UNIVERSIDADE D
COIMBRA

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PERTURBATION OF ER-MITOCHONDRIA CROSSTALK IN BIPOLAR DISORDER:
EFFECT OF SIGMA-1 RECEPTOR MODULATION

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pela Doutora Rosa Maria Branco de Matos Costa Resende e pela Professora Doutora Ana Luísa Carvalho e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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O trabalho aqui apresentado foi realizado no grupo de Sinalização Celular e Metabolismo na Doença, na linha de investigação de Resposta do Retículo Endoplasmático (RE) ao Stresse e eixo RE-Mitocôndria no Centro de Neurociências e Biologia Celular da Universidade de Coimbra, liderado pela Doutora Cláudia Pereira, e orientado pela Doutora Rosa Resende.

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The future belongs to those who believe in the beauty of their dreams

Eleanor Roosevelt

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List of abbreviations

ADHD - attention deficit hyperactivity disorder

AIS - axon initial segment

ANK3 - ankyrin G or ankyrin 3

ATF4 - activating transcription factor 4

ATF6 - activating transcription factor 6

ATF6f - activating transcription factor 6 fragment

ATP - adenosine triphosphate

BCL-2 - B-cell lymphoma 2

BD - bipolar disorder

BDNF - brain-derived neurotrophic factor

CACNA1C - calcium voltage-gated channel subunit α-1C

CHOP - C/EBP homologous protein

CoMIC - spontaneous and repetitive constriction of mitochondrial inner compartment

CSF - cerebrospinal fluid

DMSO - dimethyl sulfoxide

DOC - sodium deoxycholate

DSM-5 - Diagnostic and Statistical Manual of Mental disorders

DTT - dithiothreitol

eIF2α - eukariotic initiation factor-2α

ER - endoplasmic reticulum

ERAD - endoplasmic reticulum-associated degradation

ETC - electron transport chain

FADH2 - flavin adenine dinucleotide

FBS - fetal bovine serum

GADD34 - growth arrest and DNA damage-inducible protein 34

GRP78 - glucose-regulated protein 78

GRP94 - glucose-regulated protein 94

H_2O_2 - hydrogen peroxide

ICD-11 - International Classification of Diseases for mortality and morbidity statistics, 11th revision

IMM - inner mitochondrial membrane

IP3 - inositol 1,4,5-trisphosphate

IP3R - inositol 1,4,5-trisphosphate (IP3) receptors

IRE1α - inositol-requiring protein 1α

MAMs - mitochondria-associated membranes

MCU - mitochondrial calcium uniporter

microRNA – micro-ribonucleic acid

MMP - mitochondrial membrane potential

MOMP - mitochondrial outer membrane permeabilization

mPTP - mitochondrial permeability transition pore

mRNA - messenger ribonucleic acid

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH - nicotinamide adenine dinucleotide

OMM - outer mitochondrial membrane

PBMC - peripheral blood mononuclear cells

PDI - protein disulfide-isomerase

p-eIF2α - phosphorylated eukariotic initiation factor-2α

PERK - protein kinase R-like ER kinase

PLA - proximity ligation experiment

RIDD - IRE1α-dependent decay

ROS - reactive oxygen species

RyRs - ryanodine receptors

SDS - Sodium Dodecyl Sulfate

SEM - standard error of the mean

SERCAs - sarco-endoplasmic reticular Ca^{2+} ATPases

Sigma-1R - sigma-1 receptor

SNP - single nucleotide polymorphisms

SRP - signal recognition particle

TCA - tricarboxylic acid

TMRE - tetramethylrhodamine ethyl ester perchlorate

UPR - unfolded protein response

VDAC1 - voltage-dependent anion-selective channel protein 1

WB - Western-blotting

XBP1 - X-box-binding protein 1

Abstract

Psychiatric disorders greatly impact both the patients' quality of life and the global economy. In Europe alone, there are about 164 million people diagnosed with some type of psychiatric disorder. Bipolar disorder (BD) is a mood disorder characterised by changes between depressive and manic or hypomanic episodes. Knowledge regarding the cellular and molecular mechanisms underlying bipolar disorder are still very limited. Until then, most studies have been carried out in post-mortem tissue of patients or in animals, with major limitations for the development of therapies and biomarkers. However, the literature points to altered endoplasmic reticulum (ER) stress response, mitochondrial dysfunction, and abnormalities in the processes of inflammation, Ca^{2+} signaling, bioenergetics, autophagy, and apoptosis. These processes are regulated by mitochondria-associated membranes (MAMs) and their relevance in different brain diseases is being unravelled. Taking this into account, this work aimed to reveal whether some of the events regulated by MAMs are altered in fibroblasts derived from patients with bipolar disorder, namely in the ER stress response, in the ER-mitochondria tethering, in mitochondrial dysfunction, and in Ca^{2+} signaling.

For this purpose, dermal fibroblasts were generated from skin biopsies performed in BD patients or age- and sex-matched control subjects (n=3 males, aged 18-25 years) after informed consent in accordance with Ethics Committee's guidelines. The cells were subjected to treatments with thapsigargin, a classical inducer of ER stress, and with pridopidine, an agonist of the Sigma-1 receptor which is a protein involved in MAMs mechanisms.

Under basal and thapsigargin exposure conditions, protein levels of ER stress-induced unfolded protein response (UPR) markers were measured by Western-blotting. Cell survival was assessed by the MTT reduction assay, and the mitochondrial membrane potential was evaluated using TMRE. IP3R-mediated ER Ca^{2+} release and ER-mitochondria Ca^{2+} transfer were studied with the Fura-2 and Rhod-2 fluorescent dyes, respectively. ER-mitochondria tethering was analysed using the proximity ligation assay. In BD patients-derived fibroblasts we detected differences in the levels of proteins involved in the ER stress response, in ER-mitochondria tethering, and in Ca^{2+} signaling to mitochondria, both in basal and stress conditions. However, we found that pharmacological activation of the Sigma-1 receptor did not alter the response of bipolar patients' cells. Our studies have revealed abnormal cellular and molecular mechanisms in bipolar patients' fibroblasts that could contribute to the development of reliable biomarkers and therapeutic strategies.

Keywords: Bipolar disorder; Endoplasmic reticulum stress; Sigma-1 receptor; Mitochondria-associated membranes; Ca^{2+} signaling.

Resumo

Os distúrbios psiquiátricos têm um enorme impacto tanto na qualidade de vida dos doentes, como a economia global. Só na Europa existem cerca de 164 milhões de pessoas com diagnóstico de algum tipo de distúrbio psiquiátrico. O distúrbio bipolar é uma perturbação de humor caracterizada pelas alterações entre episódios depressivos e maníacos ou hipomaníacos. O conhecimento relativo aos mecanismos celulares e moleculares subjacentes ao distúrbio bipolar são ainda muito limitados. Até então, a maioria dos estudos foram realizados em tecido *post-mortem* de doentes ou em animais, possuindo grandes limitações para o desenvolvimento de terapias e biomarcadores. No entanto, a literatura aponta para que existam alterações da resposta ao stress do retículo endoplasmático, disfunção mitocondrial, e alterações nos processos de inflamação, sinalização de Ca^{2+}, bioenergética, autofagia e apoptose. Estes processos são regulados por zonas de junção entre o retículo endoplasmático e a mitocôndria, denominadas *mitochondria-associated membranes* (MAMs) cuja relevância em diferentes doenças do cérebro tem vindo a ser desvendada. Tendo isto em conta, este trabalho teve como objetivo investigar se alguns dos eventos regulados pelas MAMs se encontram alterados em fibroblastos derivados de doentes com distúrbio bipolar, nomeadamente na resposta ao stress do retículo endoplasmático, ao nível do contacto entre este organelo e a mitocôndria, na disfunção mitocondrial, e na sinalização de Ca^{2+}.

Para este fim, foram usados fibroblastos da derme a partir de biópsias de pele realizadas em doentes com BD ou sujeitos saudáveis (n=3 homens, com idades compreendidas entre os 18-25 anos) após consentimento informado, de acordo com as diretrizes do Comissão de Ética. As células foram sujeitas a

tratamentos com tapsigargina, um indutor clássico do stresse do retículo endoplasmático, e com pridopidina, um agonista do recetor Sigma-1, que é uma proteína envolvida nos mecanismos das MAMs. Em condições basais e em stresse induzido pela tapsigargina, analisamos os níveis proteicos de marcadores de *unfolded protein response* (UPR) por *Western-blotting*. A viabilidade celular foi avaliada pelo ensaio de redução do MTT, e o potencial da membrana mitocondrial foi avaliado utilizando TMRE. A libertação de Ca^{2+} do retículo endoplasmático mediada por IP3R e a transferência de Ca^{2+} do retículo endoplasmático para a mitocôndria foram analisados através das sondas fluorescentes Fura-2 e Rhod-2, respetivamente. A ligação do retículo endoplasmático às mitocôndrias foi analisada utilizando *proximity ligation assay*.

Em fibroblastos provenientes de doentes com distúrbio bipolar detetámos diferenças nos níveis de proteínas envolvidas na resposta ao stresse do retículo endoplasmático, na ligação entre este organelo e a mitocôndria, e ainda na sinalização de Ca^{2+} para a mitocôndria, tanto em condições basais como em stress. No entanto, observámos que a ativação farmacológica do recetor Sigma-1 não alterou a reposta das células dos doentes bipolares. Os nossos estudos revelaram mecanismos celulares e moleculares alterados nos fibroblastos de doentes relativamente aos fibroblastos controlo. Estes resultados contribuirão para o desenvolvimento de biomarcadores fidedignos e de estratégias terapêuticas.

Palavras-chave: Distúrbio bipolar; Stresse do retículo endoplasmático; Recetor Sigma-1; Mitochondria-associated membranes; Sinalização de Ca^{2+}.

Chapter I
Introduction

1.1. Psychiatric disorders

The beginning of the second decade of the 21st century forced us to put global health at the forefront. Successive social isolations were mandatory due to the *Coronavirus* pandemic. Our daily routine has transformed completely placing enormous pressure on the mental health of the general population, devastating those who already had a diagnosis of a psychiatric disorder. Psychiatric patients are more susceptible to stress, anxiety, and depression during and after social isolation (Verdolini et al., 2021; Yocum et al., 2021). In comparison to other diseases, psychiatric diseases are responsible for fewer deaths, yet mental health disorders markedly affect the patient's quality of life, augmenting the number of years lost to disability (Rehm and Shield, 2019).

The impact of mental disorders will likely be directly proportional to the demographic explosion. Seemingly, by 2030 mental health disorders will disturb the global economy with a 6 trillion US dollars burden (Bloom et al., 2012). Only in Europe, approximately 164 million people are affected by some type of mental disorder, constituting around one-third of this continent's population (Wittchen et al., 2011). Recurrently, psychiatric disorders exhibit an exacerbated social and psychological impact owed by mental disorders comorbidities (Alonso et al., 2004).

1.2. Bipolar disorder (BD) overview

Characterization of bipolar disorder (BD) endured profound changes since it was first described by Jean-Pierre Falret. He created the concept of *folie*

circulaire, which meant circular insanity, in the mid-19th century (Saunders and Goodwin, 2010).

1.2.1. General features

According to ICD-11 (International Classification of Diseases for mortality and morbidity statistics, 11th revision) and DSM-5 (Diagnostic and Statistical Manual of Mental disorders), BD is divided into type I and type II, depending on the displayed and severity of the symptoms. In type I, patients develop depressive, manic, or mixed mood episodes. It is associated with harsher symptoms that entail a shortage in the patient's life quality. Type II is also an episodic mood disorder, with depressive and hypomanic periods, severe enough to debilitate the patients' quality of life. Depressive episodes are distinguished by the presence of changes in appetite and sleep, feelings of worthlessness, and an increase in suicidal thoughts. While the decreased need for sleep and increased energy, euphoria, and augmented self-esteem are recurring in manic and hypomanic episodes (World Health Organization, 2018; American Psychiatric Association, 2013).

This disorder affects 45 million people worldwide (James et al., 2018) regardless of gender, socioeconomic status, and region of the globe (Eid et al., 2013). It has a tremendous psychological impact, being suicide the main cause of the high early mortality rate among BD patients (Gonda et al., 2012). Patients are more prone to commit suicide under certain conditions, such as being in a depressive episode, having comorbidity with borderline personality disorder, or with anxiety disorder (Schaffer et al., 2015). Psychiatric disorders that are repeatedly associated with BD are attention deficit hyperactivity disorder (ADHD),

anxiety disorder, and borderline personality disorder (Zimmerman et al., 2020; Inoue et al., 2020; Vancampfort et al., 2013; Charles et al., 2016; Kudlow et al., 2015; Schiweck et al., 2021). Additionally, male patients with high prevalence of manic episodes are predisposed to develop a substance use disorder (Messer et al., 2017). However, other non-psychiatric comorbidities decline the quality of life. Among the most common are diabetes mellitus, fibromyalgia, and metabolic syndrome.

1.2.2. Diagnosis and available treatments

Considering the mixed symptoms associated with BD patients, there are three main lines of treatments available: mood stabilizers (carbamazepine, lamotrigine, valproate), antipsychotics (olanzapine, risperidone, aripiprazole), and antidepressants (fluoxetine, imipramine, venlafaxine). Although these drugs are the most effective in preventing acute mood episodes in the long term, they also carry several side effects that put the patient's physical and mental health at risk (Baldessarini et al., 2019; Bahji et al., 2021). Albeit widely used, these treatments are not efficient and adequate for all patients. Lithium has been used for decades as the preferred drug for the treatment and prevention of acute episodes, however, not all patients respond to lithium, and it also has a high number of side effects (Kim et al., 2017).

It is typical to resort to the administration of three or more drugs for BD treatment. This practice is called polypharmacy and is highly inadvisable. BD patients who resort to polypharmacy tend to adhere less to medication, which instigates several mood episodes (Fung et al, 2019). Moreover, polypharmacy reflects the absent communication between research and the clinical field.

Lamentably, the number of reliable biomarkers and the quality of the treatments are unsatisfactory. Combined with this disease complexity, the diagnosis BD is often delayed, leading to an absent or misadjusted medication (Bobo, 2017; Resende et al., 2020). This has a direct consequence on the treatment success since early phases of the disorder seem to be more responsive to treatments (Vieta et al., 2018). Precision and personalised medicine will minimise the risk of side effects, but also improve the effectiveness of treatments. For this to function, we need a broad method of diagnosis through biomarkers, genetic testing, and neuropsychological testing (Menke, 2018). It is imperative to reinforce research in this area thus diagnosis and treatment can be more effective.

1.2.3. BD pathophysiology

The brain anatomy of patients with BD has raised great interest in the scientific community, as it may give us indications about the affected neuronal circuits. BD has been associated with reductions in cortical thickness in the frontal, parietal, and temporal regions of the brain (Altamura et al., 2018; Hibar et al., 2018). Furthermore, volume reduction of the hippocampus, thalamus, and amygdala has also been reported (Hibar et al., 2016). Despite that direct associations should not be made between volume and neuronal circuit functionality, we know that these affected regions are involved in sensorimotor integration of the mirror neuron system, emotional processing, and executive behaviour (Hibar et al, 2016; Hibar et al., 2018). These alterations may be implicated in the perception of emotion in BD and may be one of the underlying roots of mood alterations.

A considerable number of studies propose deterioration in Ca^{2+} homeostasis in BD due to identified single nucleotide polymorphisms (SNP) in Ca^{2+} channels, constituting risk factors for the development of this disorder. The ANK3 gene encodes the ankyrin G protein, a scaffold protein essential for the formation and maintenance of the axon initial segment (AIS) and nodes of Ranvier. This protein is responsible for regulation of Ca^{2+} at AIS to maintain its function and structure. Variations in this gene may imply abnormalities in the propagation of synapses and incorrect circuits wiring (Leussis et al., 2012; Schulze et al., 2009). Genome-wide association studies have also revealed that the CACNA1C gene may be a risk factor for BD. This gene encodes the α-1C subunit of the L-type voltage-gated Ca^{2+} channel, which coordinates Ca^{2+} influx and conducts dendritic development, synaptic plasticity, memory, learning, and behaviour (Bhat et al., 2012; Jogia et al., 2011; Ferreira et al., 2008).

