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CHARACTERIZATION OF METABOLISM AND MITOCHONDRIAL DYNAMICS IN ASTROCYTES DERIVED FROM INDUCED PLURIPOTENT STEM CELLS OF MULTIPLE SCLEROSIS PATIENTS

CARACTERIZAÇÃO DO METABOLISMO E DINÂMICA MITOCONDRIAL EM ASTRÓCITOS DERIVADOS DE CÉLULAS TRONCO PLURIPOTENTES INDUZIDAS DE PACIENTES COM ESCLEROSE MÚLTIPLA

SÃO PAULO
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RESUMO

GHIROTTO NUNES, B. Caracterização do metabolismo e dinâmica mitocondrial em astrócitos derivados de células tronco pluripotentes induzidas de pacientes com esclerose múltipla. 2021. 118 páginas. Dissertação (Mestrado em Imunologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

A Esclerose Múltipla (EM) é uma doença autoimune caracterizada por um quadro inflamatório crônico e progressivo no Sistema Nervoso Central (SNC), que resulta em um processo de neurodegeneração axonal, originando quadros de deficiência neurológica nos pacientes. Recentemente, estabeleceu-se que os astrócitos (células da glia) apresentam um papel chave na regulação das sinapses neuronais e na regulação dos processos inflamatórios e neurodegenerativos no SNC. Sabe-se também que os astrócitos são uma das maiores fontes de espécies reativas de oxigênio e isso está diretamente relacionado à regulação da função mitocondrial. Mitocôndrias modificam sua morfologia constantemente de acordo com as necessidades bioenergéticas da célula e alterações nos mecanismos de regulação podem desencadear processos neurodegenerativos. A EM dificilmente é representada em todas as suas apresentações clínicas por modelos animais, sendo que muitos estudos terapêuticos em modelos experimentais não conseguiram ser traduzidos para humanos devido às diferenças interespecíficas. Ainda, o estudo do papel de células residentes do SNC em pacientes de EM é dificultado por questões éticas. Nesse sentido, as células tronco pluripotentes induzidas (hiPSC), que podem ser reprogramadas a partir de amostras de sangue ou de pele dos pacientes e diferenciadas em qualquer população de células somáticas, surgem como uma abordagem poderosa para se investigar mecanismos moleculares que possam estar associados ao desenvolvimento de doenças complexas como a EM. Desta forma, neste trabalho formulamos a hipótese de que astrócitos derivados de hiPSC de pacientes com EM devem apresentar alterações de dinâmica e metabolismo mitocondrial que podem estar associadas às características fenotípicas da EM. De maneira sucinta, os astrócitos derivados de hiPSC foram obtidos e caracterizados com sucesso. Observamos diferenças basais entre os grupos tendo os astrócitos dos pacientes apresentado enriquecimento de genes associados à mitofagia e a processos neurodegenerativos, bem como de transporte de moléculas pela mitocôndria. Em seguida, observamos nesse mesmo grupo um aumento na produção de superóxido e uma diminuição da qualidade mitocondrial. Analisando funcionalmente as células por

meio da técnica de Seahorse observamos um aumento no metabolismo oxidativo e glicolítico nos astrócitos de pacientes, indicando um processo de estresse metabólico nessas células, além de uma diminuição significativa na eficiência bioenergética mitocondrial das mesmas. Por meio de microscopia eletrônica de transmissão analisamos a morfologia mitocondrial nos astrócitos e observamos um aumento significativo na fissão das mitocôndrias no grupo dos pacientes, corroborando nossos resultados de expressão gênica obtidos por PCR array. Ainda, ao encontro com os resultados anteriores, observamos uma diminuição no razão entre o DNA mitocondrial e o DNA nuclear. Como observamos um inchamento do retículo endoplasmático nos astrócitos de pacientes por microscopia eletrônica, fomos analisar a expressão de genes relacionados à via UPR (Unfolded Protein Response) bem como de genes ligados à regulação da resposta ao estresse mitocondrial e observamos que vários deles estão diminuídos significativamente nos astrócitos de pacientes, indicando um possível defeito na maquinaria celular de resposta ao estresse. Assim, nossos resultados sugerem um fenótipo de disfunção mitocondrial nos astrócitos derivados de pacientes com EM e abrem novas perspectivas para a modelagem da doença e futuras abordagens terapêuticas.

Palavras-chave: Esclerose Múltipla; neuroimunologia; células-tronco pluripotentes induzidas; dinâmica mitocondrial; metabolismo.

ABSTRACT

GHIROTTO NUNES, B. Characterization of metabolism and mitochondrial dynamics in astrocytes derived from induced pluripotent stem cells of multiple sclerosis patients. 2021. 118 pages. Dissertation (Master's degree in Immunology) - Institute of Biomedical Sciences, University of São Paulo, São Paulo, 2021.

Multiple sclerosis (MS) is an autoimmune disease characterized by a chronic and progressive inflammatory condition in the Central Nervous System (CNS), which results in an axonal neurodegeneration process, culminating in neurological deficiency in patients. Recently, it was established that astrocytes (glial cells) play a key role in the regulation of neuronal synapses, along with inflammatory and neurodegenerative processes in the CNS. It is also known that astrocytes are one of the major sources of reactive oxygen species and this is directly related to the regulation of mitochondrial function. Mitochondria constantly modify their morphology according to the bioenergetic needs of the cell and changes in these regulatory mechanisms can trigger neurodegenerative processes. MS is hardly represented in all its scope by animal models, and many therapeutic studies in experimental models have failed to be translated into humans due to interspecific differences. Still, the study of the role of CNS resident cells in MS patients is hampered by ethical issues. In this sense, induced pluripotent stem cells (hiPSC), which can be reprogrammed from patients' blood or skin samples and differentiated into any somatic cell population, appear as a powerful approach to investigate molecular mechanisms that may be associated the development of complex diseases like MS. Therefore, in this work we formulated the hypothesis that hiPSC-derived astrocytes from patients with Multiple Sclerosis should have changes in mitochondrial dynamics and metabolism that may be associated with the MS phenotype. In a brief manner, hiPSC-derived astrocytes were successfully obtained and characterized. We observed baseline differences between the control and patients' astrocytes, with enrichment of genes associated with mitophagy and neurodegenerative processes, as well as the transport of molecules through the mitochondria in MS astrocytes. Then, we observed in this same group an increase in superoxide production and a decrease in mitochondrial quality. Analyzing the cells functionally using the Seahorse technique, we observed an increase in oxidative and glycolytic metabolism in patients' astrocytes, indicating a process of metabolic stress in these cells, in addition to a significant decrease

