The Mitochondrial Permeability Transition Pore: An Evolving Concept Critical for Cell Life and Death

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ABSTRACT

In this review, we summarize the current knowledge of perhaps one of the most intriguing phenomena in cell biology: the mitochondrial permeability transition pore (mPTP). This phenomenon, which was initially observed as a sudden loss of the inner mitochondrial membrane (IMM) impermeability caused by excessive calcium, has been studied for almost 50 years and still no definitive answer has been provided regarding its mechanisms. From originally being considered an *in vitro* artifact to the current notion that the mPTP is a clear phenomenon with physiological and pathological implications, a long road has been traveled. The evolving mPTP models and mechanisms, which have involved many proposed mitochondrial protein components, also result from methodological advances and the use of more complex biological models. We described how the scientific process of thinking and methodological advances allowed to obtain some of the milestone discoveries on mPTP regulation and composition and recognize it as a valid target for drug development and a critical component of mitochondrial biology. We here summarize the role of mitochondria in cytosolic calcium control and the evolving concepts regarding the mitochondrial permeability transition / mitochondrial permeability transition pore. In this context, we describe the early experiments that led to recognizing the mPTP as a molecular entity and present evolution of methodologies and experimental protocols aimed at elucidating the structure and mechanisms of mPTP. We show the evolution of the mPTP structure and composition along the years and describe its pathological and physiological roles. Moreover, we present how scientific thinking and novel molecular biology methodologies led to creation of a novel model for the mPTP.

Keywords: Mitochondria, mitochondrial permeability transition pore, calcium, cell death

Contents:

I. Introduction

The mitochondrial permeability transition (mPT) is a pathophysiological state of the inner mitochondrial membrane (IMM). Under favorable conditions including calcium (Ca^{2+}) overload, oxidative stress, increased phosphates concentration, and decreased adenine nucleotide availability, the IMM becomes highly permeable to solutes with a molecular weight of up to 1,500 Da (Halestrap, 2009; Morciano *et al.*, 2015). Since biological and evolutionary features made the IMM relatively impermeable, the mPT leads initially to a considerable and not selective influx of solutes and to an abrupt loss of mitochondrial metabolites allowing mitochondrial homeostasis perturbation that, in different degrees, entails cell death (Izzo *et al.*, 2016). The mPT is a highly conserved phenomenon in evolutionary history, with key features conserved in yeast, mammals and plants; only the crustacean *Artemia franciscana* seems to lack a known and regulated mPT that may contribute to its long-lasting hypoxia tolerance (Menze *et al.*, 2005). The mPT is caused by the opening of proteinaceous channels at the juxtaposition of the IMM and OMM rather than a variation in the phospholipid bilayer composition (Crompton, Costi & Hayat, 1987) and mPT pores (mPTP) opening can be generally monitored by the use of fluorescent dyes or absorbance assays in living cells, isolated mitochondria and tissues. Pore opening results from relatively severe perturbations of the mitochondrial matrix and mPT consequences are dictated by several factors such as the open time of the pore, the number of forming channels in each mitochondrion at a given time, and the number of mitochondria affected by this pathophysiological event inside cells. Thus, long-lasting mPT can lead to irreversible consequences including matrix swelling, dissipation of mitochondrial potential, ATP hydrolysis to ADP and free phosphate ions, and uncoupling oxidative phosphorylation causing cell death. Modalities by which mPTP entails cell death are still debated; based on recent reports, apoptosis or necroptosis may occur depending on ATP concentration and availability (Brenner & Moulin, 2012).

mPTP opening, considered by some authors as a cellular catastrophe (Briston *et al.*, 2019), has been considered a critical mechanism in the development of several pathologies and organ damage caused by the toxicity induced by several compounds. For more than one decade, the mPTP has been associated with the cardiac dysfunction observed after an episode of ischemia/reperfusion of the heart (Borutaite *et al.*, 2003). In 1987, Steenbergen and co-workers (Steenbergen *et al.*, 1987) demonstrated an increase in cytosolic Ca^{2+} concentration after an ischemic episode. Cytosolic Ca^{2+}

overload induces the opening of mPTP and consequently, the release of cytochrome c from mitochondria to cytosol and activation of the mitochondrial-dependent apoptotic pathway was observed (Whittington *et al.*, 2018). During ischemia, it has been described that the mPTP remains closed because of cytosolic acidification, since, as described above, protons are mPTP inhibitors (Ong *et al.*, 2015). In opposition, the opening of the mPTP is strongly potentiated during reperfusion, after an ischemic episode. During reperfusion, a burst of reactive oxygen species (ROS) production occurs (Gonzalez-Montero *et al.*, 2018; Hausenloy, Duchen & Yellon, 2003; Di Lisa *et al.*, 2001). With mitochondria weakened from an ischemic episode, the reintroduction of oxygen in the system has a deleterious effect, with an increased ROS production. As discussed earlier in this review, ROS also potentiate mPTP opening, and at this moment, cardiac cell death and cardiac dysfunction may occur. In the brain, mPTP opening after episodes of ischemia/reperfusion was also observed (Halestrap, 2006). Inhibition of mPTP opening has been reported as a neuroprotective strategy to prevent cerebral ischemia reperfusion injuries (Matsumoto *et al.*, 2018; Rekuviene *et al.*, 2017; Leger *et al.*, 2011). The mPTP also associates with metabolic diseases, namely with insulin-resistance or diabetes. Indeed, Taddeo et al. (Taddeo *et al.*, 2014) demonstrated in 2013 that mPTP opening is a required phenomenon for induction of insulin resistance in skeletal muscle, establishing a link between mPTP and insulin resistance. Work performed in animal models demonstrated how the mPTP is induced in diabetic conditions, which may contribute to its complications, including diabetic cardiomyopathy or hyperglycemia (Riojas-Hernandez *et al.*, 2015; Oliveira *et al.*, 2003; Taddeo *et al.*, 2014; Lumini-Oliveira *et al.*, 2011). As another example, an accumulation of hydrophobic bile acids in hepatic cells during cholestasis induces hepatic cells apoptosis through mPTP opening (Yerushalmi *et al.*, 2001; Rolo, Palmeira & Wallace, 2003).

The opening of mPTP may also be observed during drug-induced toxicity. For example, our group demonstrated that high doses of caffeine enhance the Ca^{2+}-dependent cardiac mPTP in isolated mitochondrial fractions (Sardao, Oliveira & Moreno, 2002), alerting for the excessive consumption of high energy drinks and dietary supplements with high doses of caffeine. Doxorubicin, a potent chemotherapeutic drug used in several types of cancer but with associated cardiotoxicity, decreases the threshold for mPTP opening in cardiac cells, impairing the contracting performance of the heart (Montaigne *et al.*, 2011), as we demonstrated in rodent models (Oliveira *et al.*, 2004; Oliveira *et al.*, 2005; Pereira *et al.*, 2011). The role

of mPTP in cell toxicity induced by xenobiotic compounds, namely those that cause oxidative stress, and in different diseases, has been extensively explored by our group (Carvalho *et al.*, 2018; Teixeira *et al.*, 2018; Bernardo *et al.*, 2013). Due to the importance of mPTP for the mitochondrial function and, consequently cellular performance, the dynamics and regulation of mitochondrial permeability transition phenomenon have been one of ours research interests.

Although mPTP opening is often associated to disease conditions, there are evidences for important physiological roles in its flickering or transient opening mode, especially in the heart (cardiac development and damage protection) (Hausenloy *et al.*, 2004; Korge *et al.*, 2011) in the brain (putative or indirect involvement in synaptic efficacy and plasticity) (Mnatsakanyan *et al.*, 2017) and targeting metabolic functions (Hom *et al.*, 2011).

