation in systemic involvement can pose a diagnostic challenge (Gorman et al., 2016). Symptoms of PMD may include severe muscular weakness, progressive encephalopathy, liver malfunction, chronic fatigue, and neurodegeneration, among others (Tuppen et al., 2010; Zolkipli-Cunningham et al., 2018).

Mitochondrial DNA (mtDNA) depletion syndromes (MDS) are rare autosomal recessive disorders characterized by a reduction in the amount of mtDNA in affected tissues (Rötig and Poulton, 2009). MDS can be caused by a mutation in genes that are involved in mtDNA replication, maintenance, or stability. The reduction

of Children's Hospital of Orange County (CHOC), Research Department

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0.4 mM azide, 0.5 mM tert-butylhydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the enzyme activity was expressed as Units/mg protein.

2.8.4 Glutathione reductase activity

Glutathione reductase (GR) activity was measured at 340 nm (Carlberg and Mannervik, 1985). The NADPH consumption was determined in a reaction medium contained 0.1 M phosphate buffer, pH 7.0, 1 mM EDTA, and 0.225 mM NADPH. When adding 1 mM GSSG to the reaction media, the GR present in the sample reduces GSSG to GSH, thereby consuming NADPH. The enzyme activity was expressed as Units/mg protein.

2.9 mtDNA copy number determination

Genomic DNA was extracted using Puregene DNA extraction according to the manufacturer protocol. Quantitative PCR assay was performed in triplicate on a CFX Connect Real-Time PCR Detection System using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The ratio of mtDNA amplification for the ND1 gene vs the nuclear housekeeping gene beta-2 microglobulin (B2M) was calculated. Primers were used as previously described (Bonnen et al., 2013).

2.10 Protein determination

Protein levels were determined using DC protein assay (Bio-Rad). Protein quantifications were performed following manufacturer instructions and biological samples were prepared under the same conditions as standards.

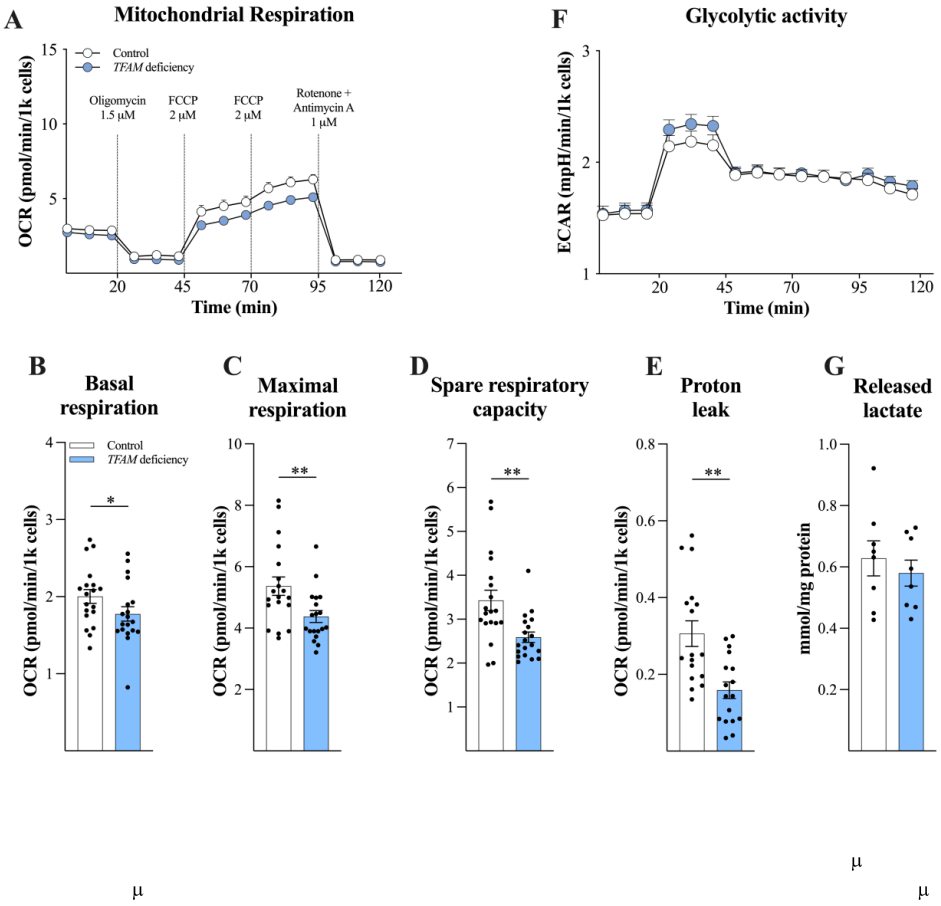
2.11 Statistical analysis

One-tailed student's *t* test was used to determine significant differences between *TFAM* deficient cells and controls. Data are presented as mean ± standard error of mean. The accepted level of significance for the tests was $P < 0.05$. Statistics were calculated and all graphs were generated using GraphPad Prism 9®.

3. Results

3.1 Mitochondrial respiration is impaired in TFAM-deficient fibroblasts

Figure 1 shows the bioenergetics profile of a *TFAM* deficient cell line. **Figure 1A** illustrates the oxygen consumption rate after the addition of inhibitors or stimulants of respiration. Basal respiration (**Figure 1B**) [$t_{(36)}$= 1.759; $P < 0.05$], and maximal oxygen consumption (**Figure 1C**) were significantly decreased in *TFAM*-deficient fibroblasts [$t_{(36)}$= 2.777; $P < 0.01$]. Importantly, spare respiratory capacity (**Figure 1D**) [$t_{(36)}$= 3.201; $P < 0.01$] and the proton leak (**Figure 1E**) [$t_{(32)}$= 3.709; $P < 0.01$] were also significantly reduced in *TFAM*-deficient cells. However, the ECAR under basal conditions (**Figure 1F**), the levels of lactate released into the cell culture media



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= 8) was measured as an index of the cell's glycolytic activity

complex IV (cytochrome *c* oxidase**; Figure 2G**) [$t_{(30)}$= 3.790; $P < 0.001$] in *TFAM*-deficient fibroblasts. No differences were observed in the activities of succinate dehydrogenase (**Figure 2D**), complex II (**Figure 2E**), or complex II-CoQ-III (succinate:Ubiquinone oxidoreductase; **Figure 2F**). The normalized activities of the complexes of the respiratory chain are shown in **Supplementary Table 1**.

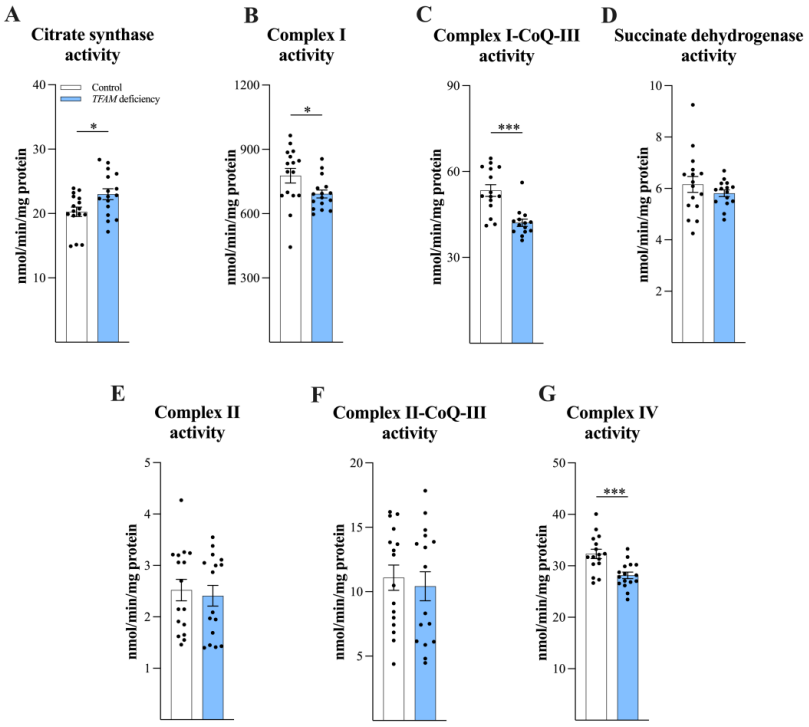
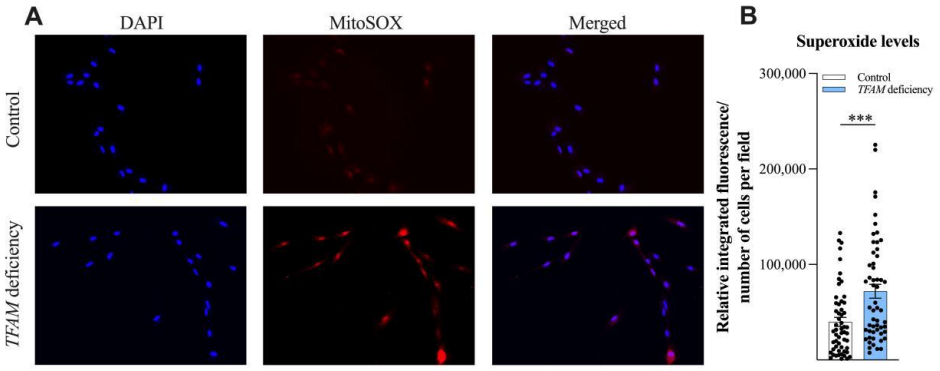


Figure 2. The activities of key respiratory chain complexes are decreased in *TFAM*-deficient fibroblasts. Fibroblasts from an individual affected by *TFAM* deficiency and from a healthy volunteer (Control) were cultured in a 6-well plate for 48 h (n=15-16). Then cells were trypsinized and resuspended in SETH buffer. Cell homogenates were used to assess the activity of (**A**) citrate synthase (Krebs cycle enzyme; n=15), (**B**) complex I, (**C**) complex I-CoQ-III, (**D**) succinate dehydrogenase (Krebs cycle enzyme), (**E**) complex II, (**F**) complex II-CoQ-III, and (**G**) complex IV, following protocols already described by our group (See M&M for details). Bars represent the mean ± standard error of mean. * $P < 0.05$, *** $P< 0.001$, Control vs. *TFAM* deficiency. One-tailed student's t-test.

3.3 TFAM-deficient fibroblasts produce higher ROS levels

The levels of superoxide anion, a toxic compound produced in excess by unhealthy mitochondria, were assessed in *TFAM*-deficient by fluorescence microscopy. Representative images of control and *TFAM*-deficient fibroblasts are shown in **Figure 3A**. **Figure 3B** shows higher oxidation of the probe MitoSox in *TFAM* deficient


A
DAPI
MitoSOX
Merged
Control
TFAM deficiency
B
Superoxide levels
Relative integrated fluorescence/ number of cells per field
300,000
200,000
100,000
Control
TFAM deficiency
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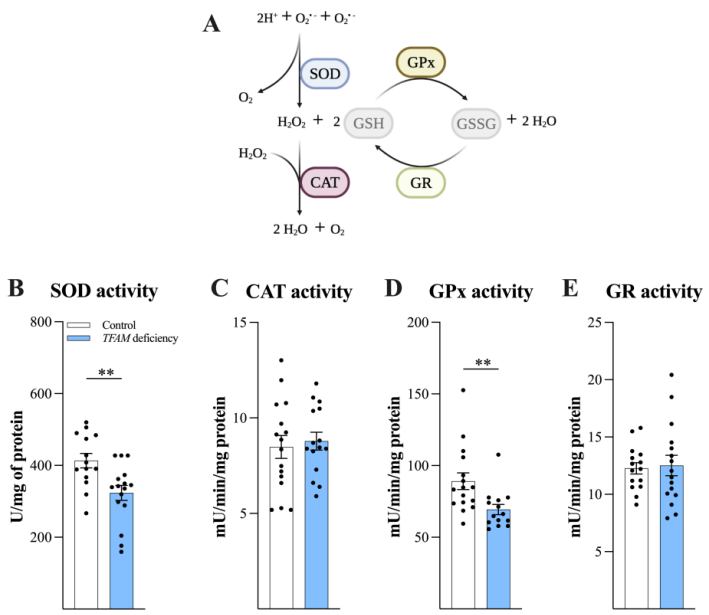

Figure 4. The enzymatic antioxidant system is compromised in *TFAM*-deficient fibroblasts. (**A**) Diagrammatic representation of the interaction of the main antioxidant enzymes to reduce reactive oxygen species: superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione oxidized (GSSG), glutathione peroxidase (GPx), and glutathione reductase (GR). Fibroblasts from an individual affected by *TFAM* deficiency and from a healthy volunteer (Control) were cultured in a 6-well plate for 24 h (n=14-16). Then cells were trypsinized and resuspended in potassium phosphate buffer (See M&M for details). Cell homogenates were used to assess the activity of (**B**) SOD, (**C**) CAT, (**D**) GPx, and (**E**) GR. Bars represent the mean ± standard error of mean. ** P< 0.01, Control vs. *TFAM* deficiency. One-tailed student's *t*-test.

4. Discussion

Human TFAM, encoded by the nuclear gene *TFAM*, is involved in vital mitochondrial processes including transcription, replication, and packaging of mtDNA into nucleoids (Kaufman et al., 2007). *TFAM* plays a crucial role in regulating mtDNA copy number by preserving mtDNA stability, and due to its interaction with mtDNA also contributes to the regulation of mitochondrial biogenesis (Picca and Lezza, 2015). Here, we investigated the bioenergetics and oxidant/antioxidant phenotype in fibroblasts of a mtDNA depletion syndrome caused by a homozygous missense variant in *TFAM*. In agreement with the biology of the disease, mtDNA levels were markedly decreased (up to ~ 70 %), which represents an empirical cut-off level for a diagnosis of a PMD (Stiles et al., 2016). This is also in agreement with a report showing reduced TFAM protein level and mtDNA content, as well as abnormal mitochondrial morphology and distribution in human *TFAM*-deficient fibroblasts (Stiles et al., 2016).

PMD are multisystem disorders affecting more than one type of cell, tissue, or organ. Because muscles and nerve cells have especially high energy needs and high mitochondrial content, muscular and neurological

symptoms are common features in PMD (Wallace, 1999). Therefore, the diagnosis is normally performed by assessing mitochondrial activity in muscle biopsies. However, we showed here that fibroblasts can be used to characterize the mitochondrial impairment in *TFAM* deficiency. This is relevant, since cell cultures can be used as valuable and affordable tools for the identification of potential novel treatments for PMD. Moreover, these cells can also be successfully reprogrammed to become induced pluripotent stem cells (iPSC) offering the opportunity to generate patient-specific, tissue-specific cell lines that can help model tissue-specific human diseases.

Reduced basal and maximal respiration, spare respiratory capacity, and proton leak were the energy parameters that characterized the mitochondrial dysfunction in the *TFAM*-deficient cell line. Reduced mitochondrial respiration was, in part, due to the inhibition of complexes I and IV of the respiratory chain, a phenotype that is in agreement with previous reports describing that *TFAM* depletion compromised mitochondrial electron transfer, biogenesis, and architecture with damaged mitochondrial cristate in mouse heart and human pluripotent stem cells (Larsson et al., 1998; Picca and Lezza, 2015; Yang et al., 2022).

We also observed that released lactate and bulk extracellular acidification, represented by protons generated by glycolysis and/or the Krebs cycle, was not increased in *TFAM*-deficient cells. This suggests that the glycolic pathway is not compensating for the energy deficiency produced by the unhealthy mitochondria. Usually, elevated levels of lactate and/or pyruvate are typically found in PMD. They only be detected when PMD patients undergo a metabolic crisis or following exercise (Debray et al., 2007).

TFAM is required for the transcription and replication of mtDNA; therefore, a deficiency of mitochondrial proteins is expected to occurred in affected tissues, leading to a decrease in respiratory complex assembly and activity (Virbasius and Scarpulla, 1994). In fact, it was already reported that the deletion of *Tfam* in mice cause reduced enzymatic activity of complex I and IV in skeletal muscle (Wredenberg et al., 2006); while the induced production of recombinant TFAM in mouse lead to global increase in mitochondrial ETC protein (Iyer et al., 2008). Moreover, a compensatory mechanism for the impairment of complex I was described, represented by increased citrate synthase activity in mouse adipose tissues (Vernochet et al., 2012).

Mitochondrial function is a complex and adaptable process, and cells have the ability to activate spare mitochondria when required, a mechanism that is linked to mitochondrial plasticity (Nicholls, 2009). Diminished levels of spare mitochondria, as those observed in this study, have been associated with pathological conditions, such as acute myeloid leukemia (Sriskanthadevan et al., 2015), motor neuron diseases (Tan et al., 2014), and Alzheimer's disease (Bell et al., 2020), resulting in energy deficit, cellular stress, and ultimately cell death (Nicholls, 2009).

It is known that under physiological conditions, 0.2 – 2 % of the electrons in the respiratory chain do not follow the normal transfer order but instead directly leak out and interact with oxygen to produce ROS (Cadenas and Davies, 2000; Turrens, 2003). Complex I is believed to be the major site for ROS generation in the mitochondria, mainly in some pathological conditions such as mitochondrial diseases (Cadenas and Davies,

2000). However, inhibition of complex IV is also known to contribute to ROS generation. The nonsequential transfer of the electrons from complex IV to oxygen, or the accumulation of electrons at the levels of complex III will also contribute to increased ROS production (Cadenas et al., 1977). Moreover, reduced proton leak, as we observed here, will enhance ROS formation due to a lack of partial physiological mitochondrial uncoupling (Starkov and Fiskum, 2003). Indeed, higher levels of oxidative stress in human fibroblasts and mouse cardiomyocytes have been demonstrated in different *in vitro* and *in vitro* systems of knockdown *TFAM* (Balliet et al., 2011; Zhang et al., 2018). In agreement, *Tfam* overexpression in mouse decreased mitochondrial ROS production (Ikeda et al., 2015).

Given that, we showed here that fibroblasts derived from a *TFAM* deficiency showed the disease's phenotype, presenting a valuable research tool for the understanding of its physiopathology and for the development of new treatments. In addition, differential responses to the same drug have been found among individuals, suggesting the need for personalized therapy, which is feasible using cultured fibroblasts.

The use of human specimens is crucial for scientific and medical progress. It has helped improve health care by leading to discoveries in diagnosis, disease progression, targeted therapies, medical procedures, and personalized medicine. Moreover, research involving human samples instead of mouse samples is important because it can provide more accurate and relevant results, specifically addressing human biology, and increasing translational value.

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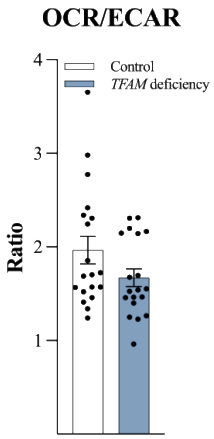
Supplementary material



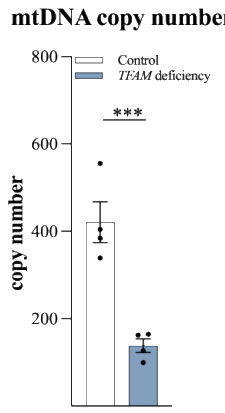
Supplementary Figure 1. The OCR/ECAR ratio is not impaired in *TFAM* deficient fibroblasts. Fibroblasts from an individual affected by *TFAM* deficiency and from a healthy volunteer (Control) were cultured in a 24-well plate for overnight and mitochondrial physiology was assessed by using Mito Stress and measured in the Seahorse XF Flux Analyzer (See M&M for details). OCR/ECAR ratio (n=19). Bars represent the mean ± standard error of mean. One-tailed student's t-test.

Supplementary Table 1. Mitochondrial complex activities normalized by the activity of citrate synthase (CS) in *TFAM*-deficient fibroblasts

Complex enzyme activity / CS activity	Control	*TFAM* deficiency	*P* value
CI/CS	39.2 ± 2.38	30.6 ± 1.40	< 0.01
CI-CoQ-III/CS	2.63 ± 0.14	1.85 ± 0.08	< 0.001
SDH/CS	0.31 ± 0.03	0.25 ± 0.01	< 0.05
CII/CS	0.13 ± 0.01	0.11 ± 0.01	ns
CII-CoQ-III/CS	0.55 ± 0.04	0.44 ± 0.04	< 0.05
CIV/CS	1.63 ± 0.08	1.25 ± 0.06	< 0.001

Fibroblasts from an individual affected by *TFAM* deficiency and from a healthy volunteer (Control) were cultured in a 6-well plate for 24 h (n=15-16). Then cells were trypsinized and resuspended in SETH buffer. Cell homogenates were used to assess the activity complex I (CI), complex I-CoQ-III, (CI-CoQ-III), complex II (CII), complex II-CoQ-III (CII-CoQ-III), complex IV (CIV) and succinate dehydrogenase (SDH). All these activities

were normalized by CS activity as an index of mitochondrial content. *P* values indicate statistical differences between Control and *TFAM* deficiency. One-tailed student's *t*-test.



Supplementary Figure 2. Mitochondrial DNA (mtDNA) copy number is reduced in *TFAM*-deficient fibroblasts. Fibroblasts from an individual affected by *TFAM* depletion syndrome and from a healthy volunteer (Control) were cultured in a 6-well plate for 24 h (n=4). Then, cells were trypsinized and collected in a DNAse- / RNAse-free tube. Bars represent the mean ± standard error of mean. *** $P<$ 0.001, Control vs. *TFAM* deficiency. One-tailed student's *t*-test.

COMPLEMENTARY DATA: TRANSCRIPTOME

The transcriptome analysis of these cells complemented the data presented in this chapter. **Table 1** shows that the main modulated metabolic pathways were associated with inflammation. These findings are also evident in the heat map presented in **Figure 1,** showing several pro-inflammatory genes upregulated in *TFAM* deficiency. The volcano plot also shows that the most upregulated genes are also involved in inflammation (**Figure 2**).

Pathway	z-score	Overlapping genes	p-value
Pulmonary Fibrosis Idiopathic Signaling Pathway	1.219	35	5.48E-09
HOTAIR Regulatory Pathway	0.853	23	1.53E-08
Osteoarthritis Pathway	0.471	28	2.17E-08
Wound Healing Signaling Pathway	0.756	28	8.91E-08
Role of Osteoclasts in Rheumathoid Arthritis	0.73	31	1.90E-07
Tumor Microenvironment Pathway	0.894	21	1.48E-06

Table 1. Metabolic pathways most affected by differentially expressed genes from *TFAM*-deficient fibroblasts.

The table presents the six most affected metabolic pathways in *TFAM*-deficient fibroblasts. It contains the name of the pathways and their z-score, overlapping genes, p-value, and -log10 of the p value.

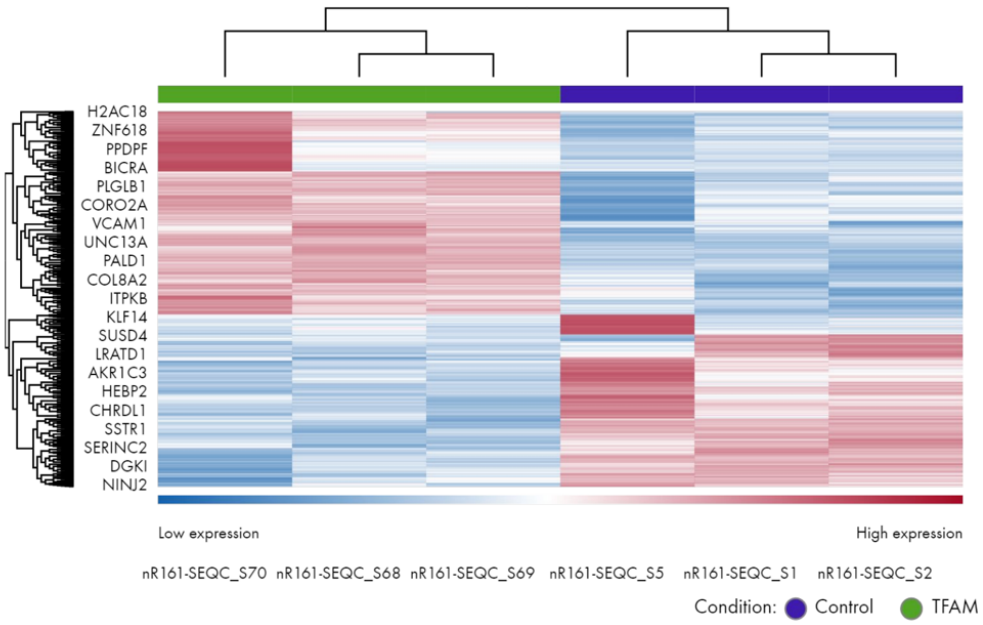


Figure 1. Gene expression of *TFAM*-deficient fibroblasts (green top bar) vs control fibroblasts (purple top bar).

Heat map of the most differentially expressed genes. The blue color represents a lower, and the red color represents a higher expression level.

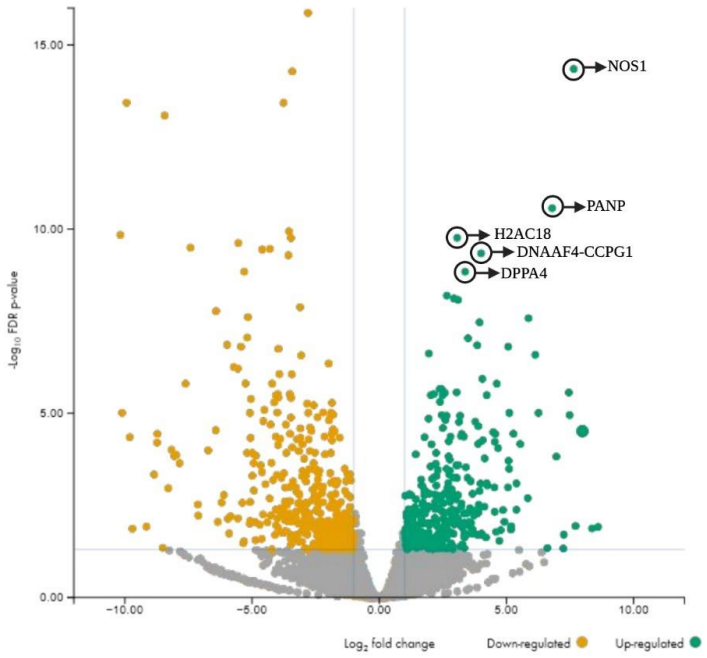


Figure 2. Volcano plot of the distribution of all differentially regulated genes in *TFAM*-deficient fibroblasts.

Volcano plot of the most differentially down-regulated (yellow dots) and up-regulated (green dots) genes. The black circles highlight the five top up-regulated genes.

A deeper analysis of the transcriptome revealed that the genes involved in BH4 synthesis were not upregulated in *TFAM*-deficient cells (**Table 2**).

Name	Ensembl ID	Fold change	FDR p-value	P-value	Biotype
GCH1	ENSG00000131979	-1.175378568	0.964586547	0.71184678	protein_coding
PTS	ENSG00000150787	-1.259561346	0.804457943	0.289575933	protein_coding
SPR	ENSG00000116096	-1.473474756	0.643562862	0.144221858	protein_coding
PCBD2	ENSG00000132570	-1.312476731	0.806849146	0.29545891	protein_coding
PCBD1	ENSG00000166228	1.042370995	0.985026035	0.860095228	protein_coding
QDPR	ENSG00000151552	1.055114496	0.980310329	0.789260688	protein_coding

Table 2. Gene expression of BH4-related enzymes in *TFAM*-deficient fibroblasts. The table contains the name of the gene, the ensembl ID, fold change, FDR p-value, P-value, and biotype. GTP cyclohydrolase 1 (GCH1), 6-pyruvoyl-tetrahydrobiopterin synthase (PTS), sepiapterin reductase (SPR), pterin-4 alpha-carbolamine dehydratase 2 (PCBD2), pterin-4 alpha-carbolamine dehydratase 1 (PCBD1), quinoid dihydropteridine reductase (QDPR).

CHAPTER II

EPIGENETIC MODIFICATIONS INDUCED BY OBESITY AND EXERCISE, AND THE DETRIMENTAL EFFECTS TRIGGERED BY HIGH FAT DIET IN MICE

This chapter provides a comprehensive overview of obesity and chronic pain, including their definitions, epidemiology, inflammatory status, and the relationship between pain and obesity. It also explores the role of BH4 metabolism in the physiopathology of pain associated with chronic inflammatory diseases. The chapter presents original data collected from mice subjected to an HFD, encompassing body composition measurements, urinary BH4 levels, locomotor activity, motivational behavior, and nociceptive responses. Furthermore, it highlights the beneficial effects of physical exercise in preventing increased visceral adipose tissue, body fat percentage, glucose tolerance, and hyperalgesia induced by HFD. Finally, a literature review article delves into the known epigenetic modifications induced by exercise and obesity.

(P3) Obesity:
Development of pain hypersensitivity and increased urinary tetrahydrobiopterin levels in mice submitted to high fat diet.
Tuany Eichwald, Leonardo Barros, Alexandre Francisco Solano, Débora da Luz Scheffer, Vivian Menegassi, Ananda Christina Staats Pires, Camila Sartor Spivakoski, Rodrigo A. Foganholi da Silva, Joana Margarida Gaspar, Marcelo Fernando Ronsoni, Alexandra Latini (Manuscript submitted to Antioxidants).

(P4) Physiology and Behavior:
Epigenetic modifications induced by exercise: Drug-free intervention to improve cognitive deficits associated with obesity.
Leonardo Barros, **Tuany Eichwald**, Alexandre Francisco Solano, Débora Scheffer, Rodrigo Augusto da Silva, Joana M. Gaspar, Alexandra Latini (Published in 11 March 2019).

2.1 OBESITY

Obesity is defined by the World Health Organization (WHO) as abnormal and excessive accumulation of fat. The increase in adipose tissue (AT) from a daily positive energy balance is associated with low-grade chronic systemic inflammation (WHO, 2016). Clinically, obesity is determined based on the combination of methods that evaluate total adiposity, such as the body mass index (BMI) and the abdominal circumference. BMI is a statistical index based on a calculation considering the individual's weight in kilograms (kg) divided by the squared height in meters (m^2). BMI defines individual status as underweight, normal weight, overweight, or obese. Also, obesity can be divided into classes I, II, or III (Table 1) (WHO, 2015).

BMI (kg/m^2)	**Classification**
< 18.5	Underweight
18.5 – 24.9	Normal Weight
25 – 29.9	Overweight
> 30	Obese
30 – 34.9	Obese Class I
35 – 39.9	Obese Class II
> 40	Obese Class III

Table 1. Obesity classification according body mass index.

Although widely used in epidemiological studies and clinical practice, more than the measurement of BMI alone is required. For example, individuals with heavier muscle bodies (such as athletes and bodybuilders) can be classified as affected by obesity according to the BMI calculation. Moreover, BMI may not be an accurate measurement for assessing body composition and health risks in different ethnic groups due to differences in body shape, muscle mass, bone density, and fat distribution patterns (GARRIDO-CHAMORRO et al., 2009; RAHMAN; BERENSON, 2010). It has been extensively shown that waist circumference strongly correlates with abdominal obesity and is widely used to measure body fat distribution (JANSSEN et al., 2002).

Obesity is considered a public health problem and a risk factor for the development of other chronic diseases such as type 2 diabetes *mellitus*, cardiovascular diseases, and some types of cancer, among others (WILLIAMS et al., 2015). Obesity also causes negative impacts on the psychological state, mood, and cognitive functions of affected individuals (JAUCH-CHARA; OLTMANNS, 2014).

The prevalence of obesity due to sedentary behavior and excessive caloric intake has increased dramatically in recent decades. The world estimative is that about 1.9 billion adults are overweight, more than 600 million are obese, and 107 million children are obese (WHO, 2016). Obesity has significantly increased in the past 20 years in Brazil, meaning that the prevalence increased from 12.2 % in 2002 to 26.8 % in 2019 (IBGE, 2020). Brazil occupies the third position in the world ranking of obesity, which means that 41.2 million Brazilians have obesity (IBGE, 2020). Moreover, the overweight affects 33 % of Brazilian children between 5 and 9 years, 20 % of teenagers, and 51 % of adults (MINISTÉRIO DA SAÚDE, 2017).