Numerous studies have revealed changes in neuroplasticity and cellular resilience in BD patients (Walker et al., 2014). Neuroplasticity is a broad term that comprises different cellular mechanisms that confer resilience, adaptation, and cognitive flexibility. Some of the most studied mechanisms are synaptic plasticity, neurogenesis, and modulation of growing neurons (Cheng and Mattson, 2010). Like in BD (Gandhi et al., 2020; Machado-Vieira et al., 2014), other mood disorders such as depression (Price and Duman, 2020), and schizophrenia (Voss et al., 2019) are associated with alterations in neuroplasticity. These mechanisms disruption may underlie the neuroprogressive nature of BD, which is manifested by shortening the intervals between each episode (Walker et al., 2014). Ample evidence implies compromised cellular resilience in BD. Additionally to brain volume decrease, there is also neuronal and glia cell atrophy, impaired

synaptogenesis, mitochondrial alterations, compromised endoplasmic reticulum (ER) stress response, changes in Ca^{2+} signaling, oxidative stress, and apoptosis (Machado-Vieira et al., 2014). These alterations are responsible for an increase in cell death, leading to abnormal brain development, shaping neuronal circuits, and undermining cellular resilience and neuroplasticity. It is urgent to conduct studies focusing on cellular mechanisms to understand the implications that these changes have on neuronal circuits in BD.

1.3. Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in BD

1.3.1 ER function overview

The ER is an exceptionally conserved organelle of eukaryotic cells (Westrate et al., 2015). It is recognised by its complex architecture maintained by integral membrane proteins and interactions with other organelles. The structure comprises a continuous membrane system composed of a network of sheets, dynamic tubules, and a nuclear envelope. The ER structure is dynamic and the adjustments it undergoes are due to changes in the cell. Indeed, the complex structure of the ER is fundamental to certain cell types, such as neuronal cells that depend on ER-associated functions (Westrate et al., 2015).

The preeminent function associated with the ER is protein synthesis, transport, and maturation (Schwarz and Blower, 2016). The canonical pathway that regulates protein synthesis involves the anchoring of the ribosome-mRNA complex to the ER membrane by the recognition of a signal recognition particle

(SRP) in the polypeptides (Schwarz and Blower, 2016). However, the complex structure also reflects the multiplicity functions of the ER as it is also responsible for lipid biogenesis (Jackson, 2019) and Ca^{2+} metabolism (Carreras-Sureda et al., 2018).

The ER is the organelle that stores the highest Ca^{2+} concentration within the cell and is responsible for regulating Ca^{2+} signaling, essential for several functions considering Ca^{2+} acts as a second messenger. It is involved in energy production, apoptosis, autophagy, and even neuronal processes such as neurotransmitter release (Schwarz and Blower, 2016). In the ER, there are a large variety of Ca^{2+} channels, such as the sarco-endoplasmic reticular Ca^{2+} ATPases (SERCAs) responsible for pumping Ca^{2+} from the cytosol into the ER, the ryanodine receptors (RyRs), which through Ca^{2+} binding induce Ca^{2+} release, and the inositol 1,4,5-trisphosphate (IP3) receptors (IP3R) that are fundamental for Ca^{2+} release from the ER (Schwarz and Blower, 2016).

1.3.2. ER stress response

ER quality control mechanisms assure protein synthesis, conformation, and maturation. Collectively, these mechanisms avoid the accumulation of misfolded proteins in the ER lumen, preventing ER stress (Walter and Ron, 2011). Ca^{2+} depletion of the ER lumen triggers ER stress, as this ion is critical for the function of ER chaperones such as calreticulin, glucose-regulated protein 94 (GRP94), glucose-regulated protein 78 (GRP78), and protein disulfide-isomerase (PDI) (Wang et al., 2012; Eletto et al., 2010; Pobre et al., 2019; Corbett et al., 1999). PDI is responsible for disulfide ligations formation, which is essential for the correct conformation of polypeptides (Weissmanand Kim, 1993). ER stress

activates signaling pathways aimed to restore cellular homeostasis that together are termed the unfolded protein response (UPR) (Walter and Ron, 2011).

The UPR has three main branches that induce distinct signaling cascades through three ER stress sensors: inositol-requiring protein 1α (IRE1α), activating transcription factor 6 (ATF6), and protein kinase R-like ER kinase (PERK) (Figure 1) (Walter and Ron, 2011). All these transmembrane transducers possess a domain in the ER lumen that interacts with GRP78. Upon ER stress, GRP78 dissociates from the stress sensors, activating them, and initiating the UPR (Bertolotti et al., 2000; Vitale et al., 2019).

IRE1α is the most conserved transducer (Mori, 2009). Under ER stress, IRE1α dimerizes and autophosphorylates, removing an intron from the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1). Active XBP1 exerts a prosurvival action by activating the expression of numerous genes responsible for the correct conformation of proteins, ensuring quality control processes. In addition, active XBP1 also leads to ER-associated degradation (ERAD), a pathway that signals proteins with incorrect conformation for ubiquitylation and consequent degradation in the proteosome. Regulated IRE1α-dependent decay (RIDD) signals the degradation of mRNAs and microRNAs. Cooperatively, IRE1α activation aims to decrease ER stress and recover cellular homeostasis (Figure 1) (Bashir et al., 2020).

ATF6 operates differently because this protein is translocated to the Golgi apparatus, which in turn induces translocation of the ATF6 fragment (ATF6f) to

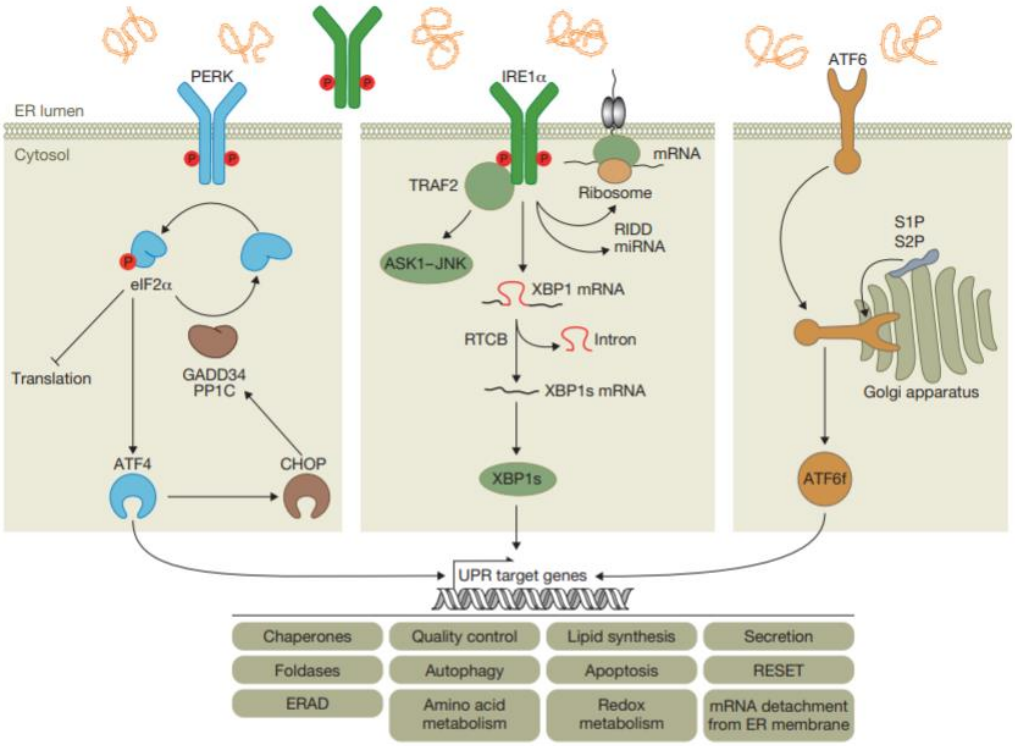

Figure 1. Unfolded protein response (UPR) pathways. The three main UPR pathways are activated by three UPR transducers when ER stress is present: PERK, IRE1α, ATF6. The PERK pathway triggers phosphorylation of eIF2α, inhibiting translation of most proteins. Even under p-eIF2α inhibition, ATF4 is translated triggering CHOP and other UPR target genes. CHOP signals the cell for ER stress-induced apoptosis and further activates GADD34 which dephosphorylates p-eIF2α, promoting cyclic availability of eIF2α to inhibit protein translation. IRE1α splices an intron of the mRNA encoding XBP1 through its RNase activity. RIDD is initiated by IRE1α activity in order to degrade mRNA and microRNA present in the cell, decreasing the ER burden. Under stress, ATF6 is translocated to the Golgi complex where the ATF6f fragment is released that induces the expression of UPR target genes in the nucleus. Together the UPR has been evolutionarily evolved for efficient combat of ER stress, which aims at restoring homeostasis or, if the insult is too severe or prolonged for the cell, induces cell death for the organism to get rid of cells that might be detrimental to its functioning. From: Hetz, Chevet, & Oakes, 2015.

the nucleus. ATF6f is involved in the expression of several genes related to ERAD, protein folding, and GRP78 (Figure 1) (Wang et al., 2000; Yang et al., 2020).

Lastly, PERK phosphorylates the eukaryotic initiation factor-2α (eIF2α), which is responsible for protein translation. The p-eIF2α inhibits protein synthesis, diminishing the number of proteins in the ER and mitigating ER stress. Nonetheless, p-eIF2α induces the synthesis of activating transcription factor 4

(ATF4), which in turn activates the C/EBP homologous protein (CHOP), known for inducing apoptosis, and the growth arrest and DNA damage-inducible protein 34 (GADD34) that dephosphorylates p-eIF2α. Therefore, GADD34 expression inhibits the action of PERK (Han et al., 2013). CHOP is also responsible for the activation of ER oxidase 1α (ERO1α), which mediates electron transfer from PDI to H_2O_2 after disulfide bond formation (Masui et al., 2011). This mechanism accompanies an increase in ROS and Ca^{2+} efflux from the ER by IP3Rs. The amount of Ca^{2+} uptaken by the mitochondria determines the activation of pro-survival or pro-apoptotic signaling pathways (Decuypere et al., 2011). If Ca^{2+} influx into the mitochondria is excessive, the mitochondrial permeability transition pore (mPTP) opens releasing cytochrome c, culminating in cell death (Figure 1) (D'Arcy, 2019).

The UPR decides the fate of the cell depending on the insult it is experiencing. If the ER stress is prolonged, there is apoptosis induction, on the contrary, if the stress is transient, the cell can restore homeostasis and cease the ER stress response. This mechanism ensures that cells that are too compromised are eliminated, not harming the organism (Hetz and Pope, 2018). Therefore, several diseases have already been associated with poor ER stress responses, such as some neurological diseases, diabetes, and cancer (Oakes and Papa, 2015; Wang and Kaufman, 2016).

1.3.3. Compromised ER stress response in BD

Both neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's, and psychiatric diseases, such as BD, schizophrenia, and major depressive disorder, have been associated with a dysfunction of the ER stress

response. The search for the underlying pathophysiology of BD has revealed a strong link between the ER stress response and the molecular dysfunctions observed in BD (Muneer, 2019). An insufficient ER stress response may determine the progressive nature of this disorder, as the frequency of acute mood episodes increases at later stages of the disorder (Fries et al., 2012).

Lithium is a mood stabiliser widely used to control mood episodes in BD patients. This compound can restore neurotransmission and brain structure, favouring neuroplasticity and euthymic periods. Some studies have revealed an association between lithium and the expression of UPR-related genes. Some of the UPR-related events that lithium regulates are ER stress, excitotoxicity, and autophagy (Puglisi-Allegra et al., 2021). As Marques et al. (2021) demonstrated, fibroblasts from early-stage BD patients appear to undergo an increase in mitophagy compared to controls, revealing some type of dysfunction in this mechanism. The activation of the UPR induces autophagy, favouring the degradation of protein aggregates and organelles (e.g., the ER and mitochondria). Lithium, by activating the UPR, promotes cellular homeostasis (Puglisi-Allegra et al., 2021; Machado-Vieira, 2018).

Moreover, the regulation of several UPR-associated genes and proteins is altered in BD. In lymphoblastoid cells and B lymphoblasts from BD patients, ER stress induced by thapsigargin and tunicamycin did not increase XBP1 and CHOP mRNA levels. It has been also suggested a decrease in GRP94 levels, an important protein for Ca^{2+} buffering (Hayashi et al., 2009; So et al., 2007). Furthermore, Kakiuchi et al. have associated BD with polymorphisms in XBP1 and GRP78 genes (Kakiuchi et al., 2003; Kakiuchi et al., 2005). Other study found

reduced levels of GRP78, p-eIF2α, and CHOP after ER stress induction in BD lymphocytes. Dysfunction of the ER stress response appears to worsen with disease progression, as in later stages the response is more impaired or even non-existent compared to early stages of the disease. Alterations in the PERK signaling cascade may be reflected in neuronal dysfunction, as the UPR is also involved in neuronal development and plasticity (Pfaffenseller et al., 2014).

1.4. Mitochondria dysfunction in BD

Mitochondria are complex organelles known as the powerhouse of the cell due to their influence on cellular bioenergetics. However, this organelle also regulates cellular adaptation in stress situations, such as DNA damage, oxidative stress, nutrient deficiency, and ER stress. Mitochondria have an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), essential for energy production in the form of ATP. Electrons from the tricarboxylic acid (TCA) cycle are in the NADH and FADH2 transporters and are subsequently transported to the electron transport chain (ETC) in the IMM, where they will be used to pump protons into the intermembrane space. ATP is only produced through ATP synthetase due to the electrochemical gradient created in this environment (Spinelli and Haigis, 2018).

In addition, mitochondria regulate apoptosis, as mitochondrial outer membrane permeabilisation (MOMP) is a crucial step in inducing cell death. Members of the B cell lymphoma 2 (BCL-2) protein family trigger MOMP which in turn leads to the release of cytochrome c and the initiation of the apoptosis signaling cascade (Bock and Tait, 2019). To regulate and induce apoptosis, Ca^{2+}

signaling is fundamental. Ca^{2+} overload in the mitochondrial matrix triggers cell death, and changes in Ca^{2+} uptake alter organelle morphology (Bock and Tait, 2019; Kowaltowski et al., 2019). Knowing that Ca^{2+} is required for the activity of several enzymes involved in ATP production, Ca^{2+} signaling also has a key role in bioenergetics (Paillusson et al., 2016).

Mitochondria are highly dynamic, regulating and adapting their morphology to environmental changes. They modulate the interaction between organelles and adjust their size through processes of fission and fusion. Fusion is an adaptive response to increased energy demands, and fission, although necessary during cell division, when excessive fragmentation of mitochondria occurs, cells tend to demonstrate increased oxidative stress, and impaired energy production (Westermann, 2012; Kowaltowski et al., 2019).

Several evidence highlight the importance of this organelle in neuronal activity. The position of mitochondria in astrocytes influences their survival and communication between neurons (de Oliveira et al., 2021). Also, in physiological conditions, mitochondrial function is crucial to maintain and improve neuroplasticity. Neuronal growth, axon growth or atrophy, and axon maintenance require mitochondria transport and/or immobilisation (Courchet et al., 2013; Rossi et al., 2019). Neuronal differentiation regulates neuroplasticity under certain circumstances and is associated with mitochondrial biogenesis. BDNF is a molecule widely involved in neuroplasticity mechanisms and also modulates mitochondria biogenesis (Cheng et al., 2010). Therefore, the balance between fusion and fission mechanisms and oxidative phosphorylation is necessary for neuron growth (Agostini et al., 2016). Synapse assembly demands high

quantities of ATP, being the synaptic vesicle cycle the most ATP-consuming process in the brain. The stabilisation and size of the synapse are dependent on the existence of resident mitochondria in the vicinity. It forms stronger synapses, relevant for learning and memory processes (Rossi et al., 2019).