in their mitochondrial bioenergetics efficiency. Using transmission electron microscopy, we analyzed the mitochondrial morphology in astrocytes and observed a significant increase in mitochondrial fission in the MS group, corroborating our gene expression results obtained by PCR array. Also, in line with the previous results, we observed a decrease in the ratio between mitochondrial DNA and nuclear DNA. As we observed a swelling of the endoplasmic reticulum in the astrocytes of patients by electron microscopy, we analyzed the expression of genes related to the UPR (Unfolded Protein Response) pathway as well as genes linked to the regulation of the response to mitochondrial stress and we observed that several of them are significantly decreased in patient astrocytes, indicating a possible defect in the cellular stress response machinery. Thus, our results suggest a phenotype of mitochondrial dysfunction in astrocytes derived from MS patients and open new perspectives for both disease modeling and future therapeutic approaches.

Keywords: Multiple Sclerosis; neuroimmunology; induced pluripotent stem-cells; mitochondrial dynamics; metabolism.

1. INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disease that affects the central nervous system and currently affects more than two million people around the world [1]. A recent study made an epidemiological forecast of the incidence of MS in Brazil, showing that on average it affects 8.69/100.000 individuals, with a much higher prevalence in the South and Southeast than in the Northeast region, given that the disease affects the Caucasian population more frequently [2]. It is characterized by neurological deficits that are partial or fully recoverable, lasting from a few days to weeks. The average age of onset of symptoms is around 30 years old and approximately 25 years after diagnosis 50% of patients are permanently confined to a wheelchair. The symptoms are heterogeneous and can include sensory, vision and motor problems, as well as fatigue, pain and cognitive impairments [3].

MS is a complex and multifactorial disease. The knowledge of the genetic, environmental and lifestyle-related factors that are associated with the development of MS has been currently expanded. Environmental factors, such as smoking, diet, obesity and vitamin D intake are more determinant in susceptibility to the disease than genetic factors [4]. However, some genetic factors such as the differential expression of class II MHC haplotypes (HLA II in humans) contribute significantly to the predisposition to MS [5].

In agreement to an important autoinflammatory component in MS, a recent study showed that genetic variants in NLRP3 and NLRC4 inflammasomes influence both the susceptibility and severity of MS. The work shows that gain of function variants in the *NLRP3* and *IL1B* genes are directly associated with the severity and progression of the disease, suggesting that a constitutive activation of the NLRP3 inflammasome may be a risk factor to the development of more severe forms of MS. In this sense, the authors report that a SNP in the promoter region of *IL1B* that results in a gain of function is more frequent in the progressive forms of MS than in the relapsing-remitting ones. Still, it is shown for the first time that a SNP in the *NLRC4* intronic region, which leads to a loss of function, is more frequent in patients whose disease progression is slower and is related to a better response to IFN-γ therapy, being also more frequent in patients who are better

responders to therapy. This polymorphism leads to a decrease in *NLRC4* transcription in addition to a decrease in serum IL-18 levels, which seems to have a protective effect in MS [6].

There are two fundamental aspects in the progression of MS that must be considered: first, the neurologic manifestations of the disease are heterogeneous; second, most of the pathology manifests itself silently. Given that the disease generally affects individuals in their 20 to 30 years, uncertainties regarding the progression of symptoms negatively impact decisions both personally and professionally. Most patients start symptoms in a picture of Relapsing-Remitting Multiple Sclerosis (RRMS), which can last from years to decades, in which neuronal deficiencies are recurrent (about 1-2 times a year) but reversible and are characterized by demyelination and destruction of oligodendrocytes and axons, which occur as a result of an inflammatory process mediated by immune cells that enter the CNS. This relapsing phase lasts for a few months until a pro-resolving remitting phase occurs, characterized by a resolution of the inflammatory process and remyelination, which allows patients to recover their clinical condition, with reestablishment of the conduction of nerve impulses within the CNS. After 8 to 20 years, patients with RRMS may transition to a condition of Secondary Progressive Multiple Sclerosis (SPMS), in which neurological damage becomes progressive and irreversible. A transition from one frame of RRMS to another frame of SPMS has severe consequences for patients, leading to extremely severe cognitive and life quality issues [5].

1.2 Multiple Sclerosis immunopathology

It is well established that the immune system plays an essential role in MS progression by detecting induced expression of class II MHC molecules in macrophages, astrocytes, and microglial cells during MS [7]. Peripheral tolerance mechanisms mediated mainly by regulatory Foxp3+ T lymphocytes (Tregs) can inhibit the effector actions of autoreactive cells by several mechanisms, such as the release of antiinflammatory cytokines (IL-10, TGF-β, IL-35), apoptosis induction or through modulation of dendritic cells (DCs) via inhibitory receptors, such as CTLA-4. However, if this tolerance is broken in any way, autoreactive lymphocytes that recognize CNS antigens can be activated and become effector cells. Studies show that this cellular activation can occur by molecular mimicry, presentation of new autoantigens, recognition of a CNS antigen previously