II. mPTP and mitochondrial Ca^{2+} uptake or overload

The concentration of free Ca^{2+} in the cell regulates an array of biochemical reactions and is crucial for signal transduction (Giorgi *et al.*, 2018a; Elustondo *et al.*, 2017; Santulli, 2017; Krebs, 2017; Giorgi, Marchi & Pinton, 2018b; Herst *et al.*, 2017; Hausenloy *et al.*, 2020; Del Re *et al.*, 2019; Glaser *et al.*, 2019; Rizzuto, Duchen & Pozzan, 2004). Mitochondria are fundamental for cellular energy metabolism not only by supplying energy in the form of ATP but also by affecting cell physiology through the regulation of Ca^{2+} homeostasis (Krebs, 2017; Picard, Wallace & Burelle, 2016). Mitochondria have a large capacity to accumulate Ca^{2+} and can transiently store that cation, a contributing process for cell calcium homeostasis. In fact, their ability to rapidly accumulate calcium for later releasing it makes those organelles very important cytosolic stores or buffers for Ca^{2+} in the context of cell physiology and pathophysiology (Ludtmann & Abramov, 2018; Elustondo *et al.*, 2017; Dedkova & Blatter, 2008; Delierneux *et al.*, 2020). Intramitochondrial free calcium plays a significant part in Ca^{2+} homeostasis in cells, being also important in cell survival and death (Picard *et al.*, 2016; Santulli, 2017; Ludtmann & Abramov, 2018; Bhosale *et al.*, 2015; Del Re *et al.*, 2019). It has been demonstrated that a basal level of Ca^{2+} in the mitochondrial matrix is needed for the correct functioning of these organelles, while the pathophysiological role of Ca^{2+} overload, which occurs for a wide range of pathologies, still should be clarified (Ludtmann & Abramov, 2018; Bertero & Maack, 2018; Burgoyne *et al.*, 2012).

Calcium accumulation in mitochondria was described in the 1960s for the first time (Deluca & Engstrom, 1961). Since then, there have been no doubts concerning the role of Ca^{2+} in the regulation of mitochondrial bioenergetics and diverse cellular functions. Calcium homeostasis in mitochondria is regulated by the complex system of mitochondrial Ca^{2+} influx and efflux mechanisms. This Ca^{2+} transport system in mitochondria comprises specific transporters in the IMM and the outer mitochondrial membranes (OMM). To access the mitochondrial matrix, Ca^{2+} firstly should pass through the OMM. This membrane is permeable to ions, in particular to Ca^{2+}, and small proteins, thanks to the presence of a large conductance channel – the voltage-dependent anion channel (VDAC), allowing the exchange of molecules of molecular weight up to 1,500 Da (Magri, Reina & De Pinto, 2018; Krebs, 2017; Colombini & Mannella, 2012; Schein, Colombini & Finkelstein, 1976; Becker & Wagner, 2018). The VDAC is responsible for Ca^{2+} transport from cytoplasm to mitochondria and its permeability is controlled by ATP and other regulatory factors. Three different VDAC isoforms have been identified: VDAC1, VDAC2 and VDAC3 (Ponnalagu & Singh, 2017; Mertins, Psakis & Essen, 2014; Krebs, 2017). Although these isoforms have some similar structural and functional properties, they appear to assume different physiological roles (Magri *et al.*, 2018; Cheng *et al.*, 2003; De Pinto *et al.*, 2010; Rostovtseva *et al.*, 2020). While limited information is available regarding the functions of VDAC2 and VDAC3 as Ca^{2+}-influx mechanism (Magri *et al.*, 2018; De Pinto *et al.*, 2010; Lemasters *et al.*, 2012), VDAC1 is highly expressed in most cells (Shoshan-Barmatz & Golan, 2012) and seems to be the most prevalent and best-studied isoform for Ca^{2+} transport into the intermembrane mitochondrial space (Krebs, 2017).

Ca^{2+} transport through the IMM is regulated via several transporters. At present, three main mechanisms of Ca^{2+} influx through the IMM are considered (Figure 1): (1) an electrogenic mitochondrial Ca^{2+} uniporter (MCU), (2) the rapid mode of Ca^{2+} uptake (RaM), and (3) the mitochondrial ryanodine receptor (mRyR). Equally, three main mechanisms of Ca^{2+} efflux through the IMM are also considered: (1) Na^{+}/Ca^{2+} exchanger (NCXm), (2) H^{+}/Ca^{2+} exchanger (HCXm), and (3) mPTP. Besides this, leucine zipper- EF-hand containing transmembrane protein (LETM1) was also proposed as a Ca^{2+}-transport system. However, the role of LETM1 in mitochondrial Ca^{2+} influx and efflux through the IMM is still under discussion. Ca^{2+} transport across the IMM was originally thought to be a single mechanism that was demonstrated to be highly sensitive to ruthenium red and lanthanides (Gunter & Pfeiffer, 1990). The molecular

identity of this ruthenium and lanthanide-sensitive Ca^{2+} transport was unclear for several decades until 2011, when this transporter was identified as the mitochondrial Ca^{2+} uniporter (MCU) complex, following the identification of the gene encoding the pore-forming subunit of the MCU, made by independent groups (Baughman *et al.*, 2011; De Stefani *et al.*, 2011).

Currently, Ca^{2+} influx through this MCU multi-protein complex is the most established and well-characterized pathway for mitochondrial Ca^{2+} uptake to mitochondria, driven by the large electrochemical gradient (mitochondrial membrane potential ~180 mV) for Ca^{2+} across the IMM (Elustondo *et al.*, 2017; Mishra *et al.*, 2017; Mammucari *et al.*, 2018; Belosludtsev *et al.*, 2019). It is now considered that this multi-protein MCU complex adapts to multiple states and is composed of several subunits, including transmembrane core components and membrane-associated regulatory subunits in the intermembrane space. Three proteins have been identified as core components of the MCU complex: the mitochondrial Ca^{2+} Uniporter (MCU), the MCU dominant negative beta subunit (MCUb) and the Essential MCU REgulator (EMRE) (Mishra *et al.*, 2017; Mammucari *et al.*, 2018; Sancak *et al.*, 2013; Raffaello *et al.*, 2013; Wang, Baradaran & Long, 2020a; Cui *et al.*, 2019). The MCU gene (previously known as *CCDC109a,* 40 kD protein) was identified through a bioinformatics screening of the MitoCarta database, i.e., a compendium of mitochondrial proteins identified by mass spectrometry analyses on mitochondrial preparations from different mouse tissues (Baughman *et al.*, 2011; De Stefani *et al.*, 2011; Mammucari *et al.*, 2018). The MCUb gene (previously known as *CCDC109b,* 33 kDa protein) was identified through an MCU sequence homology screening (Raffaello *et al.*, 2013), and the incorporation of MCUb in the MCU complex has been demonstrated also by proteomic experiments (Sancak *et al.*, 2013). EMRE (known as *C22ORF32*, 10 kDa protein), being the last identified component of the MCU pore complex (Sancak *et al.*, 2013), is essential for MCU activity, as demonstrated by experiments in the EMRE knockout cells (Patron *et al.*, 2014) and has been proposed to play a fundamental role in the interaction between the pore core subunits and the regulatory subunits (Sancak *et al.*, 2013; Mammucari *et al.*, 2018).

The family of Mitochondrial Ca^{2+} Uptake proteins (MICU 1-3), Mitochondrial Ca^{2+} Uniporter Regulator 1 (MCUR1), and SLC25A23 are now considered the main membrane-associated regulatory subunits of MCU multi-protein complex (Mishra *et al.*, 2017; Wang *et al.*, 2014; Patron *et al.*, 2014; Hoffman *et al.*, 2014; Marchi *et al.*, 2019;

Vais *et al.*, 2020). MICU1 (previously known as *CBARA1/EFHA3*, 54 kDa protein) is a soluble (or membrane-associated) protein in the intermembrane space and proposed to be pivotal in both the gatekeeping and cooperative activation of mtCU; keeping the channel closed at resting conditions (Csordas *et al.*, 2013; Patron *et al.*, 2014; Wang *et al.*, 2014; Foskett, 2020). Other isoforms of MICU family of proteins – MICU2 (known as *EFHA1*) and MICU3 (known as *EFHA2*) are also identified as the regulatory subunits of MCU multi-protein complex, displaying the EF-hand domains in their protein structure, but share only 25% sequence identity with MICU1 (Plovanich *et al.*, 2013). However, the location of the MICU proteins and the nature of the MICU-dependent regulation is still controversial (Vais *et al.*, 2020; Wu *et al.*, 2020; Wang *et al.*, 2020b). The diverse functions of the MICU family proteins maintain normal $[Ca^{2+}]m$ levels under the resting conditions and enable prompt activation of MCU to mediate rapid mitochondrial Ca^{2+} uptake (Cui *et al.*, 2019).

MCUR1 (known as *CCDC90A*, 40 kDa protein), which consists of two transmembrane domains and one coiled-coil region, was also demonstrated as a regulatory component of the MCU complex (Mallilankaraman *et al.*, 2012). More recently, it was shown that MCUR1 binds to the MCU-pore and EMRE through their coiled-coil domains that stabilize the MCU complex (Tomar *et al.*, 2016).