Obesity is a multifactorial etiology disease, and its clinical manifestations involve pain and joint dysfunction, leading to disability and affecting a significant number of individuals than any other joint disease. The physiopathology of obesity involves a complex interaction of several factors, such as socioeconomic status, genetics and epigenetics, cultural aspects, and lifestyle (GONZÁLEZ-MUNIESA et al., 2017; THE GBD 2015 OBESITY COLLABORATORS, 2017). Despite its multifactorial etiology, it is known that most cases of obesity involve increased calorie intake, facilitated mainly by greater access to and availability of diets rich in saturated fat and sugar. Associated with food intake, the decrease in energy expenditure caused by more sedentary behaviors results in an increase and excessive accumulation of adiposity (STICE et al., 2015). In Brazil, 12.6 % of men and 19.7 % of women frequently changed their main meals to a low nutritive food, like pizza and hamburger. Furthermore, the excessive ingest of saturated fat from animals is pronounced among Brazilians; 31 % of the population have the habit of consuming fatty meat every day, 53.5 % consume fatty milk frequently, and 23.3 % drink soda at least five days per week (SAÚDE, 2013).

Obesity, especially if accompanied by visceral fat, is associated with chronic low-grade systemic inflammation with AT immune infiltration and production of pro-inflammatory cytokines, like IL-1β, TNF-α, and IL-6, leading to insulin resistance and development of type 2 diabetes

(FAIN, 2006; HOTAMISLIGIL, 2017). Such a condition predisposes the development of a large number of chronic diseases, which include inflammatory and degenerative conditions of the musculoskeletal system and the peripheral and central nervous systems (Figure 1) (BERENBAUM; EYMARD; HOUARD, 2013; COPE et al., 2018; SMITH et al., 2011). Inflammatory pathways are also persistently activated in several regions of the brain, including the mid-basal hypothalamus, which exerts control over peripheral regulation of energy, glucose, and lipid metabolism (HOTAMISLIGIL; SHARGILL; SPIEGELMAN, 1993; HUNDAL et al., 2002).

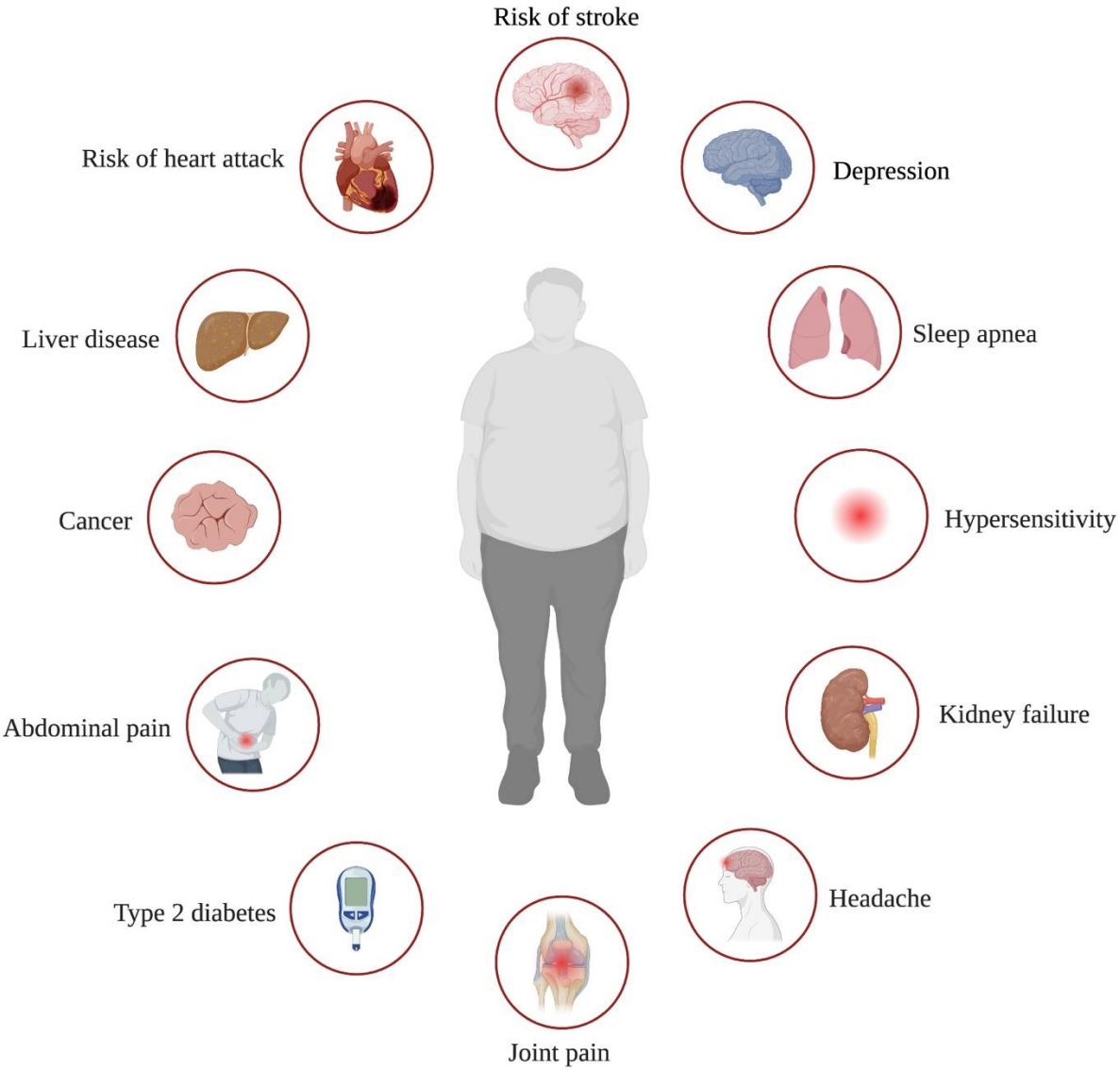


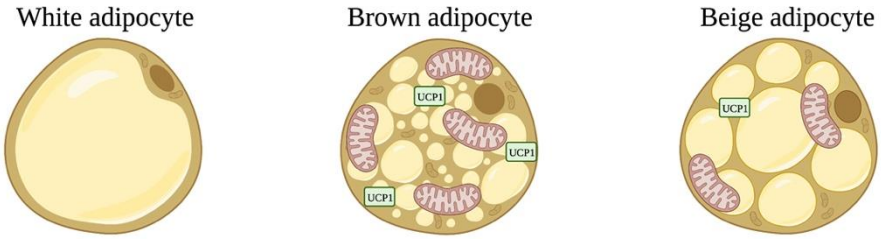
Figure 1. Obesity-related comorbidities. Obesity is a risk factor for the development of other conditions such as stroke, heart attack, depression, sleep apnea, hypersensitivity, kidney failure, headache, joint pain, type 2 diabetes *mellitus*, abdominal pain, cancer, and liver disease. Source: the author, 2023.

2.1.1 Obesity as a chronic low-grade systemic inflammatory disease

The white adipose tissue (WAT) is a complex endocrine organ that secretes lipids, bioactive peptides (adipokines), and other metabolites, modulating whole-body energy and glucose homeostasis (LONGO et al., 2019). Adipocytes act as a reservoir for energy storage and utilization, sense energy demands and secrete paracrine factors to regulate other metabolic tissues (SCHERER, 2006). It is known to all that there are two main types of mammalian AT: WAT and brown adipose tissue (BAT), which have different biological functions. White adipocytes are the most abundant and are specialized in lipid storage and mobilization, as well as in hormone secretion (SANCHEZ-GURMACHES et al., 2012), and are located in subcutaneous and visceral fat deposits (LONGO et al., 2019) (Figure 2).

On the other hand, the BAT is specialized in energy expenditure and converts chemical energy into thermal energy (LIDELL; BETZ; ENERBÄCK, 2014). It is located in the scapular region and maintains the core body temperature (GONZÁLEZ-MUNIESA et al., 2017). Brown adipocytes are characterized by multilocular lipid droplets and abundant mitochondria, which contain a unique protein called uncoupling protein 1 (UCP1) (Figure 2). In the inner mitochondrial membrane, UCP1 uncouples mitochondrial respiration from ATP synthesis resulting in thermogenesis (LIDELL; BETZ; ENERBÄCK, 2014).

A phenomenon called “browning of WAT” has been described under cold exposure or activation of β-adrenergic receptors (SEALE et al., 2007). Beige adipocytes are in the interscapular, axillary, cervical, and supraclavicular regions. Its main action is in thermogenesis, controlling low-temperature conditions (CHENG et al., 2021) (Figure 2). Moreover, brown adipocytes and beige adipocytes can uptake glucose and fatty acids to produce heat, which plays a decisive role in regulating the body’s glucose and lipid metabolism (ORAVA et al., 2013; SINGH et al., 2018).



	White adipocyte	Brown adipocyte	Beige adipocyte
Functions	Energy storage	Thermogenesis and energy expenditure	Thermogenesis and energy expenditure
Location in humans	Subcutaneous, visceral	Supraclavicular, neck, axillary	Supraclavicular, neck
Location in mice	Visceral, epididymal, retroperitoneal	Interscapular	Inguinal
Mitochondrial density	Low	High	Medium
UCP1 amount	Low/undetectable	High	Medium

Figure 2. Architecture, functions, and location of white, brown, and beige adipocytes.
White adipocytes (left column) function as of energy storage and are located subcutaneously and viscerally in humans and viscerally, epididymal, and retroperitoneally in mice, presenting low mitochondrial density and UCP1 amount. The brown adipocytes (middle column) are specialized in thermogenesis and energy expenditure. The localization in humans is supraclavicular, neck, and axillary, while in mice, the location is interscapular, presenting high mitochondrial density and UCP1 amount. The beige adipocytes (right column) have the thermogenesis and energy expenditure function and are supraclavicular and neck located in humans. In mice, the location is inguinal, presenting medium mitochondrial density and UCP1 amount. Uncoupling protein 1 (UCP1). Adapted from CHENG et al., 2021.

WAT depots contain 2 to 5 million cells, of which 65 % are leukocytes (macrophages, T cells, B cells, NK cells, and mast cells) (KANNEGANTI; DIXIT, 2012); an estimated excess of 20–30 million macrophages accumulate with each kilogram of excess fat in humans (O'ROURKE et al., 2009). Thus, the lean AT is mainly composed of M2 macrophages, innate lymphoid cells type 2 (ILC2), eosinophils, and regulatory T cells; while the fat AT is dominated by M1 macrophages, neutrophils, ILC1, and cytotoxic T cells (LUMENG et al., 2007) (Figure 3).

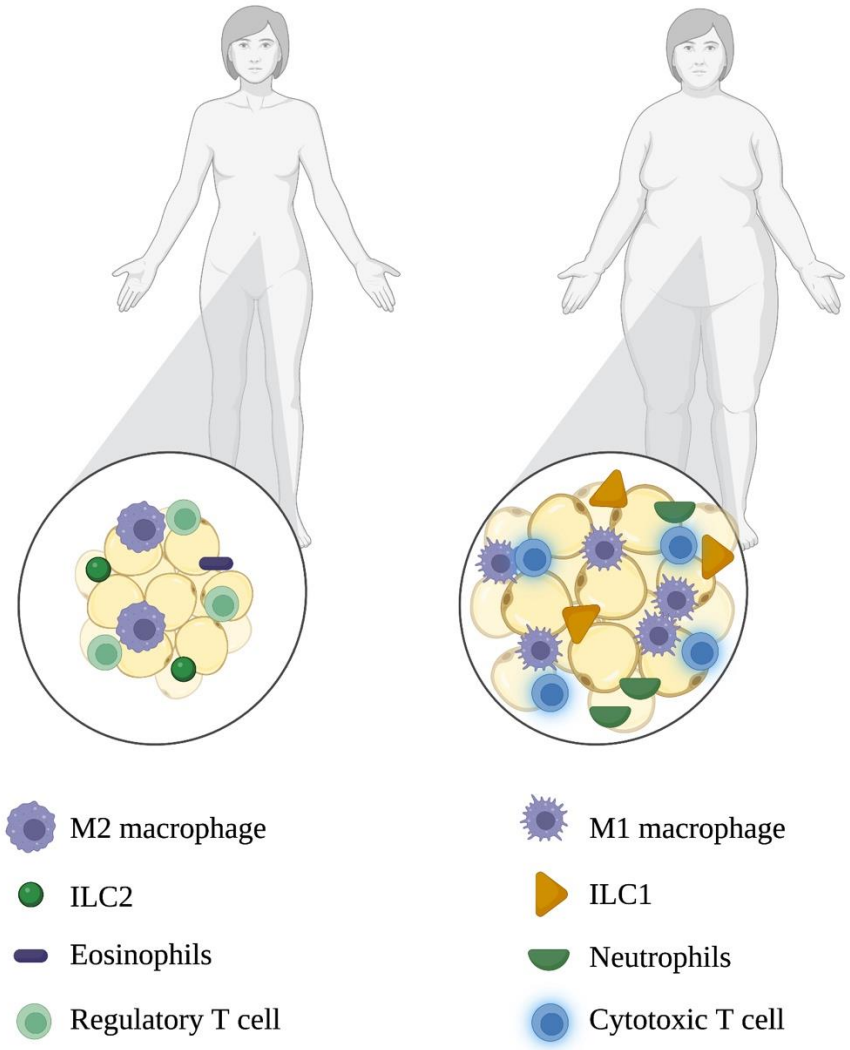


Figure 3. Adipose tissue (AT) composition in lean and obese-affected individuals. In a lean, healthy individual, the AT is infiltrated by M2 macrophages, innate lymphoid cells type 2 (ILC2s), eosinophils, and regulatory T cells (left). On the other hand, in individuals affected by obesity, the AT is composed mainly of M1 macrophages that infiltrate the AT, neutrophils, innate lymphoid cells type 1 (ILC1), and cytotoxic T cells (right). Source: the author, 2023.

The enlargement of WAT in obesity is possible due to two adipocyte growth mechanisms, hyperplasia (cell number increase) and hypertrophy (cell size increase) (CHOE et al., 2016). Hyperplastic growth appears only at the early stages of AT development, while hypertrophy occurs

prior to hyperplasia to meet the need for additional fat storage capacity in the progress of obesity (DROLET et al., 2008; SPALDING et al., 2008). The rapid hyperplasia growth in obesity can trigger an inflammatory response due to intrinsic signals, including adipocyte death, hypoxia, and mechanical stress arising from interactions between the cells and the extracellular matrix (CHOE et al., 2016).

The massive hypertrophy can also lead to loss of adipocyte expansion capacity, favoring its rupture, which in turn leads to the altered secretion of chemoattractant and immune-related genes that may promote macrophage infiltration (HENNINGER et al., 2014; KIM et al., 2015). Adipocyte-released monocyte chemoattractant protein 1 (MCP-1) and CCR2 attract circulating monocytes and favor their differentiation into M1 macrophages (ARNER et al., 2012). Once in the WAT, macrophages form “crown-shaped structures” around dead adipocytes and produce pro-inflammatory molecules such as IL-1β, IL-6, TNF-α, and C-reactive protein (CINTI et al., 2005; LUMENG; BODZIN; SALTIEL, 2007). Therefore, WAT M1 macrophages constitute a primary source of pro-inflammatory cytokines in obesity (LUMENG et al., 2007). These metabolic complications in obesity favor the onset of local and systemic inflammation (CANCELLO; CLÉMENT, 2006; CHAWLA; NGUYEN; GOH, 2011).

2.2 TETRAHYDROBIOPTERIN AND INFLAMMATION

The *de novo* BH4 pathway is regulated by the inducible enzyme GTPCH, which is controlled by pro-inflammatory mediators such as IFN-γ, TNF-α, IL-1β, hydrogen peroxide and LPS (KATUSIC; STELTER; MILSTIEN, 1998; SHIMIZU et al., 2003; WERNER et al., 1990) (for a review on the BH4 synthesis pathways see chapter 2). Thus, the synthesis and levels of BH4 are known to increase in cases of inflammation, while its blockage leads to reduced inflammation (COSTIGAN; LATREMOLIERE; WOOLF, 2012; FUJITA et al., 2020; LATREMOLIERE et al., 2015; TEGEDER et al., 2006).

While *GCH1* activity can be stimulated up to 100-fold during the inflammatory response, the activity of the downstream enzymes PTPS and SPR is only minimally increased under the same conditions, resulting in PTPS as the rate-limiting enzyme for BH4 synthesis via the *de novo* pathway during the inflammatory state (WERNER et al., 1990). The pseudo metabolic block created between enzyme activity favors the accumulation of the PTPS substrate, which is non-

enzymatically transformed into neopterin, a sensitive biomarker of inflammation and activation of the BH4 *de novo* pathway (for a review, see GHISONI et al., 2015). Moreover, the expression of *SPR* under an inflammatory condition is upregulated, suggesting that the salvage pathways might also be triggered under inflammation (DE PAULA MARTINS et al., 2018b).

Neopterin is a marker of macrophage activation, produced by macrophages stimulated by IFN-γ (HUBER et al., 1984), and is elevated in DRGs (TEGEDER et al., 2006). In this context, our group demonstrated for the first time that neopterin is also released by primary human nerve cells, including glutamatergic neurons, astrocytes, and microglia, when exposed to an inflammatory environment (DE PAULA MARTINS et al., 2018a). Elevated levels of neopterin were described in different biological fluids when immune-mediated and inflammatory disorders appear, in which T-helper 1 cells and macrophages are involved (HOFFMANN; WIRLEITNER; FUCHS, 2003; HUBER et al., 1984), such as obesity, viral meningoencephalitis, and bacterial meningitis (LENOIR DA SILVA et al., 2017; MOLERO-LUIS et al., 2020; NEDEVA et al., 2021).

The pro-inflammatory cytokine stimuli can regulate *GCH1* transcription by activating the NF-κB and the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathways (HUANG et al., 2005) in various cell types, including endothelial cells (GROSS; LEVI, 1992). Moreover, macrophages in particular, are highly responsive to inflammatory and cellular immune stimuli, leading to an elevation in BH4 levels upon exposure to these stimuli (SAKAI; KAUFMAN; MILSTEIN, 1993). This modulation of BH4 by inflammatory stimuli suggests that BH4 is involved in immunity and the response to inflammation (STAATS PIRES et al., 2020a).

2.3 PAIN

Pain is defined as "an *unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage,*" according to the International Association for the Study of Pain (IASP) (IASP, 2017). However, nociception is defined as a neural process of encoding and processing the noxious stimuli. Given that, pain is considered a perception and not just a purely sensorial modality, considering the cognitive processing to be consciously experienced (LUMLEY et al., 2011).

Pain is a complex phenomenon that involves physical, psychological, social, and cultural dimensions, resulting from the integration of several neural systems comprising the detection of

peripheral stimuli and processing by the central nervous system (CNS) (JULIUS; BASBAUM, 2001). Moreover, pain is a conscious experience that requires cortical participation and aversive nociception information. Noxious stimuli, or recognized as such, are detected by the peripheral nervous system (PNS), encoded, unconsciously transmitted, and modulated by the CNS (WOOLF; MA, 2007).

An essential property of all sensory neurons is that they sensitize (their excitability can be increased) (ABRAIRA; GINTY, 2013). Synaptic transmission can be facilitated in response to changes in transmitter release from presynaptic terminals or transmitter responsiveness on the postsynaptic membrane (MA; WOOLF, 1996; NEUMANN et al., 1996). In the specific case of pain sensation, this facilitation of the synaptic transmission reduces the pain threshold, augmenting pain responses and spreading pain sensitivity to non-injured areas (IASP, 2017). As a consequence, pain may appear to arise spontaneously from stimuli that would not typically produce pain (allodynia) and from noxious stimuli that now evoke greater and more prolonged pain sensations (hyperalgesia) (Table 2) (IASP, 2017).

Pain treshold	The minimum intensity of a stimulus that is perceived as painful.
Sensitization	Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs.
Hyperalgesia	Increased pain from a stimulus that normally provokes pain.
Allodynia	Pain due to a stimulus that does not normally provoke pain.

Table 2. The definition of pain threshold, sensitization, hyperalgesia, and allodynia by the International Association for the Study of Pain (IASP).

According to the pathophysiological mechanism, pain can also be divided into three subtypes: i) nociceptive pain, associated with the detection of potentially harmful tissue-damaging stimuli; ii) inflammatory pain, associated with tissue damage and immune cell infiltration to promote repair, causing hypersensitivity until healing occurs; iii) pathological pain, disease state

caused by damage in the nervous system (neuropathic) or abnormal function (nociplastic) (COSTIGAN; SCHOLZ; WOOLF, 2009).

Pain may be acute or chronic. Acute pain is a normal and protective physiological response to a noxious event the nervous system detects. It is often classified as a symptom of the underlying tissue damage precipitating it (WOOLF, 2010). Acute pain can be mild to severe, lasting for a moment, weeks, or a few months until the cause of the pain is resolved. In the chronic state, pain loses its protective functions. It becomes physically debilitating, impacting daily functions like mobility and sleep, adding an emotional burden that can lead to anxiety and depression disorders, which are 2.5 times more likely to affect people with chronic pain (BRENNAN; CARR; COUSINS, 2007). However, pain is considered chronic when it continues even in the absence of harmful stimuli and persists for over three months (TREEDE et al., 2015). Then it is regarded as a disease in its own right (IASP, 2019).

Chronic pain affects 1.6 billion people worldwide, meaning 1 in 5 people suffer from chronic pain (Relieving Pain in America, 2011). Moreover, it is a debilitating condition and one of the most common reasons adults seek medical care (SCHAPPERT; BURT, 2006). It represents a high economic impact disorder, estimated that about $ 635 billion is spent each year due to lost productivity and medical expenses. The economic burden of pain is higher than the total cost of treating diabetes, heart disease, and cancer combined (Relieving Pain in America, 2011). The prevalence of chronic pain in Brazil is not available. However, studies have revealed that some regions in the Southern and Southeastern showed a prevalence of chronic pain of 40 % in the population. Furthermore, the most affected population was females, with a mean age of 41 years (Figure 4) (CARVALHO et al., 2018; SOUZA et al., 2017).

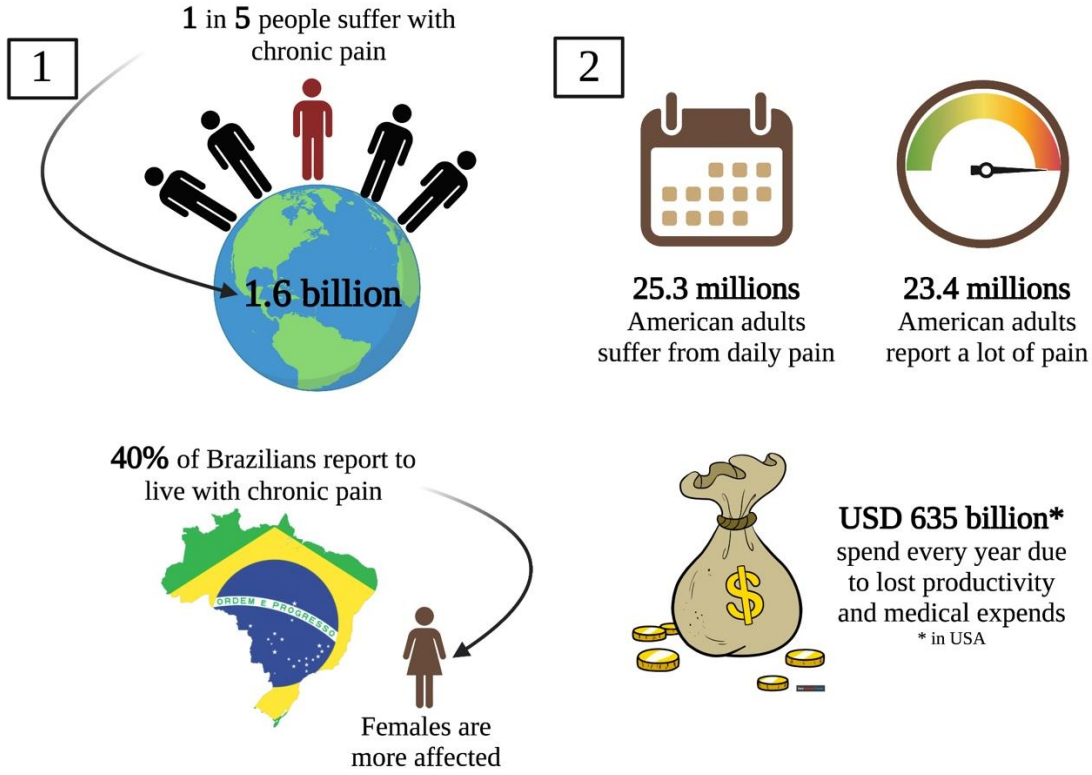


Figure 4. Chronic pain epidemiology and costs.

(1) 1 in 5 people suffer from chronic pain worldwide. It represents 1.6 billion people. In Brazil, 40 % of the population reports living with chronic pain, and women are the most affected. **(2)** 25.3 million Americans suffer daily, and 23.4 reports living with much pain. In the USA, 635 billion of dollars are spent yearly on the pain management. Source: the author, 2023

While many treatment options are available, none are universally endorsed, and many have counterproductive severe adverse effects. Opioids are the most used pharmacological therapy in the treatment of chronic pain. However, this drug class is only 30 % effective and has numerous adverse effects, mainly CNS alterations (COSTANTINO et al., 2012). Furthermore, opioids induce strong tolerance and risk of dependence, a particular concern in chronic pain management (CORDER et al., 2017).

Failure to treat chronic pain or its persistence can trigger a series of physical and psychosocial changes, such as disability (DUENAS et al., 2016), anxiety (KATZ; ROSENBLOOM; FASHLER, 2015), depression (MARGARETTEN et al., 2011) and sleep loss

(ALEXANDRE et al., 2017). There is, therefore, an imminent need to identify new biological targets and more effective therapies for managing and treating chronic pain.

2.3.1 Pain in obesity

Several studies have positively correlated the experience of pain with increased BMI (HITT et al., 2007; SOMERS; WREN; KEEFE, 2011). Obesity is hypothesized to induce pain because of excess mechanical stresses and the pro-inflammatory state, while chronic pain could contribute to obesity because of physical inactivity. Genetic, psychological, or metabolic factors may also lead to obesity and pain (SOMERS; WREN; KEEFE, 2011). However, the causal relationship between the two remains unclear: Is it obesity that causes chronic pain, or does chronic pain causes obesity? Or even other factors causing both concurrently?

Evidence suggests that people with obesity are more pain sensitive (MCKENDALL; HAIER, 1983). Obesity-related chronic pain includes widespread pain in joints and other musculoskeletal pain (YOO et al., 2014), headache (CHAI et al., 2014), abdominal pain (ESLICK, 2012), pelvic pain (GURIAN et al., 2015), and neuropathic pain (MISCIO et al., 2005), among others, and may interfere with daily functioning.

It was demonstrated in 407 adults younger than 70 years that central obesity, defined by waist circumference, was the most critical and consistent risk factor for chronic pain development (RAY et al., 2011). Moreover, individuals with a higher waist circumference were twice as likely to report chronic pain (RAY et al., 2011). In agreement, a survey of more than 1 million US residents showed that individuals with BMI class I obesity reported 68 % more pain, class II obesity had 136 % more pain, and class III obesity had 254 % more pain, compared to normal BMI individuals (STONE; BRODERICK, 2012).

Higher pain scores are observed in children (SMITH; SUMAR; DIXON, 2014), teenagers (DEERE et al., 2012), youth (HOFTUN; ROMUNDSTAD; RYGG, 2012), and adults affected by obesity (MCKENDALL; HAIER, 1983). Community studies reported the association of obesity with various pain diagnoses, including low back pain, headaches, and pelvic pain, among others. Table 3 shows the primary pain diagnoses in individuals affected by obesity, as well as age and prevalence percentage (GURIAN et al., 2015; KRUL et al., 2009; MCCARTHY et al., 2009; OKIFUJI; BRADSHAW; OLSON, 2009; TAYLOR et al., 2006; TIETJEN et al., 2007).

Age (years)	Pain	Percentage (%)	Reference
2 – 11	Musculoskeletal	63.5	(TAYLOR et al., 2006)
±10	Back	93.8	(KRUL et al., 2009)
12 -18	Knee	56.1	(TAYLOR et al., 2006)
± 40	Pelvic	54.8	(GURIAN et al., 2015)
± 42	Chronic headache	35	(TIETJEN et al., 2007)
± 44	Fibromyalgia	50	(OKIFUJI; BRADSHAW; OLSON, 2009)
≥ 70	Legs and feet	64.9	(MCCARTHY et al., 2009)
≥ 70	Harms and hands	60.1	(MCCARTHY et al., 2009)
≥ 70	Neck and shoulder	60.2	(MCCARTHY et al., 2009)

Table 3. Most frequent pain sites in individuals affected by obesity.

Obesity-affected individuals affected obesity report suffering from pain in different areas of the body, depending on their age.

Some explanations and mechanisms seek to explain the relationship between obesity and pain. The increase in body weight causes stress and mechanical pressure in the joints, predisposing to the appearance of musculoskeletal disorders (TAYLOR et al., 2006). In addition, pro-inflammatory cytokines released by the AT promote inflammation that sensitizes primary afferent neurons, contributing to hypersensitivity and exacerbation of chronic pain symptoms (GODFREY et al., 2018; STONE; BRODERICK, 2012; VOS et al., 2016; WOOLF et al., 1997).

Mechanical joint pressure is considered the leading cause of pain in individuals affected by obesity (CHRISTENSEN et al., 2006; DING et al., 2005). Joint diseases are a group of chronic conditions characterized by cartilage degeneration and changes in nearby bone structures. The long-term outcome includes decreased cartilage thickness, posture and gait changes, and pain (BLAZEK et al., 2014; NEUMANN et al., 2008). The most prevalent musculoskeletal disorder in the world is osteoarthritis (OA), which has been positively correlated with obesity (SCOTT et al., 2006). Also, it is known that the need for surgical interventions due to joint injuries in individuals with obesity is 35 % higher than in individuals with normal BMI (BOURNE et al., 2007).

Sustained overloading on the musculoskeletal structure of the lower back, hip, and knee joints prompts the development of OA in individuals affected by obesity (KING; MARCH; ANANDACOOMARASAMY, 2013). Obesity-related OA is multifactorial and involves direct joint damage and genetic, biological, and metabolic factors (DAVIS; ETTINGER; NEUHAUS, 1988). For instance, increased mechanical load changes the chondrocyte mechanotransducers signaling (HAUDENSCHILD; D'LIMA; LOTZ, 2008) and leads to the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), matrix metalloproteinases and contribute to the establishment of a pro-oxidative microenvironment (STANNUS et al., 2010). The result is the degradation of type II collagen, joint extracellular matrix, and hyaluronic acid fragmentation. All these factors can cause an imbalance between deterioration and repair of the cartilage, the apoptosis of chondrocytes, reduced synoviocytes fluid viscosity, and increased joint friction, leading to changed patients' posture and gait, reducing their mobility and increasing pain scores (JORDAN et al., 2003).

Pro-inflammatory factors suggest that only mechanical damage is insufficient to cause chronic pain in obesity (TODA et al., 1998). This hypothesis is confirmed by the fact that individuals with obesity report suffering from chronic pain not just in the body areas that load weight, i.e., hand, head, and neck, suggesting complex interactions in biomechanical and physiological factors at various levels (AGRAWAL et al., 2011).

The WAT is influenced by various chemicals and signals from the brain and nerves. These signals affect the body's energy levels and the number and characteristics of the immune, blood vessels, and structural cells (OUCHI et al., 2011). In addition to signals from the blood, adipocyte cell size, fat breakdown, and local signaling are regulated by sensory nerve terminals (BARTNESS et al., 2014).