phagocyted or even by stimulation of cytokines (bystander activation) [3]. In addition, environmental, genetic and infectious factors can contribute to this break in immunological tolerance. After activation, Th1, Th17 and $TCD8^+$ lymphocytes migrate to the CNS, leading to an inflammatory condition associated with tissue damage [3]. One of the most challenging questions regarding the immunopathology of MS concerns the origin of the immune response dysregulation, that is, if it starts directly in the immunocompetent cells of the CNS, with proinflammatory capacity (i.e. astrocytes and microglia) or if it is initiated in the periphery. In the first scenario, the first pathological event would occur within the CNS, in which an inflammatory state mediated by resident cells, especially astrocytes and microglia would lead to a release of CNS antigens that could be carried by antigen presenting cells (APCs) to the periphery. or drained directly to the lymph nodes. A proinflammatory autoimmune response would then be generated, mediated mainly by T lymphocytes, which would then migrate to the CNS and increase inflammation. The second model, in turn, suggests that the autoimmune response starts in the periphery, in the context of a systemic infection for example, and may eventually start inflammatory events in the CNS. Both scenarios result in a sort of positive feedback mechanism in which the tissue damage would lead to a greater release of CNS antigens to the periphery, causing greater activation and migration of autoreactive lymphocytes to the CNS and, consequently, increasing inflammation and tissue damage [4].

In an earlier stage of the disease, it is suggested that innate and adaptive immune cells may migrate from the periphery to the CNS via the blood-brain barrier, subarachnoid space or even via the choroid plexus. Upon entering the CNS, along with the resident cells (microglia and astrocytes), damage to axons and oligodendrocytes occurs, in addition to demyelination [3]. Recently, the existence of lymphatic vessels in the brain meninges has been discovered, characterizing another possible pathway for the activation of encephalitogenic immune cells, especially T lymphocytes, within the CNS. One study showed that removing meningeal lymphatic drainage in mice with Experimental Autoimmune Encephalomyelitis (EAE), an experimental model of MS, attenuates the clinical progression of the disease, but not does not lead to complete remission of the symptoms suggesting a direct participation of this pathway in the pathogenesis of EAE but without excluding other routes previously studied [8]. The later stage of MS progression is characterized by an exhaustion of adaptive immune cells. Therefore,

chronic inflammation, demyelination and progressive neurodegeneration damage are now mediated mainly by resident CNS cells with pro-inflammatory capacity, especially astrocytes and microglia [3].

Astrocytes, when stimulated by microglia, secrete the chemokine ligand CCL-2 and GM-CSF, contributing to an even greater recruitment and activation of microglial cells while producing reactive oxygen species (ROS), which intensify the process of neuronal degeneration. Astrocytes are still capable of inhibiting the differentiation of progenitor cells into oligodendrocytes, preventing neuron remyelination and are therefore key cells in the clinical course of MS [3]. There is a great amount of evidence in the literature supporting the existence of reactive astrocytes in patients with MS. In a study which aimed to understand the source of the chemokine MCP-1, important in recruiting T lymphocytes and monocytes, the researchers observed brain tissue slices from patients with the disease in the active and chronic demyelination phase and detected the presence of MCP-1 immunoreactive astrocytes, whereas the same result was not observed in perivascular and parenchymal macrophages [9]. Another study showed that in brain injuries in MS patients, activated astrocytes are BAFF producers, a factor that promotes the proliferation and activation of B lymphocytes, which could support an increase in the presentation of autoantigens coming from the CNS, leading to a worse MS progression [10]. Together, these studies demonstrate that astrocytes are activated in MS, especially in the phases of chronic demyelination and can directly impact disease progression.

1.3 Therapies for Multiple Sclerosis

MS is a complex disease, and no cure has been developed for it until today. For many years it was difficult to develop therapies for MS, given the limited knowledge on the pathological mechanisms that triggered it in combination with the extreme clinical variability observed in patients.

The therapeutic era in MS began with the FDA approval of Interferon-Beta-1b (IFNβ-1b) for the treatment of RRMS in 1993 [11]. A study published in 1996 [12] then showed that intramuscular injections of IFNβ-1a not only decreased the frequency of relapses in patients with RRMS but also decreased their weakness. Glatiramer acetate was the second injectable therapy approved for the treatment of RRMS in 1997, consisting of a mixture of peptides which mimic the basic myelin protein and thus competitively inhibit

the binding of immune cells to myelin [13]. Glatiramer acetate and new formulations of IFN-β constituted the therapeutic strategy adopted in the subsequent decade. The safety in the use of these drugs in humans is such that these treatments are still used to treat MS patients.

Even considering that RRMS patients responded positively to therapies with IFN-β and glatiramer acetate, their effect can be considered moderate, since they reduced the rate of relapses in only one third of the treated individuals, whereas several patients did not respond to them [11, 12, 13]. Therefore, more effective treatments have been developed over time, including therapies with monoclonal antibodies, being natalizumab the first developed. This antibody is able to block the adhesion of immune system cells, especially autoreactive T lymphocytes to the blood-brain barrier, preventing their migration to the CNS. Studies published in 1999 reported natalizumab as safe and effective in RRMS and SPMS patients, decreasing the number of active lesions in treated individuals [14]. Subsequent and larger-scale studies reaffirmed the same pattern of improvement in patients as seen in the first studies [15]. However, in 2005, three cases of patients who developed Progressive Multifocal Leukoencephalopathy (PML) during treatment with natalizumab [16] were described. In 2006, the FDA approved natalizumab therapy for patients with RRMS but with safety restrictions such as constant monitoring of patients, especially regarding the incidence of PML [17].