SLC25A23, which belongs to a family of Mg-ATP/Pi solute carriers, was also proposed as an essential component for MCU complex (Hoffman *et al.*, 2014; Bassi *et al.*, 2005; Krebs, 2017). A mutation of the EF-hand domain of SLC25A23 reduces mitochondrial Ca^{2+} accumulation, but whether this depends on a direct MCU activity regulation or whether it affects mitochondrial bioenergetics or mitochondrial Ca^{2+} buffering capacity is still debated (Bassi *et al.*, 2005; Mammucari *et al.*, 2018; Rueda *et al.*, 2015). In addition to MCU, there are other mitochondrial Ca^{2+} influx mechanisms, including the mRYR, RaM, and LETM1, which all have unique biophysical properties other than MCU (Gunter & Gunter, 2001; Beutner *et al.*, 2005; Jiang, Zhao & Clapham, 2009; Mammucari *et al.*, 2018; Elustondo *et al.*, 2017; Krebs, 2017).

mRyR is the largest known ion channel of about >2MDa, localized in the IMM, and can be an alternative mechanism for mitochondrial Ca^{2+} uptake, especially in the mitochondria of cardiac and neuronal cells (Jakob *et al.*, 2014; Beutner *et al.*, 2001; Beutner *et al.*, 2005). Three different subtypes of RyR isoforms (RyR1, RyR2, and RyR3) with different pharmacological properties and tissue-specific expression have been described. RyR1, the primary isoform in the skeletal muscle, was identified in the

IMM of isolated heart mitochondria through performing [^{3}H]ryanodine binding, immunogold labeling and Western blot techniques, and it is thought to mediate ryanodine-sensitive, rapid mitochondrial Ca^{2+} transport and believed to play a central role in mitochondrial Ca^{2+} uptake (Beutner *et al.*, 2001; Beutner *et al.*, 2005). RyR2 is mostly presented in cardiac muscle cells (Bhat *et al.*, 1999), while RyR3 is widely expressed in the endoplasmic reticulum (ER) of different vertebrate tissues (Giannini *et al.*, 1995) and may be co-expressed with RyR1 and RyR2. Besides this, there are suggestions that under certain situations (e.g. mitochondrial Ca^{2+} overload), mRyR channels may also mediate Ca^{2+} efflux (Ryu *et al.*, 2010).

In isolated heart mitochondria, RaM has been described as an additional mechanism for Ca^{2+} transport, capable of sequestering significant amounts of Ca^{2+} hundreds of times faster than the MCU (Gunter & Gunter, 2001). RaM is activated only transiently, facilitates the rapid sequestration of Ca^{2+} by mitochondria at the beginning of each cytosolic Ca^{2+} pulse, and rapidly recovers between pulses, which allows mitochondria to respond to repetitive Ca^{2+} transients (Sparagna *et al.*, 1995). In contrast to the MCU, this transporter is activated at much lower Ca^{2+} concentrations than the MCU - 50 to 100 nM vs. greater than 500 nM for the MCU (Sparagna *et al.*, 1995). However, a protein responsible for this rapid mode of Ca^{2+} uptake has not been identified, although it has been speculated that the RaM comprises a sub-state of MCU operation since both modes are inhibited by ruthenium red and RaM activity does not appear to be present in MCU-knockout mitochondria (Baughman *et al.*, 2011; De Stefani *et al.*, 2011).

LETM1 was initially identified as a K^+/H^+ exchanger; however, it was later reported as a Ca^{2+}/H^+ antiporter, localized at the inner mitochondrial membrane (Jiang *et al.*, 2009). LETM1 transports Ca^{2+} bidirectionally across the inner membrane, depending on the pH gradient, and is inhibited by ruthenium red (Jiang *et al.*, 2009). However, further studies are needed for correct characterization of its role in Ca^{2+} transport, as well as sensitivity to ruthenium red, in face of the recent demonstrations, in which LETM1 protein was reconstituted in liposomes and was demonstrated as a ruthenium red-insensitive electroneutral $Ca^{2+}/2H^+$ antiporter (Tsai *et al.*, 2014). Besides, other studies have reinforced the role of LETM1 in K^+ homeostasis and suggested LETM1 as an electroneutral proton-potassium(H^+/K^+) exchanger (Nowikovsky & Bernardi, 2014). This hypothesis was supported by other results, which showed that LETM1 was not responsible for extruding Ca^{2+} from the mitochondria (De

Marchi *et al.*, 2014b). Moreover, there are some suggestions that the role of LETM1 could change based on specific conditions (J, Pan & Sheu, 2012; Austin & Nowikovsky, 2019).

(1) Mitochondrial calcium efflux

While the biochemical characteristics and physiological functions of the mitochondrial systems for Ca^{2+}-influx have been widely studied, the understanding of the molecular nature and properties of the mitochondrial Ca^{2+}-efflux systems have just begun, although functional characterization of the release system started in the 1970s (Carafoli *et al.*, 1974). Currently, two separate mechanisms have been proposed to account for Ca^{2+} extrusion from the mitochondrial matrix: Na^{+}-dependent (NCXm) and Na^{+}-independent (HCXm). In most cells, the main mechanism of Ca^{2+} extrusion from mitochondria is the NCXm. Although Na^{+}-dependent Ca^{2+} efflux from mitochondria was first discovered in isolated rat heart mitochondria and was described as the mitochondrial Na^{+}/Ca^{2+} exchanger years ago (Carafoli *et al.*, 1974), its molecular identity was also only recently resolved (Palty *et al.*, 2010). It seems to extrude Ca^{2+} from the mitochondrial matrix to the intermembrane space and more specifically to constitute a $Na^{+}/Ca^{2+}/Li^{+}$ exchanger in the IMM (Palty, Hershfinkel & Sekler, 2012; Palty *et al.*, 2010). This Na^{+}-dependent Ca^{2+} exchange activity is benzodiazepine- and CGP-37157-sensitive and found in a wide variety of tissues and is dominant in the heart, brain, skeletal muscle, parotid gland, adrenal cortex, and brown fat (Gunter *et al.*, 2004; Takeuchi, Kim & Matsuoka, 2015), being also present in liver, kidney, and lung mitochondria, although its activity is lower (Haworth, Hunter & Berkoff, 1980). In those tissues in which the activity of NCXm is lower, HCXm activity as Ca^{2+} efflux mitochondrial system is the dominant (Gunter & Pfeiffer, 1990; Krebs, 2017). The NCX is active primarily in excitable cells and in contrast to the plasma membrane Na^{+}/Ca^{2+} exchanger, it has a unique characteristic – the ability of additional transport of Li^{+} ions (Palty *et al.*, 2004; Carafoli *et al.*, 1974). The stoichiometry (ion-exchange ratio) and the electrogenicity of the mitochondrial NCXm were controversial, but it was believed to be electroneutral (Affolter & Carafoli, 1980; Wingrove & Gunter, 1986b). By using permeabilized rat ventricular myocytes, it was demonstrated that the NCXm is voltage-dependent and electrogenic, which suggests the stoichiometry is higher than $3Na^{+}$ for one Ca^{2+} (Kim & Matsuoka, 2008). This stoichiometry and electrogenic nature of the NCXm was proved recently by using the whole-mitoplast patch-clamp technique

(Islam, Takeuchi & Matsuoka, 2020). Detailed mechanisms of the regulation of NCXm activity and sensitivity to the effectors have not yet been clarified, but some studies demonstrate its regulation by a stomatin-like protein 2 (Da Cruz *et al.*, 2010) and mitochondrial kinase PINK1 (Gandhi *et al.*, 2009). In the tissues with low NCXm activity, HCXm has a dominant effect on the release of Ca^{2+} from mitochondria (Gunter & Pfeiffer, 1990). This HCXm operation mode is relevant to the liver, kidney, lung, and smooth muscle (Takeuchi *et al.*, 2015). The molecular identity of the HCXm is still debated, but most likely its activity is electroneutral with the stoichiometry $2H^+$ for Ca^{2+} (Gunter, Zuscik & Gunter, 1991). Moreover, studies of HCXm activity in rat isolated liver mitochondria demonstrated that the rate of efflux via HCXm decreases with increasing pH gradient and suggests that this mechanism is an active Ca^{2+} for $2H^+$ exchanger and not a passive Ca^{2+} for $2H^+$ exchanger (Gunter *et al.*, 1991).