2.4 TETRAHYDROBIOPTERIN AND PAIN

The relationship between BH4 metabolism and the induction of pain hypersensitivity was discovered in humans by identifying the presence of single nucleotide polymorphisms within or near the *GCH1* gene (LÖTSCH et al., 2007). In humans, this *GCH1* gene haplotype (15.4 % population frequency) was significantly associated with less pain after discectomy for persistent

radicular low back pain. Still, healthy homozygous individuals for this haplotype showed reduced experimental sensitivity to pain (TEGEDER et al., 2006).

One of the main clinical manifestations of obesity is chronic joint pain. Sulfasalazine (SSZ) is a compound that belongs to a class of drugs described as disease-modifying anti-rheumatic drugs used as a first-line treatment in arthritis. SSZ was discovered in 1950 and approved by the FDA for treating inflammatory diseases such as inflammatory arthritis and ulcerative colitis (HUBER et al., 1984). Initially, it was believed that the anti-inflammatory action of SSZ was through inhibiting NF-kB (O'DELL et al., 2013). However, collaborators from our research group demonstrated that the molecular mechanism of SSZ action involves the inhibition of SPR activity (CHIDLEY et al., 2011). The discovery of the molecular effects of SSZ raised the possibility that its therapeutic action would be mediated, in part, inhibiting BH4 production, consequently inducing analgesia (CHIDLEY et al., 2011; HARUKI et al., 2013; LATREMOLIERE et al., 2015). However, considering SSZ does not have optimal absorption from the gastrointestinal tract, a new SPR inhibitor was developed by our group, SPR inhibitor 3 (SPRi3) (LATREMOLIERE et al., 2015).

It has been shown *in vivo* that blocking the synthesis of BH4 reduces inflammation and hypersensitivity to pain, in addition to reducing intracellular concentrations of BH4 and increasing sepiapterin in plasma and sciatic nerve, without compromising, for example, the monoaminergic system (LATREMOLIERE et al., 2015). The inhibition of the salvage pathway by SPRi3 leads to the metabolic precursor sepiapterin accumulation in the tissues and, subsequently, secretion, allowing the follow-up of the pharmacological inhibition (FUJITA et al., 2020; LATREMOLIERE et al., 2015).

Inflammatory and neuropathic pain prevalent in individuals affected by obesity may be highly related to BH4 metabolism. BH4 synthesis increases at pathological levels in peripheral neurons in response to injury or inflammation due to the up-regulation of synthesis enzymes, which can be stimulated by pro-inflammatory cytokines and macrophage infiltration into inflamed tissues (TEGEDER et al., 2006). Thus, the inflammatory scenario promoted by the accumulated AT in obesity may activate the BH4 *de novo* pathway, pathologically increasing its levels and promoting pain hypersensitivity (Figure 5).

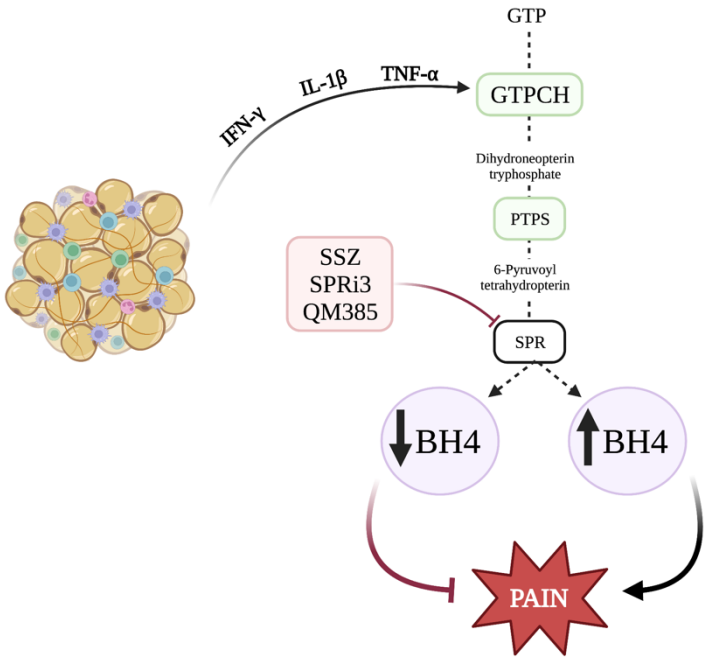


Figure 5. Increased *de novo* tetrahydrobiopterin (BH4) synthesis induced by inflammation promotes pain hypersensitivity. The expansion of adipose tissue (AT) leads to increased pro-inflammatory cytokines release. Inflammatory-inducible enzyme GTPCH is stimulated, activating the BH4 *de novo* synthesis and leading to the production of higher levels of BH4. The sepiapterin reductase (SPR) inhibitors sulfasalazine (SSZ), sepiapterin reductase inhibitor 3 (SPRi3), and QM385 inhibit SPR activity, reducing BH4 levels and resulting in analgesia. The exact molecular mechanism by which BH4 induces pronociceptive effects has yet not be fully elucidated. TNF-α (tumor necrosis factor alpha); IL-1β (interleukin-1-beta); IFN-γ (interferon gamma); GTP (guanosine 5'-tryphosphate); GTPCH (GTP cyclohydrolase I); PTPS (6-pyruvoyl-tetrahydropterin synthase); SSZ (sulfasalazine); SPRi3 (sepiapterin reductase inhibitor 3); SPR (sepiapterin reductase); BH4 (tetrahydrobiopterin). Source: the author, 2023.

2.5 PHYSICAL EXERCISE AS AN ANTI-INFLAMMATORY APPROACH

Physical exercise is considered an efficient free-drug strategy preventing and treating several chronic diseases (GARBER et al., 2011). According to the Physical Activity Guidelines for Americans, adults affected by chronic diseases should practice at least 150 to 300 min per week of

moderate-intensity exercise or 75 to 150 min per week of vigorous-intensity aerobic physical activity to improve health (SERVICES, 2018).

Regular moderate-intensity exercise enhances the immune function response, reinforces the antioxidant capacity, reduces oxidative stress, and increases the efficiency of energy generation (PETERSEN; PEDERSEN, 2005; RADÁK et al., 1999). Due to enhanced musculoskeletal function, cardiorespiratory and metabolic health, sleep, pain management, cognition, learning, memory, and more, the practice of physical exercise represents a non-pharmacological tool to reduce the incidence of inflammatory conditions, such as obesity (LLAMAS-VELASCO et al., 2016; NIEMAN; WENTZ, 2019).

It has been shown that physical exercise improves overall health and reduces all-cause mortality risk in individuals with pre-existing medical conditions, including those with a chronic inflammatory component (KELLY et al., 2014). The constant practice of physical exercise is well documented to help manage obesity and its related comorbidities due to its capacity to guide the immune system response by favoring an anti-inflammatory status and reducing the persistent pro-inflammatory response (COLBERG et al., 2010; PETERSEN; PEDERSEN, 2005). Moreover, abdominal adiposity is known to correlate positively with sedentary behavior (WEDELL-NEERGAARD et al., 2019). In this scenario, regular physical exercise reduces inflammation in obesity caused mainly by immune infiltration in WAT, and the extent of visceral AT reduction is positively correlated with the intensity and duration of exercise (WEDELL-NEERGAARD et al., 2019).

Experimental studies in mice have shown that physical exercise decreased hippocampal proliferation of microglia (VUKOVIC et al., 2012), hippocampal expression of immune-related genes (PARACHIKOVA; NICHOL; COTMAN, 2008), nuclear NF-κB activation (PARACHIKOVA; NICHOL; COTMAN, 2008), and the expression of pro-inflammatory cytokines, such as TNF-α (GOMES DA SILVA et al., 2013), IFN-γ (SVENSSON; LEXELL; DEIERBORG, 2015), and IL-1β (BOBINSKI et al., 2015). Moreover, reduction of the pro-inflammatory mediators IL-18, C-reactive protein (CRP), TNF-α and IL-1β levels (DONGES; DUFFIELD; DRINKWATER, 2010; STEWART et al., 2005), and a marked increase in IL-10 have been identified in blood from exercised individuals (Figure 6) (KADOGLOU et al., 2007).

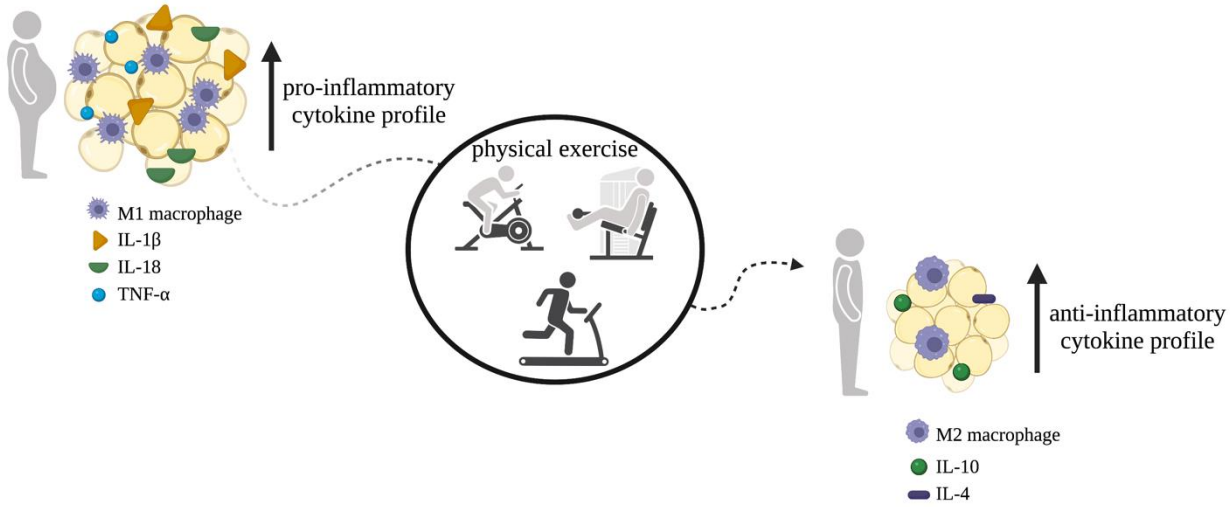


Figure 6. Role of physical exercise in obesity-linked inflammation. Chronic low-grade systemic inflammation present in obesity can be prevented and treated by the regular practice of physical exercise, which will not just reduce the size and number of adipocytes but also switch the cytokine release profile from a pro-inflammatory characterized by M1 macrophages, interleukin (IL)-1beta (IL-1β), IL-18, and tumor necrosis factor-alpha (TNF-α) to an anti-inflammatory profile with the majority presence of M2 macrophages, IL-10, and IL-4. Source: the author, 2023.

The anti-inflammatory effects of physical exercise appear to be controlled by multiple mechanisms, such as *i)* increased production of adrenaline, cortisol, and growth hormone, among others that have immunomodulatory effects by influencing leukocyte trafficking and functions (GLEESON et al., 2011; PEDERSEN; HOFFMAN-GOETZ, 2000); *ii)* visceral fat loss (WEDELL-NEERGAARD et al., 2019); *iii)* improved levels of anti-inflammatory myokines by the working skeletal muscle (PEDERSEN, 2017), and *iv)* diminishing the expression of TLRs in immune cells. Furthermore, moderate-intensity physical exercise, similar to ROS, can stimulate eustress levels, significantly promoting healthy adaptations (SANCHIS-GOMAR et al., 2012). Thus, exercise promotes cellular antioxidant defenses and optimal oxidative capacity in various tissues, including the skeletal muscle (RADÁK et al., 1999; SCHEFFER et al., 2019), the AT (KAWANISHI et al., 2010), and the brain (SPECK et al., 2014).

ORIGINAL RESEARCH ARTICLE: DEVELOPMENT OF PAIN HYPERSENSITIVITY AND INCREASED URINARY TETRAHYDROBIOPTERIN LEVELS IN MICE SUBMITTED TO HIGH FAT DIET

(P3) Obesity (impact factor 7.675):

Development of pain hypersensitivity and increased urinary tetrahydrobiopterin levels in mice submitted to high fat diet.

Tuany Eichwald[1], Leonardo Barros[1], Alexandre Francisco Solano[1], Débora da Luz Scheffer[1], Ananda Christina Staats Pires[1], Joana Margarida Gaspar[1], Alexandra Latini[1] (Manuscript submitted to Antioxidants).

Obesity-linked chronic low-grade inflammation is associated with the expansion and accumulation of WAT. AT is a complex metabolic and endocrine organ, and adipocyte hyperplasia/hypertrophy promotes the secretion of pro-inflammatory cytokines and adipokines. For example, the AT release of MCP-1 attracts monocytes. It promotes their differentiation into M1 macrophages (ARNER et al., 2012), favoring the production of a pro-inflammatory environment characterized by increased TNF-α, IL-6, IFN-γ, and IL-1β (STRISSEL et al., 2010). Furthermore, IL-1β can further activate nociceptors and induce hypersensitivity to pain (BINSHTOK et al., 2008).

In addition to the *de novo* synthesis of BH4 being controlled by inflammatory mediators, such as IL-1β and IFN-γ, excessive concentrations of BH4 are also produced by macrophages that infiltrate damaged nerves and inflamed tissue (LATREMOLIERE et al., 2015). The relationship between BH4 metabolism and the induction of pain hypersensitivity was discovered for the first time in humans by identifying the presence of single nucleotide polymorphisms within or close to the *GCH1* gene that codes for the GTPCH enzyme. These haplotypes were positively associated with a marked reduction in clinical pain scores and a decrease in *GCH1* expression (LÖTSCH et al., 2007).

This relationship led our group to study the BH4 metabolism in experimental inflammatory and neuropathic chronic pain models (CRONIN et al., 2018; FUJITA et al., 2020; LATREMOLIERE et al., 2015). Thus, the inflammatory environment promoted by the enlargement

of AT seems favorable for the over-stimulation of BH4 metabolism and, therefore, the development of pain hypersensitivity. While there is some research investigating the relationship between obesity and pain and between pain and BH4, remains a significant gap in the understanding of the correlation between BH4 levels and pain hypersensitivity in obesity. Finally, the validation of physical exercise as a tool to mitigate the detrimental effects linked to obesity, including pain, provides crucial support for its anti-inflammatory effects, underscoring the significance of exercise in preventing conditions associated with inflammation.

Key Results & Implications

It is presented here increased urinary BH4 levels and pain hypersensitivity development in mice with increased WAT induced by HFD in mice. The main results were: *i)* higher body fat and visceral WAT accumulation; *ii)* impaired glucose tolerance and higher fasting glucose; *iii)* higher chemical and thermal hyperalgesia; *iv)* augmented levels of urinary BH4; *v)* prevention of the alterations named above by physical exercise. The concomitant increase of AT, pain, and BH4 levels is a novel finding, and it is possible to suggest that BH4 levels can be considered a biomarker of inflammatory-mediated pain in obesity. Moreover, the findings presented in this study agree with the literature that proposes that the blockage of BH4 synthesis is a potential tool for pain management. Finally, the physical exercise proved to have anti-inflammatory properties, reducing or normalizing the deleterious effects elicited by HFD.

Development of pain hypersensitivity and increased urinary tetrahydrobiopterin levels in mice submitted to high fat diet

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Abstract

Obesity is characterized by excessive fat accumulation and is associated with low-degree chronic inflammation. Obesity predisposes to the development of several other chronic conditions or comorbidities, including chronic pain. We have previously shown that the levels of tetrahydrobiopterin (BH4) are increased in biological fluids and tissues of painful inflammatory conditions, in multiple animal models and in humans. This study aimed to examine the impact of obesity induced by high fat diet (HFD) on pain sensitivity and BH4 levels in mice. The effect of moderate-intensity physical exercise, used as an anti-inflammatory non-pharmacological intervention, on pain scores was also investigated. We showed that HFD increased weight gain, visceral white adipose tissue accumulation, and the percentage of body fat. These anthropometric alterations led to impaired glucose tolerance at 4 and 8 weeks of the dietary intervention. It was also observed reduced locomotor activity and higher pain scores in HFD-fed mice that were prevented by physical exercise. HFD also induced the increase of urinary BH4 levels at 4 and 8 weeks of intervention. Altogether, it is plausible to propose the use of urinary BH4 levels as a biomarker for increased nociception in obesity.

Keywords: obesity, adipose tissue, sepiapterin reductase inhibitors, chronic pain, biomarker.

1 Introduction

Obesity is defined by the World Health Organization (WHO) as abnormal and excessive accumulation of fat. The increase in white adipose tissue (WAT) from a daily positive energy balance is associated with low-grade chronic systemic inflammation, with immune infiltration in the WAT. Obesity is a multifactorial etiology disease, and the clinical manifestations involve chronic pain and joint dysfunction [1]. Obesity predisposes the development of a large number of other chronic diseases, including inflammatory and degenerative conditions of the musculoskeletal system and the peripheral and central nervous systems [2–4]. The inflammatory pathways are also persistently activated in several brain regions, including the mid-basal hypothalamus, which exerts control over the peripheral regulation of energy, glucose, and lipid metabolism [5].

BH4 is a pterin that acts biologically as an obligatory cofactor for the metabolism of phenylalanine and certain lipids, and for the biosynthesis of the transmitters dopamine, serotonin and nitric oxide (NO) [6,7]. BH4

intracellular concentrations are finely tuned by three metabolic pathways, assuring continue basal levels of the molecule to supports the different system where it is involved. However, excessively increased BH4 levels have been associated with numerous pathological conditions, including cardiovascular disease [8], cancer [9], and chronic pain [10,11]. Furthermore, inflammatory conditions positively modulate the synthesis of BH4, in immune, nerve cells and others [12,13].

Pain is a conscious experience that demands cortical participation and aversive information from nociception, which comprises the mechanisms by which harmful stimuli, or recognized as such, are detected by the peripheral nervous system, encoded, transmitted, and unconsciously modulated by the nervous system [14,15]. The immune and pain-signaling systems are evolutionarily designed to protect the organism by responding to danger [14,16,17]. Normally, the adaptive response against the stimuli that activate both systems overcome the threat and reach the resolution [18]. However, the maladaptive inflammatory reactions, in which pro-inflammatory mediators persistently activate and sensitize neurons at different levels of the nociceptive pathway, is believed to induce chronic pain [19–22]. Thus, this work aimed to identify whether obesity, a low-degree chronic inflammatory disease, may negatively modulate nociceptive thresholds and positively increase BH4 levels that can be monitored in biological fluids as a biomarker for pain.

2 Material and methods

2.1 Animals

Adult male C57BL/J6 mice (3–5 months of age; body mass 45–50 g) from the central animal house of the Centre for Biological Sciences, *Universidade Federal de Santa Catarina* (UFSC) (Brazil) were kept in a controlled environment (22 ± 1 °C, 12 h light/dark cycle) with water and food ad libitum, for ten days (acclimatation period). The experimental protocols were approved by the Ethics Committee for Animal Research (CEUA, 4401201118) of UFSC. All procedures were performed under current guidelines for the care of laboratory animals and ethical guidelines for investigations of experimental pain in conscious animals [23].

2.2 Experimental strategy

Mice were fed with a high fat rich diet (HFD) to induce increased adiposity, and to be used as a proxy of the human condition obesity. C57BL/J6 mice were fed with HFD for 8 weeks. The diet's macronutrient composition was proteins: 20 kcal %; carbohydrates: 35 kcal %; lipids: 45 kcal %; while the micronutrient composition of the HFD was corn starch: 30.3 kcal %; casein: 21.35 kcal %; l-cystine: 0.3 kcal %; dextrinized starch: 10 kcal %; sucrose: 10 kcal %; soy oil: 4 kcal %; lard: 14.3 kcal %; microcrystalline cellulose: 5 kcal %; mineral mix AIN 93: 3.5 kcal %; vit mix AIN 93: 1 kcal %; choline bitartrate: 0.25 kcal %; butylated hydroxytoluene: 0.005 kcal % (Pragsoluções Biociências, Jaú, SP). It has extensively been shown that HFD induced hyperglycemia, oral glucose intolerance, insulin resistance and white adipose tissue (WAT) accumulation in Swiss and C57BL/J6 mice [24,25]. Animals fed with standard diet were used as controls.

2.3 Intraperitoneal glucose tolerance test

The intraperitoneal (i.p.) glucose tolerance test (GTT) was performed after 6 h of fasting, without the use of anesthesia. The animals had the tip of the tail sectioned for the collection of blood drops. The first measurement was performed at time 0 (baseline). Then, a glucose solution 2 g/kg of body weight at 36 ºC was injected i.p. Blood samples from the tail were subsequently collected at 5, 10, 15, 30, 60 and 120 min to determine blood glucose concentrations (adapted from [26]). The test was performed before the dietary intervention, at 4 and 8 weeks.

2.4 Physical exercise

To prevent HFD-induced WAT accumulation, a group of animals was also submitted to physical exercise as described below.

2.4.1 Incremental test to determine the maximal capacity for exercise

The incremental test was performed to identify 60 % of the maximal capacity of the animals (**Supplementary Figure 1A**). The protocol had a habituation phase for three consecutive days where the animals remained on the treadmill at a speed of 16 m/min for 10 min without inclination. After the third day of habituation, the animals

rested for 24 h and then performed an incremental test (fifth day). The test started at a speed of 16 m/min with a 2 % inclination. The speed was increased by 2 m/min every 3 min until voluntary exhaustion, which is defined as the animal giving up physical exercise [27,28]. When exhaustion was reached, 25 μL of caudal blood was collected from the animal's tail to measure lactate levels (see item 2.4.2.1 Caudal blood collection).

During the first week of training, the animals were daily evaluated for performance and were classified according to their score: 1 for animals that refused to run; 2 for animals that ran at variable speeds, run, and stop; 3 for animals that ran regularly; 4 for animals that are runners; 5 for animals that are good runners. Only animals that scored 3 or more continued the training [29].

2.4.2 Physical exercise protocol

After the incremental test was performed (**Supplementary Figure 1A**), the mice rested for 24 h. Subsequently, they performed three consecutive days of habituation at low intensity (40 %) for 10, 20, 30 min respectively, at 2 % inclination. Then the animals had a rest of 24 h before starting the exercise protocol.

The physical exercise protocol consisted of five training sessions per week for six weeks on an adapted treadmill with an interval of 48 h each week (see Table 1) [30]. The animals performed a 5 min warm-up at low intensity (40 %) before the start of each training session. No noxious stimulus was used to encourage the animal to run. The physical exercise speed was defined as 60 % of the final speed found in the incremental test, which corresponds to moderate intensity [31] in the first 3 weeks, and with a progressive increase in training time at each session (35, 40, 45 and 45 min/day), respectively. At the end of the first 3 weeks, a new incremental test was performed to equalize the intensity of physical exercise for the last 2 weeks, thus avoiding the principle of adaptation. The last 2 weeks again had a progressive increase in training time at each session (35, and 40 min/day), respectively.

The adaptation period, incremental test, habituation, and exercise program were carried out from 6 pm, a period that corresponds to the light cycle of the animals.

Table 1. Physical exercise protocol

Training weeks	Session duration (min)	Treadmill inclination (%)	Activity intensity (%)
1	35	2	60
2	40	2	60
3	45	2	60
Effort test to equalize the intensities and avoid adaptation			
5	35	2	60
6	40	2	60

2.4.2.1 Caudal blood collection

Immediately at the end of the incremental test, 25 µL of caudal blood were collected with a heparinized capillary, through a small cut in the distal portion of the animal's tail. For this, the animal was restrained with the tail exposed. Asepsis was performed with 70 % alcohol by sliding the cotton longitudinally from the base to the end. Blood collection was done with a cross-section, ± 2cm from the end of the tail with the aid of a scalpel. Blood flow was stopped after collection by pressing the incision with sterile gauze [32]. Blood was used to measure lactate concentrations (**Supplementary Figure 1B**).

2.4.2.2 Lactate measurement

Caudal blood was collected right after the voluntary mice's exhaustion in plastic tubes containing 50 µL of 1 % sodium fluoride to inhibit the glycolytic pathway. To measure lactate, a specific analyzer YSL 2700 (YSL 2700, Yellow Springs, CA, USA) was used. High blood lactate concentrations indicated the intensity of the protocol applied.

2.5 Behavioral tests

Animals were transported to the acclimatized experiment room 1 h before the beginning of the behavioral tasks, or according to the needs of each test. Assessments were performed during the light phase of the rodent's cycle.

2.5.1 Locomotor activity

The locomotor activity was evaluated in the open field arena. The open field was made of wood covered with impermeable Formica (100 cm × 100 cm × 50 cm) and the experiments were performed in a sound-attenuated room under low-intensity light. Each animal was placed in the center of the apparatus and the exploratory activity was registered for 5 min [33]. The tests were videorecorded and analyzed by the ANY-mazy Platform™ [34]. Animals were evaluated before (baseline) and after (eight week) the induction of WAT accumulation by HFD.

2.5.2 Motivational behavior

The self-care and motivational behaviors were assessed by measuring the time spent in grooming behavior after mice being squirted with 1 mL of a 10 % sucrose solution on the dorsal coat, as previously reported [35,36]. The time spent in grooming activities was recorded for a period of 5 min in an observation device which consisted of an acrylic box (20 cm x 20 cm x 20 cm). A camera located above the box pointing down was used to observe the animal behavior. The amount of grooming was scored after the experiment had been completed from video, by an observer blind to treatment using a stopwatch [36]. Animals were evaluated before (baseline) and after (eight week) the induction of WAT accumulation by HFD.

2.5.3 Mechanical hypersensitivity

All sensory testing was performed between the hours of 9 am and 6 pm in an isolated room maintained at 22 ± 2 °C and 50 ± 10 % humidity. For mechanical threshold the von Frey filaments were used. Mice were brought from the animal colony and placed in transparent plastic boxes on a metal mesh floor with 5 x 5 mm holes (Ugo Basile, Italy). Mice were then habituated for at least 30 min prior to testing. To assess mechanical sensitivity, the withdrawal threshold was measured using a series of filaments (0.20, 0.40, 0.70, 1.6, 3.9, 5.9, 9.8 and 13.7 mN, Stoelting, Wood Dale, IL, USA; equivalent in grams to 0.02, 0.04, 0.07, 0.16, 0.40, 0.60, 1.0 and 1.4). The 50 % withdrawal threshold was determined using the ‘up-down’ method and calculated using Up-Down Reader software [37]. A brisk hind paw lift or flinch in response to von Frey filament stimulation was regarded as a withdrawal response. The 0.4 g filament was the first stimulus to be used, and when a withdrawal response was obtained, the next weaker filament was used. This process was repeated until no response was obtained, at which

time the next stronger filament was administered. All behavioral testing was performed by an investigator who was blind to the treatment of the mice [37]. Animals were evaluated before (baseline) and after 2, 4, 6, and 8 weeks after the induction of WAT accumulation by HFD.

2.5.4 Thermal hypersensitivity

To assess thermal hypersensitivity to heat, the hot plate test (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) was used. Mice were placed in an acrylic cylinder (40 cm high x 20 cm in diameter) on the surface of a previously heated metal plate (50 ± 2 °C). The latency that the animal took to stand up, shake and/or lick one of the hind legs on the previously heated plate was considered as an indication of thermal hypersensitivity. To avoid tissue damage, the maximum time the animals remained on the heated plate was 40 s [38]. Animals were evaluated after the induction of WAT accumulation by HFD.

2.5.5 Chemical hypersensitivity

Chemical hypersensitivity was assessed using the capsaicin test. A volume of 20 uL containing 1.6 µg capsaicin was injected under the skin of the dorsal surface of the right hind paw using a micro syringe. The animal was then placed in a transparent glass chamber (with a mirror behind it to allow clear observation of the paws) and the observation period began. The latency and the number of times the animals showed the behavior of licking and/or shaking the paw, fingers, or leg where the capsaicin was injected was timed. Mice were individually observed for 5 min [39]. Animals were evaluated after the induction of obesity by HFD.

2.6 Adipose tissue dissection

Animals were euthanized, following the ARRIVE guidelines, to collect blood and visceral WAT.

Supplementary Figures 2A, **2B, and 3B** show the accumulated WAT in a representative image taken from a mouse from each experimental group.

2.7 BH4 quantification by HPLC

Urinary BH4 levels were determined by high-performance liquid chromatography (HPLC) coupled with electrochemical detection as previously described with some modifications [9,11]. The HPLC measurement of BH4 was carried out in a HPLC (Alliance e2695, Waters, MA, USA) by using a Waters Atlantis dC18, reverse phase column (4.6 mm × 250 mm; 5 μm particle), with a flow rate set at 0.7 mL/min and an isocratic elution of 6.5 mM NaH_2PO_4, 6 mM citric acid, 1 mM sodium octyl sulfate, 2.5 mM diethylenetriaminepentaacetic acid, 160 μM dithiothreitol and 12 % acetonitrile, pH 3.0. The temperature of the column compartment was set at 35 °C. The identification and quantification of BH4 was performed by coupling to the HPLC an electrochemical detector (module 2465, Waters, MA, USA) with the voltage set at + 450 mV. The results were expressed as μmol/mmol of creatinine.

2.8 Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were analyzed by one-way or two-way ANOVA followed by the *post hoc* test of Šídák when *F* was significant. When comparing two independent groups, one tail Student's *t*-test was used. The accepted level of significance for the tests was $P \leq 0.05$. Statistics and all graphs were performed by using GraphPad Prism 9®.

3 Results

Figure 1 shows the effect of HFD on caloric consumption, body weight and fat accumulation. **Figure 1A** shows that caloric consumption was significantly lower in animals submitted to HFD. However, **Figure 1B** shows that the weight gain delta, calculated as the final body weight minus the initial body weight, was increased in HFD fed mice [$t_{(11)} = 1.77$; $P \leq 0.05$]. In agreement, the energy efficiency of the diets (**Figure 1C**) was shown to be higher in HFD-fed mice [$t_{(11)} = 2.28$; $P < 0.05$]. **Figure 1D** shows that the weight of the visceral WAT was significantly higher in HFD-fed animals [$t_{(11)} = 3.38$; $P < 0.01$]. Similarly, the relationship between visceral WAT and total body weight was increased in the group of animals fed with HFD (**Figure 1E**) [$t_{(11)} = 4.42$; $P < 0.001$]. **Supplementary Figures 2A** and **2B** are representative images from a mouse from each experimental group.

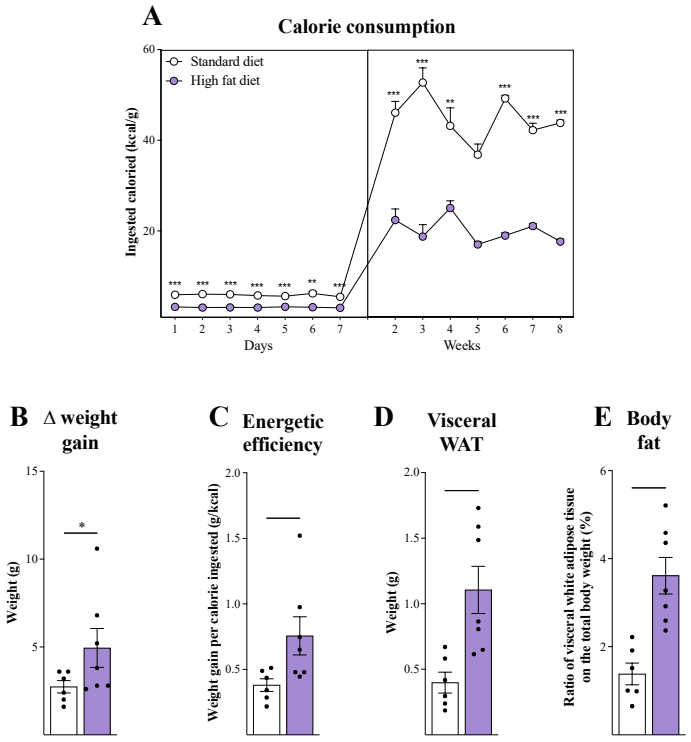

Figure 1. Visceral white adipose tissue (WAT) accumulation and higher body fat in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). Animals in the control group received a standard rodent diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). (**A**) Daily calorie intake. (**B**) Delta of weight gain between final weight and initial weight. (**C**) Energy efficiency calculated by dividing the energy content (in kcal) by weight of foods (in g) consumed. (**D**) Accumulated visceral WAT after 8 weeks of intervention. (**E**) Relationship between visceral WAT and body weight. ANOVA for repeated measures followed by Šídák *post hoc* test for multiple comparisons for A. One tail Student's *t*-test for B, C, D, and E. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (n= 6-7 animals per group).