The treatment of RRMS has improved significantly since 2010, with the development of oral and non-injectable drugs effective in treating the disease. In comparison to previously available therapies, these drugs were either as effective or more while extending the available routes of application, satisfying patients. The first approved drug was fingolimod, a sphingosine-1-phosphate receptor (S1PR) antagonist, highly expressed in leukocytes and necessary for the escape of immune cells from lymph nodes. In this sense, fingolimod induces the retention of autoreactive T lymphocytes in the lymph nodes, preventing them from migrating into the CNS [18]. The second approved drug was teriflunomide, which inhibits the proliferation of T lymphocytes by blocking the pyrimidine synthesis pathway, decreasing the number of circulating lymphocytes [19]. The third drug approved by the FDA was dimethyl fumarate (DMF), an immunosuppressant that also decreases the number of circulating T lymphocytes,

especially TCD8 + [20]. All therapies were effective in clinical trials with RRMS patients, however fingolimod increased the susceptibility of some patients to infections [18].

Another oral drug known as cladribine has been developed for the treatment of RRMS, inducing apoptosis of lymphocytes, especially B cells, in patients. The drug had a lasting effect on treated individuals, suggesting that it promotes a prolonged remission of the disease without the need for short term therapy maintenance [21].

Recently, monoclonal antibodies known as ocrelizumab and rituximab have been developed, both targeting the CD20 molecule, found mostly on the surface of B lymphocytes [22, 23]. Ocrelizumab was approved by the FDA for the treatment of RRMS in 2017, after studies which showed that the therapy was more effective than IFNβ-1a in reducing disease progression [23].

Finally, in the last two or three years, studies have been trying to develop therapeutic strategies for SPMS such as autologous hematopoietic stem cell transplantation, which has managed to inhibit disease outbreaks and the formation of new lesions in patients [24] and an oral drug known as siponimod, which was approved by the FDA in 2019 for treating SPMS. This drug acts in a similar way to fingolimod, inhibiting S1PR and preventing the migration of autoreactive lymphocytes to the CNS and had positive effects in the patients' treatment, reducing the progression of the disease and being considered relatively safe, with very few side effects [25]. Together, these studies indicate that as much as there are therapies approved for MS, there is an urgent need for better understanding the molecular mechanisms behind the different forms of the disease so that new and increasingly effective treatments with less side effects may be developed.

1.4 Modeling neurologic diseases with hiPSC-derived cells

Among stem-cells, embryonic ones have stood out in studies in the biomedical field given their ability to differentiate in all cell types derived from the germ layer. Nevertheless, given the numerous ethical implications regarding their use, several researchers around the world started to look for alternative models that had the same differentiation capacity as embryonic cells. In this sense, the discovery that the state of pluripotency could be induced in fibroblasts or peripheral blood mononuclear cells (PBMCs) with the use of specific factors has revolutionized the field of medicine and opened new perspectives for both disease modeling and drug testing. This state is induced

by the overexpression of the factors Oct3/4, Sox2, Klf4 and c-Myc, which control the signaling pathways required for cellular reprogramming to a state of pluripotency, constituting the so-called induced pluripotent stem cells (hiPSC), which have unlimited self-renewal capacity and can differentiate to any type of human somatic cell. Currently, several reprogramming protocols for hiPSC use episomal or adenovirus-based vectors, to avoid the use of the c-Myc oncogene [26].

Prior to the establishment of the hiPSC technology, research in neurologic diseases was focused mainly on animal and cell culture models. Despite many important discoveries enabled using these models, they do not fully recapitulate human diseases for several reasons. Regarding animal models, species-related differences make it hard to translate study findings into humans, especially those focused on the development of new therapeutic strategies. Primary cells are, for instance, exceedingly difficult to be obtained fresh from brain tissues due to ethical implications, being most of the times replaced by post-mortem obtained samples, which lack blood and oxygen supply and are therefore distinct from CNS cells in steady-state conditions [27].

The use of hiPSC-derived cells to study neurological diseases has overcome the aforementioned disadvantages since they are obtained from human blood or skin samples, eliminating both the ethical and species-related differences issues. Additionally, hiPSC-derived cells maintain the genetic background from the donor sample and can therefore be used to investigate the functional effects of gene mutations in human diseases. Finally, the recent discovery of the CRISPR technology now allows stem-cell researchers to edit the hiPSC genome, either correcting or introducing specific gene mutations and studying their effects on differentiated cells, aiming to unravel disease mechanisms in a more precise way [27].

Regarding the CNS, hiPSC can be differentiated into several resident cell populations, such as neurons, astrocytes, oligodendrocytes and microglia, allowing researchers to study the phenotype of these cells in the context of several neurologic diseases such as Parkinson's, Alzheimer's, Amyotrophic Lateral Sclerosis and Huntington's, searching for pathology-associated molecular targets and screening for compounds that might dampen disease effects on these cells [26]. Nevertheless, it must be taken in consideration that hiPSC-disease modeling sometimes has limitations given that genetic and epigenetic variations may occur during hiPSC reprogramming, which

could lead to phenotypic differences between cell lines that are not disease-related or between clones generated from the same original cell line [27]. These changes can be overcome through using non-integrated hiPSC reprogramming methodologies such as episomal, Sendai viral and adenoviral vectors and plasmids, which maintain genome integrity during the process, instead of lentiviruses that integrate to the DNA and make the cell susceptible to SNPs and CNVs [27, 28] or using isogenic cells as controls in functional studies where a genetic mutation is involved, which can be achieved by correcting it in hiPSC using the CRISPR editing technology [27].

Finally, recent studies using hiPSC-modeling in neurologic diseases are exploring their potential to generate human brain organoids, which consist in tridimensional culture systems that mimic the human brain in developmental, morphological, transcriptional and epigenomic aspects that cannot be fully recapitulated by animal models, allowing researchers to study neurologic disease in a physiologically relevant context. This tool can be used to analyze in situ electrophysiology and calcium imaging of neuronal populations, as well as to explore molecular signatures of disease, even at a single cell level and to unravel intercellular interactions during neurodevelopment, such as the crosstalk between neurons and astrocytes. HiPSC-derived brain organoids have been used to model several diseases, such as Alzheimer's, Parkinson's, Huntington's and Autism [29].