LETM1 is considered an additional and/or alternative mechanism of Ca^{2+} efflux from mitochondria (Takeuchi *et al.*, 2015; Krebs, 2017; Nowikovsky *et al.*, 2012; Austin & Nowikovsky, 2019). Ca^{2+} transport process through the IMM could be mediated by LETM1, since this protein functions as a Ca^{2+}/H^+ antiporter under certain conditions (Mailloux & Harper, 2011). In 2009, by using digitonin-permeabilized S2 or 293 cells expressing the mitochondrial Ca^{2+} sensor protein pericam, and by using purified 1 reconstituted in liposomes, it was found that this protein mediates H^+– Ca^{2+} exchange (Jiang *et al.*, 2009). Later, in 2014, by using LETM1 reconstituted proteoliposome, it was reported that LETM1 mediates the electroneutral $2H^+/Ca^{2+}$ antiport, which is insensitive to ruthenium red (Tsai *et al.*, 2014). Further studies, focused on the intracellular-store-dependent Ca^{2+} dynamics, provide evidence that LETM1 acts as the $2H^+/Ca^{2+}$ exchanger (Huang *et al.*, 2017). Combined with a theoretical analysis (Nowikovsky *et al.*, 2012), this leads credibility to the role of LETM1 as an important Ca^{2+} efflux mechanism.

Another important mechanism for Ca^{2+} release from mitochondria is now suggested as the transient open form of the mPTP. Under pathophysiological conditions, in which this high-conductance non-specific pore opens, it may function as a Ca^{2+}-efflux system (Biasutto *et al.*, 2016; Hurst, Hoek & Sheu, 2017; Takeuchi *et al.*, 2015; Britti *et al.*, 2018; Briston *et al.*, 2019). The opening of the mPTP is directly regulated by the concentration of free calcium level, triggering by Ca^{2+} overload and allowing for rapid Ca^{2+} efflux (Hurst *et al.*, 2017). Detailed information about the function of mPTP as the mechanism of Ca^{2+} transport is available later in this text.

In summary, it is important to note that the localization of mitochondria within the cell is a crucial factor for mitochondrial Ca^{2+} uptake. It is now well accepted that the location of mitochondria in proximity to the plasma membrane or the ER/sarcoplasmic reticulum (SR) is essential in modulating a variety of cellular functions, as well as Ca^{2+} transporting to subcellular structures, in particular to mitochondria (Stefan, 2018; Rowland & Voeltz, 2012; van Vliet, Verfaillie & Agostinis, 2014; Silva *et al.*, 2020). These interactions between mitochondria and the ER/SR have been identified and described as physiological and pathophysiological significant for Ca^{2+} crosstalk between mitochondria and other cellular and subcellular structures (Rieusset, 2018; Rowland & Voeltz, 2012; van Vliet *et al.*, 2014; Takeuchi *et al.*, 2015; Giacomello *et al.*, 2010; Yousuf *et al.*, 2020). During Ca^{2+}-flowing through the plasma membrane or its release from the ER/SR, these interactions promote Ca^{2+} influx to the neighboring mitochondria, determining the particular properties of Ca^{2+} transport into these organelles (Lawrie, Zolle & Simpson, 1997; Giacomello *et al.*, 2010; Park *et al.*, 2001). There is some evidence of Ca^{2+}channeling through plasma membrane Ca^{2+} channels to closely-located mitochondria, as well as in the opposite direction (Korzeniowski *et al.*, 2009). Mitochondria - ER/SR communication is also reported as an important regulatory factor for the variety of cellular processes, including Ca^{2+} signaling, lipid biosynthesis, or mitochondrial division (Rieusset, 2018; Friedman *et al.*, 2011; Namgaladze, Khodzhaeva & Brune, 2019; Granatiero *et al.*, 2019). It is now clear that bidirectional Ca^{2+} crosstalk between both mitochondria and the plasma membrane, and mitochondria and ER/SR, is crucial for the regulation of the wide range of cellular functions.

(2). The early observations: Ca^{2+} induces mitochondrial swelling

Hunter at al. were the first to introduce the concept of Ca^{2+}-induced mitochondrial swelling (Hunter, Haworth & Southard, 1976). In a series of seminal experiments performed on isolated heavy beef heart mitochondria, the authors observed that when low levels of Ca^{2+} were added, mitochondria would go through a configurational transition from a shrunken matrix and large intracristal spaces to a swollen matrix with decreased intracristal spaces (Figure 2). Mitochondria were described to transit from an aggregated to an orthodox configuration in this process. By using electron microscopy images, it was concluded that for each time point post- Ca^{2+} addition, the mitochondrial population was shown to be heterogeneous with only the

two different configurations mentioned. Facing these observations, the authors assumed that the transition sequence for each mitochondrion consisted of a lag phase in the aggregated form followed by a sudden transformation to the orthodox form.

To assess the correlation between this configurational change and membrane permeability, Hunter et al. measured the permeability of mitochondrial membranes of isolated heavy beef heart mitochondria to [^{14}C]sucrose in the presence of Ca^{2+}. In samples taken at the same time points like the ones used for the electron microscopy imaging, it was observed that the permeability to [^{14}C]sucrose increased simultaneously with the decrease of the number of aggregated mitochondria. This result was very important since it was concluded that Ca^{2+}increased the permeability to [^{14}C]sucrose. Important was also the notion that the swelling that follows the addition of Ca^{2+} was caused by mitochondrial osmotic water influx that accompanies sucrose entry into the matrix space (Hunter *et al.*, 1976).

Another experimental question regarded the effect of this inner membrane permeability on mitochondrial respiratory activity. The uncoupler respiratory control index, meaning the magnitude of the respiration increase, was compared with configurational changes at several time points after the addition of Ca^{2+}. From this analysis, the conclusion was that coupling in an individual mitochondrion follows an all-or-nothing law, exactly as for the configurational transition. However, experiments using hypotonically swollen mitochondria led to the conclusion that the changes in coupling are caused by changes in the permeability of the IMM, rather than changes in configurational transition (Hunter *et al.*, 1976). This clarified certain misconceptions supplied by other authors (Lehninger, 1962; W.Harman & Feigelson, 1952), who proposed that mitochondrial swelling by itself does not determine mitochondrial functioning. It was also observed in the same work that the transition was reversible, and that arsenate, phosphate and fatty acids induced that phenomenon.

In later studies by Hunter and Haworth, light scattering has been used to measure mitochondrial swelling, based on the changes in pseudo-absorbance of a mitochondrial suspension from heavy beef heart. By using this methodology, still used nowadays in isolated mitochondrial fractions, the authors determined that mitochondrial metabolites and small molecules and ions can counteract the Ca^{2+}-induced membrane transition (Hunter & Haworth, 1979a). In another seminal work that opened the way for hundreds of studies regarding the regulation of this phenomenon, the authors concluded that endogenous NADH, ADP, and Mg^{2+} prevented the mPT. Also, the same effect was

achieved with mitochondrial energization (Hunter *et al.*, 1976). Following these pioneer discoveries, the authors next explored a logical question: what would be the mechanism by which Ca^{2+} leads to this mPT? And how inhibitors would work? (Haworth & Hunter, 1979).

One important observation was that mitochondria from the heavy beef heart which had been previously submitted to Ca^{2+}-induced mPT, would lose their endogenous protection. Also, the concentration of Ca^{2+} present in the incubated medium was able to regulate in a dose-dependent manner the permeability of the IMM, as seen by measuring light scattering in suspensions titrated with different concentrations of Ca^{2+}. Along with the fact that the membrane transition needed no energy source (Hunter & Haworth, 1979a), this observation led to the conclusion that the physical binding of Ca^{2+} to units in the IMM was enough to cause the mPT. Moreover, an important observation was that each preventive agent showed an apparent mode of action when counteracting the membrane transition. For example, Mg^{2+} was observed to competitively inhibit the transition. The authors had already proposed that Mg^{2+} would bind to trigger sites, where Ca^{2+}, with an apparent higher affinity for those sites, would exclude magnesium and induce the transition (Hunter *et al.*, 1976). The effects of ADP and NADH were only investigated in more detail later (Haworth *et al.*, 1980). ADP and NADH were measured to have a synergistic effect on inhibiting the Ca^{2+}-induced transition in isolated beef heart mitochondria. ADP was thought to have an inner binding site and which, when bound to that nucleotide, would significantly inhibit binding of Ca^{2+} to the trigger site and therefore inhibit the transition. Inhibition of NADH was observed to be diminished by NADPH (Haworth *et al.*, 1980). Several other cations were also identified as inhibitors of the modulating effects of Ca^{2+} (Hunter & Haworth, 1979a). Divalent cations, such as strontium and manganese, as well as monovalent cations, potassium, sodium, and tetramethylammonium (TMA), exhibited competitive inhibition. The trivalent metal inhibitor lanthanum ion also exhibited competitive inhibition more potent than the mono and divalent cations. Another competitive inhibitor included protons, H^{+}, which would be the basis for studies that many years later involved the inhibition of the mPT by ischemia-induced cytosolic acidification (Qian *et al.*, 1997). These observations suggested that the trigger site could be the same involved in high-affinity Ca^{2+} uptake. However, Ca^{2+}-induced transition progression was found to be ruthenium red insensitive. Because high-affinity Ca^{2+} uptake is ruthenium red-sensitive (Moore, 1971), the authors assumed that another

mitochondrial site, besides the one involved in high-affinity Ca^{2+} uptake, should be involved in the transition.