At the beginning of the millennium, Kato and Kato (2000) created a hypothesis that has since gained robustness and prominence in the scientific community. They gathered several studies to propose mitochondrial dysfunction as the main altered event in BD. The high frequency of mitochondrial disorders comorbidities, the possibility of maternal heredity, the high number of mtDNA deletions, and the changes observed in brain energy were the great pillars for the development of this hypothesis.

The mtDNA is quite vulnerable to oxidative stress, leading to genome deletions. In the bipolar patients' brains, it was detected a significant increase in the 4977bp deletion of mtDNA (Kato et al., 1997). Mitochondria can increase the mtDNA copy numbers to offset defects or impairments. A study reported an increase in mtDNA copy number and mitochondrial biogenesis in BD, probably compensating for reduced mitochondrial activity (Fries et al., 2017). Another study reported that the onset of manic and depressive states results from a reduction in mtDNA copy numbers, whereas in euthymic patients there were no differences (Wang et al., 2018).

After two decades, many studies increasingly support the mitochondrial dysfunction hypothesis for BD. Different research groups revealed alterations in mitochondrial morphology in fibroblasts and post-mortem brain tissue from bipolar patients (Marques et al., 2021; Cataldo et al., 2010). Marques and

colleagues also reported an imbalance in mitochondria fission and fusion processes, an increase in mitochondrial biogenesis, an increase in mitophagy (to eliminate dysfunctional mitochondria), a decrease in mitochondrial membrane potential (MMP), and a reduced respiratory capacity in fibroblasts from early stages BD patients (Marques et al., 2021). These changes may compensate for the deficient ATP production, trying to maintain cellular homeostasis. These results are also in line with previous studies demonstrating an impaired energy production in BD (Cataldo et al., 2010).

Many studies tried to evaluate the energy deficit in bipolar patients' brains. Lactate levels are abnormally high in both brain and the cerebrospinal fluid (CSF) of patients (Regenold et al., 2009; Stephen et al., 2004). Indeed, patients in a depressive episode showed higher lactate levels which was reverted by lithium (Rooks and Garret, 2017). Under physiological conditions, lactate does not accumulate in the CSF. The increase in glycolysis at the expense of oxidative phosphorylation leads to less efficient energy production. The change to anaerobic metabolism may be the origin of the high lactate values which were associated with mood episodes (Regenold et al., 2009).

The TCA cycle is impaired on multiple levels in BD (Yoshimi et al., 2016). Alongside with reduced gene expression of mitochondrial ETC genes, it culminates in an ATP deficit (Sun et al., 2006). Taking these considerations, scientists developed a promising animal model for BD involving the administration of mitochondrial respiration inhibitors. The energy dysfunction led to behavioural changes associated with BD. Animals either spent more or less time in the open arms of the elevated maze; either increased or decreased their

mobility in the forced swim test; and consumed higher or lesser amounts of sweet solution, resembling the manic or depressive states in BD (Damri et al., 2021). These findings support the idea that there is abnormal brain energy metabolism in BD due to altered mitochondrial function.

1.5. Mitochondia-associated membranes (MAMs)

The ER and mitochondria have physical contact zones between them – the mitochondria-associated membranes (MAMs). In these platforms, the distance between the membranes of these organelles is 10 nm in the smooth ER, and 25 nm in the rough ER (Csordas et al., 2006). These lipid enriched zones have proteins responsible for the organelles to come closer and maintain contact physically and biochemically (Resende et al., 2020).

The contact zones between the ER and mitochondria are essential for maintaining the physiological conditions of the cell. MAMs are involved in signaling cascades responsible for Ca^{2+} signaling, fusion and fission processes, lipid transport, bioenergetics, and ER stress responses (Rowland and Voeltz, 2012; Vance, 2014; Herrera-cruz and Simmen, 2017).

ER-mitochondria contacts allow efficient Ca^{2+} uptake by mitochondria. In these plataforms, IP3R releases Ca^{2+} that enters mitochondria through voltage-dependent anion-selective channel protein 1 (VDAC1) (Paillsusson et al., 2016). Due to the importance of this ion in mitochondrial mechanisms, altered ER-mitochondria contacts numbers could translate into abnormalities in bioenergetics, mitochondrial dynamics, and cell survival. Upon ER stress, there

is an increase in the number of contacts to ensure the efficacy of Ca^{2+} uptake (Marchi et al., 2018). Mitochondrial fission depends on the spontaneous and repetitive constriction of the mitochondrial inner compartment (CoMIC). When the ER releases lower concentrations of Ca^{2+} into the mitochondria, CoMIC occurs culminating in mitochondrial division (Cho, 2017). These MAM-regulated events are altered in many diseases, such as diabetes, cancer, and neurodegenerative diseases (Perrone et al., 2020).

Since BD is associated with compromised mitochondrial function and impairment of ER stress response, which are events regulated at MAMs, Pereira et al. (2017) proposed the “MAM hypothesis for BD” which postulates that dysfunction of the signaling platforms MAMs can be implicated in the pathophysiology of mood disorders such as BD (Figure 2) (Pereira et al., 2017; Resende et al., 2020). Considering the relevance of MAMs for BD pathophysiology, their potential as therapeutic targets must be further explored.

1.6 Sigma-1 receptor (Sigma-1R)

The Sigma-1 receptor (Sigma-1R) is a MAM-resident chaperone that forms a complex with another chaperone, GRP78. When the complex Sigma-1R-GRP78 is assembled, Sigma-1R is in a dormant state because it does not express a chaperone activity (Hayashi and Su, 2007). This receptor regulates reactive oxygen species (ROS) production and increases BDNF levels (Hayashi, 2019).

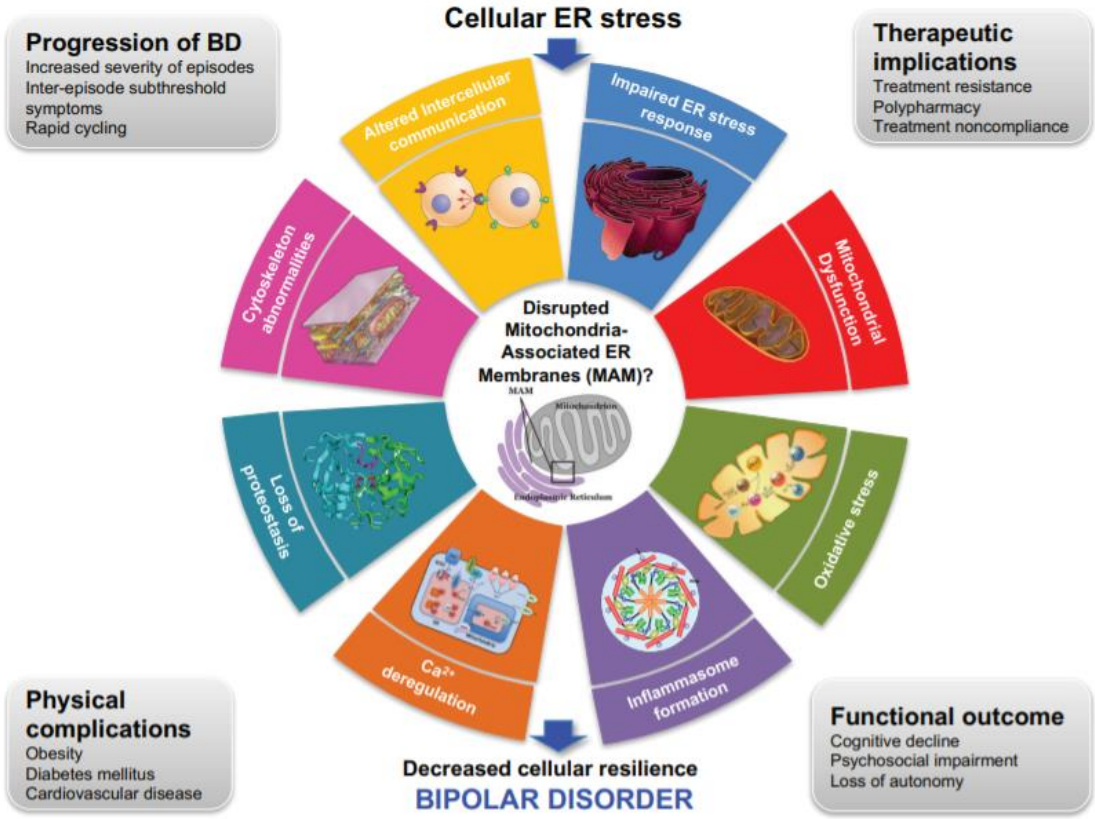

Figure 2. MAM hypothesis for the underlying BD pathophysiology. MAMs may be involved in the pathophysiology of BD, as they regulate many mechanisms that are altered in this disease. There are altered intracellular communication, impaired ER stress response leading to loss of proteostasis, multiple mitochondrial dysfunctions such as increased oxidative stress, Ca^{2+} dysregulation, inflammasome formation, and cytoskeleton abnormalities. Together, these changes may be implicated in the progression of BD, and the functional outcome of the disease. Moreover, this hypothesis indicates new therapeutic possibilities. From: Pereira et al., 2017

Several studies point to a pivotal function of Sigma-1R during ER stress. Ca^{2+} depletion led to the Sigma1R-GRP78 complex dissociation, triggering a Ca^{2+} signaling cascade between the ER and mitochondria via IP3R (Hayashi and Su, 2007). Sigma-1R is also responsible for stabilizing active IRE1, an UPR transducer, at MAMs (Mori et al., 2013). In addition, during the early stages of ER stress, Sigma-1R promotes mitochondrial bioenergetics (Koshenov et al., 2021).

A SNP of the *SIGMAR1* gene is associated as a risk factor for various psychiatric disorders such as depression, schizophrenia, and BD (Ohi et al., 2011; Mandelli et al., 2017). Many studies have demonstrated the therapeutic effect of Sigma-1R modulation due to the role it plays in the cell and the ER, particularly in diseases related to ER stress (Hashimoto, 2015).

In some brain illnesses, like stroke and ischemia or reperfusion, modulating Sigma-1R activity may decrease the affected brain areas through its ER stress-induced anti-apoptotic activity (Zhao et al., 2019; Omi et al., 2014; Ruscher et al., 2011). Polymorphism of *SIGMAR1* is associated with familial amyotrophic lateral sclerosis (ALS). Couly and colleagues (2019) generated a mutant *Drosophila* for Sigma-1R that displayed a phenotype resembling ALS. In addition to ATP deficient production and mitochondrial dysfunction, mutant *Drosophila* had motor alterations. Furthermore, they observed that activated Sigma-1R confers neuroprotection (Couly et al., 2019; Herrando-grabulosa and Navarro, 2020). Defects in Sigma-1R expression impair GABAergic neurotransmission leading to seizur. Sigma-1R activation may have a therapeutic effect by preventing seizures (Vavers et al., 2021).

Sigma-1R modulation may change the panorama of some psychiatric disorder therapies. Crouzier and colleagues (2020) recently demonstrated that Sigma-1R activation enhances neuroplasticity in mice. Moreover, its inactivation led to many impaired mechanisms involved in inflammatory responses, cell survival and ER-stress induced apoptosis. These mice presented loss of topographic memory, reduced neuronal resilience, and decreased neurogenesis (Crouzier et al., 2020).

As mentioned above in 1.2.3. subchapter, in BD there are many altered processes involving neuroplasticity. The pharmacological modulation of Sigma-1R may be another way of enhancing neuroplasticity in BD patients, resulting in BD symptoms relief (Gandhi et al., 2020). The question of whether Sigma-1R activation is a potential therapeutic strategy for BD arises.

Chapter II
Aims

The diagnosis of neuropsychiatric disorders has skyrocketed in recent decades and the trend is not stopping. These disorders put a great strain on society at an economic level, but also at the patient's psychological level. Bipolar disorder affects a large portion of the European population and is one of the disorders with the highest suicide rates. Although relatively common, the therapies developed so far have not proven effective for all patients. Therefore, understanding the underlying pathophysiological mechanisms of BD will help to identify potential therapeutic targets as well as to develop more accurate biomarkers. It will also promote earlier diagnosis which is crucial due to the neuroprogressive aspect of this disorder.

Mitochondria-Associated Membranes (MAMs) are signaling platforms that regulate a wide variety of cellular functions such as, ER stress response, ER-mitochondria Ca^{2+} transference, bioenergetics, autophagy, inflammation, and apoptosis (Resende et al., 2020; Wang et al., 2021; Yang et al., 2020; Luan et al., 2021). Thus, disrupted ER-mitochondria signaling has been implicated in numerous diseases including neurodegenerative disorders, cancer, and obesity (Eysert et al., 2020; Yu et al., 2021; Sasi et al., 2020).

Multiple pieces of evidence, including genetic and neuro-imaging studies, suggest that BD is associated with events regulated at MAMs namely, abnormalities in ER-related stress responses, mitochondrial function, and Ca^{2+} signaling.

The main goal of this work was to test the hypothesis that ER-mitochondria miscommunication at MAMs is an initial event leading to altered ER-related stress

response. We also wanted to evaluate if approaches targeting these structures can lead to new therapeutic strategies.

Specifically, we aimed to:

1) Explore ER stress responses in control- and BD patients-derived fibroblasts;

2) Analyse MAMs alterations under basal and ER stress conditions in early-stage BD patients and controls-derived fibroblasts;

3) Examine changes in MAM-modulated cellular events, namely: UPR, mitochondrial dysfunction and ER-mitochondria Ca^{2+} transfer;

4) Test the protective effect of pharmacological activation of MAM-resident chaperone Sigma-1R.

Chapter III
Methods and Materials

3.1. Cell culture

Dermal fibroblasts were obtained from three male patients in the early stages of bipolar disorder (stage 2) and three age- and gender-matched healthy controls. Both patients and controls were aged between 18 and 35 years old. The patient's diagnosis was assessed using the DSM-5 (American Psychiatric Association, 2013) and controls were selected among students and health professionals from University of Coimbra. Informed consent was provided to all patients and controls, and the study was approved by the Ethics Committee of the Centro Hospitalar e Universitário de Coimbra (150/CES, July 3rd).

Dermal fibroblasts were generated from skin biopsies (3 mm) performed in subjects who agreed to participate, having no contraindications for cutaneous biopsy (e.g., coagulation problems). After removing subcutaneous tissue and epidermis, dermal samples were cultured in 0.1 % gelatin-coated tissue culture dishes (Onofre, 2016). After 1-2 weeks, fibroblasts outgrowths reached the necessary confluence to be collected with 0.05 % trypsin and transferred to culture flasks. Characteristic spindle-shape morphology was confirmed by optical microscopy.

Fibroblasts were maintained in HAM's F10 (#31550-023 ThermoFisher Scientific, Waltham, MA, USA) medium supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS), 1 % (v/v) antibiotic solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin) and 1 % (v/v) L-Glutamine and AmnioMAX™-II Complete Medium (#11269-016, ThermoFisher Scientific) in a proportion of 1:5. Cells were cultured in 75 cm^2 flasks and maintained in a humidified 5 % CO2-95 % air atmosphere at 37 ºC. The medium was changed every 3-4 days. Cultures were passaged when cells reached 70–80 % confluence

using trypsin. Fibroblasts with less than 15 passages were used in the experiments.

3.2. Cellular treatments

3.2.1. Thapsigargin-induced ER stress

The lyophilised powder of the ER stress-inducer thapsigargin (T9033-5MG Sigma-Aldrich Chemical, St. Louis, MO, USA) was reconstituted in DMSO (DMSO-00A-2K5 Labkem, Vilassar de Dalt, Barcelona, Spain) to prepare the stock solution of 5 mM thapsigargin.