GTT and the fasting glucose test were performed at three different times, baseline (pre-intervention), 4 and 8 weeks after intervention to identify insulin resistance (**Figure 2**). Caudal blood collection for GTT was performed before glucose administration, and at 5, 10, 15, 30, 60 and 120 min after i.p. injection of 2 g of glucose per kg of body weight. **Figure 2A** shows that GTT was identical in both experimental groups before the dietary

intervention. The area under the curve (**Figure 2B**) and fasting glucose levels (**Figure 2C**) were also not different. **Figure 2D** shows that GTT was not altered after 4 weeks of intervention; however, the area under the curve shown in **Figure 2E** was significantly bigger in the HFD group [$t_{(11)}$= 4.90; $P < 0.001$], as well as fasting blood glucose levels (**Figure 2F)** [$t_{(11)}$= 1.903; $P \leq 0.05$]. **Figure 2G** shows significant increase in glycemia values at 15 min [$F_{(6,66)}$= 4.68; $P < 0.001$], and the values under the curve [$t_{(11)}$= 2.774; $P < 0.01$] (**Figure 2H**) after the glucose i.p. injection in HFD-fed mice. However, fasting glycemia at week 8 (**Figure 2I**) was not different between groups.

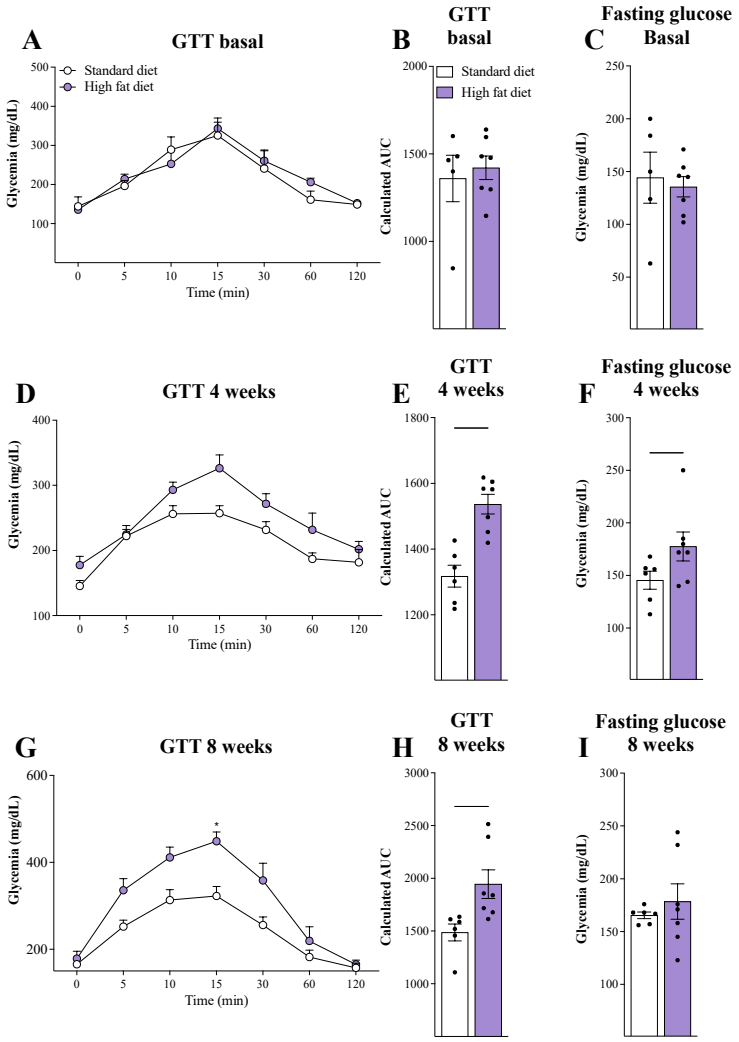

Figure 2. Glucose tolerance test (GTT) impairment in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). Control mice received a standard diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). (**A, D, G**) Glucose tolerance test (GTT), (**B, E, H**) area under the curve of the GTT, and (**C, F, I**) fasting glucose were measured before the intervention, and at 4 and 8 weeks afterwards, respectively. ANOVA for repeated measures followed by Šídák *post hoc* test for multiple comparisons for A, D and G. One tail Student's *t*-test for B, C, E, F, H, and I. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (n= 6-7 animals per group).

Figure 3 shows the influence of HFD on motivational behavior and spontaneous locomotor activity. **Figures 3A-C** show that the performance in the sucrose test, that is a task used to assess motivation, depression, and anhedonia by measuring first lifting and/or cleaning movement evoked by the challenge (**Figure 3A**), and total time spent in grooming activity (**Figure 3C**) was not altered in HFD-fed mice. However, total time spent in grooming activity was longer in the group that received the standard diet after 8 weeks of intervention [$F_{(2,20)}$= 0.80; $P \leq 0.05$] (**Figure 3B**). To identify whether the diet would cause changes in locomotor activity, the open field test was performed before the intervention and at the end of the 8 weeks of experimentation. **Figure 3H** shows that animals fed with HFD for 8 weeks spent less time in the center of the apparatus [$F_{(2,23)}$= 0.11; $P \leq 0.05$]. No significant differences were observed between groups in total distance traveled (**Figure 3D**), crossing (**Figure 3E**), average speed (**Figure 3F**), maximal speed (**Figure 3G**), and time in the periphery (**Figure 3I**).

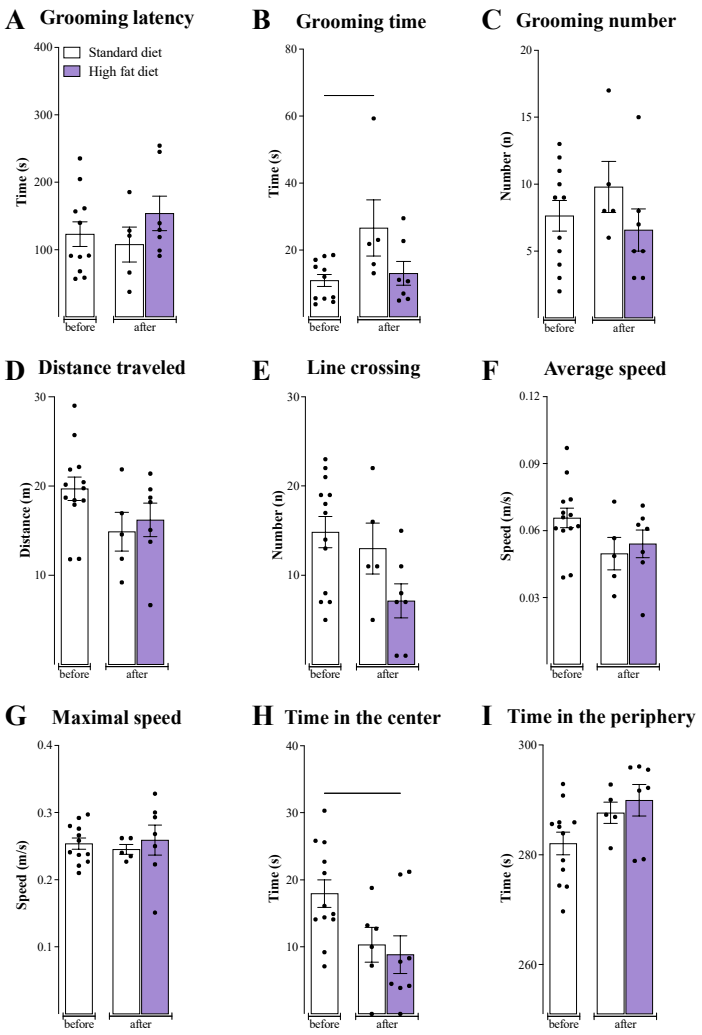

Figure 3. Motivational behavior and spontaneous locomotor activity was not altered in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). Animals in the control group received a standard rodent diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). (**A**) Latency, (**B**) total time and (**C**) number of grooming evoked by spraying sucrose on the back. (**D**) Total distance traveled, (**E**) number of intersections, (**F**) average speed, (**G**) maximum speed, (**H**) time spent in the center of the apparatus, and (**I**) time spent on the periphery of the apparatus. One-way ANOVA followed by Tukey *post hoc* test. * $P \leq 0.05$ (n= 5-11 animals per group).

To investigate whether the HFD would induce nociceptive changes under different noxious stimuli, mice were evaluated for the thresholds of chemical hyperalgesia after the injection of capsaicin, thermal and mechanical hyperalgesia. Chemical and thermal hyperalgesia were assessed after 8 weeks of intervention, while mechanical hyperalgesia was determined before the intervention, and at weeks 2, 4, 6 and 8 after intervention. **Figure 4A** shows that there was no difference between groups in the latency for the animals' first response evoked by capsaicin. However, total response time evoked by capsaicin was higher in animals fed with HFD [$t_{(9)}$= 2.11; $P \leq 0.05$] (**Figure 4B**). **Figure 4C** shows that there was no significant difference in the reflexes evoked by the thermal stimulus. HFD-fed mice showed lower thresholds for mechanical hyperalgesia at weeks 2, 6 and 8 post-intervention (**Figure 4D**). To investigate whether BH4 levels are increased when nociceptive thresholds are reduced, the levels of the pterin were measured in the urine of the animals. **Figure 4E** shows that higher levels of BH4 were found in the urine of HFD-fed animals at 4 weeks [$t_{(5)}$= 3.73; $P < 0.01$], and 8 weeks [$t_{(4)}$= 3.32; $P < 0.01$] after the intervention.

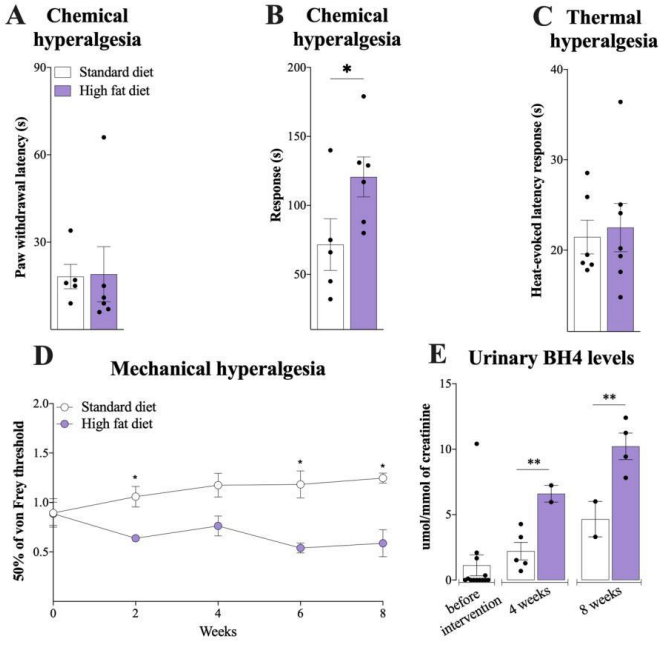

Figure 4. Induction of hyperalgesia and increased urinary tetrahydrobiopterin (BH4) levels in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein,

35 kcal % carbohydrates and 45 kcal % lipids). Control mice received a standard diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). (**A**) First response latency, and (**B**) total response time evoked by hind paw subcutaneous injection of capsaicin. (**C**) Latency for the first response evoked by the heat of the hot plate at 50 ºC. (**D**) 50 % of the von Frey threshold used to determine mechanical hyperalgesia evaluated before, and at weeks 2, 4, 6 and 8 after the intervention. (**E**) BH4 concentrations in the urine before, and at 4 and 8 weeks after the intervention. ANOVA for repeated measures followed by Šídák *post hoc* test for multiple comparisons for D. One tail Student's *t*-test for A, B, C, and E. * $P \leq 0.05$; ** $P \leq 0.01$ (n= 5-7 animals per group).

Our group has previously reported that blood mononuclear cells from children affected by obesity presented increased DNA methylation [40]. Thus, to verify whether the level of DNA methylation was responsible for the increased BH4 levels in the urine, the degree of methylation of the promoter of genes involved in BH4 biosynthesis was assessed. **Figure 5** shows that the percentage of methylation of the promoters for *Dhfr* (**Figure 5A**), *Spr* (**Figure 5B**) and *Ptps* (**Figure 5C**) was not changed in mice receiving HFD.

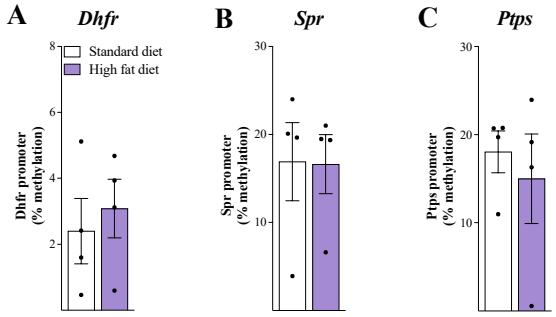

Figure 5. DNA methylation of the promoters for genes involved in tetrahydrobiopterin (BH4) biosynthesis was not altered in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). Animals in the control group received a standard rodent diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). The percentage of promoter methylation for (**A**) *Dhfr*, (**B**) *Spr* and (**C**) *Ptps* were measured after 8 weeks HFD. One tail Student's *t*-test (n=4 animals per group).

HFD-fed mice were also submitted to moderate-intensity physical exercise for 5 weeks, starting three weeks after initiating the dietary intervention. Prior to the initiation of the exercise sessions, an incremental test was conducted to determine the speed in the treadmill at which the animal is at 60 % of its maximal capacity. **Supplementary Figure 1A** illustrates the results of the incremental test, represented by the percentage of success, and **Supplementary Figure 1B** shows increased lactate concentrations after the completion of the incremental test, in agreement with the exhaustion of the mice. **Figure 6** shows the effects of physical exercise on caloric intake, body weight and visceral WAT accumulation. As shown in **Figure 6A** caloric consumption was higher after 2 weeks of performing exercise, although no differences were observed in the percentage of weight gain (**Figure 6B**), and in the weight gain delta (**Figure 6C**). Physical exercise caused a decrease in visceral WAT weight [$t_{(11)}$= 2.56; $P \leq 0.05$] (**Figure 6D**), and body fat [$t_{(11)}$= 2.64; $P \leq 0.05$] (**Figure 6E**) after 6 weeks of the physical exercise intervention. **Supplementary Figures 3A** and **3B** are representative images from a mouse from each experimental group.

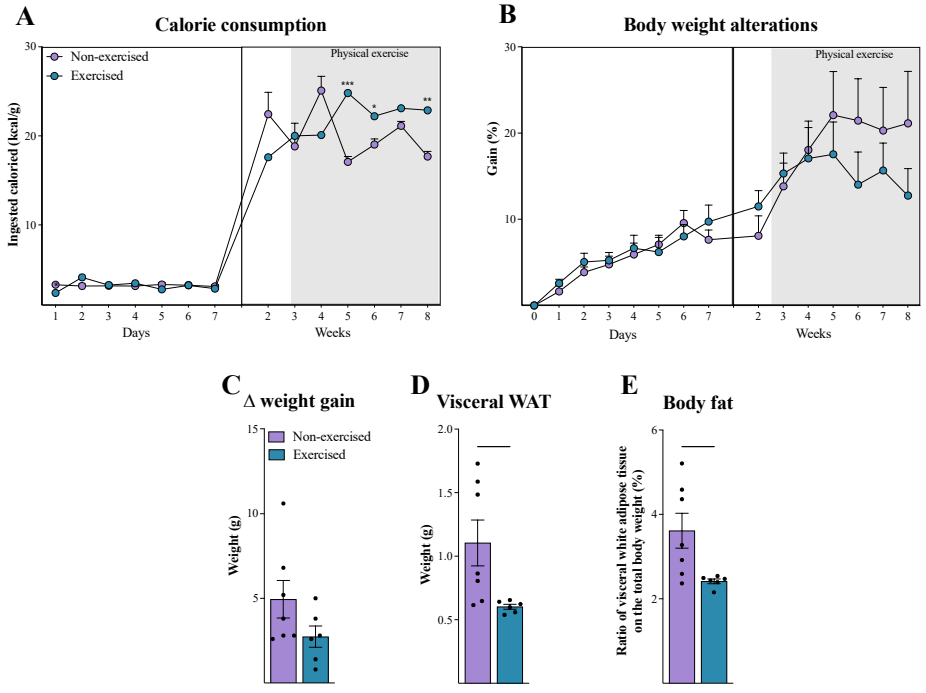

Figure 6. Physical exercise reduced visceral white adipose tissue (WAT) and body fat in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive

weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). After 2 weeks of the dietary intervention, a group of mice were also submitted to physical exercise 5 times per week for 6 weeks (for details see M&M). (**A**) Calorie intake, and (**B**) body weight gain were assessed daily for the first week of the treatment, and weekly for the next 7 weeks. (**C**) Delta of weight gain between final weight and initial weight. (**D**) Visceral WAT in grams after 8 weeks of interventions (8 weeks of dietary intervention plus 6 weeks of concomitant physical exercise). (**E**) Relationship between visceral WAT and body weight. ANOVA for repeated measures followed by Šídák *post hoc* test for multiple comparisons for A and B. One tail Student's *t*-test for C, D, and E. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (n= 6-7 animals per group).

Figure 7 shows the effects of physical exercise on GTT and nociceptive parameters after the interventions, diet plus exercise. **Figure 7A** shows that the GTT was significant decreased after 15 min of the glucose challenge in exercised mice submitted to HFD [$F_{(6,66)}= 2.74$; $P \leq 0.05$]. Similarly, the area under the curve was also reduced [$t_{(11)}= 2.41$; $P \leq 0.05$] (**Figure 7B**). However, fasting glucose (**Figure 7C**) was not different between groups at the end of the interventions. When the nociceptive thresholds were assessed, no differences were observed in (**Figure 7D**) chemical or thermal hyperalgesia (**Figure 7E**). However, mechanical hyperalgesia was reduced at week 6 (**Figure 7G**) in exercised mice submitted to HFD [$t_{(11)}= 18.21$; $P < 0.01$].

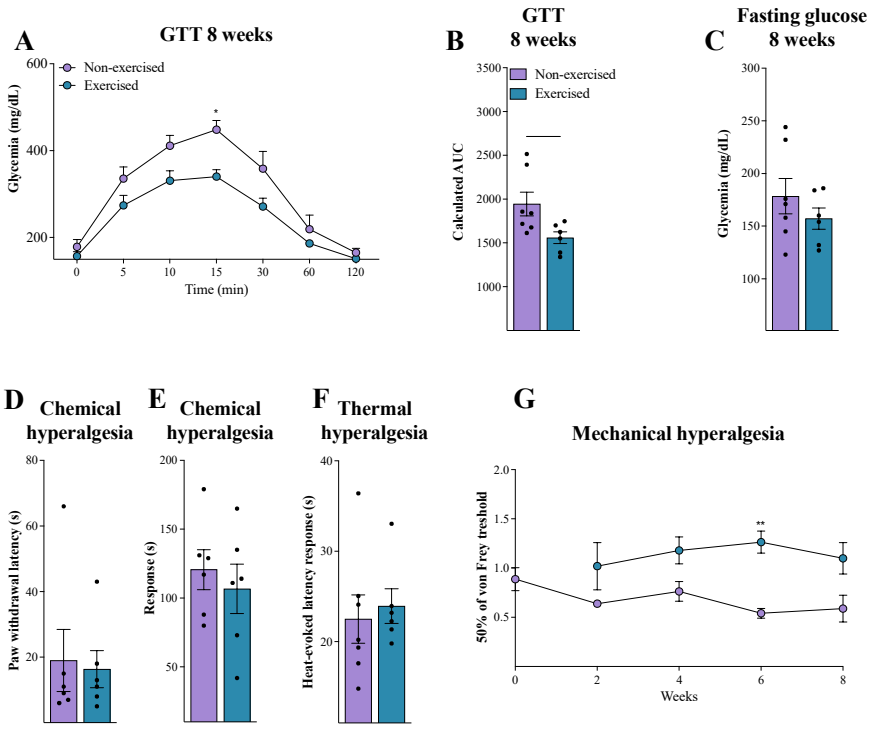

Figure 7. Physical exercise normalized the glucose tolerance test and hyperalgesia evoked by mechanical stimuli in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). After 2 weeks of the dietary intervention, mice were also submitted to physical exercise 5 times per week for 6 weeks (for details see M&M). (**A**) Glucose tolerance test (GTT), (**B**) area under the curve of the GTT, and (**C**) fasting glucose were measured after the interventions (week 8). (**D**) First response latency, and (**E**) total response time evoked by an injection of capsaicin in the right paw (for details see M&M). (**F**) Latency for the first response evoked by the heat of the hot plate at 50 ºC. (**G**) 50 % of the von Frey threshold used to determine mechanical hyperalgesia evaluated before, and at weeks 2, 4, 6 and 8 after the interventions. ANOVA for repeated measures followed by Šídák *post hoc* test for multiple comparisons for A and G. One tail Student's *t*-test for B, C, D, E and F. * $P \leq 0.05$; ** $P \leq 0.01$ (n= 6-7 animals per group).

4 Discussion

Obesity is a chronic disease characterized by excessive accumulation of WAT, which is mainly due to increased consumption of dietary fats and a lifestyle with more sedentary behaviour [41]. A better understanding of the

elements involved in the pathophysiology of obesity is fundamental, enabling the emergence of new therapies for the treatment of this disease. Animal models are essential tools for studying the pathogenesis of diseases, including the increased adiposity seen in obesity.

HFD-fed mice are commonly used to study potential treatments or the physiopathology of obesity because the induced metabolic alterations are similar to those seen in individuals affected by the disease [42]. Here we showed that HFD promoted visceral WAT deposition, increased visceral WAT to total body weight ratio, and increased body weight in adult male C57BL/J6 mice. The energy efficiency of the diets showed higher weight gain per calorie ingested in HFD-fed mice. However, the weekly food consumption showed that, on average, 24 g of standard diet and 13 g of HFD were consumed, resulting in 50 kcal/g and 20 kcal/g respectively. That resulted in a lower caloric intake in HFD group.

Obesity is evidenced by adiposity in visceral and subcutaneous WATs [42]. Considering that lipids are significant sources of energy and that adipocytes have great potential for hypertrophy and hyperplasia in response to fat ingestion, the saturated fat in HFD plays a fundamental role in the formation of large deposits of body fat [43]. Our results are in agreement with other studies that fed mice with HFD and observed increased body weight gain, visceral fat accumulation and changes in the lipid profile [44].

The adipose tissue is a complex, essential, and highly active metabolic and endocrine organ that constitutes a source of hormones, peptides, cytokines, and adipokines [5]. Adipocytes in WAT release monocyte chemoattractant protein-1 (MCP-1), which attracts monocytes and favors their differentiation into pro-inflammatory polarized M1 macrophages forming “crown-like structures” around the WAT [45]. This mechanism leads to the dead of adipocytes and increased local levels of TNF-α, IL-1β and IL-6 [46]. Furthermore, adipocytes tend to rupture during obesity due to the limited capacity for expansion, leading to apoptosis, and consequently, to sustained inflammation [47]. In this scenario, it is known that the pro-inflammatory IL-1β released by adipocytes can rapidly and directly activate nociceptors to generate action potentials and induce hypersensitivity to pain [48]. WAT is innervated by sensory neurons [49] and presents bidirectional communication with the brain through afferent and efferent sensory fibers. Adipocyte size, lipid mobilization and paracrine secretion are controlled by nerve endings in WAT [50]. Thus, the sensory neurons that innervate the WAT are involved in the

production of cytokines and the influx of immune cells, playing a central role in the low-grade inflammation observed in obesity [50]. The increase visceral WAT in mice fed with HFD that we observed plays an important role in the development of mechanical hypersensitivity. Mechanical hyperalgesia can be triggered by the process of hypertrophy and hyperplasia of adipocytes in an inflammatory state. IL-1β is also a stimulus that massively activates BH4 synthesis via the *de novo* pathway, sensitizing nociceptive fibers and contributing to pain hypersensitivity [6,9,11,51,52].

The relationship between BH4 and pain was first discovered, through the identification of an allele of the *GCH1* gene haplotype that encodes for the GTPCH enzyme, the rate-limiting enzyme for BH4 biosynthesis, associated with reduced pain scores in multiple independent neuropathic pain cohorts (for review see 52). Based in this human validation of the biological role of BH4 in neuropathic and inflammatory pain, we demonstrated that excessive BH4 levels are produced by neurons in active pain, and immune cells infiltrating damaged nerves and inflamed tissues [9,11,51]. Based on this information, we developed two inhibitors of BH4 production (SPRi3 and QM385) and showed that inflammation and pain hypersensitivity are reduced along with decreased BH4 levels in targeted tissues. Furthermore, we discovered that the BH4-related metabolite, sepiapterin, accumulates in tissues and fluids exposed to the inhibitors [51]. Urinary sepiapterin was also validated as a sensitive, specific, and non-invasive biomarker in a cohort of healthy humans receiving sulfasalazine, a pharmacological treatment approved by the FDA for treating inflammatory bowel diseases. Indeed, sulfasalazine was recently described to be an inhibitor of BH4 synthesis [54]. These ground-breaking findings allowed us to hypothesize that excessive BH4 levels may play a significant role in pain development.

The involvement of BH4 metabolism in animals models of inflammatory and neuropathic nociception showed a marked increase in BH4 synthesis in sensory neurons and nervous tissues inducing hyperalgesia [11]. The data presented here showed that increased WAT deposition also favored the synthesis of BH4, and possibly, the development of hyperalgesia. Increased transcription of *GCH1* and higher levels of BH4 have previously been reported in leukocytes infiltrating injured sciatic nerves, reinforcing the contribution of the immune system in the induction and maintenance of pain induced by the pathological production of BH4 [11].

BH4 is produced by macrophages that infiltrate tissues as a response to inflammation [55] and its overproduction correlates with hyperalgesia [11]. Thus, increased visceral WAT appears to be a favourable environment for the development of pain hypersensitivity. In fact, the results demonstrated here showed concomitant increase in WAT, mechanical hyperalgesia, and BH4 levels. Furthermore, the urinary BH4 can be proposed as a potential easy-to-access, sensitive and reliable biomarker of pain development, and a promising target for the control of pain hypersensitivity in obesity. The use of pharmacological inhibitors aimed at reducing high levels of BH4 provide a potential therapeutic tool in the treatment of chronic pain.

The literature has extensively demonstrated that the regular practice of moderate-intensity physical exercise is beneficial for health promotion, and for decreasing the risk of death from all causes (for a review see 51). The Physical Activity Guidelines for Americans points out that in individuals affected by chronic diseases, the practice of moderate-intensity physical exercise for 150 min a week, or the practice of high-intensity aerobic physical activity for 75 min a week, are beneficial in improving the health of chronic illnesses affected individuals. Also, muscle-strengthening activities involving all major muscle groups, if practiced at least twice a week, provide additional health benefits [56]. The effect of this intervention is more pronounced in the conditions in which the physiopathology is associated with persistent activation of the immune system [57].

It has been widely reported that the regular practice of moderate-intensity physical exercise reduces the systemic inflammation. Our group demonstrated that the inflammation induced by lipopolysaccharides in mice increased levels of glycemia in the GTT and led to an increase in the levels of urinary neopterin [52], a BH4-related metabolite that increases parallel to BH4, and a sensitive marker of activation of the immune system. We also shown that GTT and neopterin levels were normalised by physical exercise [58]. Additionally, we have also shown that neopterin is increased in the plasma of individuals affected by obesity type III [59], and obesity with insulin resistance, who also showed increased markers of inflammation [40]. Thus, regular practice of physical exercise positive modulates the anti-inflammatory response of the immune system normalizing the hypersensitivity scores seen in mice with increased WAT.

5 Conclusions

This study showed that increased visceral WAT elicited the overproduction and secretion of BH4 with consequent increased scores of chemical and mechanical hyperalgesias. Thus, urinary BH4 can be proposed as a biomarker of pain in obesity that is easy to access and little invasive. Finally, physical exercise can prevent the development of metabolic diseases and chronic pain, possibly by reducing WAT accumulation, BH4 overproduction, and therefore the associated inflammatory state.

6 Funding

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8 Conflicts of Interest

The authors declare no conflicts of interest.

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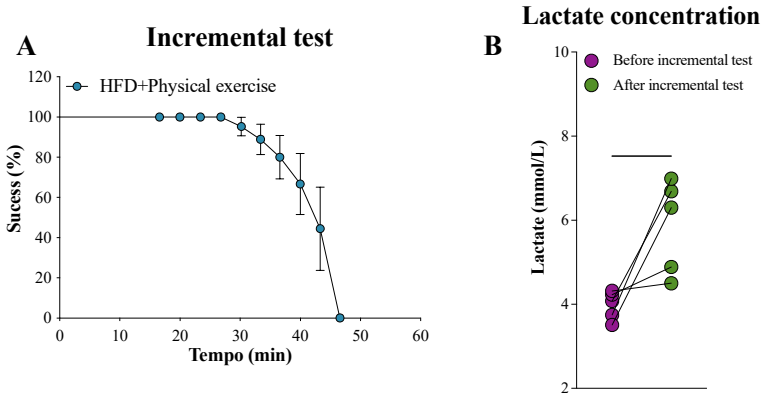
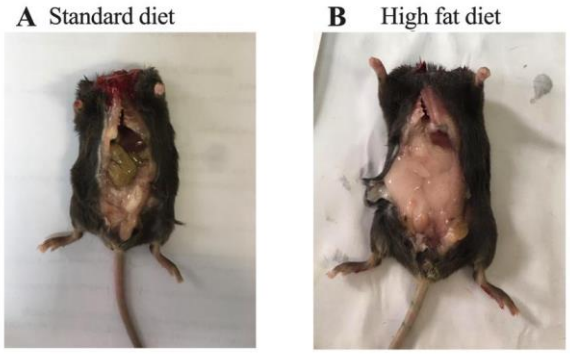
Supplementary Material

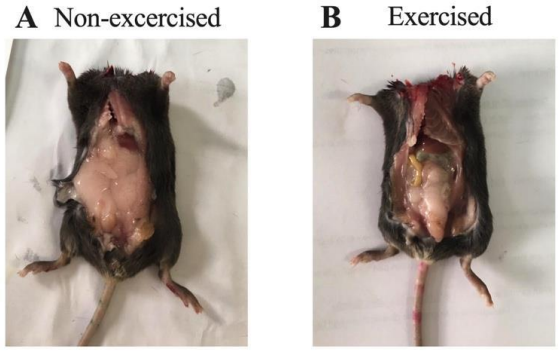


Supplementary Figure 1. Incremental exhaustion test in C57BL/J6 mice. Adult male C57BL/J6 mice were submitted to the maximum incremental test to determine the animal's voluntary withdrawal from treadmill running physical exercise. (**A**) Incremental test, (**B**) Lactate concentrations. ANOVA for multiple comparisons followed by *post hoc* Tukey test for A. One tail Student's *t*-test for B. * $P \leq 0.05$ (n= 5 animals per group).



Supplementary Figure 2. Visceral white adipose tissue (WAT) accumulation in C57BL/J6 mice submitted to high fat diet (HFD). Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). Animals in the control group received a standard rodent diet for the same period (20 kcal % protein, 70 kcal % carbohydrates and 10 kcal % lipids). (**A**) Representative image of a mouse from the control group submitted to regular rodent diet that shows no WAT accumulation. (**B**)

Representative image of a mouse from the group of animals submitted to HFD that shows increased WAT accumulation.