1.4.1 Using hiPSC-derived astrocytes to study Multiple Sclerosis

In contrast to the abundant literature available in mouse models, the number of studies analyzing the reactivity of astrocytes in humans is reduced, mainly due to ethical implications, given the difficulty in obtaining biological samples from the CNS of individuals affected by neurological diseases. It must also be stated that there are significant differences between human and mouse astrocytes at the baseline level and in the face of inflammatory stimuli. In this context, the use of induced pluripotent stem cells appears as a technology to study molecular mechanisms of complex diseases such as MS in CNS resident cells [30].

Another relevant aspect to be considered is that the heterogeneity observed in several human diseases such as MS is hardly represented in its scope in animal models.

The generation of hiPSC from the same disease in its different clinical presentations opens doors to the discovery of molecular mechanisms that may be responsible for the transition to the most severe forms of the disease. In the case of MS, the discovery of mechanisms that are behind one of the disease presentations can assist in the development of drugs for the treatment of that phenotype [31].

Unlike another neurodegenerative diseases such as Parkinson's, Amyotrophic Lateral sclerosis, Alzheimer's and Huntington that have well described genetic mutations linked to their onset and have therefore been modeled through hiPSC-derived cells many times, MS is complex and involves both genetic and environmental factors, which makes it harder to find a consistent model that recapitulates disease aspects [32]. Nevertheless, disease-related alterations have already been demonstrated in hiPSC-derived neurons from sporadic cases of Schizophrenia [33], Alzheimer's Disease [34], Parkinson's Disease [35] and Amyotrophic Lateral Sclerosis [36], which raises the possibility whether hiPSC-derived CNS cells from MS patients, which are sporadic in their majority, could also display phenotypic abnormalities related to the disease.

There are very few studies in the literature which analyzed hiPSC-derived cells in the context of MS. It has been demonstrated that hiPSC from MS patients can efficiently be differentiated into functional neurons, astrocytes and oligodendrocytes [37, 38]. Interestingly, one recent study [39] demonstrated that hiPSC-derived neural progenitor cells from progressive MS patients have increased markers of cellular senescence and HMGB1 protein levels, inhibiting oligodendrocyte progenitor cells maturation into myelinating oligodendrocytes, which reaffirms the potential of using hiPSC technology to unravel MS pathogenic mechanisms. Regarding astrocytes there are just two studies available so far [40, 41] which derived these cells from MS patients' hiPSC but they reached very contradictory conclusions in relation to each other. One of them [40] analyzed the inflammatory reactivity of astrocytes to different inflammatory stimuli and observed no differences between secretion levels of proinflammatory cytokines between cells from controls and MS patients, suggesting that genetic risks for MS should be more linked to the regulation of immune response than directly to CNS resident cells. Although the authors do not report differences between hiPSC-derived astrocytes from controls and MS patients in a basal level, they essentially focus on transcriptomic changes between these cells and do not assess another functional aspects of these cells which are very

relevant to MS pathology, such as mitochondrial dysfunction along with the metabolic profiling of these astrocytes. The other study [41] showed instead that a genetic risk variant (rs7665090^{G}) on astrocytes which leads to a gain of function in NF-κB is related to a higher risk of developing MS. Interestingly, the authors also report impairments in metabolic parameters that are important to astrocyte homeostasis including glutamate uptake and lactate secretion in hiPSC-derived astrocytes bearing the risk variant. Therefore, in opposition to the previous study [40], their results demonstrated that MS risk is also influenced by dysregulated responses of CNS resident cells such as the astrocytes, which should play a significant role in establishing autoimmune inflammation within the CNS.

Despite several advances in the field of MS, there are many questions regarding the onset of the disease, its subtypes and different responsiveness to therapies which still need to be better elucidated. Therefore, the ability to obtain a specific phenotype of an MS patient on a laboratory dish appears as an alternative capable of overcoming most of the limitations of existing animal models of autoimmune demyelinating diseases, in addition to allowing the in-depth study of human CNS resident cells which were a priori very little accessible [31]. The specific analysis of these populations at the molecular level should, in a long-term perspective, allow the development of new therapeutic strategies for each subtype of MS, increasing the effectiveness of the treatments currently available

1.5 The role of astrocytes in Multiple Sclerosis

Astrocytes are the predominant glial cells in the CNS and are essential in maintaining homeostasis of several biological factors such as glutamate and extracellular potassium [42]. In addition, these cells communicate directly with other astrocytes and oligodendrocytes through communicating junctions, forming a connection network between glial cells [43].

Currently, it is known that astrocytes are key cells in the regulation of neuronal synapses, forming the so-called tripartite synapses, being able to regulate neuronal activity by releasing neurotransmitters such as glutamate, D-serine and ATP [44]. A single astrocyte can connect to several neuronal synapses at the same time, and it can also alter gene expression in neurons by releasing exosomal vesicles containing miRNA molecules. Furthermore, astrocytes can release neurotrophic factors and are metabolically linked to

neurons, providing the latter with lactate and antioxidant factors such as glutathione and thioredoxin. Finally, astrocytes also participate in the synthesis of some neurosteroids such as estrogen and dehydroepiandrosterone (DHEA), which modulate excitability and promote neuronal myelination [43].