Hunter and Haworth further investigated mechanisms associated with the mPT induced by Ca^{2+} in isolated heavy beef heart mitochondria (Hunter & Haworth, 1979b), including the pathways for the release of Ca^{2+} that occurred during the transition. The authors concluded that Ca^{2+} release was due to Ca^{2+}-induced transition because of several indications: a) agents that blocked the transition, such as Mg^{2+} ADP and bovine serum albumin (BSA), inhibited Ca^{2+} release under steady-state respiration, b) mitochondria that accumulated Sr^{2+}, did not release it afterward, meaning that the spontaneous release mechanism was selective for Ca^{2+}, similar to the transition, c) by using light scattering and electron microscopy to assess the configurational change during Ca^{2+} release, the authors observed the transition to the orthodox state at the same time Ca^{2+} was released. Since transition implies an aggregated-to-orthodox change of configuration, this further supports the hypothesis that Ca^{2+} release is due to the progression of mPT. Another clue was that the kinetics of Ca^{2+} release was similar to the transition one. By measuring $^{45}Ca^{2+}$ fluxes, it was observed that mitochondria release their entire pool of Ca^{2+} all at once, following a lag phase without any release, just as the transition. In the same paper, the authors investigated the release of Ca^{2+} induced by the addition of an uncoupler, a molecule which dissipated the transmembrane electric potential ($\Delta\Psi$) (Hunter & Haworth, 1979b). It was observed that as the amount of Ca^{2+} accumulated in mitochondria increased, so did the amount of Ca^{2+} released by the addition of the FCCP uncoupler. The addition of the uncoupler provoked a configurational change from the aggregated form to the orthodox, i.e. a permeability transition was observed. However, the percentage of mitochondria that underwent the transition was higher in populations with higher levels of Ca^{2+} accumulation. Similar to the spontaneous release and the kinetics of the transition, also uncoupler-induced Ca^{2+}-dependent Ca^{2+} release is sudden and total and follows a lag phase of no release. Also, in conditions where the NADH protective mechanism was lost, mitochondria were observed to transition to an orthodox conformation without the requirement of additional Ca^{2+}, and to release Ca^{2+} much faster than mitochondria, which still contained a NADH-dependent protective mechanism, thus implying that the release depended on the characterized permeability transition.

Moreover, Sr^{2+} release was seen to be dependent on Ca^{2+} accumulation and was ruthenium red insensitive, both facts being characteristic of the transition. Because of all

these reasons, the uncoupler-induced release of Ca^{2+} was found to be another consequence of Ca^{2+}-induced transition, but dependent on the level of Ca^{2+} accumulated.

Furthermore, the work explored whether the release of Ca^{2+} caused by Na^{+} was mediated by the Ca^{2+}-induced transition (Hunter & Haworth, 1979b). Because no mitochondrial configurational changes upon addition of Na^{+} and consequent Ca^{2+} release were observed, it was concluded that not all release of Ca^{2+} from mitochondria depends on mPT. In this particular case, Na^{+}-induced Ca^{2+} release was later well-described as originating from a protein membrane exchanger (Wacquier *et al.*, 2017).

III. From the initial observations to the composition and regulation of the permeability transition: an evolving model

Looking back over the past three decades, the mPTP structure was an evolving entity; nowadays, only some modulators are known and pore-forming parts including what dynamics they take shape, remain elusive. Overall, the knowledge of the mPTP complex (mMPTP) composition and assembly has been improved according to the technology used. Similarly, the understanding of its regulation in intact cells and tissues was almost completely absent in early works, as useful techniques have become available only recently. Thus, first findings have been obtained mainly from the use of isolated mitochondria from animal organs (e.g. heart and liver from mice, rats, and beef). Studies by J. Raaflaub (Raaflaub, 1953) and Albert L. Lehninger (Lehninger & Remmert, 1959; Lehninger, 1962) have shown that isolated liver mitochondria can take up water, increasing its volume. At that time this process was easily followed by gravimetric or optical methods. The latter ones based on measuring either light transmittance (transparency) or light scattering (opacity, turbidity) of mitochondrial suspensions. Interestingly, with the same methods, A.L. Lehninger also observed the reverse process, for which he coined the name contraction (Lehninger, 1959). This effect consisted of the extrusion of water from mitochondria, resulting in a decrease of mitochondrial volume manifested by increased turbidity (light scattering) of mitochondrial suspensions.

Mitochondrial swelling described by Lehninger and co-workers, and subsequently studied by Azzi and Azzone (Azzi & Azzone, 1965a) could be termed “large amplitude swelling” as it eventually led to complete depletion of the OMM and the formation of IMM ghosts of low light absorbance. In contrast “low amplitude

swelling", first observed by Chance and Packer (Chance & Packer, 1958) and later on studied by Azzi and Azzone (Azzi & Azzone, 1965b), was a fully reversible and depicted variation in the energy and metabolic state of mitochondria. These low amplitude changes in the mitochondrial volume and structure could also be observed inside living cells (Hackenbrock *et al.*, 1971). The term 'permeability transition' of the IMM was probably first used by Wingrove and Gunter (Wingrove & Gunter, 1986a) in 1986. The large- and (most likely) the low-amplitude mitochondrial swelling could be interpreted as resulting from an unspecific increase of the permeability of the inner mitochondrial membrane to low molecular weight solutes. As effect, their concentrations inside and outside mitochondria equilibrated, whereas the concentration of high molecular weight compounds, in particular, soluble intramitochondrial proteins, remained not much changed. This led to a higher osmotic pressure (the so-called colloidal or oncotic pressure) inside mitochondria, resulting in the influx of water.

In contrast to the mitochondrial swelling, mitochondrial contraction was assumed to be an active process, connected with the energization of these organelles (Lehninger, 1959). While mitochondrial swelling was accompanied by release of non-esterified fatty acids, their contraction was paralleled by re-incorporation of these fatty acids into mitochondrial phospholipids. This was demonstrated using isotopically labeled both [^{14}C]oleate and glycerol 3-[^{32}P]phosphate (Wojtczak, Wlodawer & Zborowski, 1963). These studies suggested that contraction was enabled not only due to the removal of free fatty acids that have accumulated but also by restoration of some lipidic compounds indispensable for the original low permeability of the inner membrane.

A systematic study using electron microscopy revealed that a swelling-related increase of the mitochondrial matrix volume is accompanied by the rupture of the OMM (Wlodawer *et al.*, 1966) (Figure 2). The ATP-induced contraction was reflected by matrix condensation, which, however, never led to the restoration of the original structure of intact mitochondria. Impermeability of the OMM to cytochrome c (Wojtczak & Zaluska, 1969; Wojtczak & Sottocasa, 1972) led to the development of an assay for its intactness in preparations of isolated mitochondria that was based on oxidation of externally added reduced cytochrome c (Wojtczak *et al.*, 1972). The same protocol can be used to detect release of cytochrome c from mitochondria that might occur during swelling of that organelle. In living cells, release of cytochrome c to the cytosol accompanied by the rupture of the outer membrane (Vander Heiden *et al.*, 1997;

Petit *et al.*, 1998) can be investigated with the use of antibodies against cytochrome c; however, this requires isolation of cytosolic fractions. It is also important to mention that other possible ways of releasing cytochrome c from mitochondria into the cytosol without swelling and rupture of the OMM have also been proposed (Wieckowski *et al.*, 2001).