Supplementary Figure 3. Visceral white adipose tissue (WAT) accumulation in C57BL/J6 mice submitted to high fat diet (HFD) and physical exercise. Adult male C57BL/J6 mice received HFD ad libitum for 8 consecutive weeks (20 kcal % protein, 35 kcal % carbohydrates and 45 kcal % lipids). After 2 weeks of the dietary intervention, mice were also submitted to physical exercise 5 times per week for 6 weeks (for details see M&M). (**A**) Representative image of a mouse from the non-exercised group submitted to HFD that shows increased WAT accumulation. (**B**) Representative image of a mouse from the exercised group submitted to HFD that shows no WAT accumulation.

LITERATURE REVIEW ARTICLE: EPIGENETIC MODIFICATIONS INDUCED BY EXERCISE: DRUG-FREE INTERVENTION TO IMPROVE COGNITIVE DEFICITS ASSOCIATED WITH OBESITY

(P4) Physiology and Behavior (impact factor 3.742):
Epigenetic modifications induced by exercise: Drug-free intervention to improve cognitive deficits associated with obesity.
Leonardo Barros, **Tuany Eichwald**, Alexandre Francisco Solano, Débora Scheffer, Rodrigo Augusto da Silva, Joana M. Gaspar, Alexandra Latini (Published in 11 May 2019).

Obesity is a widespread global health issue associated with chronic diseases and disabilities. The prevalence of obesity is increasing, and it is a significant risk factor for metabolic conditions and cognitive impairments, presenting a higher risk of developing dementia, lower cognitive performance, decreased blood-brain barrier integrity, and brain atrophy, among others (COPE et al., 2018; SMITH et al., 2011). Changes in lifestyle, including calorie reduction and increased physical activity, are recommended for preventing and treating obesity and related disorders.

Physical exercise has been shown to have beneficial effects on body mass, metabolic health, cardiovascular risk, and cognitive function in individuals of all ages (STRASSER, 2013). In recent years, research has focused on understanding the underlying mechanisms, particularly epigenetic pathways, through which exercise exerts its positive effects on obesity, cognition, and other conditions with chronic progression (GOMEZ-PINILLA; HILLMAN, 2013; HILLMAN et al., 2009). Given that, physical exercise and epigenetic modifications profoundly impact an individual's health and disease susceptibility.

While it is clear and well described the benefits of the regular practice of exercise (SCHEFFER & LATINI 2020), there is still a gap in understanding whether physical exercise modulates epigenetics and whether chromatin compaction may be the underlying mediator of its beneficial effects, mainly in obesity-related cognitive decline.

Therefore, reviewing the relationship between exercise and epigenetic mechanisms can shed light on the management of complex diseases and can help uncover potential therapeutic

strategies for disease prevention since epigenetic modifications are reversible and can be influenced by interventions.

Key Results & Implications

It is presented here a literature review regarding exercise-induced epigenetic modification as a non-pharmacological approach to enhance cognitive impairments linked to obesity. It highlights how obesity has a detrimental impact on gene transcription pathways that play a role in cognitive processes, leading to compromised levels of critical mediators like BDNF and REST in the hippocampus. To counteract the detrimental effects associated with obesity, physical exercise is recommended as a primary drug-free treatment. Further research on the molecular mechanisms underlying the positive impact of exercise on cognition, especially focusing on epigenetic changes, will enable the development of targeted exercise protocols to achieve enhanced cognitive outcomes.

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Review

Epigenetic modifications induced by exercise: Drug-free intervention to improve cognitive deficits associated with obesity

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ABSTRACT

Obesity and metabolic disorders are increasing worldwide and are associated with brain atrophy and dysfunction, which are risk factors for late-onset dementia and Alzheimer's disease. Epidemiological studies demonstrated that changes in lifestyle, including the frequent practice of physical exercise are able to prevent and treat not only obesity/metabolic disorders, but also to improve cognitive function and dementia. Several biochemical pathways and epigenetic mechanisms have been proposed to understand the beneficial effects of physical exercise on cognition. This manuscript revised central ongoing research on epigenetic mechanisms induced by exercise and the beneficial effects on obesity-associated cognitive decline, highlighting potential mechanistic mediators.

1. Introduction

Obesity is a complex condition that has reached epidemic proportions worldwide and has become a major contributor to the global burden of chronic diseases and disabilities. Obesity coexists with undernutrition in developing countries, with serious social and psychological dimensions, affecting virtually all ages and socioeconomic groups. According to the World Health Organization (WHO), 1.9 billion adults were overweight and at least 650 million were clinically obese in 2016 [1].

Obesity is a risk factor for several metabolic diseases, such as insulin resistance and type 2 diabetes, whose prevalence is also increasing globally. A growing body of evidence has demonstrated that obesity, insulin resistance, and diabetes are associated with impairment of hippocampal-dependent cognition and memory, presenting a higher risk for the development of dementia [2,3]. In addition, obesity and higher body mass index (BMI) over the course of a lifetime are associated with lower cognitive performance, cognitive decline, reduced white matter, decreased blood brain barrier integrity, brain atrophy and increased risk for late onset Alzheimer's disease (AD) [4].

Changes in lifestyle, including reduced caloric intake and increased physical activity, are recommended for prevention and treatment of obesity and associated metabolic disorders [5]. According to the Physical Activity Guidelines for Americans [6], adults with chronic health conditions such as cancer, osteoarthritis, hypertension, multiple sclerosis, type 2 diabetes, dementia and other cognitive disorders should practice 150 to 300 minutes a week of moderate-intensity aerobic activity, or 75 to 150 minutes a week of vigorous-intensity aerobic activity. With additional health benefits, these individuals should associate aerobic activity with moderate/higher intensity muscle-strengthening activities, that involve all major muscle groups. Lifestyle interventions can benefit not only loss of body mass and improve metabolic health or cardiovascular risk [7], but they can also improve cognitive function in children, adolescents and adults [8–10].

During the last decade, several biochemical pathways have been investigated to understand the underlying mechanisms involved in the beneficial effects of physical exercise on cognition, obesity and many other conditions with chronic progression. While performance, muscle size and insulin sensitivity may decline after a few weeks of physical inactivity [11], some other exercise-induced effects last for considerable time, to the point of affecting future generations as recently shown in experimental rodents [12]. This mini-review summarizes

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fundamental ongoing research on epigenetic mechanisms induced by physical exercise and the beneficial effects on obesity-associated cognitive decline, highlighting potential contributors and mechanistic mediators.

2. Epigenetics

The term epigenetics was originally conceived by Conrad Waddington to describe the existence of processes that alter gene activity without changing the DNA sequence, leading to modifications that can be transmitted to daughter cells [13]. These processes are natural and essential to the function of many organisms, but if they occur improperly, they can induce major adverse health and behavioural effects. Epigenetic modifications can influence gene activity at the transcriptional and post-transcriptional levels and/or at the translation and post-translational modifications. The most characterized epigenetic modifications are DNA methylation that includes methylation and hydroxymethylation and post-translational modifications of histones namely, acetylation, methylation, phosphorylation, poly-ADP ribosylation, ubiquitination, deamination, sumoylation and isomerization of proline. All these processes regulate gene activity and involve chemical modifications in the DNA [14].

2.1. DNA methylation

DNA methylation is the main form of epigenetic alteration, consisting of the covalent addition of a methyl group, transferred from S-adenosylmethionine to the carbon 5 of a cytosine that usually precedes a guanine (CpG dinucleotide), forming 5-methyl cytosine (5mC; Fig. 1). This reaction is catalysed by DNA methyltransferases enzymes (DNMTs) [15] resulting in methylation of the DNA predominantly in promoter regions of functional genes called CpG islands, usually leading to the inhibition of gene expression [16]. In mammals, five members of the DNMT family have already been identified. DNMTs 1 and 2 are responsible for maintaining the epigenetic pattern and have high affinity for hemimethylated DNA strands and for the maintenance of DNA methylation patterns during replication. DNMTs 3A, 3B and 3L, are classified as *de novo* DNMTs and are responsible for the occurrence of *de novo* methylation of sequential waves in regions of the genome with no previous indication of methylation [17].

DNA can be demethylated by opposite mechanisms; by inhibition of DNMT or by active 5mC removal. Ten-eleven translocation (TET) proteins (2-oxoglutarate, ascorbate and Fe (II) dependent dioxigenases) convert 5mC into 5-hydroxymethylcytosine (5hmC) [18,19], then 5hmC is transformed into 5-formylcytosine and finally into 5-carboxylcytosine (5caC) [20,21]. The subsequent decarboxylation of 5caC leads to DNA demethylation [22] (Fig. 2). These changes lead to the activation of gene expression by the destabilization of the DNA structure, which allows the access of the transcriptional machinery to their respective transcription initiation sites.

2.2. Histone modifications

The nucleosome consists of two histone H3-H4 dimers surrounded by two dimers of H2A-H2B. Their N-terminal tails protrude from the nucleosomes to the nuclear lumen where they can undergo post-

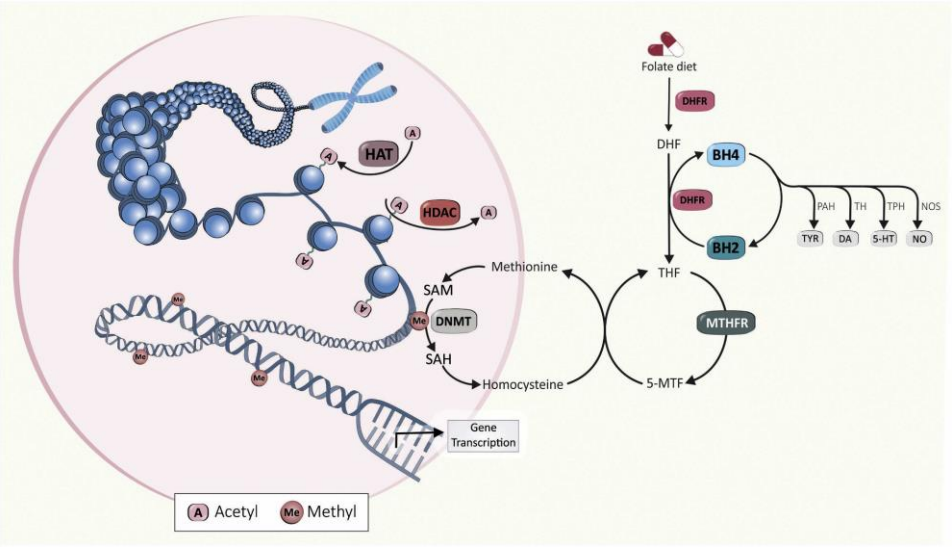

Fig. 1. Role of tetrahydrobiopterin (BH4) on chromatin remodeling. The main epigenetic profiles are represented by *a)* methylation of the DNA, controlled by DNA methyltransferases (DNMT) enzymes and ten-eleven translocation (TET) proteins, and *b)* post-translational modifications of histones, represented mainly by acetylation and regulated by histone acetyltransferases (HAT) and histone deacetylases (HDAC). The DNA methylation results from the close communication between methionine, folate and biopterin cycles. The methylation pathway is formed by the interaction of three essential metabolic routes: the methionine, folate and biopterin cycles. The methionine cycle involves the methylation of homocysteine into methionine, which is further transformed into S-adenosylmethionine (SAM), the most important methyl donor. SAM can donate the methyl group in more than a hundred biological reactions, including DNA methylation, to yield S-adenosylhomocysteine (SAH) and the methylated acceptor (methyl-DNA). 5-Methyltetrahydrofolate (5-MTHF) functions as a methyl donor for homocysteine methylation. The resulting tetrahydrofolate (THF) is transformed back to 5-MTHF by methylenetetrahydrofolate reductase (MTHFR). Dietary folic acid is transformed into THF (active folate) by dihydrofolate reductase (DHFR), the same enzyme that catalyzes the reduction of dihydrobiopterin (BH2) into tetrahydrobiopterin (BH4). BH4 is a mandatory enzyme cofactor for the synthesis of nitric oxide (NO) and the neurotransmitters dopamine (DA) and serotonin (5-HT), and for the transformation of phenylalanine (PHE) into tyrosine (TYR). The transformation of BH2 intro BH4 by DHFR occurs in the BH4 salvage pathway. Abbreviations: PAH, phenylalanine-4-hydroxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

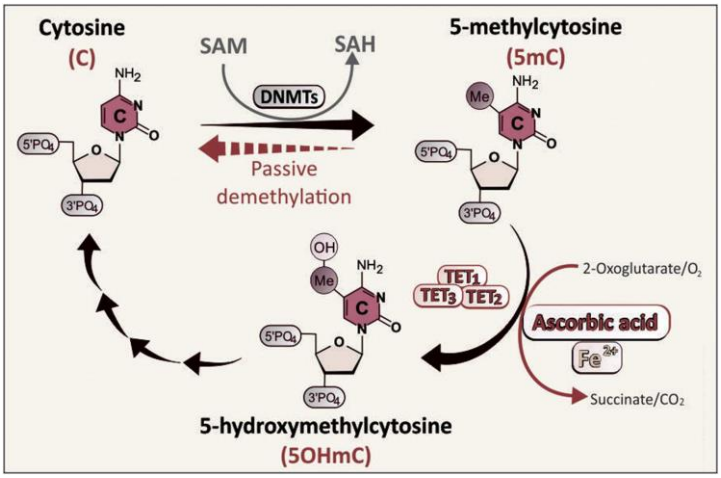

Fig. 2. DNA demethylation involves passive and active processes. Active DNA demethylation is achieved through ten-eleven translocation (TET1, 2, 3) proteins, by the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) in a reaction dependent on ascorbic acid, iron, oxygen and 2-oxoglutarate. TET enzymes can further oxidize 5-hmC to 5-formylcytosine and 5-carboxylcytosine, which can eventually be removed from the genome. Reproduction with authorization of Aline Pertile Remor.

translationally modified modifications, such as phosphorylation, ubiquitination, sumoylation, acetylation and methylation [23,24]. These modifications alter chromatin structure to influence gene expression and can interfere with chromatin condensation and nucleosome positioning. In general, tightly folded chromatin tends to be shut down, or not expressed, while more open chromatin is functional, or expressed. Therefore, chromatin structure and gene accessibility to transcriptional machinery are regulated by modifications to both DNA and histone tails [24].

The histone acetylation at the ε-amino group of lysine residues in H3 and H4 tails is most consistently associated with the promotion of transcription. Histones can be acetylated and deacetylated by the action of histone acetyltransferases (HAT) and histone deacetylases (HDACs), respectively. Acetylation is targeted to regions of chromatin by the recognition and binding of DNA sequence-specific transcription factors that recruit one of a growing family of HAT cofactors, such as the CREB binding protein, which promotes an essential pathway for cognition and memory [25,26]. Deacetylation of histones performed by HDACs correlates with CpG methylation and the inactive state of chromatin. The HDAC proteins are themselves subject to regulation by acetylation, phosphorylation, and sumoylation, which can affect their function, subcellular distribution, and protein-protein associations [27] (Fig. 1).

Histone lysine methylation patterns and their effects on transcription are more complex than acetylation, since some methylation sites are associated with transcriptionally permissive chromatin (euchromatin) and some are repressive, fostering heterochromatin formation. In addition, ε-amino groups of lysine residues can be mono-, di-, or tri-methylated. Histone methylation is catalysed by histone methyltransferase (HMT) and demethylated by histone demethylase (DMH) [28]. The addition of the acetyl group to lysine (K) residues catalysed by HAT enzymes in the histone N-terminal tail, weakens the electrostatic interactions between histones, promoting an opening of the chromatin and consequently the activation of gene expression [29]. However, the addition of the methyl group by HMTs in the histone N-terminal tail can promote both activation and gene repression, depending on the type of amino acid modified and also on the amount of modifications (trimethylated, dimethylated or monomethylated) [30,31]. Overall, the methylation of lysine 9 and lysine 27 of the histone H3 are associated with gene silencing, whereas methylation of lysine 4 and lysine 36 of the same histone are transcriptionally permissive modifications [32].

2.3. Nonprotein-coding RNAs (ncRNAs)

Diverse classes of RNA, ranging from small to long ncRNAs, have emerged as regulators of gene expression, genome stability and defence against foreign genetic elements. ncRNAs are RNA molecules transcribed from genomic DNA that are not translated into proteins [33]. Some ncRNAs are represented by tRNA (transfer RNA), rRNA (ribosomal RNA), miRNA (micro RNA), snoRNA (small nucleolar RNAs), and pi-RNA (piwi-interacting RNA), etc. [34]. Small ncRNAs typically modify chromatin structure and silence transcription by guiding Argonaute-containing complexes to complementary nascent RNA scaffolds and then mediating the recruitment of HMT and DNMTs [35]. Long ncRNAs exhibit their biological functions by acting as *cis*- or *trans*-regulators in biological processes [36]. The long ncRNAs that control chromatin structure interact with nucleosome remodeling factors as well as chromatin modifying enzymes [37]. In general, long ncRNAs are found to play an important role in gene expression regulation of various diseases including cancer.

DNA methylation, histone modification and ncRNA regulation are the main epigenetic mechanisms that modulate the gene expression in physiology and several pathological processes [38,39].

2.4. Role of tetrahydrobiopterin (BH4) on DNA methylation

BH4 is an essential cofactor for aromatic amino acid hydroxylase, nitric oxide synthases, and alkylglycerol monooxygenase, making it indispensable for synthesis of serotonin, epinephrine, norepinephrine, dopamine, nitric oxide, and metabolism of glycerol ethers [40]. Physiological intracellular levels of BH4 are regulated by the combined action of three metabolic pathways, namely *de novo* synthesis, recycling, and salvage pathways. The *de novo* via generates BH4 from GTP through a three-step enzymatic metabolic route starting with the rate-limiting enzyme guanosine triphosphate cyclohydrolase I, followed by 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase [41]. The BH4 recycling pathway is a mechanism that economizes intracellular energy (without consuming GTP) and sustains the appropriate levels of BH4 in tissues with a high requirement of this pteridine. After BH4 participates as a mandatory enzyme cofactor, quinonoid dihydrobiopterin redutase (qDHPR) is formed by quinoid dihydropteridine reductase and reduced back to BH4 in a NADH-dependent reaction [41]. Alternatively, intracellular BH4 levels can be generated via the salvage pathway using sepiapterin and 7,8-dihydrobiopterin (BH2)

as metabolic intermediates. Although the salvage pathway is not fully understood, sepiapterin reductase and dihydrofolate reductase (DHFR) appears to be key generating BH4 enzymes [40,42].

DHFR is a ubiquitous enzyme that normally peaks at the G1/S cell cycle boundary, playing a key role in DNA methylation. DHFR catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (THF) needed for several one-carbon transfer reactions, like in DNA methylation (Fig. 1) and in purine and pyrimidine synthesis [43]. DHFR is also required for the intracellular conversion of synthetic folic acid, consumed in supplements and fortified foods, into the THF forms that can participate in folate/homocysteine metabolism. Therefore, DHFR appears to be critical for the maintenance of BH4 and THF pools.

Reduction of DHFR enzymatic activity diminishes the THF pool inside the cell, affecting therefore, folate-dependent enzymes [44]. Folate deficits have been extensively associated with increased risk of cardiovascular diseases, multiple cancers, and neural tube defects, due to one-carbon metabolism impairment, which is required for DNA methylation [45–48]. However, the impact or relationship of BH4 metabolism on the THF pathway has not yet been studied. Excessive levels of BH4 have been associated with increased sensitivity to pain [49], immune cell proliferation [50], tumor angiogenesis [51] among other pathological processes, in humans and experimental systems. However, BH4 non-hereditable or genetic deficiencies have also been related to conditions where folate metabolism was reported to be compromised, including brain maturation defects [52] and cardiovascular diseases - however, in this case the systemic and local vascular regulation of BH4 levels has been reported to be different - (for a review see [53]), pointing to an intricate relationship between these two pathways, and possibly to BH4 regulating DNA methylation. In this scenario, it has been reported that DHFR can replace qDHPR activity in qDHPR-null mouse, under increased BH2 levels, generating therefore BH4 [54]. Furthermore, our group recently demonstrated that BH4 production is essential for proper mitochondrial activity [50], and compromised mitochondrial one-carbon metabolism has been shown to develop neural tube defects with 100% penetrance [55,56], strengthening the view that BH4 and folate metabolisms interact to control DNA methylation. Finally, DHFR inhibition is essential to the action of antifolate medications used to treat cancer and some inflammatory diseases, and it is well described that methotrexate reduces BH4 levels [57].

Methylenetetrahydrofolate reductase (MTHFR) is another key enzyme in folate metabolism that participates in DNA methylation, and the presence of MTHFR polymorphisms (C677T variants) is a risk factor for neural tube defects [58]. MTHFR catalyses the irreversible reaction that establishes the balance between 5,10-methyltetrahydrofolate (5,10-MTHF) and 5-methyltetrahydrofolate (5-MTHF) at the cellular level. 5,10-MTHF is used for DNA synthesis, while 5-MTHF is used for DNA methylation. 5-MTHF donates one carbon (methyl group) for the methylation of homocysteine into methionine, which is further transformed into S-adenosylmethionine (SAM). SAM can donate the methyl group in more than a hundred biological reactions (*i.e.* DNA methylation), to yield S-adenosylhomocysteine and the methylated acceptor, including DNA [59]. SAM also donates a methyl group to catechol-O-methyltransferase (COMT), which is involved in the catabolism of dopamine, epinephrine, and norepinephrine, catecholamine neurotransmitters, which biosynthesis if fully dependent on appropriate BH4 cellular levels. In this context, it has been demonstrated that 5-MTHF regenerates oxidized BH4 in the absence of adequate amounts of the pterin [54,60,61].

The structure of 5-MTHF is very similar to BH4; the direct infusion of 5-MTHF into the brachial artery of patients with hyperlipidemia mimicked the effects of BH4 on endothelial function, suggesting a direct interaction with endothelial nitric oxide synthase [62]. This fact further supports the idea of an intimate interplay between these two pathways, regulating the methylation of DNA (Fig. 1).

3. Epigenetic factors controlling the expression of metabolic and cytotoxic/neuroprotective genes in human obesity

Obesity is a complex condition that has reached epidemic proportions worldwide and has become a major contributor to the global burden of chronic diseases and disabilities. Obesity has a multifactorial aetiology which results from a complex interaction of social factors, culture, environment, epigenetics and individual genetic predisposition. Environmental factors such as socioeconomic status, lifestyle and cultural aspects are determinant for the establishment of this disease. High caloric intake associated with low energy expenditure due to sedentary lifestyle leads to an "obesogenic" environment that plays a fundamental role in predisposition to the development of chronic diseases [5].

Genetic factors are believed to be essential for the development of obesity, diabetes, AD, Parkinson's disease (PD), etc. [63–65]. However, gene modifications alone cannot explain the development of these chronic diseases or their related long-term complications. In this scenario, epigenetic alterations might strongly increase predisposition to the development of obesity-induced cognitive impairments. The main epigenetic alterations in physiology and several pathological processes, including DNA methylation, histone modification and ncRNAs have been demonstrated in obese individuals.

3.1. DNA methylation in obesity

Individuals affected by common chronic metabolic diseases present altered patterns of DNA methylation [63–65] and compromised expression of neuroprotective genes, which will favor neurotoxicity and cognitive impairment or dementia [66]. However, when comparing global DNA methylation profiles in obese subjects vs. lean controls, several studies failed to demonstrate strong associations between obesity and global methylation [67]. DNA methylation in large-scale studies is routinely assessed in DNA from peripheral blood mononuclear cells (PBMC). Since blood samples consist of a mixture of different cell types with different methylation profiles, the available data in the literature might lead to limited interpretations about global DNA methylation patterns in obesity [68]. Moreover, blood cell methylation profiles obtained from adults may not report the epigenetic state in specific tissues (including the brain), or whether the changes are causes or consequences of the obese state. Furthermore, there are numerous factors, including sex, ethnic background, age, exposure to toxins and diet that might also contribute to the lack of evident association [69,70].

Clear and defined patterns of global DNA methylation are observed, however, when at least one clinical variable is included in the statistical analysis of the obese and lean populations. BMI as a measure of adiposity was shown to positively correlate with global DNA methylation in adults affected by diabetes, metabolic syndrome and heart diseases, all conditions linked to obesity [71]. Moreover, two studies performed in obese children demonstrated that the correlation between obesity and epigenetics (global DNA methylation) may be stratified in obese and severely obese groups vs. lean controls [72,73]. Furthermore, genome-wide quantification of site-specific DNA methylation has led to the identification and validation of multiple adiposity-associated differentially-methylated sites and regions [74]. The candidate genes implicated in obesity are related to appetite control and/or metabolism, insulin signaling, immunity, growth, brain activity, circadian clock regulation and imprinted genes [67] (some examples are shown in Table 1). For example, increased methylation of proopiomelanocortin in whole blood [75], and of PGC-1α in muscle and PBMC [76,77] have been shown in obese adults. Our group also demonstrated increased methylation in the promoter region of the repressor element 1 (RE1) silencing transcription factor (REST) in PBMC from obese children [72]. REST is a master transcription factor that, in differentiated neurons, down-regulates genes linked to cell-death and enhances the expression

Table 1
Example of genes with increased DNA methylation and compromised expression in human obesity

Gene	Biological function	Biological sample
Proopiomelanocortin (POMC) [75]	Appetite; POMC neuron stimulation results in satiety	whole blood
PGC-1α [76]	PGC-1α; master regulator of mitochondrial biogenesis	muscle; PBMC
PDK4 [76]	Regulation of metabolism	muscle
Glucocorticoid receptor [80]	Inhibition of inflammation	PBMC
IGF2/H19 gene cluster [82]	H19; gene for a lncRNA with negative regulation of body weight and cell proliferation IGF2; cell proliferation, metabolism and neuroprotection	whole blood
HIF3A [74]	Metabolism adaptation to hypoxia; related to endurance exercise	PBMC
UBASH3A and TRIM3 [67]	UBASH3A; suppressive effect on T-cell signaling and activation – promotion of inflammation TRIM3; autoimmunity	PBMC
REST [72]	Neuroprotective transcription factor in the adult and aging brain	PBMC
BDNF [82]	Neuroprotection	PBMC
Serotonin transporter [79]	Serotonin uptake	PBMC
CREB3L3 [67]	Hepatic regulation of metabolism	PBMC

PBMC: peripheral blood mononuclear cells

of other genes that induce resistance to the ageing process and the preservation of cognitive function [78]. The increased DNA methylation was accompanied by reduced expression and content of REST in the obese group [72]. In addition, other associations between BMI, adiposity, and waist circumference with methylation in PDK4 in muscle [76], the serotonin transporter gene [79], glucocorticoid receptor [80] and IGF2/H19 imprinting region in blood cells [81], and hypoxia inducible factor 3, alpha subunit (HIF3A) [74], ubiquitin associated and SH3 domain containing A (UBASH3A) and tripartite motif containing 3 (TRIM3) [67], cAMP responsive element binding protein 3-like 3 (CREB3L3), and BDNF (brain-derived neurotrophic factor) in PBMC [82] have also been reported. BDNF is a protein synthesized in neurons that participate in cognitive processes, including neuroplasticity, neurogenesis and neuroprotection of the hippocampus. It may also enhance learning, memory and cognition [83,84]. Obesity negatively regulates hippocampal BDNF expression [85,86], and low blood BDNF content has been observed in obese individuals and its reduction has been extensively associated with cognitive impairment [87].

Collectively, these studies provide evidence that obesity is associated with altered epigenetic regulation of a number of genes involved in metabolism and cytoprotection. The association between DNA methylation and gene expression is dependent on the region of CpG that is methylated. The best understood relationship is the methylation of CpG islands (300 to 3000 base pairs in length with a CpG -cytosine and guanine separated by one phosphate- content greater than 0.6), a DNA region where many mammalian promoter regions are located. Under a simplistic view, hypermethylation, a characteristic of obesity, will induce stable silencing of genes. The opposite has been described in several cancers where repressed oncogenes are hypomethylated, therefore promoting its expression.

3.2. Histone post-translational modifications in obesity

In addition to DNA methylation, histone post-translational modifications, in particular altered histone acetylation, have also been demonstrated in obesity and in many other human diseases.

HATs require the central mitochondrial metabolite of metabolism, acetyl-coA, as the acetyl-donor, connecting metabolism to the transcriptional regulation of many genes, including pro-inflammatory genes. Histone acetylation, in general terms, allows interconversion between repressive and permissive chromatin structures and domains, and it is the major regulator of gene expression [88]. For example, acetylation of histone H3 at the promoters of several cytokines and chemokines after inflammation results in the increased recruitment of NF-κB to these regions, thus promoting NF-κB-dependent inflammatory gene expression in human obesity [89]. In this context, obesity, described for the first time by Hippocrates (460 BC–370 BC) as a surplus of four humors (fluids): blood, black bile, yellow bile, and phlegm that induces early mortality (reviewed in [90]), was understood as a low-grade chronic inflammation disease after more than two thousand years. However, inflammation at the chromatin level results from the acetylation status of histones, as a balance between the activities of HATs and HDAC; *i.e.* the increased H3 acetylation of the promoters that drive inflammation can be reverted by increased histone acetylation of the glucocorticoid receptor promoter or increased HDAC2 activity [89].

Mammalian HDACs are divided into 4 classes (I, IIa, IIb, III, and IV) [27]. Class III HDACs, known as sirtuins due to the shared homology with yeast Sir2 (Silent information regulator 2), are structurally distinct from Classes I, II, and IV. Mammalian sirtuins (SIRT 1-7) deacetylate lysine residues on targeted proteins, utilizing a mechanism that requires NAD+ as a co-substrate, releasing nicotinamide (NAM), O-acetyl ADP ribose, and deacetylated protein [27,91]. The list of SIRTs targets in mammals includes, among others, those involved in aging, cognition, neuroplasticity, memory, calorie restriction, thermogenesis, malignancy, stress responses, metabolic and transcriptional regulations, and in genome maintenance [27,91]. Most of the SIRT enzymes are highly sensitive to several environmental factors, including calorie restriction, exercise and cold exposure, due to fluctuations in intracellular NAD^+ levels in response to nutrient availability [92].

Numerous studies performed in obese individuals have shown reduced availability of SIRTs in different tissues (see Table 2), when comparing with normal-weight controls [93–96]. Thus, considering the inflammatory component of obesity and the main biological functions of these type III HDACs (Table 2), increasing SIRT activity seems to be a promising target for the treatment of obesity and obesity-associated comorbidities. In this context, the non-pharmacological treatment of obesity, the regular practice of physical exercise, is known to increase the activity of sirtuins. For example, the first effort to demonstrate increased activity of SIRTs in humans induced by exercise was performed in PBMC from athletes of the Italian male rowing team [97].

Other HDACs have also been shown to be altered in human obesity. For example, HDAC5, HDAC6 and HDAC10 (class II HDACs) are markedly reduced in adipose tissue from obese subjects [103,104]. These class II HDACs have been proposed to be negative regulators of obesity development [104]. However, the best understood relationship between HDACs and obesity (cause or consequence?) is the compromised content of SIRTs in tissues from obese individuals.

3.3. ncRNAs in obesity

Only 1.2% of the human genome encodes protein, and a large fraction of it is transcribed. About 98% of the transcriptional output in humans consists of ncRNA from the introns of protein-coding genes and the exons and introns of non-protein-coding genes. The function of ncRNA has begun to be elucidated in recent years, and it has been shown to be involved in various aspects of gene expression and in the physiopathology of many diseases, including obesity [105].