After 20 hours of plating the fibroblasts, cells were incubated with 5 µM thapsigargin for 3 or 24 hours in a humidified 5 % CO_2-95 % air atmosphere, as indicated in the experimental protocol. Cells treated with DMSO were used as control.

3.2.2. Pharmacological Sigma-1 Receptor activation

The pridopidine lyophilised powder (#HY-10684 MedChemExpress, Monmouth Junction, NJ, USA), the Sigma-1 receptor agonist, was reconstituted in DMSO to prepare a 5 mM stock solution.

Once plated for 20 hours, cells were incubated with 5 µM pridopidine for 24 hours in a humidified 5 % CO_2-95 % air atmosphere at 37 ºC. Cells incubated with DMSO were used as controls.

3.3. MTT Reduction assay

To evaluate cell viability, fibroblasts were plated on 96-well plates at a density of $0.3x10^5$ cells/cm^2.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (#M2128, Sigma-Aldrich Chemical) solution was prepared in Krebs solution (132 mM NaCl, 4 mM KCl, 1.2 mM Na_2HPO_4, 1.4 mM $MgCl_2$, 6 mM glucose, 10 mM Hepes, 1 mM $CaCl_2$, pH 7.4). After treatments, the cell culture medium was replaced by 0.5 mg/mL MTT solution. The plate was properly protected from light and incubated for 4 hours at 37 °C. Finally, the MTT solution was removed and replaced by 100 µL DMSO to dissolve the formazan crystals. After 15 minutes of stirring, the absorbance was read at 570 nm in spectrophotometer (Spectrophotometer Spectramax plus 384, ThermoFisher Scientific). The reduction of MTT was expressed as percentage of the absorbance value obtained in control cells (untreated cells), which was considered to be 100%.

3.4. Protein extraction and quantification

Cells were plated on a 6-well plate at a density of $0.3x10^5$ cells/cm^2. Depending on the experimental protocol, they were incubated with 5 µM thapsigargin for 3 or 24 hours. At the end of the incubation time, fibroblasts were lysed in radioimmunoprecipitation assay (RIPA) buffer [250 mM NaCl, 50 mM Tris base, 1 % (v/v) Nonidet P-40, 0.5 % (v/v) sodium deoxycholate (DOC), 0.1 % (v/v) Sodium Dodecyl Sulfate (SDS), pH 8.0], supplemented with 2 mM DTT, 1 % (v/v) of the protease inhibitor cocktail (#P2714 Sigma-Aldrich), 100 µM PMSF, 2 mM sodium orthovanadate and 50 mM sodium fluoride.

Cells were scraped using plastic scrapers and collected into 1.5 mL tubes. After 20 minutes on ice, cell lysates were centrifuged at 17,968 g (Sigma 2-16PK, Sigma Laboratory centrifuges, Osterode am Harz, Germany) for 10 minutes at 4 ºC. The pellet was discarded, and the supernatant collected and stored at -20 ºC. Total protein concentration was determined using Pierce-BCA Protein Assay Kit (#23225 ThermoFisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Cells were incubated at 37 ºC, protected from light, for 30 minutes. Then, absorbance values were read at 562 nm in a spectrophotometer (Spectrophotometer Spectramax plus 384, ThermoFisher Scientific)

3.5. Western-blotting

After protein quantification, each sample was prepared to contain approximately 30 µg of protein to ensure an equal amount of protein loading. Samples were denatured with 6x SDS sample buffer [0.5 M Tris, 30 % (v/v) glycerol, 10% (w/v) SDS, 0.6 M dithiothreitol, 0.012 % bromophenol blue] at 95 ºC for 5 minutes. Proteins were separated by electrophoresis in 12 % (v/v) SDS-polyacrylamide gels (SDS/PAGE) at 100 V for the first 20 minutes, and 125-130 V for 100 minutes for proper bands. Proteins were electrotransferred to PVDF membranes (#ipvh00010 Merck Millipore, Burlington, MA, USA) previously activated with methanol for 20 seconds. Transference took 2 hours at 4 ºC, with stirring at constant amperage of 750 mA. Afterwards, membranes were blocked with 5 % (w/v) bovine serum albumin (BSA) in TBS-T (25 mM Tris-HCl, pH 7,6, 150 mM NaCl, 0,05 % Tween-20) for 1 hour at room temperature, followed by overnight incubation at 4 ºC with primary antibodies (Table 1) prepared in 5 %

(w/v) BSA in TBS-T solution. After six washes with TBS-T, membranes were incubated for 2 hours at room temperature with the corresponding secondary antibodies prepared in TBS-T (Table 2). The protein immunoreactive bands were visualized by chemiluminescence with the Pierce ECL Western-blotting Substrate (#32106 ThermoFisher Scientific) in a Chemidoc Imaging System (Bio-Rad Laboratories, Lda, Algés, Portugal). The optical density of the bands was quantified with the Image Lab Software (Bio-Rad). The results obtained were normalized to β-actin or eIF2α.

Table 1. List of primary antibodies used for Western-blotting.

Antibodies	Source	Dilution	Reference
Anti-GRP78	Mouse monoclonal	1:2000	#610978 BD Biosciences
Anti-Sigma-1R	Goat polyclonal	1:1000	#22948 Santa Cruz Biotechnology
Anti-PDI	Rabbit polyclonal	1:1000	#2446S Cell Signaling Technology
Anti-MCU (D2Z3B)	Rabbit monoclonal	1:1000	#14997 Cell Signaling Technology
Anti-VDAC1	Mouse monoclonal	1:1000	#390996 Santa Cruz Biotechnology
Anti-Ero1-Lα	Mouse monoclonal	1:1000	#100805 Santa Cruz Biotechnology
Anti-p-eIF2α (Ser51) (D9G8) XP®	Rabbit monoclonal	1:1000	#3398S Cell Signaling Technology
Anti-eIF2α (D7D3) XP®	Rabbit monoclonal	1:1000	#5324S Cell Signaling Technology
Anti-β-actin	Mouse monoclonal	1:10000	# A5316-2ML Sigma-Aldrich

Table 2. List of secondary antibodies used for Western-blotting.

Antibodies	Source	Dilution	Reference
Anti-goat	Rabbit	1:20000	#31402 Invitrogen
Anti-mouse	Goat	1:20000	#31432 Invitrogen
Anti-rabbit	Goat	1:20000	#31462 Invitrogen

3.6. Fluorimetric analysis of cytosolic and mitochondrial Ca^{2+} levels

To evaluate mitochondrial and cytosolic Ca^{2+} levels we used the fluorescent Ca^{2+} indicators Rhod-2, AM (R1245MP, Invitrogen, ThermoFisher Scientific) and Fura-2, AM (F1225, Invitrogen, ThermoFisher Scientific), respectively. These probes exhibit an increase in fluorescence when they bind to Ca^{2+}. For Rhod-2, AM, fluorescence was read at 552 nm (excitation) and 581 nm (emission) wavelengths, and it accumulates selectively in mitochondria, allowing the analysis of the variation of Ca^{2+} levels in mitochondria. Fura-2, AM is an intracellular Ca^{2+} ratiometric indicator (349/380 nm) and is excitable under UV light. The fluorescence intensity of excitation at 340 nm is directly proportional to cytosolic Ca^{2+}, while excitation at 380 nm is inversely proportional.

Both probes were reconstituted in DMSO, and the fluorescent probes solutions were prepared in Buffer A (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 5.5 mM glucose, and 1 mM $CaCl_2$). Fura-2, AM solution was supplemented with 0.1 % (w/v) BSA.

Fibroblasts were previously plated on 96-well clear bottom black polystyrene plates at a density of $0.3x10^5$ cells/cm^2 and incubated with the corresponding protocol treatments. After incubation, cells were washed twice with buffer A and the medium was replaced by 10 µM Rhod-2, AM or 2 µM Fura-2, AM. Cells were protected from light and incubated for 30 minutes (Fura-2, AM) or 45 minutes (Rhod-2, AM) in a humidified 5 % CO_2-95 % air atmosphere at 37 ºC. Then, cells were washed twice with Buffer B (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.5 mM EGTA, 5.5 mM

glucose). Basal Fura-2, AM and Rhod-2, AM fluorescence was recorded for 90 seconds in buffer B. ER Ca^{2+} release was induced upon stimulation with 100 µM histamine (#H7125, Sigma-Aldrich) and fluorescence was measured for 600 seconds (Figure 1) in a microplate reader (SpectraMax iD3, Molecular Devices, CA, USA).

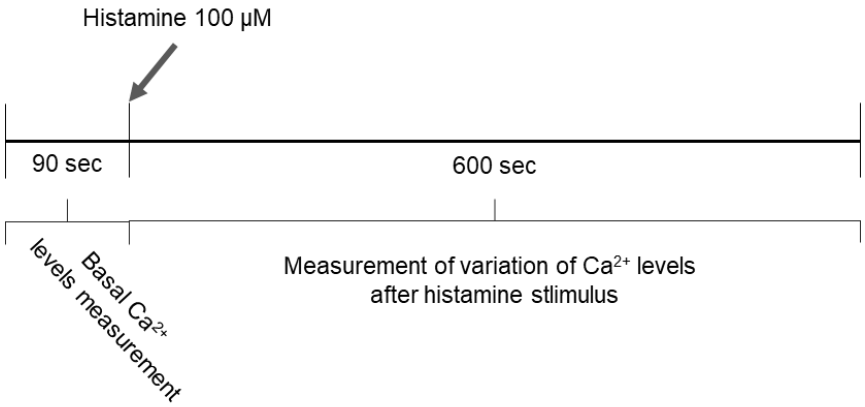

Figure 3. Experimental design for Ca^{2+} measurements

3.7. Fluorimetric analysis of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was assessed using the tetramethylrhodamine ethyl ester perchlorate (TMRE) probe (#87917 Sigma-Aldrich). Human fibroblasts were plated in 96-well clear bottom black polystyrene at 37 ºC and 5 % CO_2-95 % air, at a density of $0.3x10^5$ cells/cm^2. After treatments, the cell culture medium was replaced by 1 µM TMRE prepared in Krebs solution. Cells were incubated for 30 minutes at 37 ºC and 5 % CO_2-95 % air. The fluorescence was read at 540 nm (excitation) and 595 nm (emission) wavelengths in a microplate reader (SpectraMax Gemini EM fluorocytometer, Molecular Devices, CA, USA).

3.8. Proximity ligation assay

The proximity ligation experiment (PLA) is based on the specificity of secondary antibodies and the sensitivity afforded by rolling circle amplification. Thus, PLA probes (Table 3) only generate a signal when the primary antibodies are in proximity (<40 nm). Thus, allowing the analysis of colocalization of two proteins.

Fibroblasts were plated on nitric acid-treated coverslips at a density of $5.0x10^4$ cells/cm^2 and incubated with the respective treatments for a total of 48 hours at 37 ºC and 5 % CO_2-95% air. After thapsigargin and pridopidine treatments, cells were fixed with 4 % (w/v) paraformaldehyde for 15 minutes, followed by permeabilization with 1 % (v/v) TritonX-100 in PBS (140 mM NaCl, 1.5 mM KH2HPO4, 8.1 mM Na2HPO4.7H2O, and 2.7 mM KCl) for 5 minutes at room temperature. Next, cells were blocked with 3 % (w/v) BSA prepared in 0.2 % (v/v) Tween 20, for 1 hour at room temperature. Between each step two washes of 5 minutes each were performed with buffer A (0.01 M Tris base, 0.15 M NaCl, 0.05 % Tween 20). VDAC1 and IP3R primary antibodies (Table 3) were prepared in blocking solution and the cells were incubated overnight at 4 °C in a humidity chamber. After diluting 1:5 plus and minus probes in PLA kit diluent (Table 3), cells were incubated in a humidity chamber for 1 hour at 37 °C. For ligation, the coverslips were incubated with ligase (PLA kit) in the humidity chamber for 30 min at 37ºC. For amplification, cells were incubated with polymerase (PLA kit) in the humidity chamber for 100 minutes at 37 °C. At the end, coverslips were washed twice for 5 minutes in buffer B (0.2 M Tris base, 0.1 M NaCl, pH 7.5) and once for 1 minute in 0.01 % (v/v) buffer B. For nuclei staining, fibroblasts were incubated with 15 µg/mL Hoechst 33342 (H21492, Molecular

probes, OR, USA) prepared in PBS for 10 minutes at room temperature in a concentration of 15 µg/mL for 10 minutes at room temperature. The coverslips were mounted using Aqua-Poly/Mount mounting medium (#18606 Polysciences Inc., Warrington, PA, USA).

Images of fibroblasts were obtained using a Zeiss LSM 710 confocal microscope (Zeiss Microscopy, Germany) with a 40x oil objective and analysed by Fiji software (ImageJ, National Institute of Health, Bethesda, MA, USA). For each experimental condition at least ten images were analysed for the colocalization of VDAC1 and IP3R.

Table 3. List of antibodies used in proximity ligation assay.

Antibodies	Source	Dilution	Reference
Anti-VDAC1	Mouse monoclonal	1:250	#390996 Santa Cruz Biotechnology
Anti-IP3R	Rabit polyclonal	1:1000	#ab5804, Abcam, Cambridge, UK
Anti-mouse MINUS	Donkey	1:5 (in diluent)	DUOS2004-100RXN, Sigma-Aldrich
Anti-rabbit PLUS	Donkey	1:5 (in diluent)	DUOS2002-100RXN, Sigma-Aldrich

3.9. Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Data were tested for Gaussian distribution (D'Agostino & Pearson test, Shapiro-Wilk test, Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilie for P-value). One-way ANOVA with Dunn's multiple comparisons test was used for multiple comparisons. Two-way ANOVA with Tukey's multiple comparisons test was used

for multiple comparisons with more than one variable. In all cases, $p<0.05$ was considered significant and the analysis was performed with GraphPad Prism Software (Version 8.0).

Chapter IV
Results

4.1. Thapsigargin-induced ER stress in control and BD fibroblasts

Thapsigargin is a classical inducer of ER stress by inhibiting SERCA (Zhao and Lytton, 1996). Disruption of Ca^{2+} homeostasis triggers the UPR that aims to restore the defects caused by ER stress. However, if UPR activation is prolonged, apoptosis is induced to eliminate overly compromised cells (Krebs et al., 2015; Walter and Ron, 2011).

The MTT reduction assay was performed to determine cell viability upon thapsigargin exposure in fibroblasts obtained from healthy controls (Figure 4A) and BD patients (Figure 4B). A dose-response experiment was performed with increasing concentrations (0.1, 0.25, 0.5, 1, 2.5, and 5 µM) of thapsigargin at different exposure times to the drug (0.5, 3, 6, and 24 hours). In both controls and BD patients-derived fibroblasts, only the highest tested concentrations (2.5 µM, and 5 µM) of thapsigargin significantly decreased cell viability at all exposure times. The reduction in cell viability indicates that cells are under stress. The thapsigargin concentration of 5 µM was chosen to induce ER stress in the subsequent experiments.

The UPR activates three main stress sensors, ATF6, PERK, and IRE1, and their main function is to restore cellular homeostasis (Walter and Ron, 2011). These proteins possess a luminal domain capable of interacting with the chaperone GRP78 (Bertolotti et al., 2000). The activation of the stress sensors, and consequently of the UPR, depends on the dissociation of GRP78 of their luminal domain (Kopp et al., 2019). Also, it is noteworthy that the activation of PERK leads to phosphorylation of eIF2α at residue 51. This protein inhibits the

translation of mRNAs to proteins, attenuating the accumulation of proteins, and ameliorating ER stress (Harding et al., 1999).