Largely, experiments on isolated mitochondria have been carried out by Haworth and Hunter in the late 1970s (Hunter & Haworth, 1979a; Haworth & Hunter, 1979; Hunter & Haworth, 1979b) in the effort to understand molecular mechanisms underlying the mPTP, its regulation, as well as the biological function of the transition in a "controlled" environment represented by the isolated and de-energized organelles. Indeed, although they are artificially devoid of endogenous substrates, de-energized mitochondria preserved a permeability transition leading to the analysis in an ideally variable-free environment. Methods applied to isolated mitochondria had partly replaced previous enzymatic reactions performed, for instance, to ashed preparations from rat heart preparations that were often inaccurate and difficult to performed (Slater & Cleland, 1953). Although they allowed Cleland and Slater to observe a Ca^{2+}-dependent swelling effect already in 1953 (Slater & Cleland, 1953), studies on isolated mitochondria led Haworth and Hunter to the demonstration of a sudden opening of a reversible permeability state of the IMM, termed as "Ca^{2+}-induced transition" (Hunter *et al.*, 1976), and enhancement of that effect when concentration of phosphates, arsenate and fatty acids increased (Hunter & Haworth, 1979a). The possibility to add different substrates to mitochondrial preparations to identify regulatory properties allowed understanding also protective mechanisms against mPT opening such as Mg^{2+} treatment, that competes with Ca^{2+} for a shared binding site inside mitochondria. Thus, Ca^{2+} was considered the main positive modulator for mPT (and still is) (Hunter *et al.*, 1976; Azzi & Azzone, 1966). Other protective mechanisms included the reduction status of mitochondrial NAD^{+}, to the compound bongkrekic acid and to the energetic state of mitochondria (Hunter *et al.*, 1976). This evidence was confirmed in the early 1990s by a paper in which Ca^{2+}-dependent swelling was efficiently inhibited by bongkrekic acid administration, ADP-based medium and the immunosuppressant Cyclosporin-A (Halestrap & Davidson, 1990) giving evidence the technique used was useful to study the opening of this unselective pore and highlighted a regulatory role for the adenine nucleotide translocator (ANT) and cyclophilin D (CypD), the molecular target of Cyclosporin-A.

Critical for modern studies was the description of two ways by which a non-specific pore opens: the one caused by low physiological Ca^{2+} concentrations involving PPi-dependent mechanism, insensitive to Cyclosporin-A, and the second followed by higher Ca^{2+} concentrations and Cyclosporin-A-sensitive (Davidson & Halestrap, 1987), and the conjecture of a model by which the pore opens, based on the CypD-ANT protein interaction in the presence of Ca^{2+} overload (Halestrap & Davidson, 1990). In this scenario, Cyclosporin-A, by binding CypD, would promote its dissociation from the translocator and block the pore. This notion, together with the reported ability for ADP, ATP, and bongkrekic acid to strongly inhibit mPTP opening, their feature to bind the ANT carrier and reversing its conformation caused by Ca^{2+} addition, led researchers to the understanding that this carrier may physically form and thus influences Ca^{2+}-dependent mPT. In support of that model, the combination of isolated mitochondria preparations and patch-clamp techniques allowed monitoring mPTP properties of ANT oligomers in artificial membranes (Brustovetsky & Klingenberg, 1996).

It was also suggested that the mPTP might result from structural and functional 'cooperation' between the inner and the outer mitochondrial membranes (McEnery *et al.*, 1992; Kinnally *et al.*, 1993; Kottke *et al.*, 1988). This view represented, among others, by Dieter Brdiczka pointed to the contact sites between the two membranes as possible loci regulating not only the permeability but also metabolic and energetic functions of mitochondria (Nicolay *et al.*, 1990; Bucheler, Adams & Brdiczka, 1991; Wieckowski, Brdiczka & Wojtczak, 2000). Arguments that mPTP is located at the junction between the two membranes were essentially based on observations that proteins supposedly participating in this pore came from both the OMM and IMM.

As previously reported, early studies on isolated mitochondria identified putative proteinaceous channels between the IMM and the OMM formed by the ANT and VDAC as the core components of the mPTP; encompassed by a plethora of regulators, including the OMM 18-kDa peripheral benzodiazepine receptor (TSPO), glycogen synthase kinase 3β (GSK3β), hexokinase II (HKII) and creatine kinase (CK) proteins (Morciano *et al.*, 2015; Tanaka *et al.*, 2018; Ong *et al.*, 2014). Although the VDAC enables the transport of most solutes via IMM, the new era of genetic studies challenged this idea on the pore structure of mPTP (Baines *et al.*, 2007) demonstrating that mitochondria isolated from VDAC-KO mice still exhibit a Ca^{2+}-dependent mPT very similar to that found in wild-type mitochondria. Similarly, the cell death was unaltered in VDAC KO cells. Nevertheless, gene inactivation studies from the Wallace

group based on knockout (KO) animal models reconsidered this model, confirming the presence of a functional mPTP also in ANT-KO mice excluding it as pore-forming part of mPTP (Kokoszka *et al.*, 2004) and conferring to CypD more important modulatory roles (Schinzel *et al.*, 2005; Hurst *et al.*, 2020). These groundbreaking experiments taking advantage of genetic models have pointed out in a direct way that VDACs (believed up to then to form the "core" of the pore) are not an essential component of the mPTP. About ANT, the innovative generation in 2019 of a triple KO for Ant1, Ant2 and Ant4 revisited once again the contribution of the translocator in the mPTP cascade; indeed, the progressive deletion of all isoforms led to an equal decrease of the sensibilization of the pore to open (Karch *et al.*, 2019) hazarding the statement that ANT, under favorable biochemical conditions and in a given tissue, may constitute the right alternative to ATP synthase to form the mPTP (see later).

From the Brdiczka's model, CypD remained the only protein which involvement in the mPTP formation remained unquestioned because experiments performed with transgenic mice lacking the peptidylprolyl isomerase f (*Ppif*) gene have confirmed that this protein is the key element of mPTP that is responsible for its sensitivity to Cyclosporin-A (Basso *et al.*, 2005). However, it should be pointed out that CypD plays rather a regulatory role and is not involved in the pore formation (Fayaz, Raj & Krishnamurthy, 2015). The elucidation of this regulatory role in mPTP opening is twenty years old, facilitated by the discovery that the gold-standard inhibitor of mPTP, CsA targeted such protein. To date multiple investigations have repeatedly reported that CypD inactivation (by means of genetic or pharmacological approaches) severely impairs mPTP induction and cell death in a number of *in vitro* and *in vivo* models. In addition, signaling events can target CypD to ultimately regulate mPTP.

In the early 1990s, TSPO, has been found to interact with ANT and VDAC proteins (McEnery *et al.*, 1992). In those years, studies on IMM-OMM contact sites were of great interest in this field, and ligands to TSPO with nanomolar affinity and joined to VDAC/ANT proteins have been shown to own mPTP-like channel activities, as recorded by patch-clamp (Kinnally *et al.*, 1993). However, biochemical attempts to identify the core complex of the pore vanished also for the TSPO translocator when its conditional deletion led the researchers to conclude that TSPO is dispensable for mPTP regulation and does not participate in mPTP-dependent cell death (Sileikyte *et al.*, 2014).

As a next step to understand the architecture of the mPTP channel, it has been proposed independently by two laboratories (Leung, Varanyuwatana & Halestrap, 2008; Alcala *et al.*, 2008) that the phosphate carrier (PiC) could be a good candidate to form the "core" of mPTP. The concentration-dependent inhibitory effect of NEM, UQo, and Ro 68-3400 on mPTP and PiC suggested that PiC provides a pore-forming component. This was reinforced by the fact that phosphates greatly enhanced mPTP opening, so it was believed that an additional protein of the IMM would represent the putative pore-forming part, the PiC. Not without surprise, later on, Halestrap (Varanyuwatana & Halestrap, 2012) questioned his concept showing that decreasing expression of the PiC in HeLa cells by 70% or more did not affect mPTP opening. This was also confirmed by other authors using KO models (Gutierrez-Aguilar *et al.*, 2014). Interestingly, the involvement of the PiC in PTP could not be fully excluded because a complete genetic deletion of this carrier in mouse cardiac mitochondria desensitizes the mPTP (Kwong *et al.*, 2014). The PiC was suggested to be, not a core component of the mPTP, but rather a regulatory component (Kwong *et al.*, 2014).

In another perspective, Molkentin's group provided evidence for Bax and Bak members of the Bcl-2 family as mPTP components (Karch *et al.*, 2013); taking advantage of the use of transgenic animal models, they demonstrated that the absence of both proteins promoted resistance to mitochondrial swelling and resulted in the lack of channel activity, as evidenced by patch-clamp on mitoplasts (Karch *et al.*, 2013). Thus, even though Bax and Bak play a structural role in the OMM part of the mPTP complex (mPTP), an increase in solutes amount of the mitochondrial matrix requires also the permeability of the IMM. This model of the pore suggested a subdivision between IMM- and OMM-pore formation, taking up some concepts formulated by Brdiczka's group in the 1990s on the IMM-OMM contact sites in mPTP assembly (Beutner *et al.*, 1998). Despite solid data supporting the contact site model of mPTP, another model of mPTP formation has been proposed (Kowaltowski, Castilho & Vercesi, 2001; He & Lemasters, 2002). This model assumed that the pore could be formed by misfolded mitochondrial proteins modified, among other, by oxidative damage and not connected with the opening of an already pre-existing inner membrane pore. Moreover, it has been proposed that opening of such unregulated pores occurs when the number of amphipathic protein clusters exceeds the number of chaperones available to block their conductance.