Table 2
Reduced levels of sirtuins in biological samples from obese subjects

Sirtuin	Tissue (cellular localization)	Biological functions	Biological sample
SIRT1	Ubiquitous (nucleus[a], cytosol)	Cell survival; metabolism; DNA damage repair; lipidand glucose homeostasis; stress resistance; insulin secretion; mitochondrial biogenesis; antioxidant; antiapoptotic; global health	adipose tissue [93,95,96,98] PBMC [93,94] liver [93]
SIRT2	Muscle[a], brain, spinal cord, testes, heart (cytosol)	Cell cycle control; thermogenesis; metabolism; neuroprotection; DNA repair	PBMC [94]
SIRT3	Heart, brain, testes, liver, kidney, muscle, and adipose tissues (mitochondria[a], nucleus, cytosol)	Metabolism; mitochondrial healthy; neuroprotection; antioxidant; longevity	PBMC [93] liver [93]
SIRT4	Ubiquitous, pancreas[a] (mitochondria[a], nucleus, cytosol)	Metabolism; insulin release, mitochondrial ATP synthesis	adipose tissue [98]
SIRT5	Ubiquitous, brain[a], kidney[a] (mitochondrial matrix[a], cytosol)	Regulation of cellular homeostasis, mitochondrial function; metabolism; reactive species detoxification	adipose tissue [99]
SIRT6	Ubiquitous (nucleus)	DNA repair; lifespan	PBMC [93] liver [93]
SIRT7	Proliferative tissues[a] including spleen, ovary, thyroid, liver and testis. Absent or low in muscle (nucleolus)	Cellular growth; metabolism; cell survival	increased in adipose tissue [98]

SIRT: Sirtuin; PBMC: Peripheral blood mononuclear cells.
[a] Denotes predominant localization. Localization and biological functions of sirtuins are reviewed in [27,100–102].

3.3.1.1. Long ncRNAs in obesity. Long ncRNAs are reported to play an important role in the control of adipogenesis and obesity [106], and small ncRNAs, in particular miRNAs, have been shown to be involved in human adipocyte differentiation, lipid metabolism, and obesity [107,108].

Table 3 shows the main long ncRNAs associated with human obesity and obesity-associated comorbidities. These long ncRNAs were shown to participate in adipocyte differentiation and function in humans. However, it should be considered that long ncRNAs on metabolism is still an open field for research.

3.3.1.2. Small ncRNAs in obesity. More than two thousand miRNAs have been described in humans. Once generated, miRNA represses the expression of target genes contributing to the regulation of many biological processes. Numerous miRNAs are present in human adipose tissue; however, the expression of only a few is altered in individuals with obesity and type 2 diabetes mellitus, or differentially expressed in various adipose depots. miRNAs can also be secreted from cells into the circulation and serve as markers of a pathological condition, *i.e.* disturbed adipose tissue function. The miRNA concentration in biological fluids is relatively stable and can be used as a biomarker for disease diagnosis [109]. For example, the plasma level of miR-375 could potentially be used to estimate the beta-cell mass in patients with insulin resistance [110]. Moreover, multiple studies have suggested that circulating miRNAs, including miR-122, are strongly associated with the risk of obesity and insulin resistance in young adults [111]. Tables 4a and 4b show the main miRNAs increased in biological samples obtained from obesity-related diseases.

With the proper development, miRNA- based biomarkers have the potential to identify metabolic problems during disease latency (pre-clinical), assess the severity of disease, identify patients with a predisposition to metabolic disease (assess risk, for example, for obesity-driven cognitive decline [2]), address disease etiology, confirm diagnosis/ reduce misdiagnosis on the basis of current clinical markers and monitor response to interventions.

Table 3
Long ncRNAs differentially expressed in adipose tissue of obese individuals

Long ncRNA	Disease condition
CATG00000111229.1 (ASMER-1)	Obese + IR
CATG00000072281.1	Obese
CATG00000000027.1	Obese + IR
ENSG00000235609.4 (ASMER-1)	Obese + IR
ENSG00000229807.5	Obese
ENSG00000231528.2	Obese

IR: insulin resistant.

4. Epigenetic biomarkers of human cognitive impairment

A growing body of evidence has demonstrated that obesity, insulin resistance, and diabetes are associated with impairment of hippocampal-dependent cognition and memory, presenting a higher risk for the development of dementia [2,3]. In addition, obesity and higher BMI over the course of a lifetime are associated with lower cognitive performance, cognitive decline, reduced white matter, decreased blood brain barrier integrity, brain atrophy and increased risk for late onset AD [4].

Although there is a higher risk for obese individuals to develop cognitive impairment or memory loss, and obesity is characterized by several epigenetic modifications in tissues and biological fluids, there is no set or unique epigenetic hallmark in obesity linking or predicting the development of cognitive decline in humans. ApoE is the strongest genetic risk factor for late onset Alzheimer's disease. ApoE encodes three common alleles (ε 2, ε 3, ε 4). ApoEε 4 is associated with an increased cumulative AD risk, such that one ApoEε 4 allele increases AD risk 3-fold, and two ApoEε 4 alleles increase AD risk 12-fold [121].

In an effort to associate epigenetic markers of obesity with cognitive performance, Table 5 shows the main epigenetic modifications identified in blood or *postmortem* tissues from individuals affected by AD or mild cognitive impairment.

5. Beneficial role of physical exercise on obesity-driven cognitive impairment: Supporting data obtained mainly from animal studies

Data generated from studies performed in experimental systems, including rodents, non-rodents, primates, iPSC (induced pluripotent stem cells), primary cultured cells, etc., have contributed to increase the knowledge on the processes involved in cognitive decline. It is clear, that the main objective to perform pre-clinical studies in this field of cognition and memory is to contribute to finding solutions to this medical question. Decline of cognitive abilities is a major risk factor for age-associated neurodegenerative disorders, in particular AD. Two main conditions favor this outcome: *i)* ageing, which is a consequence of the increased life expectancy, and *ii)* obesity and the related biggest pandemic in human history, the type 2 diabetes. In this context, calorie restriction and the regular practice of physical exercise increases longevity and prevent cognitive decline. In addition, long-term regular exercises may slow the loss of muscle mass and prevent age-associated increases in body fat.

Although, pre-clinical data might lead to negative or inconsistent

Table 4a
Main miRNAs *increased* in biological samples obtained from obesity-related diseases

miRNA	Pathological condition	Biological sample
miR-23a; miR-27a; miR-130; miR-195; miR-197; miR-320a; miR-509-5p [112]	Metabolic syndrome	serum
let-7g; miR-221 [113]	Female-specific miRNA in metabolic syndrome atherogenesis; adipogenesis	serum
miR-625 [114]	Pre-gestational and gestational obesity	plasma
miR-140-5p; miR-142-3p; miR-222 [115]	Morbidly obese patients	serum
miR-222 [116]	Insulin resistance, obesity, metabolic syndrome, type 2 diabetes, and an adverse lipid profile	plasma
miR-122; miR-199a [117]	Childhood obesity	serum
miR-486; miR-146b; miR-15b [117]	Risk of type 2 diabetes in obese children	serum
miR-132; miR-181a; miR-17-5p, miR-155 [118]	Correlation with macrophage infiltration	Adipose tissue

Table 4b
Main miRNAs *reduced* in biological samples obtained from obesity-related diseases

miRNA	Pathological condition	Biological sample
miR-126 [119]	Type 2 diabetes	plasma
miR-17-5p; miR-132 [120]	Obesity	serum
miR-532-5p; miR-125b; miR-130b; miR-221; miR-15a; miR-423-5p; miR-520c-3p [115]	Morbidly obese patients	serum

results when projecting to the human condition, a number of major breakthroughs in basic science and medical research have been possible because of observations and testing on animal models. Indeed, the discoveries in which animal models played a critical role are numerous and have led to many Nobel Prizes.

The regular practice of physical exercise has been used as a non-pharmacological treatment for preventing the development of many diseases with chronic progression, including cognitive decline [143]. During the last decade, several biochemical pathways have been investigated to understand the underlying mechanisms involved in the beneficial effects of physical exercise on cognition. The Physical Activity Guidelines for Americans states that regular practice of physical activity is one of the most important things people can do to improve their health [6]. The Guidelines stressed that the beneficial effects are highly dependent on overload, progression, and specificity; *i.e.* the physiologic effects of walking are largely specific to the lower body and the cardiovascular system. This implies that this free-drug treatment will require a specific dose and administration time to achieve its maximal therapeutic effect. In agreement, rehabilitation and/or exercise prescription for humans relies on different exercise intensities and the dose-response relationships, specificities that will lead to diverse effects over disease/health states [144]. Unfortunately, most of the data generated in this field so far, lack of essential details about the protocols applied to induce the neuroprotection. Thus, it is not clear whether physical activity, physical exercise, or exercise training (definitions commented in [145]) is driving the cognition-enhancing responses, leading therefore, to confounding results. However, having this in mind, the literature is extremely robust regarding the neuroprotective of exercise induced, *i.e.* by increased activity of BDNF driven by epigenetics alterations [146–152].

5.1. Epigenetics and cognition

5.1.1. Histone acetylation is involved in learning, cognition and memory

It has been shown that cognitive activity relies on the three main types of epigenetic modifications observed in obesity, including DNA methylation, histone modifications and ncRNAs. It has also been shown that histone acetylation is most robustly associated with promoting memory formation. However, the process works in concert with other histone modifications and DNA methylation processes to regulate memory formation and synaptic plasticity [153,154]. Two well-characterized epigenetic modifications involved in cognition and memory are the co-existence of *i)* the acetylation of lysine residue 14 on H3 with the phosphorylation of serine 10 and the trimethylation of H3K36 [155,156], and *ii)* H3 acetylation with DNA methylation [154]. When these modifications are induced, cognition and memory are enhanced.

H3 acetylation is rapid and reversible, as it is controlled by the activities of HAT and HDAC. HATs are often transcriptional coactivators that contain bromodomains, whereas histone deacetylase are corepressors. A bromodomain is comprised of about 110 amino acids that recognize acetylated lysines on the N-terminals of histone tails [157].

Class I HDACs, HDAC2 and HDAC3, negatively regulate learning and memory, while HDAC1 plays a role in memory extinction. For example, the overexpression of HDAC2 in neurons provoked dendritic synaptic plasticity and memory formation impairment, while HDAC2 deletion enhanced memory [158]. Also, mutations in CBP, a well-known transcriptional coactivator that has HAT activity, contributes to the pathology of Rubinstein-Taybi syndrome, a neurodevelopment disorder characterized by cognitive impairment [159]. The cognitive enhancement provoked by the deletion of HDAC was accompanied by increased hippocampal histone acetylation, specifically at the promoter region of genes involved in synaptic plasticity and memory, such as synaptophysin and BDNF. Acetylation of H3 and H4 around BDNF promoters is characterized by fear conditioning memory [160]. It has also been shown that CBP is recruited to the c-fos gene promoter in an activity-dependent manner, which promotes memory formation and consolidation [38].

5.1.2. Cognition and the methylation of the BDNF promoter IV

BDNF is a member of the nerve growth factor family of neurotrophins, and plays central roles in the development, physiology, and pathology of the nervous system. BDNF induces neuroprotective activities, including survival and differentiation of neurons, by binding and activating the tropomycin receptor kinase B (TrkB), a member of the larger family of Trk receptors, localized both on the pre-synaptic and post-synaptic membranes. TrkB-binding orchestrates the stabilization and strengthening of synaptic connections by the induction of several signaling cascades that are involved in the regulation of synaptic plasticity and cAMP-responsive, element-binding, protein (CREB)-dependent gene expression [161]. One of the downstream targets of BDNF signaling is the activity-regulated, cytoskeleton-associated protein (ARC), which is a member of the immediate-early gene family and is important in synaptogenesis and synaptic plasticity as well as anxiety and alcohol intake phenotypes [162]. Therefore, BDNF regulates synaptic transmission and plasticity and also plays a fundamental role in learning and memory during development and in adults [163]. In the adult rodent brain, BDNF is widely distributed with the highest mRNA and protein levels in the hippocampus, amygdala, cerebral cortex, and hypothalamus [164]. In humans, BDNF has a similar brain distribution, and it is also present in peripheral tissues and blood [165]. Blood BDNF levels reflect brain concentrations [165].

Table 5
Epigenetic modifications identified in blood or *postmortem* tissues from individuals affected by reduced cognitive performance.

DNA methylation	Cognitive impairment	Biological sample
Hypomethylation of pro-inflammatory genes: NF-kB; COX-2	AD	frontal cortex [122]
Hypermethylation of neuronal genes: BDNF and synaptophysin with reduced content of BDNF (neuroplasticidade; cognition)	AD	frontal cortex [122,123]
Global hypomethylation in neuronal nuclei in neocortex	AD	temporal neocortex and cerebellum [124]
Global hypomethylation and hydroxymethylation in glia and neurons	AD	hippocampus [125]
Genome-wide analysis: 918 differentially methylated genes (CpG sites); highest ranking genes: TMEM59 and TMEM2	AD	frontal cortex [126–128]
Hypomethylation of S100A2 gene	AD	temporal neocortex [129]
Hypermethylation of SORBS3 gene (cytoskeleton organization)	AD	temporal neocortex [129]
Hypomethylation of APP gene (amyloid deposit)	AD	frontal cortex [130]
Hypermethylation of OPRK1 OPRM1-CpG1 hypermethylation and OPRM1-CpG2-4 hypomethylation (risk factors for MCI is specific Chinese populations; opioid receptor)	MCI	blood [131]
Hypermethylation of SORL1 gen (receptor of APOE in the brain; higher methylation in MCI + T2D than in T2D or MCI alone)	MCI	PMBC [132]
Hypermethylation of the SORL1 gene promoter region	AD	hippocampus [133] frontal cortex [133]

Histone modification	Cognitive impairment	Biological sample
Decreased acetylation of H3 (repression ofgene expression)	AD	temporal lobe [134]
Increased phosphorylation of H3 in neurons (cytoplasmic)	AD	hippocampus [135]
Increased levels of HDAC2 in CA1 neurons (negative regulation of synaptic function)	AD	hippocampus [136]
Reduced SIRT1 levels (AD: 4X reduction and MCI: 2X reduction vs. young controls and 2X and 1.3X vs. geriatric controls, respectively)	AD / MCI	plasma [137]

ncRNA	Cognitive impairment	Biological sample
hsa-miR-34a[&]; hsa-miR-579; hsa-miR-181b[#]; hsa-miR-520h; hsa-miR-155; hsa-miR-517*[&]; hsa-let-7f[&]; hsa-miR-200a[&]; hsa-miR-37[#] (downregulate APP catabolism; hsa-let-7f: activation TLR7 and causes neurodegeneration)	AD [#]AD + positive for ApoEε4 [&]AD + negative for ApoEε4	PBMC [138]
hsa-let-7d-5p; hsa-let-7g-5p; hsa-miR-15b-5p; hsa-miR-142-3p; hsa-miR-191-5p; hsa-miR-301a-3p; hsa-miR-545-3p (AD signature)	AD	plasma [139]
Reduced expression of hsa-miR-27a-3p	AD	CSF [140]
Reduced expression of miR-9 and miR-132 (miR-9: neurogenesis; miR-132: BDNF expression, neurite extension and neurogenesis)	AD	Hippocampus [140]
Increased expression of miR-146b; miR-181a; miR-142 (miR-146b: innate immunity; miR-181a and miR-142: T-cell activation and differentiation)	AD	CSF [140]
miR-34c (repression of Bcl2 and Sirt1 involved in cell survival and oxidative defense pathways)	AD	plasma [141] PBMC [141]
Top 10 upregulated long ncRNAs: lnc-FAM53B-2_1, ENST00000615844.1, lnc-MYL2-2_1, lnc-NKD2-1_1, lnc-EVC-3_1, lnc-SLC5A7-1_1, NR_024101, lnc-DCP1B-6_1, lnc-MNAT1-8_1, and lnc-TNFRSF19-4_1	POCD	serum [142]
Top 10 downregulated long ncRNAs: NR_024187, lnc-PTPRU-7_1, lnc-LIPC-6_1, lnc-CTD-2144E22.5.1-26_1, lnc-OTX1-7_1, ENST00000618386.1, lnc-PRL-9_1, lnc-PAG1-9_1, lnc-ZNF729-4_1, and lnc-RAP2C-5_1	POCD	Serum [142]

AD: Alzheimer's disease; APP: β-amyloid precursor protein; MCI: Mild cognitive impairment; T2D: Type 2 diabetes; POCD: Postoperative cognitive dysfunction

The BDNF gene has a complex regulatory and genetic structure, with 9 different promoters encoding at least 20 different splice variants, sharing one common exon that encodes the BDNF protein (known as exon IX) [166]. A BDNF-antisense transcript has been described to be a negative regulator of BDNF exon IX expression and to compromise neurogenesis and cognition [167–169]. Each exon is regulated by its own unique promoter, conferring temporal and spatial control of BDNF expression in an activity-dependent manner. The eight upstream exons encode promoters regulating regional and cell-type-specific expression. Exon IV has been the most extensively characterized, since the containing-promoter elements regulate activity-dependent BDNF expression. Elevated BDNF exon IV DNA methylation is a mechanism for BDNF down-regulation demonstrated in rodents and in humans, leading to lower content of BDNF in the brain (hippocampus and pre-frontal cortex) and in the blood [170–173].

5.1.3. Cognition and the methylation of the REST promoter

REST [RE1-silencing transcription factor (otherwise called neuron-restrictive silencer factor)] is widely known to work as a master transcription factor that represses neuronal genes in non-neuronal cells during differentiation [174]. However, the better understanding of the REST-binding motifs increased the number of potential REST targets, at all stages of cell differentiation (by several thousand), and now it is known that REST also promotes gene expression at all stages of cell differentiation [175,176].

REST down-regulates genes linked to cell-death and enhances the expression of others that induce resistance to ageing and the preservation of cognitive function in adult neurons [66,177]. For example, it was reported that REST positively modulates CREB expression, inhibits reactive oxygen species production, preserves mitochondrial integrity, physiology and distribution and prevents apoptosis [78,178,179]. In this scenario, it has been proposed that REST may play a role in protecting against neurodegenerative processes [78,177,180].

In the adult rat brain, REST mRNA has been detected at higher levels in cells displaying neuronal properties, including the olfactory system (in the granule cell layer and the plexiform layers), cerebral cortex, hippocampal formation, diencephalon (thalamus and hypothalamus), midbrain (such as substantia nigra pars compacta), brain stem (such as pontine nuclei), and cerebellum, with the highest levels detected in the neurons of the hippocampus, pons/medulla, and midbrain

Table 6
Main epigenetics alterations induced by exercise to promote enhanced cognition

Exercise paradigm (model)	Epigenetic mechanism (brain region)	Mediators of enhanced cognition (brain region)
Voluntary wheel running (adult rats; 7 days of exercise) [187]	DNA demethylation at the BDNF promoter IV; increased levels of MeCP2; increased H3 acetylation; reduction of HDAC5 mRNA levels and protein (hippocampus)	Increased mRNA and content of BDNF (hippocampus)
Voluntary wheel running (adult and aged mice; 8 weeks of exercise) [177]	Possibly hypomethylation of REST promoter (hyperglycemia-induced hypermethylation of REST promoter reduces REST and BDNF levels [72]) (hippocampus)	Increased mRNA of BDNF and REST; attenuation of inflammation (hippocampus)
Voluntary wheel running (young mice; 4 weeks of exercise) [149]	Reduced HDAC2 and HDAC3 expression; reduced HDAC3 occupancy (binding) (hippocampus)	Increased D-β-hydroxybutyrate levels (HDAC inhibitor); increased BDNF promoter I and II expression; increased BDNF content (hippocampus)
Voluntary wheel running (adolescent mice; 7 days of exercise) [188]	Increased global H3 acetylation; reduction activity of DNMTs; reduction activity of DNMTs; (hippocampus; cerebellum)	Increased BDNF gene expression (hippocampus)
Voluntary wheel running (adult rats with blocked BDNF action; 3 days) [189]	TrkB-IgG chimera antagonist of BDNF (hippocampal injection)	Reduction of exercised-induced increased synapsin I and synaptophysin (hippocampus)
Voluntary wheel running (adult mice; 15 days) [190]	Upregulation of 20 miRNAs; downregulation of 12 miRNA (hippocampus)	Reduced miR-21; reduced miR-34a (hippocampus)
Voluntary wheel running (adult mice; 4 weeks) [191]	Increased glucocorticoid receptor (Nr3c1) expression; increased methylation of Nr3c1-1F promoter (hippocampus)	Reduced miR-34a (hippocampus)
Treadmill running (adult senescent mice; 60 min a day, 5 days a week; 4 weeks) [192]	Activation of HAT and HDAC (1-11) (hippocampus)	Increased BDNF expression; reduced p75 transcription (apoptosis signaling) (hippocampus)
Treadmill running (adult and aged rats; 20 min, 2 weeks) [193]	Increased global H4 activity in aged rats (hippocampus)	Reduced inflammation (hippocampus)
Treadmill running (adult paternal rats; 60% of VO2 max, 22 days)	Unknown (paternal exercise-induced changes in the offspring)	Reduced global DNA methylation (adult male offspring hippocampus)
Swimming exercise (adult mice; 4 sessions of 5 min per day; 4 weeks) [194]	Increased acetylation of H3K9, H3K14, H4K5, H4K8, and H4K12 1h after the first session and 24 h after the fifth session; increased acetylation of H3K9 up to 4 weeks (hippocampus)	Increased cAMP-response element-binding protein (CREB)-binding protein (CBP) levels (CA1 – hippocampus)

[181]. Increased expression of REST could be induced acutely by toxicants as a compensatory neuroprotection [181,182]. However, our group recently demonstrated that REST expression could be physiologically induced in the adult hippocampus by voluntary exercise. The increased REST gene expression induced by physical exercise was shown to occur in parallel to the increased BDNF expression and inversely to the pro-inflammatory status in the aged mouse hippocampus [177].

It has been reported that REST is lost from the nucleus of hippocampal and cortical neurons in many diseases of chronic progression with cognitive impairments, including several forms of dementia, such as AD, frontotemporal dementia, and dementia with Lewy bodies [66]. However, REST levels were shown to be associated with a significant induction of REST in neuronal nuclei together with increased REST binding to target genes in post-mortem samples of prefrontal cortices (PFC) from aged individuals. REST expression is significantly higher in aged individuals (73-106 years) than in younger adults (20-35 years) and also in aged mice [66,177].

The relationship between enhanced cognition and REST activity appears to be related to the repression of cell death-associated genes, suggesting that REST might be neuroprotective. Indeed, mice lacking REST exhibited cognitive impairment and progressive neurodegeneration, including neuronal loss in the hippocampus and cortex.

Our group recently demonstrated that the REST promoter is hypermethylated, leading to lower expression and content of REST specifically in the hippocampus of hyperglycemic rats with cognitive impairment [72]. Hypermethylated DNA has been linked to reduced binding of many transcription factors, including phosphorylated CREB [183], and REST is known to bind promotor regions with low methylation levels [184]. The increased methylation of REST was not due to increased methylation activities by DNMTs, but due to defective active demethylation. In addition, our group also demonstrated that the correction of the metabolic disturbances by administering insulin prevented the cognitive alterations in hyperglycemic rats and also rescued compromised REST expression and levels in the hippocampus [72]. Lower levels of REST were also observed in PBMC from obese children, and were more compromised in severely obese children, linking REST promoter methylation to BMI, adiposity and obesity.

5.2. Exercise promoting epigenetic neuroprotective modifications

It is well understood that the regular practice of physical exercise improves health by contributing to disease prevention and helping recovery from illness. It has been extensively demonstrated that exercise, with or without dietary intervention, can cause modification in the genome allowing gene expression/repression of genes that will enhance, *i.e.*, learning, cognition and memory. These processes are accompanied by increased cell proliferation and survival in the hippocampus of rodents [185,186]; effects that are mediated, in part, by increased production and secretion of BDNF and possibly by hypomethylation of REST promoter. Table 6 shows the main epigenetics alterations induced by exercise to promote enhanced cognition.

As shown in Table 6, exercise has the extraordinary potential to promote immediate and stable changes in hippocampal gene function that enhances cognition (upregulating mnemonic and repressing harmful pathways), independently of the exercise paradigm. Although Table 6 denotes the main epigenetic modifications induced by exercise (DNA methylation and histone modifications, and miRNAs) and the increased hippocampal biochemical mediators of cognition, enhanced mnemonic activity driven by exercise also provokes increased levels of hippocampal IGF-1, β-CaMKII and Akt [195], pCREB [194], glutamate [196], growth factors [197], lactate [198], irisin [199,200] or IL-6 [201], which might also be controlled by epigenetics modifications. Altogether, the exercise-induced adaptations, specifically in the hippocampus, remodeled chromatin, facilitating the access of cognitive mediators to BDNF promoter regions, resulting in greater BDNF expression and content.

5.2.1. Exercise modifications induced by the ergogenic drug caffeine

Caffeine (1,3,7-trimethylxantine) is a widely consumed social psychoactive drug in western society that increases physical and cognitive performance [202–205]. It is the main ergogenic resource used by elite

athletes [206]. Besides ergogenic and psychoactive effects, several epidemiologic studies have shown that regular consumption of caffeine, mainly in infusions, delays the progression of dementia [207–209] and also decreases the probability of developing neurodegenerative diseases, including AD and PD. Studies of dementia in animal models have also highlighted the neuroprotective effects of caffeine [210–213].

Most biological effects of caffeine are mediated through the antagonism of all types of adenosine receptors (AR). Caffeine antagonizes mostly A1R and A2R subtypes in the CNS. Caffeine is also an inhibitor of phosphodiesterases, which provokes increased levels of cAMP, induces the release of calcium from intracellular storages, and acts as a non-competitive inhibitor of acetylcholinesterase [214–216]. These effects appear to underlie caffeine-driven enhanced learning and memory. However, there is some evidence that caffeine may also inhibit HDAC activity, allowing for higher BDNF expression and content in the hippocampus of rats [217,218].

5.3. Epigenetic benefits of exercise on cognition in obesity

Individuals affected by common chronic metabolic diseases present altered patterns of DNA methylation [63–65] and compromised expression of neuroprotective genes, which favors neurotoxicity and cognitive impairment or dementia [66]. Although the negative impact of this pandemic chronic disease on cognition has been investigated extensively in humans and experimental animals, its prevention or treatment is far from being successful. It is known that obesity negatively regulates hippocampal BDNF expression [85,86], and low blood and brain BDNF content has been observed in obese individuals and animal models with cognitive impairment [87]. Therapy for obesity, including GLP-1 signaling or physical exercise, among others, is known to increase BNDF levels (see Table 6) [219]. An increasing number of identified harmful pathways induced by obesity compromise cognition by promoting specific epigenetic profiles (see Table 1, Table 4a and Table 4b). Some of these alterations, including the induction of oxidative stress, inflammation, mitochondrial dysfunction, and reduced mnemonic activity have been linked to epigenetic modifications (Table 1, Table 4a and Table 4b). Physical exercise can potentially revert or prevent many of them; however, the understanding of chromatin remodeling induced by this drug-free intervention in obesity-driven cognitive impairment has not been extensively elucidated or confirmed.

6. Conclusion

The high caloric intake associated with low-energy expenditure is a major risk factor for the development of obesity in individuals from all age groups. It is well established that insulin resistance and obesity are primary factors for cognitive impairment and are associated with higher risk of developing neurodegenerative diseases. Obesity and peripheral metabolic alterations negatively modulate gene transcription pathways that interplay to compromise BDNF and REST levels in the hippocampus, two primordial mediators of cognitive processes. To counterbalance the harmful scenario induced by obesity, physical exercise has been prescribed by doctors as the first line of non-pharmacological treatment. A better understanding of the molecular mechanisms involved in enhanced cognition induced by exercise, in particular those related to reversible epigenetic alterations, will allow for the prescription of appropriate exercise protocols, which will ultimately result in better cognitive outcomes.

Conflict of interest

The authors declare no conflict of interest.

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CHAPTER III

ANTI-INFLAMMATORY EFFECT OF CAFFEINE ON MUSCLE UNDER LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION

This chapter is original research that provides evidence of the anti-inflammatory effects of caffeine. It includes new information regarding the effects of caffeine administration on the expression of inflammatory cytokines, inflammasome components, epigenetic profile, and oxidative metabolism-linked genes in the *vastus lateralis* muscle of mice submitted to LPS-induced inflammation.

(P5) Antioxidants:
Anti-inflammatory effect of caffeine on muscle under lipopolysaccharide-induced inflammation. **Eichwald, T.**; Solano, A.F.; Souza, J.; de Miranda, T.B.; Carvalho, L.B.; dos Santos Sanna, P.L.; da Silva, R.A.F; Latini, A. (Published in 23 Feb 2023).

4.1 CAFFEINE AND ITS ANTI-INFLAMMATORY PROPERTIES

Caffeine is the most consumed psychoactive drug in the world. Caffeine can be extracted from over sixty species of plants and is present in various foods such as coffee, tea, soft drinks, energy drinks, and chocolates (ZUCCONI et al., 2013). After its chemical isolation in 1819 and synthetic production in 1895, caffeine also began to be explored by the pharmaceutical industry. Now, it can be found in cold medicines, painkillers, appetite suppressants, and stimulants (BUERGE et al., 2003), and it is also used in the treatment of conditions like headaches, postprandial hypotension, respiratory depression in neonates, among others (WALDVOGEL, 2003).

On the other hand, the acute administration of caffeine can induce hypertension, arrhythmias, increased plasma catecholamine levels, increased urine output, gastric acid secretion, and changes in mood and sleep patterns (RODAK; KOKOT; KRATZ, 2021). However, no clear evidence exists that excessive caffeine consumption causes significant health risks in healthy individuals. Caffeine is rapidly and completely absorbed by the gastrointestinal tract and distributed throughout all body tissues. Due to its ability to cross the blood-brain barrier and cell membranes, it has been established that the CNS and the muscle are the main sites of action to promote its ergogenic effects (MIELGO-AYUSO et al., 2019).

Even though caffeine is not considered an essential nutrient to promote health, its ergogenic properties have led to the inclusion of these compounds in diets to improve performance (GRAHAM; SPRIET, 1995). The capacity of caffeine to improve muscle work has been widely studied over the years. Its ergogenic effects have been demonstrated in aerobic and anaerobic exercises, including muscular strength (ASTORINO; ROHMANN; FIRTH, 2007), running (GRAHAM; SPRIET, 1991), cycling (SPRIET et al., 1992), team sports (SCHNEIKER et al., 2006), among others, before or during competitions (DEL COSO; MUÑOZ; MUÑOZ-GUERRA, 2011).

Caffeine is a non-selective competitive adenosine receptor (AR) antagonist (ONGINI; FREDHOLM, 1996), and ARs are expressed in the whole body, including in the immune system cells. Caffeine can modulate monocytes and macrophage function through these receptors depending on the receptor subtypes and the environment in which the cells are exposed (HASKÓ et al., 2007). In consonance, it has been reported that caffeine decreases the levels of inflammatory cytokine in cord blood monocytes (CHAVEZ-VALDEZ et al., 2009, 2016) as well as augments the

instruction of anti-inflammatory macrophages in mesenchymal stem cells (SHUSHTARI; ABTAHI FROUSHANI, 2017).

It has been suggested that caffeine intake may act synergistically with exercise on cytokine response, repressing the release of pro-inflammatory cytokines and increasing the production of anti-inflammatory ones (CECHELLA et al., 2014; ROSSI et al., 2017; TAULER et al., 2013). For example, it was shown that caffeine administration inhibited LPS-induced nitric oxide production in zebrafish (HWANG et al., 2016). Similar results were observed in the *in vitro* model, suggesting that caffeine inhibited LPS-induced inflammatory response in a macrophage-like cell line by downregulating NF-κB activation and MAPK phosphorylation (HWANG et al., 2016). The anti-inflammatory action of caffeine was also demonstrated in human whole blood cells culture exposed to LPS (HORRIGAN; KELLY; CONNOR, 2004).