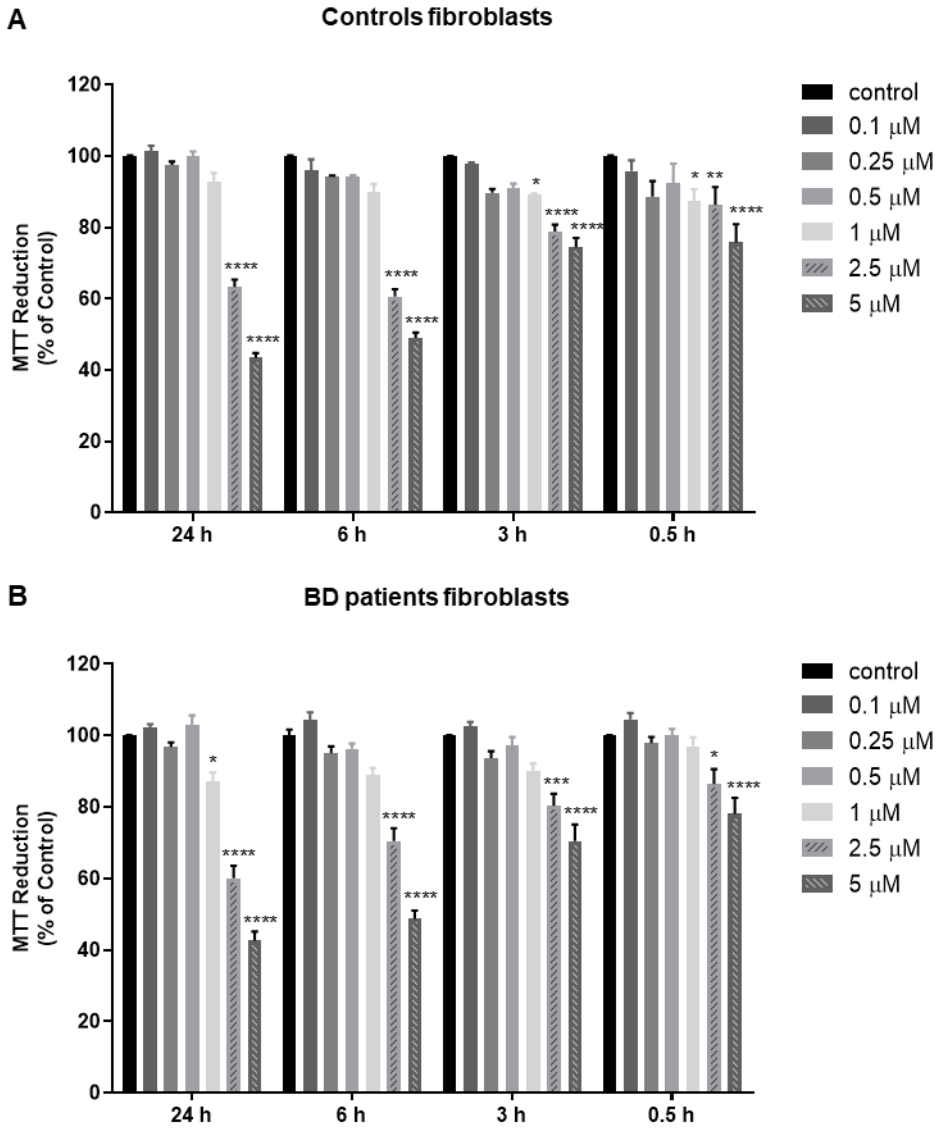

Figure 4. Cellular viability of fibroblasts upon thapsigargin exposure. Control and BD patients' fibroblasts were incubated with thapsigargin (0.1, 0.25, 0.5, 1, 2.5, and 5 μM) for 0.5, 3, 6, or 24 hours. The fibroblasts vulnerability was tested with the MTT reduction assay, as described in the materials and methods chapter. **(A)** MTT reduction in controls fibroblasts and **(B)** in BD patients' fibroblasts. Data are presented as mean ± SEM of 3 independent experiments for n=3 BD patients and n=2 controls performed in triplicate. Statistical significance was determined using the two-way ANOVA test, followed by Dunnet's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, significantly different from control.

Pfaffenseller et al. (2014) demonstrated a downregulation of GRP78 and p-eIF2α under ER stress in advanced-stage BD patients' peripheral blood mononuclear cells (PBMC). To explore ER response to stress in early-stage BD patient-derived and controls- fibroblasts, protein levels of ER stress-induced UPR markers, such as the chaperone GRP78 and eIF2α, a downstream signaling mediator of PERK activation, were measured by WB under basal and thapsigargin exposure conditions (Figure 5A-D).

Fibroblasts were incubated with 5 µM thapsigargin for 3 or 24 hours to evaluate the effects of mild and prolonged ER stress. GRP78 protein levels were significantly increased when cells were incubated with 5 µM thapsigargin for 24 hours, but not for 3 hours (Figure 5C). This response was similar in both controls and BD patients. Although the results were not statistically significant for p-eIF2α, a slight increase in its protein levels was observed upon 5 µM thapsigargin exposure (Figure 5D). It is worth noting that these results were generated from a low number of samples, and with a small number of replicates. This leads to higher SEMs and lower statistical power.

Protein disulfide isomerase (PDI) is responsible for disulfide bonds formation between polypeptides, which is essential for the correct conformation of ER proteins (Weissman and Kimt, 1993). There is a reduction of PDI state when it catalyses this reaction, and ERO1 is responsible for reoxidising it (Frand and Kaiser, 1998). Although these reactions generate an increase in ROS, the ER has an oxidative environment that promotes oxidative protein folding at physiological levels (van der Vlies et al., 2003). Together, these proteins prevent the accumulation of misfolded proteins, attenuating ER stress. To investigate

whether cells from BD patients exhibit an effective response to ER stress, PDI and ERO1-α protein levels were assessed by WB (Figure 5E-H).

No significant differences in PDI levels were detected under ER stress (Figure 5G). However, PDI activity increases in the presence of high concentrations of Ca^{2+}, and the ER is depleted of Ca^{2+} due to thapsigargin exposure. Perchance, this leads to stable protein levels, even upon ER stress. Regarding ERO1-α, although Tukey's multiple comparisons test showed no significant differences between conditions, the disease factor of the two-way ANOVA test has a p value=0.047. This means that the mean ERO1-α levels of all treatments are different between controls and BD patients. The graph suggests that there is an increase in ERO1-α protein levels in controls incubated with 5 µM thapsigargin for 24 hours, but this is not the case in BD patients-derived fibroblasts.

In conclusion, thapsigargin induces ER-stress in dermal fibroblasts from controls and BD patients. Furthermore, it appears that ERO1-α protein levels are altered in the early stages of BD in comparison with controls, in contrast to GRP78, p-eIF2α, and PDI.

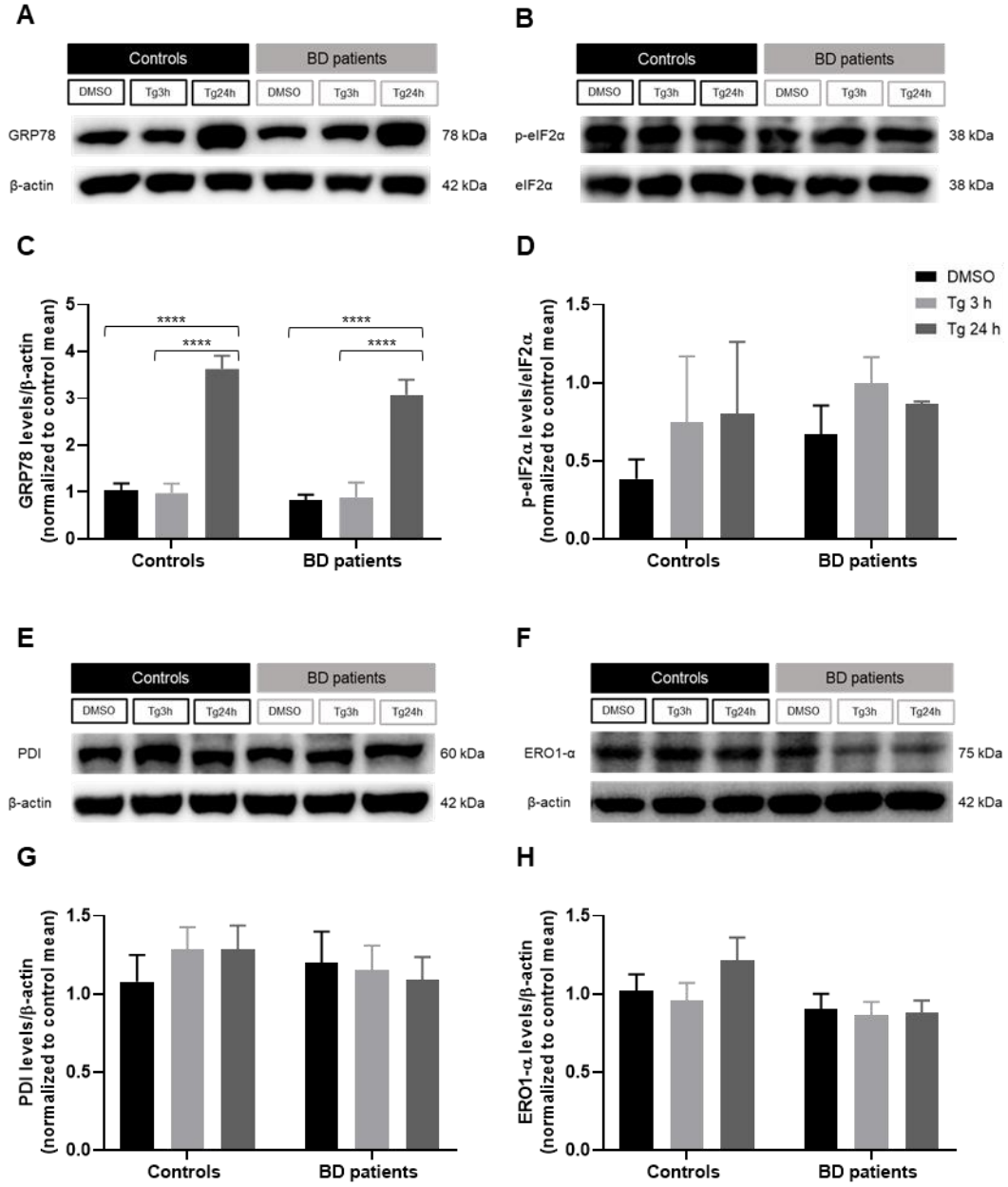

Figure 5. Levels of ER stress response markers upon thapsigargin exposure. Control and BD patients' fibroblasts were incubated with 5 μM thapsigargin for 3 or 24 hours. Protein levels were assessed by WB. **(A)** Representative immunoblot of GRP78 and β-actin protein levels at different thapsigargin-exposure time points. **(B)** Representative immunoblot of p-eIF2α and eIF2α protein levels at different thapsigargin-exposure time points. **(C)** Protein levels of GRP78 upon thapsigargin-induced ER stress. GRP78 levels were normalized with β-actin levels. **(D)** Protein levels of p-eIF2α upon thapsigargin-induced ER stress. p-eIF2α levels were normalized with eIF2α levels **(E)** Representative immunoblot of PDI and β-actin protein levels at different thapsigargin-exposure time points. **(F)** Representative immunoblot of ERO1-α and β-actin protein levels at different thapsigargin-exposure time points. **(G)** Protein levels of PDI upon thapsigargin-induced ER stress. PDI levels were normalized with β-actin levels **(H)** Protein levels of ERO1-α upon thapsigargin-induced ER stress. ERO1-α levels were normalized with β-actin levels. For GRP78, PDI, and ERO1- α three samples obtained from controls and BD patients' fibroblasts were used (n=3) in two independent experiments. For p-eIF2α fibroblasts from 2 controls and 2 BD patients were used in two independent

experiments. Statistical significance was determined using the two-way ANOVA test, followed by Tukey's multiple comparisons test. ****p<0.0001.

4.2. Effect of ER stress on mitochondrial membrane potential in control- and BD patient-derived fibroblasts

Mitochondria produce large amounts of ATP through oxidative phosphorylation. During this process, there is proton pumping into the intermembrane space. The uneven distribution of protons gives rise to the MMP (Cowan et al., 2019). Ca^{2+} accumulates in the mitochondria due to ER stress signaling responses. If mitochondrial Ca^{2+} levels exceed a critical concentration, it triggers the mitochondrial membrane permeabilisation culminating in cell death by apoptosis (Rizzuto and Pozzan, 2006).

Under basal conditions, Marques et al. (2021) demonstrated a decrease in MMP, an inefficient ATP production, and an increase in glycolytic capacity of BD patients-derived fibroblasts when compared to controls. These results indicate impaired oxidative phosphorylation. Therefore, it was evaluated the MMP in control- and BD patients-derived fibroblasts under ER stress conditions by stimulating these cells with 5 µM thapsigargin for 3 or 24 hours (Figure 6).

The results concurred with the previous study, as DMSO-treated BD patients-derived fibroblasts showed a reduction in the MMP when compared with control-derived fibroblasts. As expected, controls treated with 5 µM thapsigargin have a significant reduction in MMP. Although there is a greater reduction when incubated for 3 hours, there are no statistically significant differences between controls incubated with 3 hours and controls incubated with 24 hours (Figure 6). However, the response of BD patients is quite different. There are only significant

differences between fibroblasts incubated with DMSO and 5 µM thapsigargin for 3 hours, and this reduction is less abrupt than the observed in controls. This suggests that thapsigargin does not have such a prominent effect on the reduction of MMP in BD patient-derived fibroblasts. This effect may be linked to the depolarization of mitochondria observed under basal conditions (Figure 6; Marques et al., 2021).

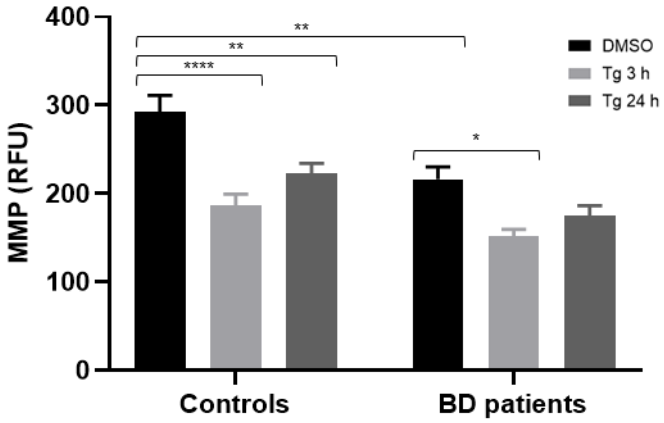

Figure 6. Mitochondrial membrane potential upon thapsigargin-induced ER stress in fibroblasts. Control- and BD patients-derived fibroblasts were incubated with 5 µM thapsigargin for 3 or 24 hours and the mitochondrial membrane potential was assessed fluorimetrically using the TMRE probe. Data are presented as mean ± SEM. Three independent experiments were performed in triplicate for each sample (n=3 controls and n=3 BD patients). Statistical significance was determined using a two-way ANOVA test, followed by Tukey's multiple comparisons test. *p<0.05, **p<0.01, ****p<0.0001.

4.3. ER-mitochondria tethering in controls and BD fibroblasts during ER stress

Depolarisation of the mitochondrial membrane hinders Ca^{2+} influx to mitochondria (Gunter and Pfeiffer, 1990). Additionally, Ca^{2+} is crucial for the correct functioning of mitochondrial metabolism and energy production (Rizzuto et al., 2012), as discussed above in chapter I. The ER is a Ca^{2+} buffer, being a key supplier to other organelles, like mitochondria. Efficient transference is conducted by biochemical and physical contacts between the ER and the mitochondria which are called the MAMs. At MAMs, the Ca^{2+} transport is ensured by IP3R in the ER, and by VDAC in the OMM (Lock and Parker, 2020; Rosencrans et al., 2021).