Although Ca^{2+} overload is widely recognized as the first inducer of mPT, other positive regulators have been reported. In a seminal paper by Halestrap et al., it has been demonstrated the presence of an additional and fine mediator of mPT regulation in rat heart and liver mitochondria, pH (Halestrap, 1991). One of the main methods that can be performed with isolated mitochondria, the mitochondrial swelling assay, records a decrease in the absorbance at 520/540 nm, which indicates mitochondrial swelling, when Ca^{2+} has been administered to preparations at pH 7.4. In contrast, mPTP opening was inhibited at acidic matrix pH because of the displacement of Ca^{2+} in the trigger site by H^{+} ions (Halestrap, 1991). Atractyloside for instance, a natural toxic glycoside, is an agent that increases mPTP opening by modifying ANT conformational state locking in its cytoplasmic-side open conformation; then ROS production and low mitochondrial membrane potential (MMP) are two others main inducers. Mitochondria are an important source of ROS (Giorgi *et al.*, 2018c) and we know that conditions with increased oxidative stress in cells, such as hypoxia-reoxygenation, strongly sensitize mPTP opening and consequent cell demise (Assaly *et al.*, 2012). Thus, it is a classical notion that the mPT is related with the redox state of mitochondria, including that of coenzyme Q (Kowaltowski, Castilho & Vercesi, 1995), and NADPH (Bernardes *et al.*, 1994), with mitochondrial oxidative stress playing an important role even in de-energized mitochondria (Kowaltowski, Castilho & Vercesi, 1996; Vercesi *et al.*, 2018).

A noteworthy role is also played by thiol groups of proteins localized in the IMM. In this regard, Halestrap et al. showed in 1994 how the exogenous administration of thiol oxidizing reagents to purified mitochondria preparations promoted key modifications in Cys56, Cys159 and Cys256 residues of the ADP/ATP translocator; particularly, Cys56 oxidation, in turn, potentiated the binding of mitochondrial CypD with the IMM leading to pore opening (Connern & Halestrap, 1994). Otherwise, the other sites are described to be involved in antagonizing ADP inhibitory properties via the nucleotide-binding site on ANT protein. This was observed by adding phenylarsine oxide (PheArs) to de-energized mitochondria with an effect independent of CypD binding previously mentioned (Halestrap, Woodfield & Connern, 1997). Not surprisingly, all these effects were reversed by the use of antioxidants (Kowaltowski *et al.*, 2001; Vercesi *et al.*, 2018). Further, the evidence given by Fontaine's group on mPTP inhibition operated by the use of rotenone and that mPT is sensitive to inorganic phosphates (Pi) prompted to the idea that NADH:ubiquinone oxidoreductase (Complex I) could be implied in mPTP negative modulation, establishing a novel level of

regulation. This would occur via conformational changes of Complex I with consequent interaction with the mPTP, depending on the availability of Pi and CypD expression (Li *et al.*, 2012).

The open conformations of the mPTP just indicated may assume a low or a high conductance. The ability to switch from a low to a high conductance has been thoroughly studied in the 90's by Ichas et al., who also reported the irreversibility nature of the transition once mPTP reached the high state (Ichas & Mazat, 1998). Historically, physiological roles have been attributed to the lower open conformation; indeed, mitochondrial functions are preserved while the so-called flickering mode guaranteed cellular Ca^{2+} homeostasis (Gunter & Pfeiffer, 1990; Altschuld *et al.*, 1992) with very limited diffusion of solutes through the IMM (cutoff <300 Da) and a fine regulation by matrix pH changes and mitochondrial Ca^{2+} uptake (Ichas, Jouaville & Mazat, 1997). Conversely, when mPTP switches to the high conductance state, it leads to consequences that are not compatible with cell life such as the irreversible dissipation of mitochondrial membrane, the activation of the apoptotic cascade and the rupture of the OMM. This conformation was the first to be identified for the mPTP thanks to Hunter and Haworth's studies on isolated mitochondria in 1976-1979, giving the IMM an increased and unselective permeability to solutes of 1500 Da that determines mitochondrial swelling.

The understanding process leading to the association of ATP synthase to mMPTP, which would occur under favorable conditions, started with the discovery of some regulatory analogies between the two complexes. Two mPTP inhibitors (ADP and Mg^{2+}) interact and block the hydrolytic activity of the Complex V (Feniouk, Suzuki & Yoshida, 2006), while phosphates, known positive regulators of the pore opening, reverted this state. In addition, the previously mentioned mPTP subunits, ANT, and PiC, form the so-called ATP synthasome by interacting with ATP synthase (Ko *et al.*, 2003). Similarly, mitochondrial CypD, the target of the known mPTP inhibitor Cyclosporin-A, has been demonstrated to interact with the peripheral stalk of ATP synthase, the oligomycin sensitivity conferring protein (OSCP) (Giorgio *et al.*, 2009). This protein is able to modulate ATP synthase activity by decreasing it,when mtCypD is anchored and to finely regulate mPTP opening (and cell death) by the C141 and H112 residues, respectively involved in the sensibility of the pore to oxidation and to be dependent from H^+ binding (Carraro *et al.*, 2020; Antoniel *et al.*, 2018).

In recent years, many efforts have been conducted on the identification of putative component(s) of the IMM with channel properties. In 2013, the c subunit of Fo-ATP synthase, an IMM-resident protein, has been identified as a key mPTP component with voltage-sensitive channel properties in the ATP synthase monomeric form and in presence of both CypD binding and high Ca^{2+} concentrations (Alavian *et al.*, 2014). Overall, studies from three independent research groups contributed to the knowledge that c subunit plays a pivotal role in mPTP opening linking the physio-pathological effect to its intracellular expression (Bonora *et al.*, 2013) and phosphorylation status (Azarashvili *et al.*, 2014). How mPTP complex takes shape and which other proteins contribute to the structure of the pore-forming part remain elusive. Biochemical studies by Pavlov's group (Pavlov *et al.*, 2005; Elustondo *et al.*, 2016) reported the presence of increasing amounts of c subunits in mitochondria where mPT is induced, rather than those unstimulated; this scenario would be due to Ca^{2+}overload and is independent of endogenous levels of c subunit. This suggested the possibility that c subunit modifies the interaction with F_I/F_O ATP synthase dimers. They described the essential role of the c subunit-polyP-PHB axis in the association of a nonspecific pore channel during Ca^{2+}-induced stress. These results could explain how the c subunit, may form water-filled pores with polyP possibly serving as the hydrophilic coating of the pore, despite being a hydrophobic protein. Findings on the importance of the c subunit role in mPTP activity have also been confirmed very recently (Neginskaya *et al.*, 2019).

Two opposite hypotheses justify the contribution of Complex V to the mPTP assembly and activity: first, the dimerization of ATP synthase would be essential for the generation of the pore opening (Giorgio *et al.*, 2013), second and related to the c subunit findings, the dissociation of ATP synthase dimers and a proper C-ring (c subunits arrangement in the IMM) conformation would constitute the pore-forming part (Bonora *et al.*, 2017). Although literature proposes these two theories for mPTP "outfit", data from both models present some critical issues to be addressed in the next future (Bauer & Murphy, 2020). Concerning the first one (Giorgio *et al.*, 2013), the dimeric (and oligomeric) state of the ATP synthase owns irrefutable proofs of its participation in improved bioenergetic functions of mitochondria, partially dependent on the ability to ensure the correct curvature of the IMM (Daum *et al.*, 2013), thus dimers of ATP synthase cannot be mediator of Ca^{2+}-dependent cell death. To support the second hypothesis, it should be fully provided the structural model by which a non-specific current occurs in the C-ring. The controversial nature of the mPTP was highlighted by

two more recent publications in which knock-out experiments showed that the subunit c and the peripheral stalk subunit of the ATP synthase are dispensable for mPT opening (He *et al.*, 2017a; He *et al.*, 2017b). However, a deeper electrophysiological analysis of isolated mitoplast knock-out for c subunit demonstrates that in the absence of c subunit the current recorded was different from what expected for mPTP. Indeed, the knock-out cells for c subunit have a Ca^{2+} inducible current, which can be inhibited by CsA, but remarkably lower than in wt cells (0.3nS vs 1.3nS). Moreover, the authors provide data suggesting that, in C subunit-KO cells, a second current could be generated by ANT (Neginskaya *et al.*, 2019).These findings confirm thus the key role of the c subunit in mPTP activity.The different proposed mPTP structure which evolved along time is shown in Figure 3. Still, fully reconstituted active ATP synthase in liposomes was responsive to Ca^{2+}, converting dimers, but not monomers, in a channel. Interestingly, the activity was sensitive to adenine nucleotides, but not to ligands of the ANT or VDAC (Urbani *et al.*, 2019). The last decades of mPT research led to the discovery of a large number of inhibitors (Morciano *et al.*, 2018) and inducers (see Figure 4 for an incomplete list), using different methodologies including the one referred to in the next section.