Healthy astrocytes contribute to the maintenance of an antiinflammatory microenvironment in the CNS by the constitutive release of IL-10 and TGF-β, expression of the Fas ligand (FasL) molecule and induction of the expression of the inhibitory co-stimulation molecule CTLA-4 on the surface of T lymphocytes [43]. Astrocytes can be activated and become reactive upon the recognition of damage-associated molecular patterns (DAMPs) or pathogens (PAMPs), the first being the results of tissue damage to the CNS and the last from viral or bacterial infections, for example. When stimulated, astrocytes shift to a pro-inflammatory profile, increasing the release of cytokines such as TNF-α, IL-1β and IL-6, several chemokines including CCL2, CCL20 and CXCL10, neurotrophic factors (such as BDNF), adhesion molecules (LFA-1, ICAM-1 and VCAM-1), vascular endothelial growth factor (VEGF), ROS and reactive nitrogen species. Astrocytes activated with IFN-γ combined or not with TNF-α showed increased expression of MHC class II, co-stimulatory molecules CD80 and CD86 and adhesion molecules, suggesting that upon neuroinflammation these cells may contribute by displaying antigens to T lymphocytes. Finally, a cascade of proinflammatory cytokines in the CNS disrupts the metabolic and homeostatic functions of astrocytes, directly impacting neuronal activity due to changes in the availability of lactate and glutamate in the medium [43].

Like macrophages, astrocytes also receive a specific classification regarding their metabolic polarization profiles, being divided into A1, proinflammatory and A2, antiinflammatory [43, 45]. A1 astrocytes are induced by inflammatory stimuli and are abundant in diseases such as MS, being characterized by the abundant expression of the Complement System C3 protein and the release of ROS, TNF-α and CCL2, which promote neurodegeneration, inflammation and leukocyte recruitment to SNC, respectively. A2 astrocytes, in turn, are related to post injury and inflammatory processes neuronal regeneration processes, releasing neurotrophic factors such as BDNF and GDNF, in addition to their capacity to form astroglial scars [43].

Recent studies emphasize the proinflammatory role of astrocytes during neuroinflammation and suggest a key role for mitochondria in the regulation of these processes. One of them [46] showed that sphingolipid metabolism in astrocytes activates cytosolic phospholipase A2 (cPLA2) which is translocated to the mitochondrion, inducing the activation of a protein called MAVS (Mitochondrial Antiviral Signaling Protein), leading to its oligomerization and activation of subsequent downstream signaling pathways, which induce astrocyte proinflammatory activity via activation of the NF-κB transcription factor. Furthermore, the authors showed that the cPLA2-MAVS interaction controls the metabolism of astrocytes, decreasing the activity of the enzyme hexokinase 2 and consequently reducing glycolysis and lactate production, the latter essential for the metabolic regulation of neurons. The study identified that the drug Miglustat, which interferes with sphingolipid metabolism by inhibiting glucosylceramide synthesis, can suppress the activation of the cPLA2-MAVS-NF-κB pathway axis and consequently decrease the pathogenic activity of astrocytes, improving the progression of EAE, the animal model of MS [46]. The second study [47] by the same group performed an RNA sequencing of astrocytes from EAE mice and MS patients, demonstrating that they have a decrease in the expression of the antioxidant response regulating factor NRF2 (Nuclear factor erythroid 2- related factor 2) together with an increase in the expression of the MAFG transcription factor, which helps the MAT2α factor in DNA methylation and consequent inhibition of anti-inflammatory and antioxidant responses in cells. The inactivation of MAFG and MAT2α in astrocytes using the CRISPR technology resulted in improvement in the progression of EAE, with a decrease in the clinical score of the disease in mice, in addition to increasing antioxidant activity and decreasing proinflammatory signaling pathways in the cells. Interestingly, an analysis of the proinflammatory astrocyte clusters in both EAE and MS using bioinformatics tools identified the most significantly altered signaling pathways in these cells in the context of these diseases, with mitochondrial dysfunction appearing in both, although it was not deeply explored in this study [47]. These works together suggest that astrocytes actively participate in the pathology of MS and that mitochondria are closely linked to this process, indicating that there must be a better investigation and characterization of mitochondrial changes in astrocytes in these diseases, aiming at the discovery of new therapeutic targets.

1.6 Oxidative Stress and Neurodegeneration in Multiple Sclerosis

A possible mechanism to explain the neurodegeneration observed in the most advanced stages of MS is mitochondrial dysfunction, since mitochondria participate in intracellular calcium homeostasis and in the production of ATP and ROS and therefore failures in these organelles that occur as a secondary effect to the inflammatory process observed in the disease, results in energy imbalance whereas increasing the neuronal degeneration process [48, 49].

The CNS has a high-energy demand resulting mainly from neuronal oxidative metabolism to maintain its proper functioning, which makes it highly susceptible to mitochondrial dysfunctions, evidenced by the huge number of neurodegenerative diseases whose primary cause involve mutations in the mitochondrial DNA (mtDNA) or mutations in protein-coding genes that are essential for mitochondrial function, being examples Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis [48, 50, 51].

The release of excessive amounts of ROS can be a consequence of mitochondrial dysfunction. The two major sources of ROS production by mitochondria are the complexes I and III of the mitochondrial electron transport chain in a process called reverse electron transport, which is induced in situations where there is a hyperpolarization of the mitochondrial membrane, which occurs during an inflammatory response for example. In this sense, stimulation of a cell with bacterial LPS induces an increase in the $\Delta\psi m$ that leads to the accumulation of succinate. This succinate starts to be oxidized by the enzyme succinate dehydrogenase, leading to an excessive production of ROS by the complex I of the mitochondrial electron chain, which, in turn, stabilizes the transcription factor HIF-1α, that acts by promoting an increase in glycolytic metabolism combined with a greater induction of pro-inflammatory cytokines such as IL-1β while suppressing the transcription of cytokines with a role in regulating the immune response, such as IL-10 [52].