By assessing the colocalization of IP3R with VDAC, it is possible to understand whether ER stress induction by thapsigargin leads to changes in Ca^{2+} transport platforms, namely MAMs. To assess whether ER stress led to changes in the number of contacts between ER and mitochondria, fibroblasts were incubated with 5 µM thapsigargin for 3 hours (Figure 7) and the IP3R/VDAC1 colocalization was analysed by PLA. Under these conditions it was observed a significant increase in the number of ER-mitochondria contacts in controls-derived fibroblasts whereas BD patients-derived fibroblasts showed a slight increase, but not statistically significant, in the ER-mitochondria contacts upon thapsigargin exposure (Figure 7B). Under basal conditions, it was observed a significant increase in the number of ER-mitochondria contacts in BD patients' fibroblasts when compared to fibroblasts derived from control individuals (Figure 7B).

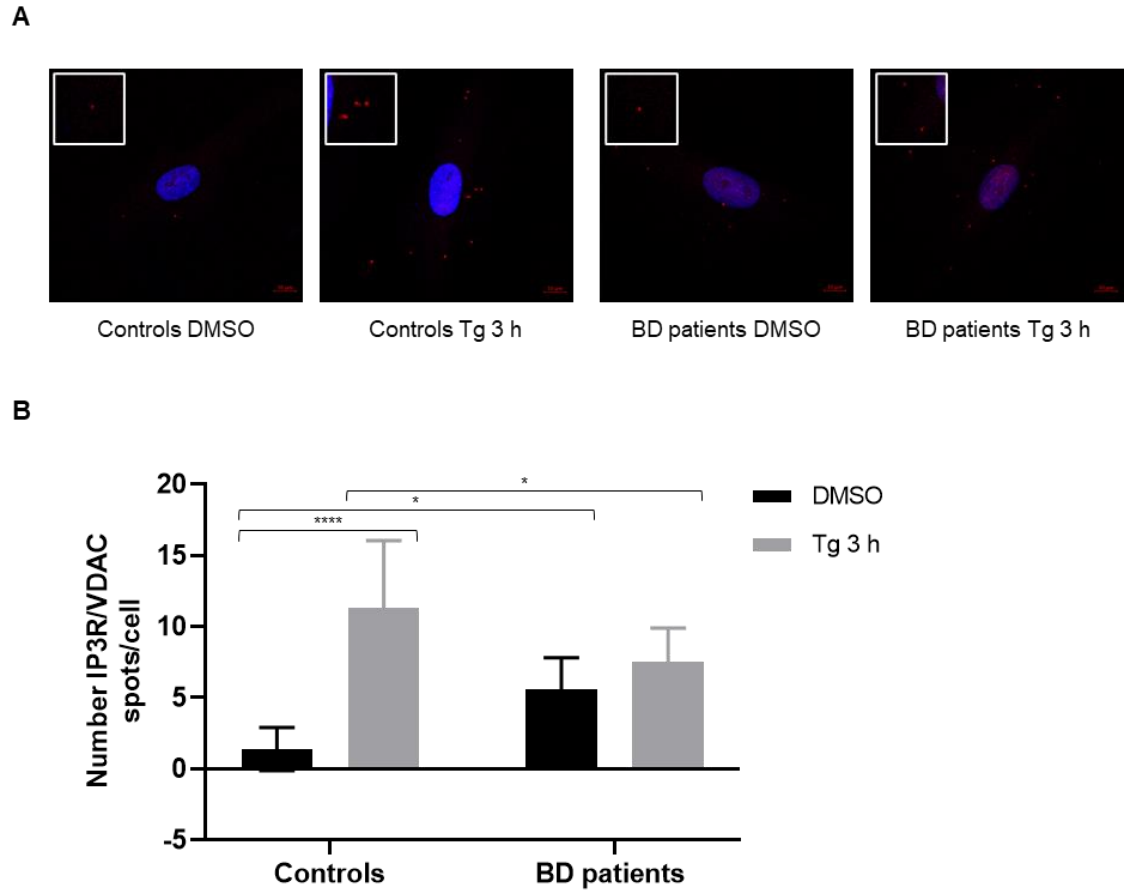

Figure 7. Effect of thapsigargin-induced ER stress in IP3R/VDAC1 colocalization. Fibroblasts from controls and BD patients were incubated with 5 µM thapsigargin for 3 hours. The IP3R/VDAC1 colocalization was analysed by the proximity ligation assay (PLA). These results inform about the tethering between the ER and the mitochondria upon ER stress. **(A)** Representative images of stained fibroblasts with the PLA probes. Each red dot represents the colocalization of IP3R and VDAC1. Scales represent 10 µm. **(B)** Plot of the analysis of the contacts number between ER and mitochondria under ER stress conditions. Data are presented as mean ± SEM of 10 cells per condition. Statistical significance was determined using the two-way ANOVA test, followed by Tukey's multiple comparisons test. *p<0.05, ****p<0.0001

4.4. ER stress-induced alterations in Ca^{2+} signaling to mitochondria

In addition to the structural alterations, in ER-mitochondria contacts, it was investigated if these translated into functional changes in Ca^{2+} transport between both organelles. To explore Ca^{2+} transfer under ER stress conditions, fibroblasts

were incubated with 5 µM thapsigargin for 3 or 24 hours. Next, cells were incubated with the fluorescent probes Fura-2, AM, and Rhod-2, AM, which bind to cytosolic and mitochondrial Ca^{2+}, respectively (Figure 8). Firstly, fluorescence values were read under basal conditions (Figure 8A, B). Then, 100 µM histamine was added to promote IP3R activation and induce Ca^{2+} release from the ER via the IP3R activation (Figure 8C, D) (Tilly et al., 1990). The difference between the maximum fluorescence peak and the mean of the basal values was calculated and demonstrated in Figures 8C and D. Figures 8E and F represent the fluorescence values over the total time of the experiment.

Thapsigargin usually induces the rise of cytosolic Ca^{2+} concentration due to ER Ca^{2+} depletion. Cell death associated with 5 µM thapsigargin for 24 hours might be the reason behind the observed reduction (Figure 4, Figure 8A). The analysis of time-course of Fura-2 fluorescence indicates that only the DMSO conditions reached a cytosolic fluorescence peak after stimulation with 100 µM histamine (Figure 8E). This is reflected in the difference between the maximum peak and the mean of the basal values that are plotted in the Figure 8C. Nevertheless, this difference is more statistically significant in controls-derived fibroblasts when compared to BD patients-derived cells (Figure 8C).

Thapsigargin inhibits Ca^{2+} entry into the ER, contributing to a decrease in ER Ca^{2+} accumulation. Consequently, Ca^{2+} influx into mitochondria declines. When exposed to thapsigargin, fibroblasts from BD patients showed a more significant reduction in basal mitochondrial Ca^{2+} concentration (Figure 8B). This suggests that under ER stress, the uptake of Ca^{2+} into the mitochondria is compromised in BD cells. Once again, only the DMSO conditions reached a Rhod-2 fluorescence peak after stimulation with 100 µM histamine (Figure 8F).

There are no statistically significant differences Rhod-2 fluorescence in histamine-induced ER Ca^{2+} release between controls- and BD patients-derived fibroblasts (Figure 8D).

4.5. VDAC and MCU levels upon ER stress in controls- and BD patients-derived fibroblasts

Ca^{2+} influx into mitochondria is due to the orchestration of the activity and expression of different Ca^{2+} channels and transporters, namely VDAC and MCU (Drago et al., 2011). Therefore, the protein levels of VDAC and MCU under ER stress were assessed by WB (Figure 9). It was observed an increase in VDAC protein levels in fibroblasts from BD patients when compared to controls, both in basal and in thapsigargin-induced ER stress conditions (Figure 9B). As shown in Figure 9C and D, there were no significant differences in the levels of MCU between controls- and BD patients-derived fibroblasts in any condition (Figure 9D).

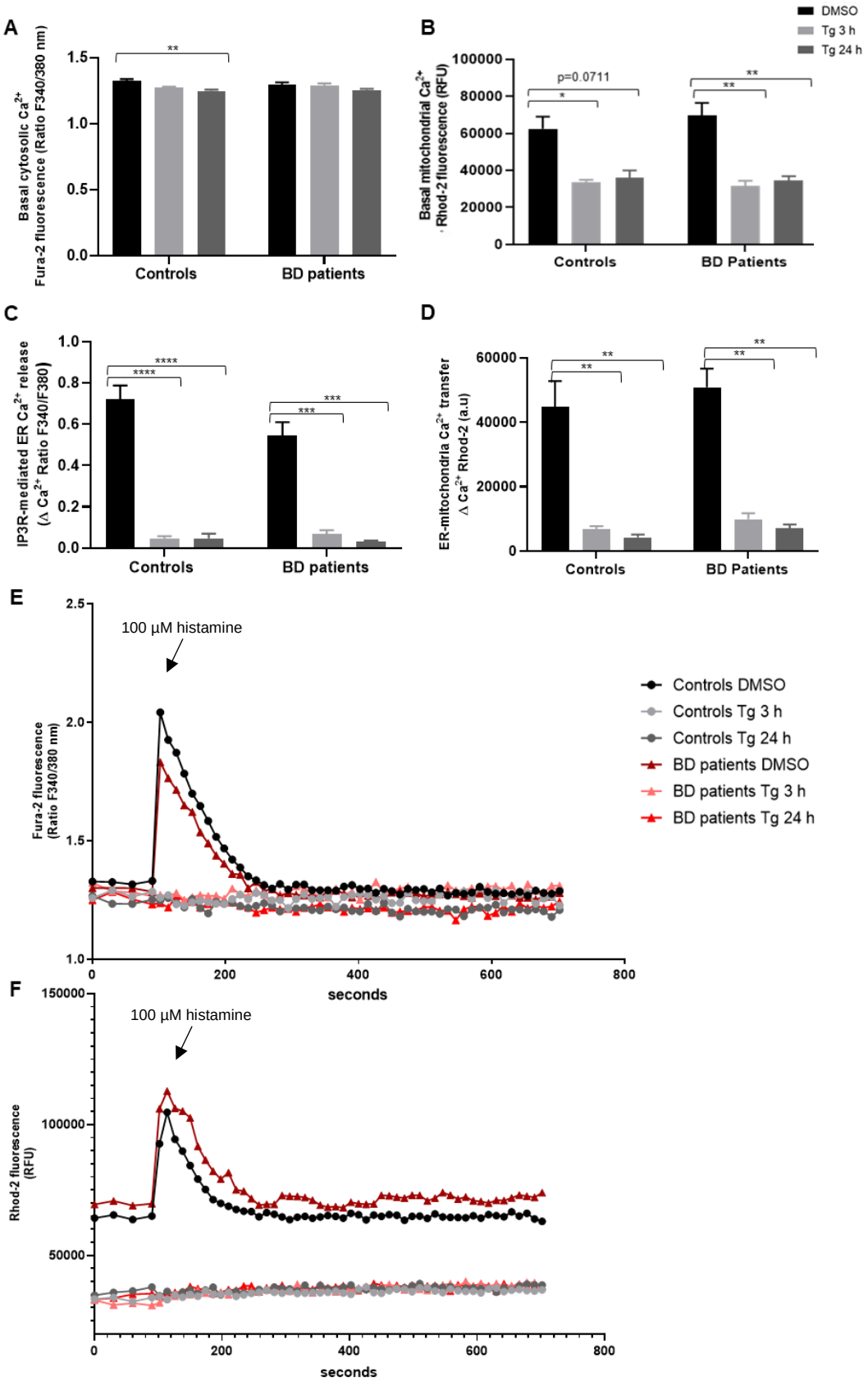

Figure 8. Analysis of ER-mitochondria Ca^{2+} transfer upon ER stress. Control- and BD patients-derived fibroblasts were incubated with 5 µM thapsigargin for 3 or 24 hours to evaluate alterations in Ca^{2+} transfer

between the ER and the mitochondria. Ca^{2+} levels were determined with two fluorescent probes, Fura-2, AM, and rhod-2, AM. Fura-2, AM binds to Ca^{2+} ions in the cytoplasm, while rhod-2, AM binds Ca^{2+} in mitochondria. **(A)** Cytoplasmic Ca^{2+} levels upon thapsigargin-induced ER stress. **(B)** Mitochondrial Ca^{2+} levels under thapsigargin-induced ER stress. Histamine induced Ca^{2+} depletion from the ER creating a maximum peak of Ca^{2+} fluorescence. **(C)** Cytosolic Ca^{2+} levels upon histamine-induced Ca^{2+} release. **(D)** Mitochondrial Ca^{2+} levels upon histamine-induced Ca^{2+} release. **(E)** Representative traces depicting the variation of Fura-2 fluorescence. **(F)** Representative traces depicting the variation of Rhod-2 fluorescence. Data are presented as mean ± SEM of n=3 controls and n=3 BD patients from 3 independent experiments. Statistical significance was determined using the two-way ANOVA test, followed by Tukey's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

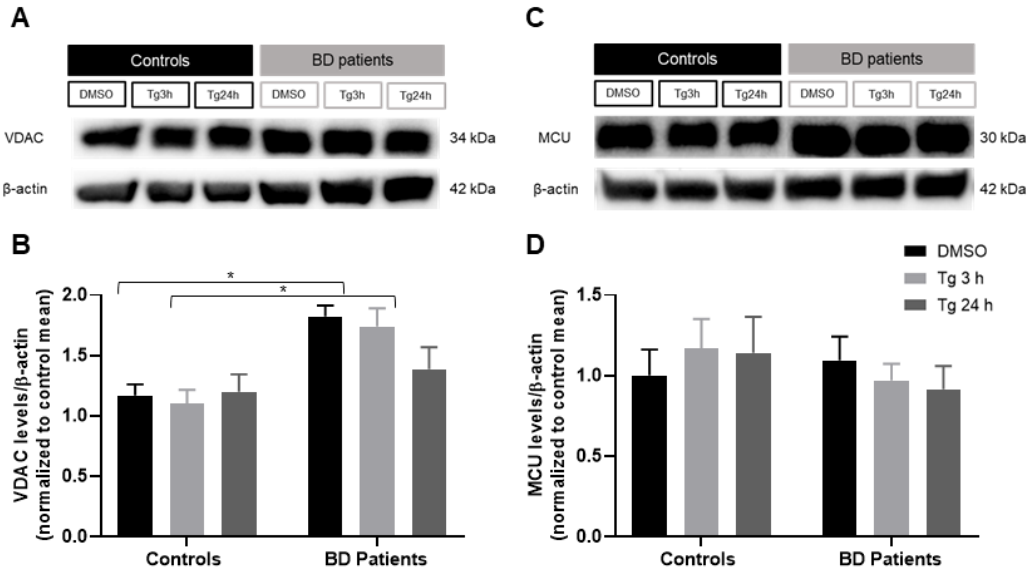

Figure 9. Protein levels of mitochondrial Ca^{2+} transporters VDAC1 and MCU. Control and BD patients' fibroblasts were incubated with 5 µM thapsigargin for 3 or 24 hours. Protein levels were assessed by WB. **(A)** Representative immunoblot of VDAC1 and β-actin protein levels at different thapsigargin-exposure time points **(B)** Protein levels of VDAC1 under thapsigargin-induced ER stress. VDAC1 levels were normalized with β-actin levels. **(C)** Representative immunoblot of MCU and β-actin protein levels at different thapsigargin-exposure time points **(D)** Protein levels of MCU under thapsigargin-induced ER stress. MCU levels were normalized with β-actin levels. Data are presented as mean ± SEM. Three samples obtained from controls and BD patients fibroblasts were used (n=3) and each experiment was performed twice. Statistical significance was determined using the two-way ANOVA test, followed by Tukey's multiple comparisons test. *p<0.05.