The potential effects of caffeine on inflammasome NLRP3 activation (for a review in inflammation and inflammasome, see chapter II) have also been demonstrated in the human monocyte leukemia cell line (ZHAO et al., 2019). Indeed, macrophages treated with different doses of caffeine (100-800 μM) for six h significantly reduced the NLRP3 expression. Moreover, the A_{2A} receptor silencing evoked by caffeine reduced the caspase-1 expression in macrophages by reducing ROS production (ZHAO et al., 2019).

ORIGINAL RESEARCH ARTICLE: ANTI-INFLAMMATORY EFFECT OF CAFFEINE ON MUSCLE UNDER LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION

(P5) Antioxidants (impact factor 7.675):
Anti-inflammatory effect of caffeine on muscle under lipopolysaccharide-induced inflammation. **Eichwald, T.**; Solano, A.F.; Souza, J.; de Miranda, T.B.; Carvalho, L.B.; dos Santos Sanna, P.L.; da Silva, R.A.F; Latini, A. (Published in 23 Feb 2023).

While moderate-intensity physical exercise has anti-inflammatory properties, it is known that high-intensity physical exercise promotes a pro-inflammatory scenario characterized by increased levels of pro-inflammatory cytokines that are related to the development of fatigue, resulting in reduced performance (HARGREAVES; SPRIET, 2020).

Caffeine is the most consumed psychoactive drug in the world (ZUCCONI et al., 2013). It is a non-selective competitive adenosine receptor antagonist (ONGINI; FREDHOLM, 1996), and it has been proposed that the main sites of action to promote its ergogenic effects are the CNS and the muscle (MIELGO-AYUSO et al., 2019). Even though it is unclear what molecular mechanisms are behind caffeine consumption and its ergogenic responses, its capacity to improve exercise performance and delay fatigue has led to the inclusion of these compounds in diets (GRAHAM; SPRIET, 1995). In addition, caffeine has been shown to promote changes in gene expression that are related to altered epigenetics (CHUANG et al., 2017)

Thus, investigating the anti-inflammatory effects of caffeine administration in the *vastus lateralis* muscle of animals submitted to inflammation may offer relevant information about the molecular mechanisms involved in the ergogenic effects of caffeine.

Key Results & Implications

It is described in this chapter anti-inflammatory effects induced by caffeine administration in the skeletal muscle of mice exposed to LPS. The main results demonstrated here allowed us to propose the use of caffeine as an inflammation-reducing agent since *i)* reduced gene expression of pro-inflammatory cytokines (*Il1b* and *Il6*); *ii)* increased gene expression of anti-inflammatory cytokine

(*Il10*); *iii)* decreased gene expression of the Nlrp3 inflammasome components *Asc* and *Casp1* were demonstrated. Moreover, along with LPS, caffeine administration evoked changes in the epigenetic profile, promoting *de novo* DNA methylation. These findings suggest that the inflammation mediated by the LPS can be attenuated by caffeine. The involvement of caffeine in epigenetics opens a new avenue for investigating caffeine's potential roles in the DNA methylation of different genes impacting biological processes.

antioxidants

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Anti-Inflammatory Effect of Caffeine on Muscle under Lipopolysaccharide-Induced Inflammation

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Abstract: Evidence has shown that caffeine administration reduces pro-inflammatory biomarkers, delaying fatigue and improving endurance performance. This study examined the effects of caffeine administration on the expression of inflammatory-, adenosine receptor- (the targets of caffeine), epigenetic-, and oxidative metabolism-linked genes in the *vastus lateralis* muscle of mice submitted to lipopolysaccharide (LPS)-induced inflammation. We showed that caffeine pre-treatment before LPS administration reduced the expression of *Il1b*, *Il6*, and *Tnfa*, and increased *Il10* and *Il13*. The negative modulation of the inflammatory response induced by caffeine involved the reduction of inflammasome components, *Asc* and *Casp1*, promoting an anti-inflammatory scenario. Caffeine treatment *per se* promoted the upregulation of adenosinergic receptors, *Adora1* and *Adora2A*, an effect that was counterbalanced by LPS. Moreover, there was observed a marked *Adora2A* promoter hypermethylation, which could represent a compensatory response towards the increased *Adora2A* expression. Though caffeine administration did not alter DNA methylation patterns, the expression of DNA demethylating enzymes, *Tet1* and *Tet2*, was increased in mice receiving Caffeine+LPS, when compared with the basal condition. Finally, caffeine administration attenuated the LPS-induced catabolic state, by rescuing basal levels of *Ampk* expression. Altogether, the anti-inflammatory effects of caffeine in the muscle can be mediated by modifications on the epigenetic landscape.

Keywords: cytokines; inflammasome; epigenetics; DNA methylation; adenosine receptors; bioenergetics

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1. Introduction

Regular, moderate-intensity exercise has been proven to promote an anti-inflammatory state that helps prevent the development of chronic diseases (for a review see [1]). Strenuous exercise can lead to increased levels of blood proinflammatory cytokines, which are linked to fatigue, and therefore, to reduced performance [2]. This scenario has led to caffeine administration being used to increase alertness [3], to accelerate metabolism [4], and to delay fatigue development in aerobic and anaerobic exercises, including muscular strength [5], running [6], cycling [7], and team sports [8], among others.

While it is unclear what molecular mechanisms are behind caffeine consumption and its ergogenic responses, evidence is mounting that caffeine may induce anti-inflammatory effects in both humans, and animals. For example, it has been demonstrated that caffeine supplementation reduced inflammatory markers in the blood of athletes [9–11]. In the case of animal models, reduced pro-inflammatory and increased anti-inflammatory markers were not only seen in the blood of trained rats, but also in key tissues linked to exercise performance, such as the brain, the lung, the heart and the skeleton, of rodents exposed

to caffeine [12–16]. Furthermore, an elegant study involving 114 participants showed that caffeine intake is associated with lower inflammation and activation of the inflammasome, which resulted in less production of the pro-inflammatory cytokine interleukin-1 beta (IL-1b) [17].

In addition, caffeine supplementation has been shown to cause changes in gene expression that could be linked to improved exercise performance [18–20]. These modifications have been related to altered epigenetics, a term conceived to describe the possible causal processes acting on genes that regulate phenotype [21]. Some of the reported effects of caffeine are associated with DNA methylation [22], a major epigenetic factor influencing gene activities. Considering that epigenetics can change the activity of a DNA segment without changing the sequence, it is plausible that caffeine can modulate inflammatory processes by changing the epigenetic landscape. When DNA methylation is increased in a gene promoter, it will typically act to repress gene transcription, including the expression of inflammatory mediators. Altogether, we aimed to understand whether caffeine can modulate epigenetics to induce an anti-inflammatory scenario in the mouse skeletal muscle.

2. Materials and Methods

2.1. Animals

Adult Swiss male mice (3–5 months of age; body mass 45–50 g) from the central animal house of the Centre for Biological Sciences, *Universidade Federal de Santa Catarina* (Brazil) were kept in a controlled environment (22 ± 1 °C, 12 h light/dark cycle) with water and food ad libitum, for ten days (acclimatation period). The experimental protocols were approved by the Ethics Committee for Animal Research (PP00760/CEUA) of the Federal University of Santa Catarina (Brazil). All efforts were made to minimize the number of animals used and their suffering. Five mice were included per experimental group, unless otherwise stated.

2.2. LPS-Induced Inflammation

Acclimatized mice were randomly divided into the following 4 groups (5 animals per group): Vehicle: Animals that received an intraperitoneal (i.p.) injection of 0.9 % sodium chloride (injection volume of 0.1 mL for every 10 g of body weight); Caffeine: Animals that received an i.p. injection of caffeine (6 mg/kg of body weight); LPS: Animals that received an i.p. injection of LPS (0.33 mg/kg of body mass; *E. coli* LPS, serotype 0127:B8), and Caffeine+LPS: Animals that received an i.p. injection of caffeine and 15 min later received the injection of LPS. Twenty-four hours after the treatment mice were euthanized by cervical dislocation and the vastus lateralis muscle was immediately collected and processed in Trizol as previously published by our group [23]. The dosage of LPS used was based on previously published data [24,25].

2.3. RNA Extraction and cDNA Synthesis

For total RNA extraction, the *vastus lateralis* muscle was collected 24 h after the administration of the different treatments (vehicle, caffeine, LPS and Caffeine+LPS), and immediately homogenized with Ambion TRIzol Reagent (Life Sciences, Fisher Scientific Inc., Waltham, MA, USA). After adding 200 µL of chloroform, and followed by centrifugation at 17,000× *g* for 15 min at 4 °C, the upper aqueous layer containing the RNA was collected and transferred to a new tube. Then, 800 µL of chilled isopropanol were added and after light agitation, the RNA was precipitated by centrifugation at 17,000× *g* for 15 min at 4 °C. The supernatant was removed by inversion and the precipitated RNA was washed with 1 mL of 70 % alcohol and again centrifuged at 17,000× *g* for 5 min at 4 °C. Supernatants were discarded and 50 µL of nuclease-free H_2O was added to the tube. The quantity and purity of the extracted RNA was estimated by using the NanoDrop spectrophotometer, at 260 and 280 nm. The synthesis of the cDNA was performed after treating the total RNA with DNase I (Invitrogen, Carlsbad, CA, USA), and with high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

2.4. Real-Time Reverse Transcription and Quantitative PCR (RT-qPCR)

Real-time reverse transcription and quantitative PCR (RT-qPCR) reactions were performed using SYBR Green Master Mix (PowerUp™ SYBR™ Green Master Mix-Applied Biosystems, Foster City, CA, USA) with specific primers shown in Table 1. All the reactions were carried out in a total of 10 µL, containing 5 µL of specific primers (0.4 µM of each primer), 50 ng of cDNA and nuclease-free H_2O in a QuantStudio® 3 Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Oligonucleotide primers and PCR conditions used in quantitative real-time PCR.

Gene (ID)	Primer	5′-3′Sequence	Reaction Condition	Product Size (bp)
Il1b (16176)	Forward Reverse	GAC CTT GGA TGA GGA CA AGC TCA TAT GGG TCC GAC AG	95 °C-15 s; 60 °C-30 s; 72 °C-30 s	183
Il6 (16193)	Forward Reverse	AGT TCG CTT CTT GGG ACT GA CAG AAT TGC CAT TGC ACA AC	95 °C-15 s; 60 °C-30 s; 72 °C-30 s	191
Tnfa (11647)	Forward Reverse	CCA CAT CTC CCT CCA GAA AA AGG GTC TGG GCC ATA GAA CT	95 °C-15 s; 60 °C-30 s; 72 °C-30 s	259
Il10 (21926)	Forward Reverse	CCA AGC CCT TAT CGG AAA TGA TTT TCA CAG GGG AGA AAT CG	95 °C-15 s; 60 °C-30 s; 72 °C-30 s	163
Il13 (16163)	Forward Reverse	CAG TCC TGG CTC TTG CTT G CCA GGT CCA CAC TCC ATA CC	95 °C-15 s; 60 °C-30 s; 72 °C-30 s	165
Adora1 (11539)	Forward Reverse	AGA ACC ACC TCC ACC CTT CT TAC TCT GGG TGG TGG TCA CA	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	227
Adora2A (11540)	Forward Reverse	ATC CCT CAGAGA AGG GAA GC AGC TTC CCA AAG GCT TTC TC	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	300
Dnmt1 (13433)	Forward Reverse	CCT TTG TGG GAA CCT GGA A CTG TCG TCT GCG GTG ATT	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	240
Dnmt3A (13435)	Forward Reverse	GAG GGA ACT GAG ACC CCA C CTG GAA GGT GAG TCT TGG CA	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	216
Dnmt3B (113436)	Forward Reverse	AGC GGG TAT GAG GAG TGC AT GGG AGC ATC CTT CGT GTC TG	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	91
Tet1 (52463)	Forward Reverse	GAG CCT GTT CCT CGA TGT GG CAA ACC CAC CTG AGG CTG TT	95 °C-15 s; 65 °C-30 s; 72 °C-30 s	367
Tet2 (214133)	Forward Reverse	AAC CTG GCT ACT GTC ATT GCT CCA ATG TTC TGC TGG TCT CTG TGG GAA	95 °C-15 s; 65 °C-30 s; 72 °C-30 s	211
Tet3 (194388)	Forward Reverse	GTC TCC CCA AGT CCT ACC TCC G GTC AGT GCC CCA CGC TTC A	95 °C-15 s; 63 °C-30 s; 72 °C-30 s	137
b-actin (11461)	Forward Reverse	TCT TGG GTA TGG AAT CCT GTG AGG TCT TTA CGG ATG TCA ACG	95 °C-15 s; 58 °C-30 s; 72 °C-30 s	82
Gapdh (14433)	Forward Reverse	AGG CCG GTG CTG AGT ATG TC TGC CTG CTT CAC CAC CTT CT	95 °C-15 s; 58 °C-30 s; 72 °C-30 s	530

2.5. DNA Extraction

Genomic DNA (gDNA) was extracted from the mouse muscle 24 h after the treatments. Tissues were homogenized in extraction buffer (10 mM Tris pH 3.0; 0.5% SDS, 5 mM EDTA) and then digested with proteinase K solution at 65 °C for 16 h. Additionally, 500 µL of equilibrium phenol was transferred to the tube and thus the mixture was spun down at 17,000× *g* for 15 min. The upper aqueous layer containing the target DNA was preserved and mixed with 200 µL of chloroform. The mixture was centrifuged at 17,000× *g* for 15 min and the supernatant was collected and transferred to a new tube. Then, 800 µL of isopropanol and 150 µL sodium acetate 3 M was added to the mixture. Next, the mixture was centrifuged at 17,000× *g* for 15 min. The supernatant was removed, and the pellet was

washed with 500 µL of 70 % alcohol, centrifuged at 17,000× *g* for 5 min. The supernatant was then completely discarded and 50 µL of nuclease free H_2O was added to the tube. The quantity and purity of extracted gDNA was estimated by using the spectrophotometer apparatus NanoDrop, at 260 and 280 nm.

2.6. Enzymatic gDNA Treatment

After confirming the quantity and purity by spectrophotometry (OD 260/280 $\geq$ 1.8 and OD 260/230 $\geq$ 1.0), the gDNA was treated with T4-β-glucosyltransferase (T4-BGT) and subsequently with MspI and HpaII (New England BioLabs, Beverly, MA, USA). For this, three tubes (A, B and C) containing 400 ng gDNA of each sample were treated with 40 mM UDP glucose and T4-BGT (1 unit) for 1 h at 37 °C, followed by enzyme inactivation for 10 min at 65 °C. Next, the samples were digested with H_2O (tube A), MspI (tube B) and HpaII (tube C) for 2 h at 37 °C according to the manufacturer's instructions.

2.7. Methylation-Specific qPCR (MS-qPCR)

MS-qPCR methylation data were derived from 5 independent animals and a technical duplicate. The pattern of methylation (5-meC) and hydroxymethylation (5-hmeC) of the promoter regions of Adora1 (island 1 (F: 5′ AAG GAG CTC ACC ATC CTG 3′); (R: 5′ GTG GGT GGG CAC AGG GTA G 3′) and island 2 (F: 5′ CGA GAC TCC ACT CTG GC 3′); (R: 5′ CAC CTC GGT ACT GTC CCT GT 3′)) and Adora2A (F: 5′ AGG GTG CGC CCA TGA GCG GC 3′); (R: 5′ CAA CCC GAG AGT CTG ACC CGC CT 3′) were determined in qPCR reactions containing 2x SYBR Green I Master (5 µL), 0.4 µM specific primers (1 µL), 25 ng of treated gDNA (1.5 µL-3 conditions: H_2O, MspI and HpaII) and q.s.p of nuclease-free H_2O (2.5 µL). Primer sequences were designed in regulatory regions with CpG islands within regions of hypersensitivity to DnaseI, regulated by histone modification markers and with transcription factor binding sites using the Primer3 Input program (version 0.4.0) [26]. All primers sequences were blasted to confirm chromosomal location by the in-silico PCR tool (https://genome.ucsc.edu/, accessed on 15 June 2022) and the secondary structures and annealing temperatures analyzed using the Beacon Designer program (http://www.premierbiosoft.com/, accessed on 15 June 2022).

2.8. Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). Data were analyzed by two-way ANOVA followed by the post hoc test of Tukey when *F* was significant. When comparing two independent groups, Student's *t*-test for independent samples was used. The accepted level of significance for the tests was $p < 0.05$. Statistics and all graphs were performed by using GraphPad Prism 9®.

3. Results

3.1. Caffeine Administration Reduced LPS-Mediated Inflammation in the Mouse Muscle

Figure 1 shows the effect of caffeine and/or LPS administration (i.p.) after twenty-four h on pro-inflammatory cytokines gene expression in the mouse *vastus lateralis* muscle (Figure 1A). LPS exposure significantly increased the expression of the pro-inflammatory cytokine *Il1b* ($F_{(1,16)} = 6.46$, $p < 0.01$) (Figure 1B). Moreover, the expression of the anti-inflammatory cytokines *Il10* ($F_{(1,16)} = 6.73$, $p < 0.05$) (Figure 1E) and *Il13* ($F_{(1,16)} = 5.36$, $p < 0.01$) (Figure 1F) were also upregulated, possibly as a physiological compensatory response elicited by LPS-induced inflammation. Figure 1B shows that the expressions of *Il1b* and *Il6* (Figure 1C) were downregulated, and *Il10* (Figure 1E) upregulated when caffeine was administered in association with LPS. Furthermore, caffeine *per se* positively modulated the expression of *Tnfa* ($F_{(1,16)} = 19.32$, $p < 0.001$) (Figure 1D) and decreased the *Il6* levels ($F_{(1,16)} = 2.20$, $p < 0.05$) (Figure 1C). However, no differences were observed in the levels of *Il1b* (Figure 1B), *Il10* (Figure 1E), and *Il13* (Figure 1F).

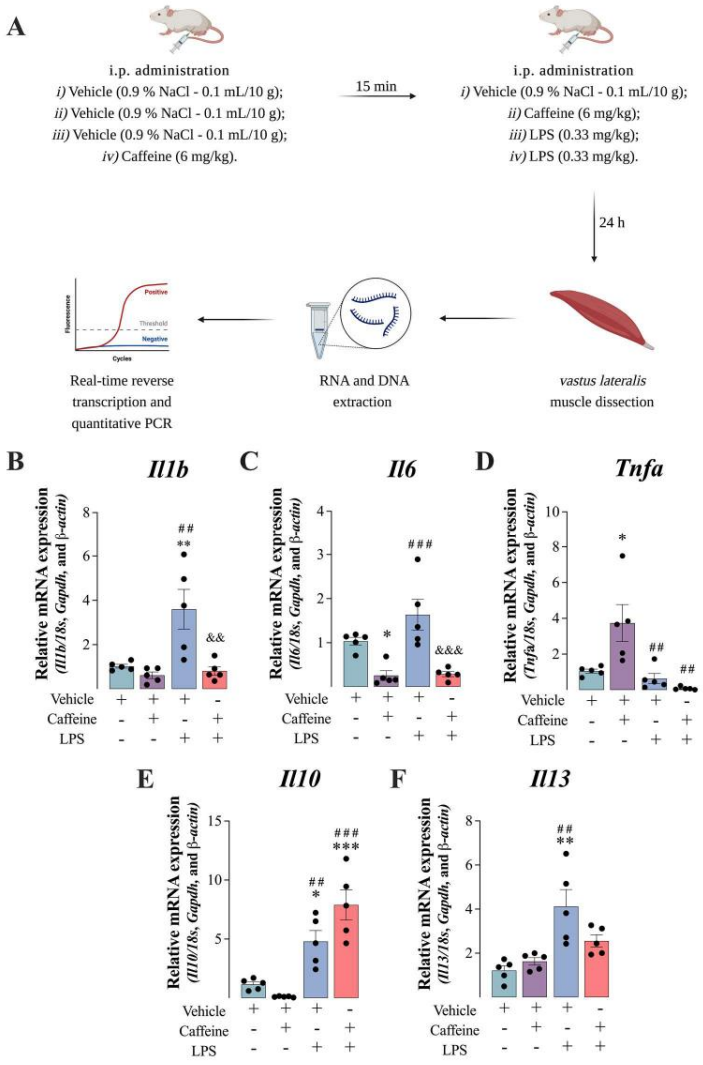

Figure 1. Caffeine administration reduced lipopolysaccharide (LPS)-mediated inflammation in the mouse *vastus lateralis* muscle. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (i.p.) injection of caffeine and/or LPS (See Materials and Methods for details). Schematic representation of the experimental protocol used to induced LPS-mediated inflammation in mouse, the muscle dissection and RNA/DNA extraction for the PCR analysis (**A**). The transcriptional profile of the cytokines *Il1b* (**B**), *Il6* (**C**), *Tnfa* (**D**), *Il10* (**E**) and *Il13* (**F**) were evaluated by qPCR after the total RNA extraction (RNAzol® Cinnabar, Molecular Research Center, Inc., Cincinnati, OH, USA, according to the manufacturer's instructions). Gene expression data were normalized by the average of the *Ct* of the *18s*, *Gapdh*, and *β-actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars represent the mean ± standard error of mean of five independent experiments (animals) performed in technical duplicates. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ vs. vehicle; ## $p < 0.01$; ### $p < 0.001$ vs. to caffeine, and && $p < 0.01$ and &&& $p < 0.001$ vs. LPS. Two-way ANOVA followed by Tukey's test.

$p < 0.001$ vs. vehicle; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ vs. to caffeine, and $^{\&\&}$ $p < 0.01$ and $^{\&\&\&}$ $p < 0.001$ vs. Two-way ANOVA followed by Tukey's test.

3.2. The Anti-Inflammatory Effect of Caffeine was Mediated by Downregulating Nrlp3 Inflammasome Components

Figure 2 shows the effect of the administration of caffeine and/or LPS (i.p.) on NLRP3 inflammasome components gene expression in the mouse *vastus lateralis* muscle (Figure 2A). Figure 2 shows that LPS administration elicited the upregulation of the inflammasome assembly components *Asc* ($F_{(1,15)} = 28.90$, $p < 0.001$) (Figure 2C) and *Casp1* ($F_{(1,15)} = 58.57$, $p < 0.001$) (Figure 2D) in the mouse muscle, which was prevented by the administration of caffeine (Caffeine+LPS experimental group). Although, LPS treatment *per se* did not alter the levels of *Nlrp3* expression 24 h after the administration, the combination with caffeine provoked its upregulation ($F_{(1,16)} = 34.78$, $p < 0.001$) (Figure 2B) in the mouse muscle. No differences were induced by caffeine administration alone. Similar results were found in the absolute gene expression (Figure S1).

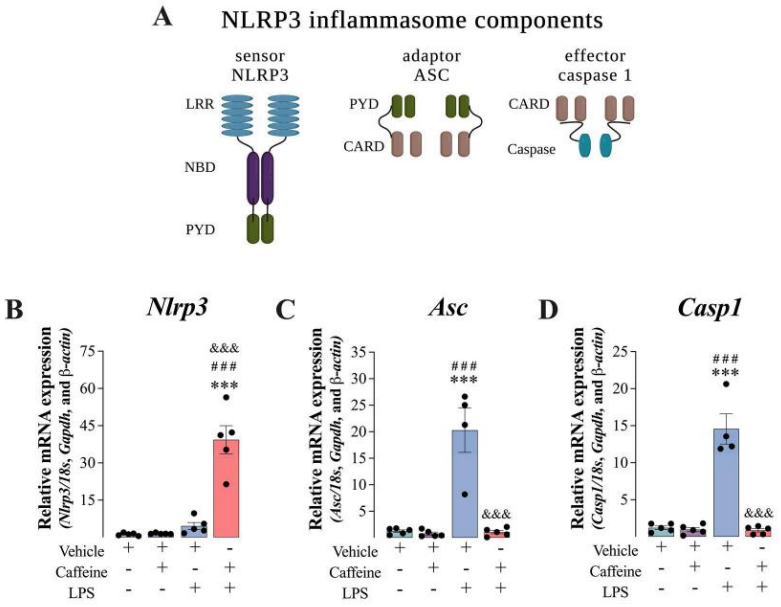

Figure 2. Caffeine administration rescued lipopolysaccharide (LPS)-induced Nrlp3 inflammasome components upregulation in the *vastus lateralis* muscle of mice. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (ip) injection of caffeine and/or LPS (See Materials and Methods for details). NLRP3 inflammasome consists of a sensor (Nlrp3), an adaptor (Asc) and an effector (caspase-1) (**A**). The transcriptional profile of the components of the NLRP3 inflammasome *Nlrp3* (**B**), *Asc1* (**C**), and *Casp1* (**D**). Gene expression raw data were normalized by the average of the Ct of the *18s*, *Gapdh* and β-*actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars represent the mean ± standard error of the mean of five independent experiments (animals) performed in technical duplicates. *** $p < 0.001$ vs. vehicle, $^{\#\#\#}$ $p < 0.001$ vs. to caffeine, and $^{\&\&\&}$ $p < 0.001$ vs. LPS. Two-way ANOVA followed by Tukey's test.

3.3. Caffeine Administration Enhanced the Expression of Adenosinergic Receptors in the Vastus Lateralis Muscle Mice

Figure 3 shows the effect of caffeine administration on adenosinergic receptors gene expression and gene methylation. Caffeine administration *per se* increased the expression of *Adora1* ($F_{(1,16)} = 10.03$, $p < 0.05$) (Figure 3A) and *Adora2A* ($F_{(1,16)} = 16.26$, $p < 0.001$) (Figure 3B) in the mouse muscle, which was prevented by the administration of LPS alone and combined with caffeine. LPS per se increased the levels of *Adora1* ($F_{(1,16)} = 10.03$,

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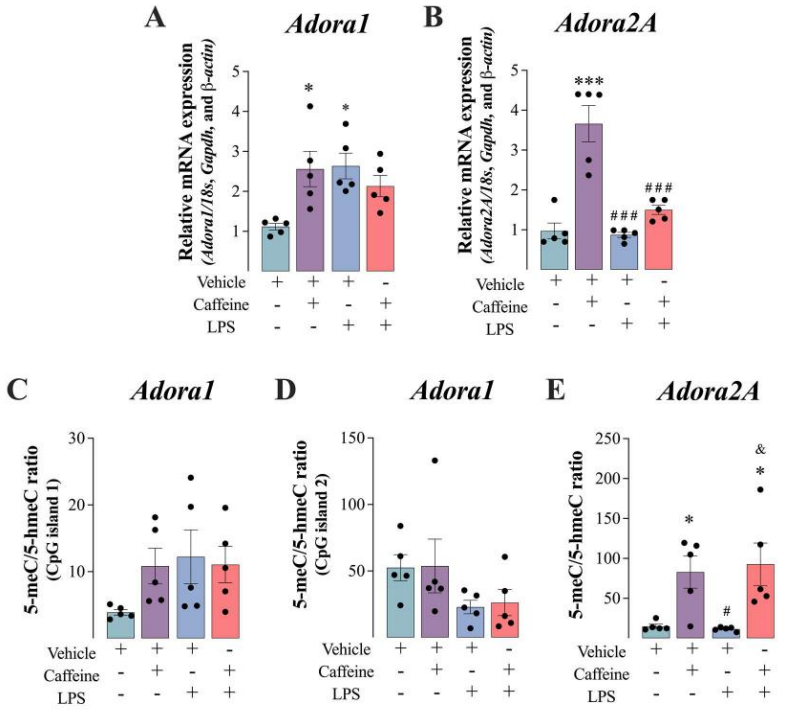

Figure 3. Caffeine administration induced the upregulation of genes encoding adenosinergic re-

Figure 3. Caffeine administration induced the upregulation of genes encoding adenosinergic re ceptors in the *vastus lateralis* muscle of mice. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (i.p.) injection of caffeine and/or LPS (See Materials and Methods for details). The transcriptional profile of the components of the adenosine recep *Adora1* (**A**), and *Adora2A* (**B**). DNA methylation and demethylation at *Adora1* CpG island 1 (**C**), *Adora1* Cpg island 2 (**D**), and *Adora2A* (**E**). Gene expression raw data were normalized by the a the Ct of the *18s*, *Gapdh* and *β-actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars repre mean ± standard error of the mean of five independent experiments (animals) performe duplicates. * $p < 0.05$; *** $p < 0.001$ vs. vehicle; # $p < 0.05$; ### $p < 0.001$ vs. to caffeine, and & $p < 0.05$ vs. LPS. Two-way ANOVA followed by Tukey's test.

3.4. Caffeine+LPS Exposure Enhanced the De Novo DNA Methylation in the Vastus Lateralis Muscle Mice

Figure 4 shows the effects of caffeine and/or LPS administration on the epigenetic profile in the mouse *vastus lateralis* muscle. Caffeine+LPS significantly upregulated the expression of the maintenance methylation gene *Dnmt1* ($F_{(1,16)}$ = 101.05, $p < 0.001$) (Figure 4A), and de novo methylation gene *Dnmt3A* ($F_{(1,15)}$ = 1.26, $p < 0.001$) (Figure 4B), while the expression of de novo methylation gene *Dnmt3B* was significantly downregulated ($F_{(1,16)}$ = 0.06,

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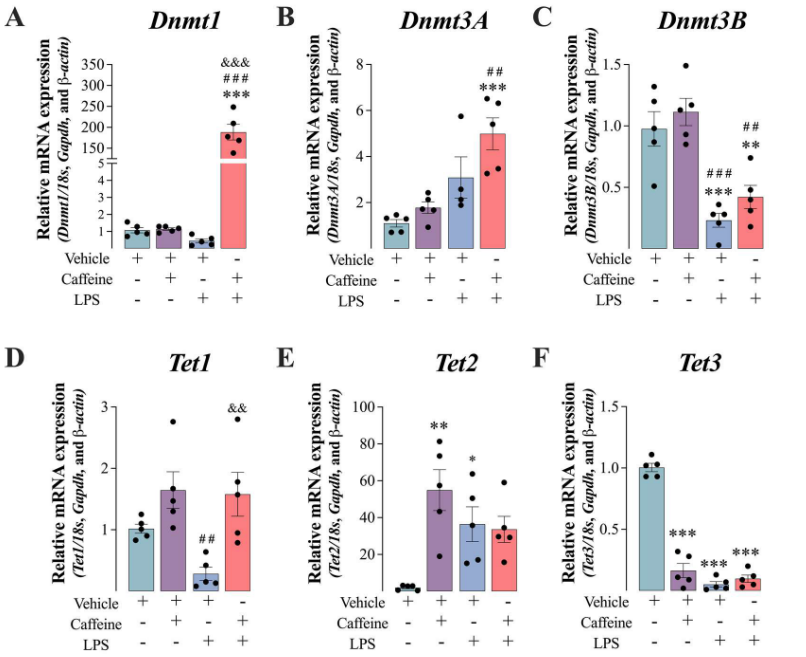

Figure 4. Caffeine plus lipopolysaccharide (LPS) administration induced *de novo* DNA methylat in the *vastus lateralis* muscle of mice. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (i.p.) injection of caffeine and/or LPS (See Materials and Meth for details). The following groups were studied: Vehicle, Caffeine, LPS, and Caffeine+LPS. The transcripts of the *de novo* DNA methylation enzymes *Dnmt1* (**A**), *Dnmt3A* (**B**), *Dnmt3A2* and *Dnmt3B* (**C**). The status of the DNA demethylation enzymes *Tet1* (**D**), *Tet2* (**E**), and *Tet3* (**F**). Gene expression raw data were normalized by the average of the Ct of the *18s*, *Gapdh* and β-*actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars represent the mean ± standard error of the mean of five independent experiments (animals) performed in technical duplicates. * p < 0.05; ** p < 0.01; *** p < 0.001 vs. vehicle; ## p < 0.01; ### p < 0.001 vs. caffeine and && p < 0.01 and &&& p < 0.001 vs. LPS. Two-way ANOVA followed by Tukey's test.