In opposite to neurons, astrocytes have shown to display a higher glycolytic profile, producing high levels of lactate that are released and uptaken by neurons in a process known as the astrocyte-neuron lactate shuttle, a very important process in the regulation of brain energy metabolism [53]. Astrocytes in a steady-state condition possess a very efficient antioxidant machinery coordinated mainly by the Nrf2 transcription factor, with increased levels of glutathione precursors and enzymes that can detoxify ROS molecules

[53]. In the case of neurodegenerative diseases such as MS, mitochondrial dysfunctions in astrocytes directly impact the production of ROS and the metabolism of these cells, which can alter the regulation of neuronal synapses and increase the process of axonal neurodegeneration, worsening disease pathology [43]

1.7 Mitochondrial dynamics in the regulation of cell metabolism

Mitochondria are organelles with a highly-organized architecture. The mitochondrion is composed of two membranes, one external and one internal, the latter in which the electron transport chain is located. The main role of mitochondria in cells is the coupling of substrate oxidation by the tricarboxylic acid (TCA) cycle to oxidative phosphorylation (OXPHOS), which results in a high rate of ATP production (up to 36 molecules of ATP for each molecule of glucose). In addition, another important role of these organelles is the use of TCA cycle intermediates in anabolic or regulatory reactions. Nevertheless, mitochondrial metabolic activity also culminates in the production of mitochondrial ROS through monoelectronic reactions using molecular oxygen and generating anion superoxide and its dismutation product, hydrogen peroxide. These molecules can either function as cell signalers or promote cell damage, mainly through DNA damage, depending on their relative concentrations [54].

Contradictory to the original thoughts, mitochondria are not static organelles but highly dynamic, changing their bioenergetic patterns according to the metabolic needs of cells. Changes in mitochondrial morphology are coordinated by two key processes, fusion and fission. They guarantee the effective segregation of mitochondrial DNA as well as regulate the production levels of mitochondrial ROS, calcium homeostasis and OXPHOS. Mitochondrial fusion is a process still poorly understood, but it happens in 2 stages: first, the fusion of the outer membrane is regulated by the interaction between two GTPases proteins called mitofusins 1 and 2 (Mfn1 and Mfn2), while fusion of the inner membrane depends on another protein, Opa1. Mitochondrial fission is, in turn, induced in sites marked by actin or the endoplasmic reticulum, with the action of the adapter protein Mitochondrial fission factor, which recruits another protein, Drp-1 and the interaction between these proteins forms spirals that fragment the structure of the mitochondria [54].

Mitochondrial dynamics is also regulated by upstream transcription factors such as PGC-1α and HIF-1α. PGC-1α mediates mitochondrial biogenesis through the co-activation of Mfn2, one of the key mediators of mitochondrial fusion, recycling dysfunctional mitochondria and increasing the organelle numbers within the cells. HIF-1α, in the opposite direction, favors mitochondrial fission through phosphorylation at serine 616 of Drp-1, facilitating translocation this protein into the mitochondria and concomitant activation [55]. In the context of neurodegeneration, mitochondrial dysfunction is a hallmark of the pathology of several diseases, in which PGC-1α has shown to exert protective effects by upregulating mitochondrial biogenesis whereas HIF-1α is possibly related to increased mitochondrial fragmentation and stress, having therefore pathogenic effects [55, 56, 57]. It is important to point out that mitochondrial dynamics is an emerging field of research in neurodegenerative diseases and therefore the exact role played by these aforementioned transcription factors in regulating this process is still poorly understood.

In terms of cell metabolism, it has been originally demonstrated in T lymphocytes that mitochondrial dynamics regulate metabolic profiling of these cells. T memory cells rely mainly on β-oxidation to perform their functions, having increased number of fusioned mitochondria with elongated shape, which sustains the high mitochondrial metabolism demand of this subpopulation. On the other hand, T effector cells rely mainly on glycolysis, which might be facilitated by the increased number of fragmented mitochondria in this subpopulation. Genetic deletion of mitochondrial dynamics regulators in these immune cells resulted in metabolic alterations, indicating a role of this process in the regulation of cellular metabolism [54]. The role of mitochondrial dynamics in shaping the metabolism of other immune and non-immune cells should be further elucidated.

Mitochondria also play a key role in regulating calcium metabolism in cells. These organelles have the capacity to capture an enormous amount of calcium, albeit with a lower affinity than that presented by the endoplasmic reticulum, acting as buffers in cases of accumulation of the ion in the cytosol, which is extremely harmful to cells. The uptake of mitochondrial calcium occurs through a transporter known as MCU, a process that is also regulated by the mitochondrial membrane potential. However, an excessive accumulation of calcium in the mitochondria is extremely harmful to cells, since it induces

a phenomenon known as mitochondrial permeability transition, leading to the loss of the impermeability of the inner mitochondrial membrane together with the opening of a pore known as mtPTP, which leads to a release of the contents of the organelles into the cytosol, culminating in exacerbated cell death. This phenomenon has been reported in several diseases, such as stroke and in cases of liver inflammation. Mitochondrial dynamics play a key role in maintaining mitochondrial calcium transport, so that the Mfn2 protein mediates the interaction between mitochondria and the endoplasmic reticulum, regulates mitophagy and can increase the bioenergetic efficiency of cells. Also, changes in the morphology of mitochondria have been directly associated with the ability to capture cellular calcium, so that an increase in mitochondrial fusion, resulting in more elongated organelles through an increase in the expression of Mfn2, increases their efficiency in capturing calcium. On the other hand, induction of mitochondrial fission led to a decrease in baseline calcium levels as well as a decrease in calcium retention by the endoplasmic reticulum [58].

A failure in the mechanisms that regulate mitochondrial dynamics can result in dysfunctions in the mitochondria, associated with neurodegenerative processes. Fragmented mitochondria, which have undergone mitochondrial fission, generate more ROS, leading to increased neuronal degeneration. Astrocytes play an important role in the metabolism of glutathione, an important antioxidant that protects cells from the effects of free radicals and express the transcription factor Nrf2, which is also directly associated with the antioxidant response and is a molecular target of one of the main therapies indicated for patients with MS, dimethyl-fumarate [47, 59]. A study showed that upon proinflammatory stimuli such as LPS + IFN-γ and IL-1β, astrocytes modify their mitochondrial dynamics profile by increasing mitochondrial fission, with higher expression of Drp1, which results in greater production of ROS. In addition, the increased expression of Drp1 was shown to be dependent on iNOS activity, since inhibition of this enzyme inhibited the transition of mitochondria to a more fragmented state. Finally, this iNOS pathway, leading to the production of NO (RNS) and Drp1 expression, has been shown to act by signaling transduction of inflammatory stimuli into changes in mitochondrial morphology to a state of fission, which in turn further accentuates the NO production by astrocytes, contributing to increase the degenerative process [60].