4.6. Effect of pharmacological activation of Sigma-1 receptor on mitochondrial membrane potential

Sigma-1R activation to ameliorate neuropsychiatric disorders symptoms has been the subject of various studies due to its neuroprotective and neuroplasticity properties. Pridopidine, initially known to be a dopaminergic stabiliser, is a Sigma-1R agonist (Sahlholm et al., 2013). Sigma-1 receptor has a chaperone activity and is highly expressed at MAMs. Under physiological conditions, this chaperone forms a complex with GRP78. However, upon ER stress, Ca^{2+} depletion triggers this complex dissociation. Sigma-1R is responsible for the stabilization of IP3Rs at MAMs, fundamental to prolonged Ca^{2+} signaling to mitochondria (Hayashi and Su, 2007). Therefore, it was examined by WB whether Sigma-1R levels change upon thapsigargin-induced ER stress. No statistical differences were found in all experimental conditions (Figure 10).

The MTT reduction assay was used to evaluate the absence of toxicity of pridopidine in fibroblasts (Figure 11). Cells were incubated with 1 and 5 µM of pridopidine for 24 hours, and none of the concentrations reduced cell viability (Figure 11).

Then, it was investigated the effect of Sigma-1R activation on MMP under ER stress since thapsigargin was shown to induce MMP loss in both controls- and BD patients-derived fibroblasts. Fibroblasts were incubated with 5 µM thapsigargin for 3 hours, and 5 µM pridopidine for 24 hours. As showed in Figure 12, Sigma-1R agonist did not prevent mitochondrial depolarisation induced by thapsigargin neither in BD patients- nor in controls-derived fibroblasts (Figure 12).

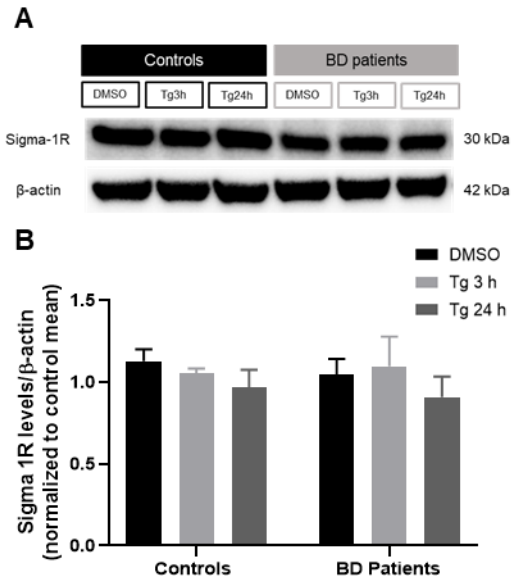

Figure 10. Sigma-1R levels under thapsigargin-induced ER stress. Control and BD patients' fibroblasts were incubated with 5 µM thapsigargin for 3 or 24 hours. Protein levels were assessed by WB. **(A)** Representative immunoblot of Sigma-1R and β-actin protein levels at different thapsigargin-exposure time points **(B)** Protein levels of Sigma-1R during thapsigargin-induced ER stress. Sigma-1R levels were normalized with β-actin levels. Data are presented as mean ± SEM of n=3 controls and n=3 BD patients from 2 independent experiments. Statistical significance was determined using a two-way ANOVA test, followed by Tukey's multiple comparisons test. These tests showed no statistical differences

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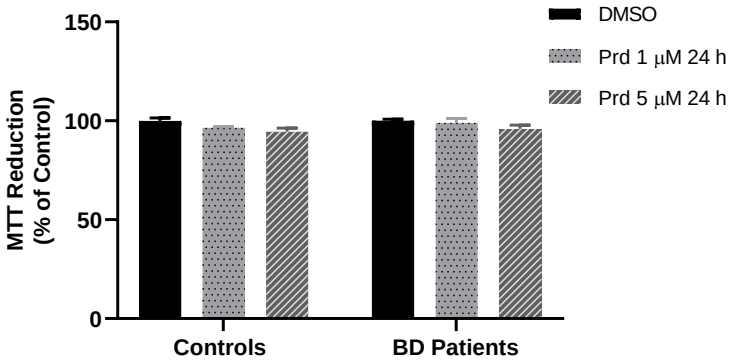

Figure 11. Fibroblasts viability upon stimulation with pridopidine (Prd). Control and BD patients' fibroblasts were incubated with pridopidine (1 µM or 5 µM) for 24 hours. The fibroblasts vulnerability was tested with the MTT reduction assay, as described in the materials and methods chapter. Data are presented as mean ± SEM of n=3 controls and n=3 BD patients from 2 independent experiments. Statistical significance was determined using the two-way ANOVA test, followed by Tukey's multiple comparisons test. These tests showed no statistical differences.

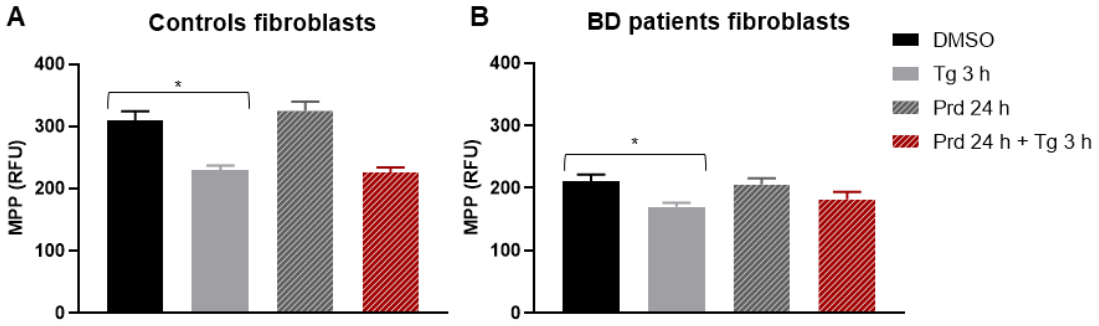

Figure 12. Effect of pridopidine on mitochondrial membrane potential upon thapsigargin-induced ER stress in fibroblasts. Control and BD patients' fibroblasts were incubated with 5 µM thapsigargin for 3 hoursin the presence or in the absence of pridopidine (5 µM). (A) Mitochondrial membrane potential changes in control and (B) in BD patients' fibroblasts. Data are presented as mean ± SEM of n=3 controls and n=3 BD patients from 2 independent experiments carried out in triplicate. Statistical significance was determined using the one-way ANOVA Kruskal-Wallis test, followed by Dunn's multiple comparisons test. *p<0.05.

4.7. Role of pharmacological Sigma-1 receptor activation on ER-mitochondria tethering and Ca^{2+} transfer

Given the stabilising effect of IP3R upon Sigma-1R activation, it was investigated whether there were changes in IP3R colocalization with VDAC. Once again, fibroblasts were incubated with 5 µM thapsigargin for 3 hours after pre-incubation with 5 µM pridopidine for 24 hours. PLA allowed to evaluate whether the number of ER-mitochondria contacts at MAMs underwent any change upon pharmacological activation of the Sigma-1R (Figure 13).

In agreement with previous results (Figure 7), ER stress led to a significant increase in the number of interactions between IP3R and VDAC in controls but not in BD patients' cells. However, Sigma-1R activation did not affect the colocalization of IP3R-VDAC upon thapsigargin stimulation, both in controls- and BD patients-derived fibroblasts (Figure 13).

As the IP3R-VDAC interaction may not translate into a functional structure, Ca^{2+} transfer into mitochondria was investigated using the Rhod-2, AM. Cells

were incubated with 5 µM thapsigargin for 3 hours after 5 µM pridopidine pre-incubation for 24 hours. In both controls- and BD patients-derived fibroblasts, pridopidine did not cause changes in basal Rhod-2 fluorescence levels in the mitochondria. That is, upon Sigma-1R activation, the decreased basal mitochondrial Ca^{2+} levels triggered by ER stress are not prevented in control and BD patients-derived fibroblasts (Figure 14A, B). When control and BD cells were under ER stress conditions, the decreased Rhod-2 fluorescence induced by the histamine pulse was not affected by the presence of 5 µM pridopidine (Figure 14C-F). According to these results, activation of the Sigma-1R is not able to prevent the inhibition of mitochondrial Ca^{2+} uptake upon ER Ca^{2+} release by the IP3R under ER stress conditions. However, the Kruskal-Wallis test revealed statistical differences with a determined p value of 0.0034 (Figure 14C) and 0.0032 (Figure 14D). This means that the null hypothesis can be rejected and that the data of all conditions do not have identical means. There also appears to exist a greater difference between the maximum peak and basal mean fluorescence values of BD patients' fibroblasts incubated with 5 µM pridopidine and 5 µM thapsigargin (Figure 14D).

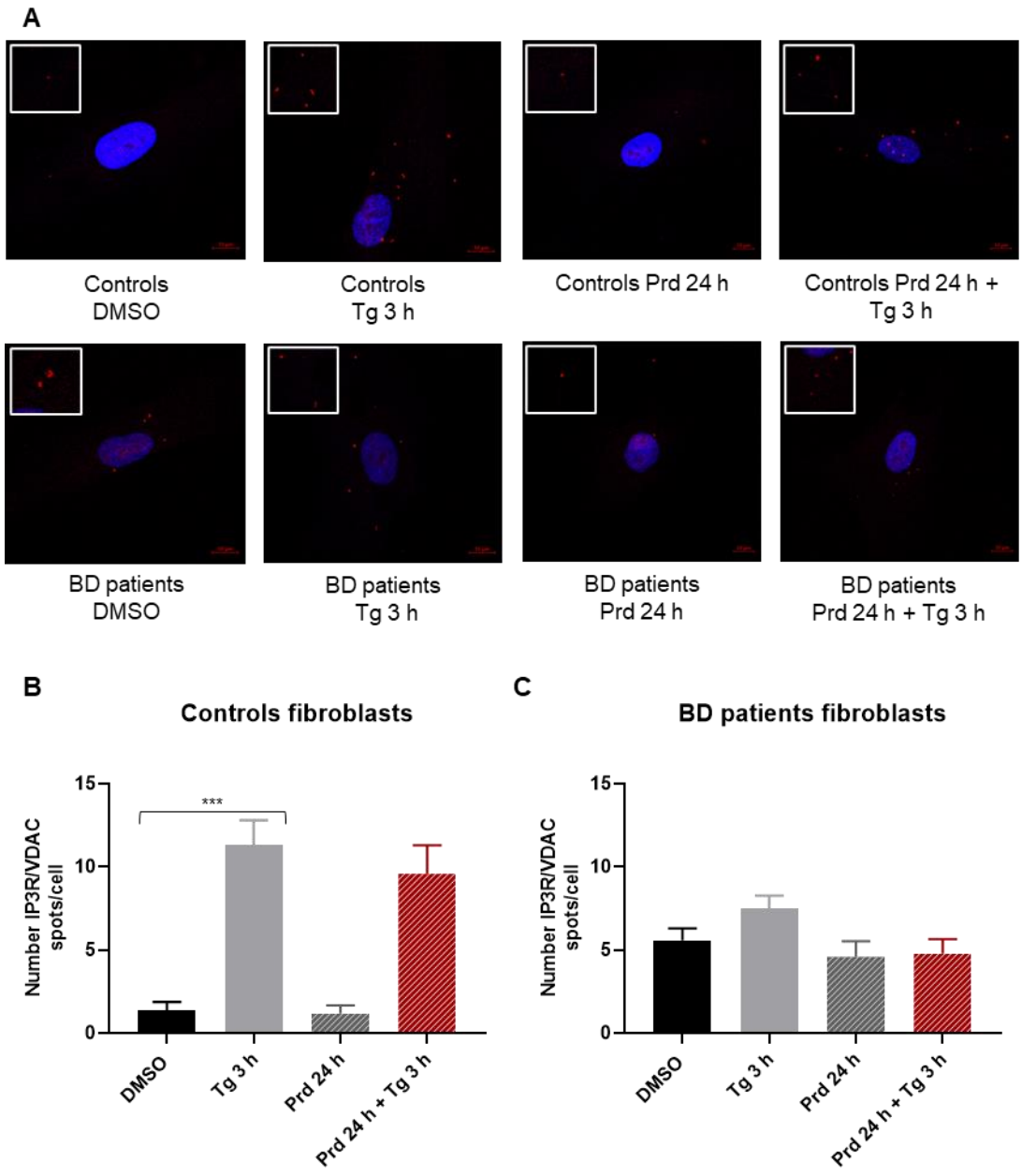

Figure 13. Effect of Sigma-1R activation on ER-mitochondria contacts upon ER stress. Control and BD patients' fibroblasts were incubated with 5 µM thapsigargin for 3 hours in the presence or in the absence of 5 µM pridopidine. The IP3R/VDAC1 colocalization was analysed by the proximity ligation assay (PLA). (A) Representative images of stained fibroblasts. Each red dot represents the colocalization of IP3R and VDAC1. Scales represent 10 µm. Plot of the number of contacts between ER and mitochondria under these conditions in control fibroblasts (B) and in BD fibroblasts (C). Data are presented as mean ± SEM of 10 cells per condition. For control fibroblasts, statistical significance was determined using the one-way ANOVA Kruskal-Wallis test, followed by Dunn's multiple comparisons test. For BD patients' fibroblasts, statistical significance was determined using the one-way ANOVA followed by Holm-Sidak's multiple comparisons test. ***p<0.001.

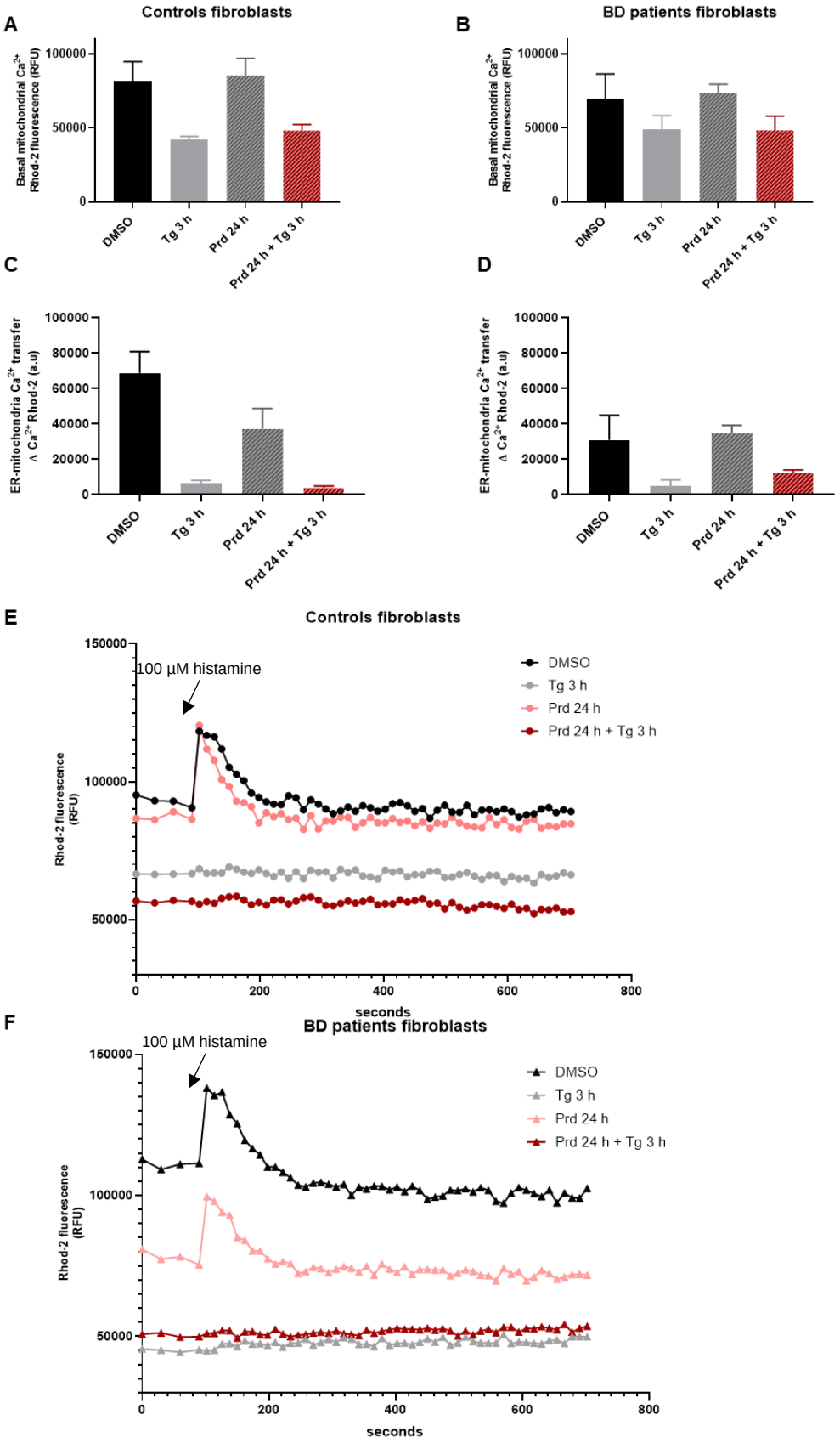

Figure 14. Analysis of Ca^{2+} transfer between ER and mitochondria under ER stress upon pharmacological Sigma-1R activation. Control and BD fibroblasts were incubated with 5 µM thapsigargin