3.5. Caffeine Administration Attenuated the Catabolic State Induced by LPS Administration in the Mouse VASTUS lateralis

Figure 5 shows the effects of caffeine and/or LPS catabolism in the mouse muscle. LPS treatment significantly elicited the upregulation of the energy status sensor gene *Ampk* ($F_{(1,16)}$ = 5.35, p < 0.01) (Figure 5). The coadministration of caffeine significantly reverted the effect induced by LPS.

3.3. Caffeine Administration Attenuated the Catabolic State Induced by LPS Administration in the Mouse *VASTUS lateralis*

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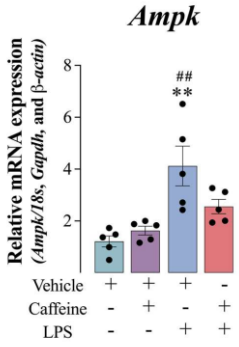

Figure 5. Caffeine administration reverted the catabolic effect induced by the administration of lipopolysaccharide (LPS) in the *vastus lateralis* muscle of mice. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (i.p.) injection of caffeine and/or LPS (See Materials and Methods for details). *Ampk* gene expression raw data were normalized by the average of the Ct of the *18s*, *Gapdh* and β-*actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars represent the mean + standard error of the mean of five independent experiments (animals) performed in technical duplicates. ** $p < 0.01$ vs. vehicle; ## $p < 0.01$ vs. caffeine. Two-way ANOVA followed by Tukey's test.

Figure 5. Caffeine administration reverted the catabolic effect induced by the administration of lipopolysaccharide (LPS) in the *vastus lateralis* muscle of mice. Adult Swiss male mice (3–5 months of age; body mass 45–50 g) received a single intraperitoneal (i.p.) injection of caffeine and/or LPS (See Materials and Methods for details). *Ampk* gene expression raw data were normalized by the average of the Ct of the *18s*, *Gapdh* and β-*actin* genes and calculated by the method ($2^{-\Delta Ct}$). Bars represent the mean + standard error of the mean of five independent experiments (animals) performed in technical duplicates. ** $p < 0.01$ vs. vehicle; ## $p < 0.01$ vs. caffeine. Two-way ANOVA followed by Tukey's test.

4. Discussion

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Caffeine is a stimulant drug widely known and used due to its psychoactive and ergogenic effects [27]. The effects of caffeine on physical exercise, endurance performance, and fatigue stalling are well documented [28–30]. However, the molecular mechanisms behind these modulations are still under study. To the best of our knowledge, this is the first study to show that epigenetics is involved in the anti-inflammatory effects of caffeine on the *vastus lateralis* muscle of resting mice. Here, we showed that treatment with caffeine prevented an increase of the gene expression of LPS-induced pro-inflammatory cytokines *Il1b* and *Il6* and promoted the upregulation of the anti-inflammatory genes *Il10* and *Il13* in the mouse muscle. The anti-inflammatory state observed in the caffeine experimental group occurred with decreased gene expression of the NLRP3 inflammasome components, *Asc* and *Casp1*. Indeed, the activation of caspase 1 mediates the cleavage of pro-IL-1β to generate and release its biologically pro-inflammatory active form, IL-1β [31]. Moreover, caffeine administration promoted the upregulation of the adenosinergic receptors *Adora1* and *Adora2A*, the signaling of which is known to induce vasodilatation, healing and anti-inflammation, promotion of tissue blood flow and cellular homeostasis in different cell types [32]. Thus, in order to maintain homeostasis, the upregulation of *Adora2A* might have been responsible of triggering the methylation of its promoter. While DNA methylation patterns were not altered by caffeine treatment, the DNA methylating status was increased after Caffeine+LPS administration, suggesting that the observed adaptation to inflammation induced by caffeine was due to epigenetics.

Caffeine is the most commonly consumed social drug to increase alertness, arousal and energy [33]. Its consumption has been related to improvement in cognitive performance and mood in healthy population [34,35], and is the main ergogenic resource used by athletes to enhance exercise performance, extend time to exhaustion, and to delay fatigue [36].

Moreover, it has been shown that caffeine upregulated dopamine metabolism and signaling, and increased the synthesis and turnover of noradrenaline, being closely associated with an improvement in both peripheral and central fatigue [37]. Furthermore, the ergogenic effects of caffeine consumption have also been shown to be more evident in fatigued than in well-rested subjects [38,39]. These effects are proposed to be mediated by the non-selective antagonism of Adora1 and Adora2A [33]. Adora1 are widely expressed in the cortex, hippocampus, cerebellum, and thalamus [33], and in the adipose tissue, stomach, kidney, and heart [40]. Due to the capacity for lowering cAMP intracellular

levels, the activation of Adora1 promotes bradycardia, inhibition of lipolysis, antinociception, reduction of sympathetic and parasympathetic activity, neuronal hyperpolarization, among others [41]. In contrast, Adora2A has a more restricted distribution, being more expressed in the striatum, nucleus *accumbens*, and olfactory tubercle [33]. Skeletal muscle, bladder, and the immune system are the tissues with the highest density of these receptors in the periphery [42]. The activation of Adora2A triggers neurotransmitter release, anti-inflammatory immune responses, and vascular smooth muscle cell relaxation, due to the activation of signaling pathways mediated by increased cAMP intracellular levels [36,41]. Therefore, the typical effects of adenosine, the natural agonist of Adoras and the final catabolite of ATP, that are associated with tiredness and drowsiness are counterbalanced by caffeine.

The effective dose of caffeine to antagonize Adoras and to lead to increased exercise time to fatigue ranges from 3 to 9 mg/kg in humans [28,43,44] and rodents [45,46]. These doses have been shown to increase performance in endurance, intermittent and resistance exercises in humans [5–8,47–50].

These effects have been associated with enhanced peripheral energy metabolism, activation of ryanodine channels for quicker release of calcium, and oxidant system in the muscle, improving muscle speed and strength (for a review see [16]). However, a large body of evidence suggests that caffeine can also mediate its ergogenic effects by inducing an anti-inflammatory status, preventing excessive endogenous catabolism and oxidative stress [1,16,51]. The anti-inflammatory effect of caffeine is also supported by the fact that individuals who suffer from cancer, obesity or liver, metabolic or neurodegenerative disorders and for whom persistent inflammation has been reported, developed fatigue when the symptoms appeared [52–58]. Moreover, high circulating levels of caffeine have been associated with delayed onset or reduced risk of dementia in individuals with mild cognitive impairment [59]. Furthermore, healthy individuals receiving an acute dose of caffeine showed reduced levels of pro-inflammatory markers and delayed development of fatigue [54,60]. Indeed, one of the first and most common symptoms associated with system immune activation is fatigue [61]. In addition, regular coffee consumption has also been associated with a reduced risk of low-grade inflammation in clinical conditions such as type 2 diabetes *mellitus* [62], and metabolic syndrome [63].

Considering that fatigue is characterized by temporary reductions in voluntary muscular force production, and cognitive and motivational changes that induces poor physical performance [64], we aimed to study whether caffeine could induce anti-inflammatory effects in the inflamed mouse muscle, and whether these effects are associate with epigenetic modifications.

It has been suggested that the development of fatigue may activate pathways that promote the activation of nuclear factor kappa b (NF-κB), which is considered a prototypical pro-inflammatory signaling pathway (for a review see [65]). NF-κB is known to be activated by a wide array of mediators, including LPS, inflammatory cytokines such as IL-1b and TNF-a, and reactive oxygen species, which in turn activates several signal transduction cascades and induces changes in transcription factors that promote a pro-inflammatory status. NF-κB's activation induces the synthesis of pro-IL-1β and the activation of caspase 1 that induces the proteolytic maturation of pro-IL-1β [31]. Caspase 1 and ASC are key components of NLRP3 inflammasome, a multiproteic complex that induces inflammatory responses and cell death in response to various danger signals [66], including pro-inflammatory cytokines, reactive species, oxidized compounds that are known to accumulate in the muscle and blood during exhaustive physical exercise [67–69]. Therefore, the effect of caffeine on the inflammatory response induced by LPS that we observed in the mouse muscle suggests that part of its ergogenic effects might be mediated by the inhibition of the inflammasome assembly.

The anti-inflammatory effect might also be related to the positive effects we observed on Adoras' increased expression in the inflamed muscle. Thus, increasing the antagonism of Adoras will potentiate the anti-inflammatory effect on different immune cell populations,

that are cells known for their expression of high levels of Adora2A. Accordingly, it has been proposed that caffeine is an immunosuppressor since it has shown to inhibit proliferation, activation, and cytokine secretion by lymphocytes [70]. For example, it has been shown that caffeine reduced TNF-a secretion and enhanced the expression of Adora2A in LPS-activated human macrophages [70]. In addition, reduced ATP/AMP ratio, which occurs during the inflammatory response [71], is a key modulator of enhanced AMPK signaling, which denotes energy deficit. Although, we did not measure the levels of phosphorylated AMPK, the restoration of basal *Ampk* gene expression suggests that caffeine also protects the inflamed muscle by improving energy metabolism as previously proposed [72].

DNA methylation is also known to repress gene expression by blocking the promoter sites at which activating transcription factors are bound [73]. The reduced global DNA demethylation could be responsible for the upregulation of *Ampk* under LPS treatment, which was rescued when caffeine was also administered. This is also in agreement with the increased expression of pro-inflammatory cytokines, which was negatively modulated after the coadministration of caffeine in our study. This effect has also been reported in other tissues and cells. For example, LPS-challenged human peripheral blood mononuclear cells exposed to caffeine at different concentrations (10–100 μM) for 24 h, negatively modulated the production of TNF-α [74]. Similarly, mouse splenocyte cultures stimulated with concanavalin A (a pro-inflammatory agent) showed reduced production of TNF-α, IL-2, and IFN-γ when co-treated with 3.75 and 10 mM of caffeine for 24 h [75].

The DNA methylation machinery requires DNMT3a and DNMT3b for the de novo [76], and DNMT1 for the maintenance [77] of DNA methylation. In general, when methylation occurs in the promoter region of a particular gene, the gene expression is expected to be repressed. DNA can also be demethylated by the action of ten-eleven translocation (TET) enzymes TET1, TET2, and TET3 [78], which may result in enhanced gene expression. Therefore, the balance of these processes may regulate the expression of different genes, including the ones involved in inflammation and adenosine signaling as shown here. Indeed, genome-wide meta-analyses identified several genes positively associating caffeine consumption and DNA methylation [22,79,80]. While previous studies have shown that caffeine intake is positively correlated with higher DNA methylation [22], we have shown in this study that caffeine *per se* can be responsible for the negative modulation of the expression of inflammatory genes in animals submitted to acute inflammation.

5. Conclusions

This study provides evidence for the anti-inflammatory effect of caffeine in the mouse muscle. The immune system activated by LPS induced the release of pro-inflammatory cytokines that was prevented by caffeine administration, an effect also observed in the reduction of the inflammasome components, possibly by a modulatory effect of caffeine on epigenetics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12030554/s1, Figure S1: Caffeine administration prevented lipopolysaccharide (LPS)-induced Nrlp3 inflammasome components upregulation in the vastus lateralis muscle of mice; Figure S2: Absolute gene expression of adenosine receptors in the mouse vastus lateralis muscle.

Author Contributions: T.E., A.F.S., R.A.F.d.S. and A.L. conceived and designed the study, performed the experiments, and wrote the first draft and the edited versions. J.S.; T.B.d.M., L.B.C. and P.L.d.S.S. contributed significantly to the literature review and the writing of the manuscript; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with Ethics Committee for Animal Research (PP00760/CEUA) of the *Universidade Federal de Santa Catarina* (Brazil).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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CHAPTER IV

DISCUSSION & CONCLUSIONS

The overarching aim of this thesis was to investigate whether BH4 metabolism is influenced by chronic diseases characterized by inflammation and whether exercise and caffeine can create anti-inflammatory environments that restore normal intracellular BH4 levels. This chapter will discuss and synthesize the findings investigated in this thesis. Finally, the main conclusions for this thesis will be outlined.

4.1 DISCUSSION

Sustained inflammation is a common component of the physiopathology of many diseases, either as a primary cause or as a secondary consequence of energy deficiency and/or oxidative stress. Tissues and organs can be damaged by chronic inflammation and contribute to the development and progression of various medical conditions (OKIN; MEDZHITOV, 2012). The potential role of BH4 metabolism as a key target for modulating inflammation in conditions were BH4 levels can be significantly increased or impaired was investigated in this thesis. Two different experimental designs were included as biological systems: one characterized by the genetic deficiency of a mitochondrial protein essential for mitochondrial translation, human *TFAM*-deficient fibroblasts; and the second approach involved animals submitted to a HFD to induce visceral WAT accumulation, a hallmark of obesity. Additionally, a protocol of physical exercise was included in HFD-fed mice since previous work from our group revealed that physical exercise, a non-pharmacological approach for treating obesity, can negatively modulate the activity of BH4 metabolism. On one hand, the results obtained in this doctoral thesis demonstrated that the deficient energy phenotype expected to occur in the most affected tissues, i.e., the liver, the brain, and the skeletal muscle, was evidenced in *TFAM*-deficient fibroblasts. It was also shown that oxidative stress is present in these cells as another component of the physiopathology caused by the genetic deficiency.

The diagnosis of acquired mitochondrial dysfunctions or genetic mitochondrial diseases is complex and requires a multidisciplinary approach, involving a battery of multidimensional measurements, including clinical evaluation, genetic testing, biochemical analysis, and imaging studies. One common procedure for evaluating mitochondrial activity is the isolation of a tissue (biopsy) that would represent the disease. Muscle biopsy is commonly collected to be examined under a microscope and to assess abnormalities in mitochondrial structure and distribution. In some cases, if the biopsy is large enough, functional studies such as oxygen consumption or the activities of the main proteins of the ETC can also be performed. Considering that muscle biopsies are invasive, expensive, that they offer high risk of complications during the procedure (FORNY et al., 2021), and that cannot be saved for future analyses, skin biopsies represent a less invasive, easily accessible, and low-risk alternative to muscle biopsies (BAILLEUX; COLLINS; NIKKELS, 2022). Furthermore, skin biopsies have high proliferative capacity and provide a renewable source

of cells, reducing the need for multiple biopsies for different studies required for diagnosis. Additionally, cultured fibroblasts can serve as the basis for personalized medicine, for example for PMDs, where medical treatments and therapies can be customized based on the individual characteristic of each patient.

The mitochondrial deficiencies observed in the genetic disorder, *TFAM*-deficient fibroblasts, were characterized by a significantly lower oxygen consumption rate in basal, maximal, spare respiratory capacity, and proton leak, indicating a low efficiency of mitochondria in utilizing oxygen for ATP production. The compromised oxygen consumption was attributed to deficient activities of complexes I, I-CoQ- III, and IV. It is known that proper functioning of ETC is crucial for cellular energy metabolism and overall cellular health. Complex I, which is the largest and first enzyme in the ETC, plays a pivotal role in facilitating NADH-linked electron transfer and ATP synthesis, while complex IV is essential for efficient oxygen reduction (LEHNINGER; SMITH, 1949). Additionally, we demonstrated that *TFAM*-deficiency led to higher levels of superoxide anion, a toxic ROS produced by unhealthy mitochondria, which can damage biomolecules such as proteins, DNA, lipid membranes, etc. (CADENAS; DAVIES, 2000). Indeed, reduced activity of two proteins, GPx and SOD , was observed in this human cell line, indicating a reduced capacity of cells to maintain a healthy antioxidant environment required for proper mitochondrial physiology and dynamics.

Although PMDs are not considered inflammatory diseases themselves, it is known that inflammation is part of their physiopathology. The presence of inflammation can be detrimental, as it can further compromise mitochondrial health. Additionally, dysfunctional mitochondria can activate the immune system by activating the inflammasome (TSCHOPP, 2011), resulting in a sustained inflammatory response and a cyclic pattern of mitochondrial dysfunction and inflammation (LÓPEZ-ARMADA et al., 2013). In this context, the persistent inflammatory response, mediated by pro-inflammatory mediators such as TNF-α, IL-1β, and NO, as observed in the transcriptome analysis of *TFAM*-deficient fibroblasts, has the potential to trigger mitochondrial damage, leading to reduced activity of complex I, ATP production, and mitochondrial membrane potential. Furthermore, it can promote the production and accumulation of substantial levels of ROS, facilitating the assembly and activation of the inflammasome (ZHOU et al., 2011). NLRP3 is the most extensively described and studied inflammasome, which can be activated by ROS, the

cytosolic release of mtDNA, and the activation of inflammatory pathways such as NF-κB (for more details, refer to chapter II) (TSCHOPP, 2011; ZHOU et al., 2011). In this scenario, it has been demonstrated that *Tfam* deficiency was characterized by the upregulation of several interferon-stimulated genes in mouse embryonic fibroblasts. This upregulation was proposed to occur due to the cytosolic release of mtDNA, a processes facilitated by aberrant mtDNA packaging (WEST et al., 2015). Another study showed that bone marrow-derived dendritic cells from *Tfam* knockout mouse exhibited higher levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (LU et al., 2023). It should be emphasized here that increased IL-1β is typically associated with the activation of the inflammasome (MARTINON; TSCHOPP, 2004).

Persistent inflammation can be contributed to by the disruption of cellular systems that maintain mitochondrial function or enable adaptation to energy demands. One example of a metabolic pathway that can result in inflammation-induced cell dysfunction is the BH4 metabolism, where deficiencies or excesses can lead to inflammation and the impairment of normal cellular functions. The cofactor activity of BH4 is well-known for phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and all NOS isoforms. Thus, the maintenance of appropriate BH4 levels is essential for optimal physiological function in the hydroxylation of aromatic amino acids, resulting in the catabolism of phenylalanine and the synthesis of the catecholaminergic neurotransmitters dopamine and serotonin. Moreover, BH4 is necessary for the cleavage of ether lipids and the biosynthesis of NO (WERNER; BLAU; THÖNY, 2011).

Recently, additional fundamental physiological roles for basal BH4 levels have been discovered by our group. These roles include antioxidant and anti-inflammatory properties, as well as functioning as a mitochondrial activator and memory enhancer (DE PAULA MARTINS et al., 2018a; GHISONI et al., 2015; LATINI et al., 2018). Thus, mitochondrial activity, ATP production, the antioxidant system, and the content of iron and iron-related proteins (essential for mitochondrial complex formation) were impaired by deficient BH4 levels, along with increased levels of ROS, lactate, and pyruvate. These effects were observed in cells highly dependent on mitochondrial function for generating cellular energy, including nerve, enteric, and immune cells (CRONIN et al., 2018, 2021; DE PAULA MARTINS et al., 2018a).

The SPR knockout, a genetic mouse model of BH4 deficiency, exhibits a shortened life span, altered cardiac OXPHOS, decreased transcription of mitochondrial biogenesis regulatory

genes, including *Tfam*, and lower mitochondrial mass (KIM et al., 2019). It has also been demonstrated that the lack of SPR activity, and the consequent BH4 deficiency, results in impaired mitophagy in brain, liver, muscle, and lung of mice, which negatively affects the quality and quantity of healthy mitochondria (KWAK et al., 2011). The molecular mechanisms that compromise mitophagy are believed to be linked to the limited availability of the amino acid tyrosine, which is BH4-dependent synthesis. In this way, lymphocytes derived from human PKU individuals showed a higher phenylalanine/tyrosine ratio with insufficient mitophagy, revealing an intimate relationship between BH4 and mitophagy, the mitochondrial quality control process by which damaged or dysfunctional mitochondria are selectively targeted for degradation and removal (KWAK et al., 2011).

In agreement, this thesis also showed that the upregulation of BH4 metabolism cannot occur as expected under deficient metabolic conditions, indicating that BH4 production might be compromised under mitochondrial dysfunction and oxidative stress. In this way, BH4 has been identified as essential for proper mitochondrial activity and antioxidant activation (CRONIN et al., 2018; LATINI et al., 2018). Preclinical studies have demonstrated that by enhancing intracellular BH4 levels, key brain antioxidant, anti-inflammatory, and mitochondrial-related pathways are activated, resulting in increased mitochondrial activity, dynamics, and memory (CRONIN et al., 2018; DE PAULA MARTINS et al., 2018a; GHISONI et al., 2015, 2016; GHISONI; LATINI, 2015; LATINI et al., 2018). It has been shown that a single i.c.v. administration of BH4 enhances hippocampal cognition through a mechanism linked to the activation of glutamatergic neurotransmission and the reduction of cell threshold, which facilitates LTP triggering in Swiss mice and Wistar rats (LATINI et al., 2018). Consistent with this, BH4 administration in PKU-affected individuals has been reported to improve working memory and brain activation (CHRIST et al., 2013). The antioxidant properties of this pterin have been also demonstrated, as BH4 is a more potent scavenger of superoxide, hydroxyl, and thiyl radicals, as well as peroxynitrite, compared to ascorbate at the physiological blood pH (KUZKAYA et al., 2003; PATEL et al., 2002). Furthermore, it has been shown that BH4 enhances mitochondrial activity when thiol compounds, such as glutathione, are reduced (FOXTON; LAND; HEALES, 2007).

Mechanistically, it is demonstrated here that boosted BH4 metabolism results in an increase in the number of mitochondria and upregulation of *Tfam* and *Nrf-2* gene expression, favoring

mitochondrial biogenesis. In agreement, BH4 administration (20 mg/kg/day, i.p.; 2 weeks) in the mouse heart induced increased *Tfam* expression, along with increased expression of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (*Pgc-1α*) and estrogen-related receptor alpha, which are nuclear transcription factors that cooperate with *Tfam* for mitochondrial biogenesis (KIM et al., 2019). Furthermore, the restoration of BH4 to optimal levels rescues mitochondrial activity, attenuates ROS production, and normalizes cardiac systolic function, as well as body size and weight (KIM et al., 2019). In this context, normalization of impaired mitophagy observed in SPR knockout mice is also achieved through BH4 treatment (KWAK et al., 2011).

Considering that PMDs have no effective treatments, the utilization of cultured fibroblasts obtained from skin biopsies can be useful for testing different compounds that could increase residual mitochondrial function and BH4 metabolism. Therefore, the confirmation of mitochondrial impairment in fibroblasts affected by *TFAM* deficiency is crucial for understanding the metabolic consequences in PMDs and for the development of future targeted therapies, such as potential treatment represented by BH4 supplementation. It should be noted that sapropterin dihydrochloride (commercial BH4) received FDA approval on 13 December 2007, and since then, over 7800 individuals have been treated in the US, demonstrating the safety and tolerance of the compound (MUNTAU et al., 2021). Overall, the essentiality of BH4 for proper mitochondrial activity and antioxidant function is evident. Hence, BH4 supplementation in patients affected by PMDs could serve as an innovative, effective, and safe strategy to treat these disorders, leading to improved bioenergetics and reduced oxidative stress and inflammation.

While poor mitochondrial function is associated with deficient BH4 metabolism, the induction of pain hypersensitivity has been linked to the pathological elevation of this pterin. In this study, it was also demonstrated that excessive levels of BH4 can be detected in biological fluids of mice exhibiting pain hypersensitivity and visceral WAT accumulation. The main characteristic of obesity is increased adiposity (WEST; YORK, 1998). The combination of the high potential for hypertrophy and hyperplasia in adipocytes, along with the significant energy contribution from lipids, highlights the crucial role of saturated fats present in the administered HFD in the development of substantial body fat accumulation and consequent weight gain (TOWNSEND; LORENZI; WIDMAIER, 2008). Furthermore, excessive weight and body fat are associated with

numerous health issues, including the onset of type 2 diabetes *mellitus*, high blood pressure, heart disease, stroke, and certain types of cancer (CANCELLO; CLÉMENT, 2006; DIRAT et al., 2011; HENNINGER et al., 2014; LONGO et al., 2019). Consistent with these findings, the intervention resulted in increased GTT levels, indicating impaired glucose tolerance and underlying metabolic issues in HFD-fed mice.

It is widely accepted that obesity is a chronic low-grade inflammatory disease, known as metainflammation. The development of secondary conditions is thought to be contributed by tissue and organ damage over time, impaired function, and increased risk of complications (LONGO et al., 2019). The enlargement of AT in obesity is also associated with the contribution of dysfunctional, hypertrophic, and/or hyperplasic adipocytes to inflammation (DROLET et al., 2008) and consequent insulin resistance (HE et al., 2011). Metainflammation is characterized by the persistent activation of the immune system and the release of pro-inflammatory mediators. It has been demonstrated that TNF-α in AT promote lipolysis in individuals with obesity, leading to the release of free fatty acids into the bloodstream and reduced insulin sensitivity (HOTAMISLIGIL et al., 1996). Additionally, IL-6 has been shown to stimulate the production of CRP, a biomarker of systemic inflammation, in women with abdominal obesity (TANGVARASITTICHAI; PONGTHAISONG; TANGVARASITTICHAI, 2016). Similarly, IL-1β can activate other immune cells such as T-cells and NK cells, promoting inflammation in AT (GAO et al., 2014). Overall, the production of pro-inflammatory cytokines in AT contributes to chronic inflammation and metabolic dysfunction, and the reduction of inflammation may serve as a therapeutic strategy to mitigate the risk of metabolic disorders associated with obesity.

Importantly, BH4 synthesis can be upregulated by the inflammatory stimuli through the *de novo* pathway (for review see GHISONI et al., 2015). Thus, the mechanical and chemical hyperalgesia observed in HFD-fed mice in this study can be attributed to the inflammatory state induced by AT accumulation, which leads to the release of pro-inflammatory cytokines. These cytokines play a crucial role as stimuli for the upregulation of BH4 synthesis, thereby contributing to pain hypersensitivity in our model (LATREMOLIERE et al., 2015; WERNER et al., 1990).

This phenomenon has already been described in conditions characterized by inflammation, including rheumatoid arthritis (FUJITA et al., 2020), neuropathic pain (LATREMOLIERE et al., 2015), and inflammatory bowel diseases (CRONIN et al., 2018) in multiple animal models, as well

as in humans affected by type 1 diabetes with neuropathy (STAATS PIRES et al., 2020b). If pain is associated with elevated levels of BH4, a decrease in pain scores should be expected upon reducing excessively high BH4 levels. In fact, experimental models have demonstrated that the genetic ablation of *Gch1* resulted in decreased hypersensitivity in sensory neurons (KIM et al., 2009) and mouse DRG (LATREMOLIERE et al., 2015), preventing excessive BH4 production. Furthermore, significant analgesic effects and reduced inflammation have been observed in animal models of granulomatous inflammation and joint inflammation with the use of pharmacological SPR inhibitors (SPRi3 and QM385) (FUJITA et al., 2020; LATREMOLIERE et al., 2015). The inhibition of SPR mediated by SPRi3 and QM385 has also shown to reduce T-cell proliferation and decrease type 2 autoimmune and allergic inflammation (CRONIN et al., 2018).

Elevated levels of neopterin, a BH4 metabolite, have been found in several diseases associated with both central and peripheral inflammation (LENOIR DA SILVA et al., 2017; NEDEVA et al., 2021). Neopterin production is induced by IFN-γ-stimulated macrophages (HUBER et al., 1984) and primary human nerve cells exposed to an inflammatory environment (DE PAULA MARTINS et al., 2018a). A positive correlation between pain and neopterin levels has been revealed in 13 adult individuals with diabetic neuropathic pain (STAATS PIRES et al., 2020b). In the context of obesity, higher levels of neopterin were found in the obese group compared to the overweight control in the plasma of 30 individuals with class III obesity (LENOIR DA SILVA et al., 2017). Similar results were described in the hyperglycemic plasma of a population sample of 9 individuals affected by type 1 diabetes *mellitus*, where higher levels of neopterin were found in the diabetic individuals (REMOR et al., 2018). Therefore, neopterin, in addition to being a marker of immune system activation, can be considered a marker of inflammation and pain, which potential clinical use for the sensitive evaluation of the inflammatory state induced by enlarged WAT.

Thus, the results obtained here suggest that urinary BH4 could also be a potential biomarker if pain development that is easy to access, sensitive and reliable. Taken together, BH4 is being shown as a promising target for pain hypersensitivity control, and the use of pharmacological BH4 inhibitors aimed at reducing pathological high levels of BH4 offers an effective therapeutic tool for treating chronic pain in various conditions, including those affecting individuals with obesity.

As a primary line of non-pharmacological treatment for obesity, a combination of diet and physical exercise is indicated by clinicians. Notably, physical exercise has been demonstrated to not only contribute to weight management but also to the prevention of systemic inflammation. It is known that the amount of visceral fat is reduced by exercise, and it increases the production of anti-inflammatory cytokines while decreasing the production of pro-inflammatory mediators. Additionally, the activity of NK cells and T-cells, which are important components of the immune system, can be enhanced by exercise, thereby helping to reduce inflammation (NIEMAN; WENTZ, 2019). Furthermore, the antioxidants system can be stimulated by exercise, contributing to the reduction of oxidative stress and the associated inflammation. This effect can ultimately lead to improved overall health and a lower risk of developing chronic diseases, including obesity, diabetes, heart disease, and cancer. Overall, regular exercise has been shown to improve immune function, which can further contribute to a better quality of life (for a review see SCHEFFER; LATINI, 2020). Our results are in agreement with the literature, showing that the practice of physical exercise prevents the deleterious effects evoked by HFD. In fact, it was observed that physical exercise reduced WAT expansion and normalized GTT and the nociceptive threshold.

Caffeine, a widely consumed drug, is known to enhance the performance of physical exercise. It has been described that one of the main effects mediated by caffeine is the reduction of inflammation (FURMAN et al., 2017). However, the molecular mechanisms involved in this process are not well understood yet. Therefore, to better understand the effect of physical exercise on inflammation, which is a key pathological component of obesity, we investigated whether caffeine – as a surrogate for exercise - could be responsible for chromatin compaction that negatively regulates the expression of pro-inflammatory cytokines. Thus, the anti-inflammatory effect of caffeine on the *vastus lateralis* muscle from mice under LPS-inflammation was investigated in order to find a way to manage inflammatory responses. It was demonstrated that caffeine administration prevented relevant pro-inflammatory aspects induced by LPS, including the gene expression of *Il1β* and *Il6,* and the NLRP3 inflammasome components *Asc* and *Casp1,* while promoting the upregulation of the anti-inflammatory cytokine *Il10*. Moreover, the DNA methylating status was increased after caffeine administration, suggesting that the observed adaptation induced by caffeine was due to epigenetic mechanisms.

In summary, contributions have been made by this project to the understanding of BH4 metabolism in different metabolic conditions, one characterized by low energy production and the other characterized by excessive nutrient exposure. The opposite scenarios observed in the two conditions presented in this thesis, a genetic mitochondrial deficiency and pathological visceral WAT accumulation that compromised the physiological nociceptive threshold, suggest that meticulous regulation of BH4 metabolism is necessary to prevent cytotoxicity. Various biological systems are compromised when this tightly controlled balance is disrupted, leading to impaired neurotransmission, immune responses, metabolism, and vascular activity. Mitochondrial dysfunction, increased oxidant status, and heightened inflammation can result from reduced BH4 levels. Conversely, elevated BH4 levels are associated with chronic pain, impaired memory and learning, and the progression of inflammatory diseases. Therefore, the normalization of BH4 levels, either by increasing deficient levels or inhibiting excessive levels, holds potential for mitigating mitochondrial disorders and chronic pain. BH4 plays multiple crucial roles, and its metabolism represents a potential therapeutic target for modulating various pathways, highlighting the dual nature of BH4 metabolism as a double-edged sword where both deficiency and excess can be toxic.

4.2 CONCLUSIONS

- Fibroblasts derived from human individual with *TFAM* deficiency exhibited the expected dysfunctions, therefore pointing them a suitable model for testing potential treatments.

- Inflammation, mediated by increased visceral WAT, leads to the overproduction of BH4 and an increase in nociception scores.

- Urinary BH4 levels can be quantified in urine and used as a marker of pain hypersensitivity in obesity.

- Normalizing BH4 levels can represent an innovative and effective treatment for chronic pain and PMDs.

- Caffeine administration present anti-inflammatory properties, providing an alternative approach to treat inflammatory conditions.

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