In this sense, here we hypothesized that MS-derived astrocytes might have alterations in mitochondrial dynamics and metabolism that could be somehow related to the disease phenotype.

6. CONCLUSIONS

In summary, our findings indicate that:

- hiPSC-derived cells can be used as a reliable tool for MS studies;

- MS hiPSC-derived astrocytes mimic several disease features *in vitro*;

- MS hiPSC-derived astrocytes have increased oxidative stress;

- MS hiPSC-derived astrocytes have enhanced oxidative and glycolytic metabolism;

- MS hiPSC-derived astrocytes have increased mitochondrial fragmentation;

- MS hiPSC-derived astrocytes can have shared molecular disease mechanisms with other neurologic diseases;

- Since the balance between mitochondrial fusion and fission is a key process in the regulation of astrocyte metabolism, we propose that mitochondrial dynamics and metabolism in astrocytes can be targeted in future MS therapeutic approaches.

A summary of the findings of this study is shown below (Figure 21):

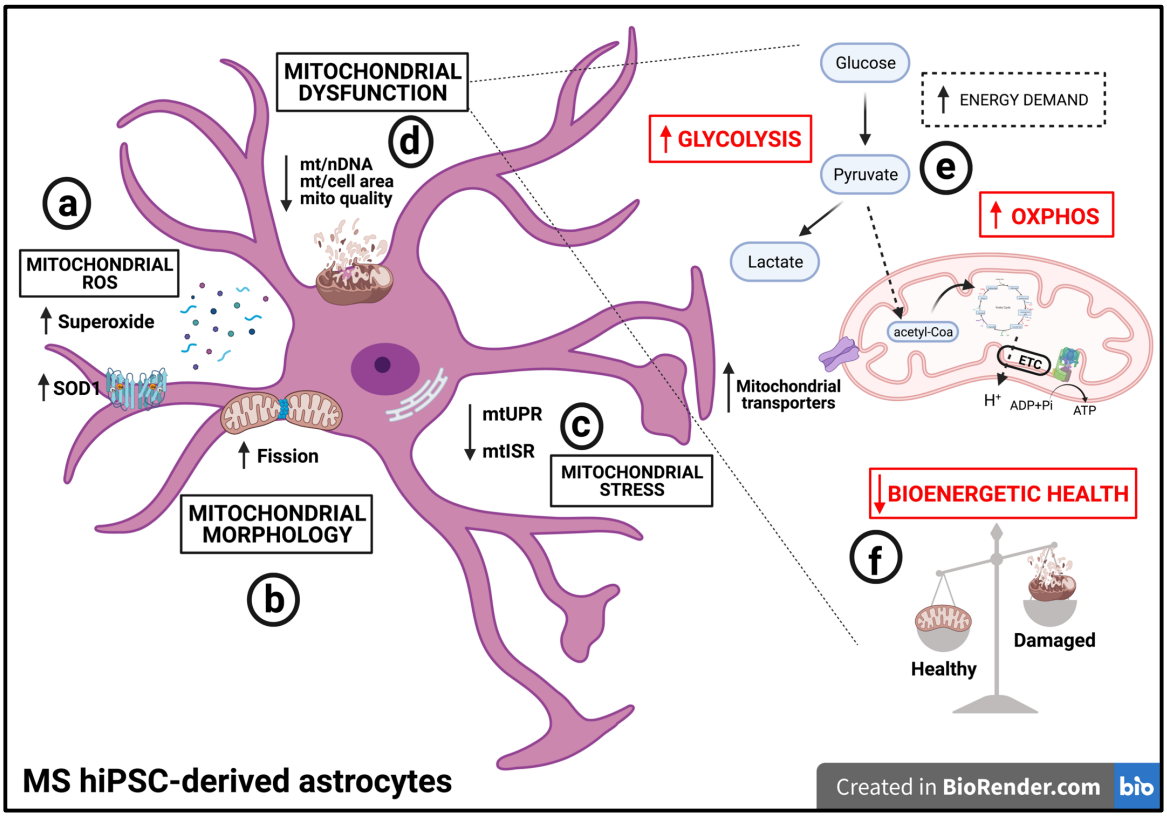

Figure 21. Summary of study findings. In this study we were able to successfully derive astrocytes from Multiple Sclerosis patients using the iPSC technology. When compared to controls, we observed in these cells changes in mitochondrial ROS, including increased superoxide production and SOD1 expression (**a**); changes in mitochondrial morphology (**b**), with increased mitochondrial fission; changes in mitochondrial stress (**c**), with a decreased expression of mtUPR and mtISR related genes, suggesting impaired activation of these protective pathways; mitochondrial dysfunction (**d**), with decreased mitochondrial/nuclear DNA ratio, mitochondrial/cell area ratio and mitochondrial quality. This mitochondrial dysfunction could be demonstrated in metabolic alterations, with enhanced glycolysis and OXPHOS in MS astrocytes, as well as increased expression of mitochondrial transporters, suggesting higher energy demands in these cells (**e**). It also was characterized by a reduced cell bioenergetic health with increased mitochondrial damage (**f**). SOD1 – superoxide dismutase 1; mtUPR – mitochondrial Unfolded Protein Response; mtISR – mitochondrial integrated stress response; mt/nDNA – mitochondrial to nuclear DNA ratio; mt/cell area – mitochondrial to cell area; mito quality – mitochondrial quality; ETC – electron transport chain; Pi – inorganic phosphate. The figure was generated using the Biorender software.

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