for 3 hours in the absence or presence of pre-incubation with 5 μM pridopidine for 24 hours to evaluate alterations in Ca^{2+} transfer from the ER to mitochondria. Ca^{2+} fluorescence was quantified with rhod-2, AM which binds to mitochondrial Ca^{2+}. Mitochondrial Ca^{2+} levels were evaluated using the fluorescent indicator Rhod-2, AM. These conditions were assessed in controls- **(A)** and BD patient-derived fibroblasts **(B)**. Variation of mitochondrial Ca^{2+} upon ER Ca2+ release through the IP3R induced by histamine in controls- **(C)** and BD patients-derived **(D)** fibroblasts. Representative traces depicting the variation of Rhod-2 fluorescence in controls- **(E)** and BD patients-derived **(F)** fibroblasts. Data are presented as mean ± SEM of n=3 controls and n=3 BD patients from 3 independent experiments. Statistical significance was determined using the one-way ANOVA Kruskal-Wallis test, followed by Dunn's multiple comparisons test. This last test showed no significant differences

Chapter V
Discussion

Previously neuropsychiatric disorders studies focused primarily on abnormalities in the anatomy of patients' brains. Nonetheless, researchers are increasingly seeking answers about the underlying pathophysiology of the disorders at a cellular and molecular level, opening new doors for research, creating countless new questions, and proposing new therapeutic targets. It is essential to study the disorders' pathophysiological mechanisms to understand the modifications that occur in the brain and possible dysfunctions of neuronal circuits. In bipolar disorder, numerous studies point towards the disruption of the ER stress response, mitochondrial function, Ca^{2+} signaling, inflammation, cell resilience, oxidative stress, and cytoskeleton (Pereira et al., 2017). These processes are regulated by physical and biochemical bridges between the ER and the mitochondria, the MAMs. Researchers unravelled the importance of these interorganellar contacts in cellular functions that regulate homeostasis, thus creating new hypotheses of how MAMs dysfunction may be involved in numerous disorders, including BD (Pereira et al., 2017). Sigma-1 receptor is a chaperone found mostly at MAMs, which has been studied for its neuroprotective properties by enhancing cellular resilience in stress conditions. Upon ER stress, Sigma-1R dissociates from another chaperone, GRP78, and stabilizes the IP3R and IRE1 at MAMs, ensuring an appropriate response to restore homeostasis (Hayashi and Su, 2007). Studies indicate that Sigma-1R activation is responsible for improved neuroplasticity in mice, which is highly impaired in BD (Crouzier et al., 2020). Given the above, this study was designed to understand the effects of Sigma-1R activation on BD underlying pathophysiology, specifically upon ER stress conditions.

We used patient-derived human fibroblasts that have gained relevance in the neuropsychiatric research of pathophysiological mechanisms. Although post-mortem and animal studies have contributed a lot to the understanding of this disorder, they have many limitations. On one hand, post-mortem studies modestly impact the discovery of new diagnostic methods and therapies, although they are widely used to find out anatomical changes and to study the transcriptome, proteome, and metabolome of patients' brains. On the other hand, animal studies examine the structural, functional, and behavioural changes that arise from genetic modifications. Nevertheless, neuropsychiatric disorders have a uniquely human context, and it is not possible to replicate the complexity of these symptoms' disorders only through genetic alterations (Kálman et al., 2016). Conversely, by using patient-derived fibroblasts we can establish a uniform cell line that is relatively easy to obtain, store and culture. Furthermore, fibroblasts create a microenvironment and express many of the receptors and signaling pathways that are found in neurons (Kálman et al., 2016). Using fibroblasts in this context also has downsides, as they are much more resilient to stress than neuronal cells, are self-sufficient unlike neurons, and have a very different structure and function (Auburger et al., 2012; Lundgaard et al., 2014). Proper use of patient-derived fibroblasts is advantageous to understand conserved signaling pathways, and cellular functions, and therefore the most suitable cell type for the study we conducted. Our project aimed to evaluate differences in UPR PERK pathway between controls and BD patients-derived fibroblasts. We also ran several experiments to understand how alterations in ER-mitochondria tethering impacted MAMs-regulated events like Ca^{2+} transfer during ER stress in the presence or absence of the Sigma-1R agonist.

Taking into consideration the impairments in the ER stress response that have been proposed for BD, we wanted to ascertain whether, upon thapsigargin-induced ER stress, the protein levels of GRP78 and p-eIF2α were altered. We found that there was an equal increase of GRP78 in both control and BD patient-derived fibroblasts when exposed to thapsigargin for 24 hours (Figure 5A, C). However, a study conducted by Pfaffenseller and colleagues uncovered a reduction in protein levels of GRP78, p-eIF2α, and CHOP in BD lymphocytes. Nonetheless, these cells were derived from patients in advanced stages, unlike the fibroblasts used in our study. Despite being distinct cell types, these results reinforce the idea that BD is a neuroprogressive disorder, and that perhaps the PERK signaling pathway dysfunction reflects in an undermined neuronal function. The PERK signaling pathway is involved in the inhibition of protein translation but also induces cell death. Dysregulation of this protective mechanism has a negative outcome in the combat of ER stress that could lead to prolonged stress and eventually apoptosis. We also investigated other proteins involved in this response and saw an increase in protein levels of ERO1α in control fibroblasts, although not significantly different in Tukey's multiple comparisons test (Figure 5F, H). This increase may be due to a demand for PDI activity to restore homeostasis. However, in BD it appears that the protein levels of ERO1α did not increase upon ER stress, which may constitute an earlier failure in the ER stress response. It is noteworthy that we only analysed protein levels and that in the future it would be beneficial to assess protein activity as well as analyse the other UPR pathways. Considering the brain atrophy seen in post-mortem studies, it would be interesting to evaluate apoptosis markers in early-stage patient-derived fibroblasts. Apoptosis is an essential mechanism to maintain organisms'

homeostasis and for the correct formation of synapses in the early stages of development, but an unbalanced apoptosis mechanism may lead to tissue atrophy or accumulation of defective cells that cannot recover from stress, jeopardising the organism's balance (Hollville et al., 2019).

Normal values of MMP are essential for the entry of cations, such as Ca^{2+}, into the mitochondria. However, BD patient-derived fibroblasts have a significant reduction in MMP under basal conditions when compared with controls-derived fibroblasts (Figure 6), which is in line with the results obtained by Marques et al. (2021). This may be hindering Ca^{2+} flux into the mitochondria that have consequences on ATP synthesis since Ca^{2+} is crucial for the activity of some enzymes involved in oxidative phosphorylation (Paillusson et al., 2016). Marques et al. (2021) also found that ATP levels were reduced and that there was an increase in glycolytic capacity in BD patient-derived fibroblasts, suggesting the existence of abnormalities in oxidative phosphorylation.

BD patient-derived fibroblasts may counterbalance the reduced Ca^{2+} entry into the mitochondria by increasing the protein levels of VDAC (Figure 9A, B). The unique environment generated at MAMs is essential for the influx of this ion into mitochondria. Therefore, the colocalization of IP3Rs and VDAC at MAMs is crucial for Ca^{2+} transfer into mitochondria. Under basal conditions, when compared to control cells, BD patient-derived fibroblasts show a higher number of ER-mitochondria contacts demonstrated by the increased colocalization between IP3R and VDAC (Figure 7B). Conversely, mitochondrial Ca^{2+} levels at basal conditions did not show any differences between BD patient-derived and control fibroblasts (Figure 8B). This suggests that at basal conditions the

increased ER-mitochondria contacts may be a compensatory mechanism to overcome mitochondria dysfunction.

Under thapsigargin-induced ER stress, this mechanism appears to be inefficient. Upon ER stress, there is depolarisation of mitochondrial membrane in both control and BD patient-derived fibroblasts (Figure 6). However, in controls-derived fibroblasts there was a very significant increase in IP3R and VDAC colocalization, whereas in BD there were no alterations (Figure 7B).

Under ER stress conditions, we observed a higher reduction in the ER-mitochondrial Ca^{2+} transfer upon histamine-induced ER Ca^{2+} release in controls in comparison to BD patient-derived fibroblasts (Figure 8C). This could be explained by the increased ER-mitochondria contacts observed in controls treated with thapsigargin (Figure 7B). Nevertheless, we did not see an increase in MCU levels, which may obstruct Ca^{2+} into mitochondrial matrix. These results suggest an inefficient response to stress in BD that could lead to mitochondrial dysfunction and impaired energy metabolism. This follows the results obtained by Marques et al. (2021), considering that they showed an increased mitochondrial biogenesis to respond to impaired ATP depletion.

Upon ER stress, BD patients-derived fibroblasts increased VDAC protein levels (Figure 9A, B), although simultaneously, the number of contacts between the ER and the mitochondria does not change when compared to basal conditions (Figure 7B). Therefore, we hypothesized that the IP3R stabilisation at MAMs upon ER stress might be impaired in BD. Due to IP3R stabilisation effect of Sigma-1R activation we assessed the Sigma-1R levels in basal and ER stress conditions. The results showed no differences under any condition (Figure 10).

To explore the neuroprotective role of Sigma-1R, we proceed to the pharmacological activation of Sigma-1R by pridopidine. Unfortunately, this had no effect on thapsigargin-induced mitochondria depolarisation neither in controls nor in BD patients-derived fibroblasts (Figure 12). Furthermore, there were also no consequences on the number of ER-mitochondria contacts (Figure 13), as well as in Ca^{2+} transfer between ER and mitochondria (Figure 14A, B) under ER stress upon Sigma-1R activation. Even with Sigma-1R activation and subsequent IP3R stabilisation, the compromised mechanisms did not ameliorate. However, in the future, it would be interesting to evaluate the protein levels of IP3Rs, as well as their activity because the formation of the contacts does not imply a functional Ca^{2+} transfer. Another aspect that should be evaluated is the protein levels of GRP75 as well as its activity, since this protein is essential for the IP3R-VDAC interaction and, without it, this interaction is not functional (Marchi et al., 2018), negatively impacting Ca^{2+} signaling.

Briefly, pharmacological activation of Sigma-1R did not show improvements in the abnormalities suggested for Ca^{2+} transfer between the ER and mitochondria upon ER stress. Inadequate Ca^{2+} signaling response upon ER stress may have more deleterious consequences in neurons, as they are less stress-resilient than fibroblasts.

After showing a Ca^{2+} signaling alteration in BD patient-derived fibroblasts, it would be interesting to understand if these alterations occur in neurons derived from BD patients' fibroblasts. Despite the similarities between these cell types, the response and consequences arising may be different due to the specialized function of the neurons. Ca^{2+} signaling is implicated in plentiful mechanisms that modulate brain function. For the correct functioning of neuronal circuits, there is

a high energy demand, and the dysregulation of ATP synthesis results in devastating consequences for the normal functioning of the brain and may even be implicated in cell death (Khacho and Slack, 2018). Marques et al. (2021) saw an increase in fission processes in fibroblasts derived from BD patients which is related to both impaired ATP production and Ca^{2+} signaling. The CoMIC is a process dependent on Ca^{2+} influx into mitochondria and determines mitochondrial fission events (Cho et al., 2017). Due to the polarised structure and unique functions of neurons, the distribution of mitochondria is crucial for modulating neurotransmission-related processes. Mitochondrial motility is regulated by mitochondrial matrix Ca^{2+}, and in other words, Ca^{2+} signaling influences the mitochondria position in neurons and hence neurotransmission (Jackson and Robinson, 2015; Chang et al., 2011). Another important aspect involved in ER Ca^{2+} release by IP3R is the transport of the GluR1 subunit of AMPA receptors to post synaptic sites, also influencing axonal growth (Johnstone and Mobley, 2020). Lastly, ER-mitochondria coupling is involved in dendritic Ca^{2+} homeostasis (Hirabayashi et al., 2017). Ca^{2+} signaling abnormalities may have other implications in the central nervous system because neurons are not the only brain cell type regulated by this mechanism. Impaired Ca^{2+} transfer can also affect astrocytes, leading to direct consequences on neurons since the survival of this cell type is regulated by glial cells. Overall, mitochondria regulate Ca^{2+} signaling, which affects astrocytes' survival and communication between neurons (de Oliveira et al., 2021).

As well as other neuropsychiatric disorders, the amount of diagnosis of BD is thought to increase even more by 2030. This study hypothesized alterations in Ca^{2+} signaling in BD patient-derived fibroblasts, that might be involved in the

pathophysiological mechanisms proposed previously by other groups. Ca^{2+} signaling is fundamental for correct cellular function, however, it deserves special attention in a neuronal context. The dysregulation of these mechanisms may be involved in abnormalities in neurotransmission, with consequences in brain circuits wiring, in the cell death seen in post-mortem studies in BD patients' brains, in the decreased resilience to environmental stressors, and in the decreased ATP production. Future studies should focus on dysregulated Ca^{2+} signaling in iPSCs reprogrammed from BD patients and controls and differentiated in neurons. If successful, these studies will improve the knowledge of the underlying pathophysiology of BD and reveal signaling pathways that could constitute a new therapeutic target or to develop reliable biomarkers.

Chapter VI
Conclusion

Altogether, our study shed further light for the understanding of the cellular and molecular compromised mechanisms in bipolar disorder. Taken together, our results revealed perturbations in the PERK signaling pathway of unfolded protein response to ER stress, in tethering of the ER and mitochondria, and in Ca^{2+} signaling of fibroblasts derived from BD patients.

Our results are in line with existing literature conjecturing that BD is a neuroprogressive disorder, as they indicate that impairment in the ER stress response is more attenuated in early stages. Furthermore, BD patients-derived fibroblasts have more contacts between the ER and mitochondria under basal conditions when compared to control fibroblasts. However, under stress conditions, controls-derived fibroblasts have an adaptive response by increasing the number of ER-mitochondria interactions while BD patients-derived fibroblasts did not show an increase. These changes also impact Ca^{2+} signaling in BD, which is critical for numerous cellular processes and may contribute to changes in neuronal circuits in the brain. Finally, despite the neuroprotective properties of Sigma-1R activation, we did not observe any improvements in the processes assessed in the presence of Sigma-1R agonist pridopidine under ER stress conditions.

The limited knowledge about the pathophysiological mechanisms of this disease hinders the development of therapeutic strategies and biomarkers able to identify BD at early stages, since it is essential to start the treatment as early as possible. This study contributed to the general knowledge of BD underlying pathophysiological mechanisms and revealed alterations in the ER and the mitochondria functions in dermal fibroblasts.

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