IV. Understanding of the mPTP with the use of electrophysiological studies

Very important information about the PTP structure and functioning has been obtained with the use of patch-clamp technique. To record channel activity by means of patch-clamp from the IMM, it is necessary to remove the outer membrane and obtain single membrane objects - mitoplasts. In general, two methods were used to achieve this: the French press (Decker & Greenawalt, 1977) or osmotic swelling (Gupte *et al.*, 1984).

The first patch-clamp recordings of mitochondrial channel activity from the IMM were reported back in 1987 by Sorgato et al. where she described a single, slightly anion-selective channel of 107 pS conductance in 150 mM KCl. In addition, a 350 pS channel attributed to the OMM was also observed in this study (Sorgato, Keller & Stuhmer, 1987). Just a couple of years later, in a study carried out by Kinnally et al. (Kinnally, Campo & Tedeschi, 1989) on mouse liver mitochondria, a variety of conductances of 10-20, 45, 80, 120-150, 200, 350, and 1000 pS were reported, with the latter one not characterized due to infrequent appearance (Kinnally *et al.*, 1989). In a parallel study on rat liver mitochondria, Petronilli et al. (Petronilli, Szabo & Zoratti,

1989) observed similar large-conductance activity of about 1.3 nS. This activity exhibited flickering, and the main subconductance state, around 0.63 nS at +20 mV, was observed. However, a range of other subconductance levels was observed at different voltages, for instance at +30 mV they were 450, 350, 860 pS while at -40 mV they were 650, 450, 1000 pS. It is worth noting that this study was carried out in 150 mM KCl with 0.1 mM $CaCl_2$, while in the study of Kinnally et al. (Kinnally *et al.*, 1989) no $CaCl_2$ was added. This observation was very soon (in 1991) followed up by Szabo and Zoratti (Szabo & Zoratti, 1991), who found out that this large-conductance activity characterized by multiple subconductance states, for which they coin the term "mitochondrial megachannel" or MMC is inhibited by 100-200 nM Cyclosporin-A - already known at that time as an inhibitor or desensitizer of the mPT (Broekemeier, Dempsey & Pfeiffer, 1989). The kinetic features of the single-channel events also supported the idea that MMC is composed of cooperating subunits. Properties of MMC were further characterized in the follow-up paper, in which Szabo and Zoratti showed that MMC was non-selective, activated by Ca^{2+} and such Ca^{2+}-activated channels were inhibited by Mg^{2+}, Cyclosporin-A, and ADP, probably acting at matrix-facing sites (Szabo & Zoratti, 1992). In the next work joined by Bernardi, it was proven that Mg^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+} and Cyclosporin-A are, in fact, competitive inhibitors for Ca^{2+} (Szabo, Bernardi & Zoratti, 1992). For the first time, it was also shown that MMC is regulated by pH in the physiological range. Lower pH values caused MMC closure in a Ca^{2+}-reversible manner. For instance, MMC activity blocked by pH=6.5 in the presence of 0.5 mM Ca^{2+} could be reopened by 1.2 mM Ca^{2+}. Patch-clamp experiments were carried out in so-called inside-out configuration in which the matrix side of the membrane patch is exposed to the bath. Therefore, the patch-clamp experiments allowed for the conclusion that the modulating sites involved in these effects are located on the matrix side of the IMM. At the same time, Bernardi et al. (Bernardi *et al.*, 1992) provided evidence that the Ca^{2+}-induced mPT is affected by above-mentioned agents, supporting the identification of MMC by patch-clamp as responsible for mPT phenomenon. This pharmacological characterization of MMC/PTP was set aside in subsequent studies in which the voltage dependence of the megachannel was re-investigated (Szabo & Zoratti, 1993). It was noticed that the closed state(s) of MMC were favored at negative (physiological) transmembrane potentials. MMC conductance was 4.35 nS in symmetrical 0.5 M KCl with gating events involving a flickering half-size conductance (2.2 nS), which corresponded to that of the fully open VDAC in this

conditions (Szabo, De Pinto & Zoratti, 1993). Based on this, it was assumed that MMC would consist of two cooperating porin (VDAC) molecules. This idea was carried further by Beutner et al. 1996 (Beutner *et al.*, 1996), who characterized high molecular weight complexes isolated by Triton X-100 extraction from rat brain homogenate. These complexes were tested with specific antibodies and shown to contain hexokinase, creatine kinase, VDAC, and ANT. After incorporation into artificial bilayers channel activity of 6 nS in 1 M KCl with the asymmetrical voltage, dependence was recorded.

The claim that ANT was responsible for the mPTP was made a couple of years before by Tikhonova et al. (Tikhonova *et al.*, 1994). In their studies, purified ANT was incorporated into liposomes and fused into BLM in the presence of 800 mM urea. Channel activity was induced by mersalyl. They observed a range of conductance levels from 200 pS to 2.2 nS in 180 Na_2SO_4, 20mM MES-NaOH, pH 7.0, in the presence of 3 mM Mg^{2+}. ANT, a molecule responsible for MMC activity, was also proposed in studies from Klingerberg group (Brustovetsky & Klingenberg, 1996; Brustovetsky *et al.*, 2002). In the first study, bovine heart ANT was purified and reconstituted into giant membrane vesicles (Brustovetsky & Klingenberg, 1996). Large conductance channels of 300 to 600 pS (in the buffer containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM potassium gluconate, 5 mM MES, and 5 mM Tris at pH 7.2) were observed. These channels exhibited low cation selectivity (PK^+/PCl^- = 4.3 +/- 0.6), were activated by Ca^{2+} (1 mM), inhibited by protons (pH=5.2) and by a combination of bongkrekate and ADP. Channel closing was induced at extreme voltages. In a follow-up study, recombinant AAC (rAAC) from *Neurospora crassa* was expressed in *Escherichia coli* (Brustovetsky *et al.*, 2002). Purified rAAC was reconstituted and its activity recorded by patch-clamp. Its behavior was similar to that observed for ANT from bovine heart. In addition, it was shown that cyclophilin, isolated from *Neurospora crassa*, suppressed channel gating, thus increasing channel open probability, while Cyclosporin-A abolished the cyclophilin effect. When ADP was applied on cyclophilin-activated channels it induced flickering of the channel effectively decreasing channel open probability. In contrast, channel gating was diminished by the pro-oxidant tert-butyl hydroperoxide (Brustovetsky *et al.*, 2002).

Although attempts to characterize mPTP by means of various essays i.e. mitochondria swelling or mitochondrial Ca^{2+} accumulation were carried out, only a few electrophysiological studies on MMC were published at this time. In the study carried out by the Zoratti group, it was observed in patch-clamp experiments with rat liver

mitochondria that ubiquinone 0 and decylubiquinone inhibited the activity of MMC, what was in line with an earlier observation on mPTP (Fontaine, Ichas & Bernardi, 1998). Inhibition by these compounds was reversed by increasing [Ca^{2+}], the behavior observed with several other MMC inhibitors. Classical MMC activity was observed in human hepatoma HepG2 cells. This channel had high conductance of 1.23 nS (in 150 KCl), and frequently occupied 640 pS sub-conductance level; it was active in high (1 mM) and closed in low (1 μM) Ca^{2+} and was inhibited by 10 μM Cyclosporin-A (Loupatatzis *et al.*, 2002). Biochemical studies suggested that BAX cooperates with ANT in apoptotic events and per se would be a component of the mPTP (Marzo *et al.*, 1998). However, Campello et al. (Campello *et al.*, 2005) showed in 2005 that activity of MMC does not require BAX protein. In this study, human HCT116 cancer cell line was used and MMC appeared in 10-20% of patches from both Bax^{+} and Bax^{-} cells indicating that the mPTP was independent of Bax. The MMC activity was recorded in 150 mM KCl, 0.5 mM $CaCl_2$, 1 mM Pi, 20 mM HEPES, pH 7.35 and under these conditions had a conductance of about 1 nS (within 0.9–1.3 nS range) with a hallmark flickering substate of about half the size. Here, weakly anionic or no selectivity (PCl^{-}/PK^{+} = 1.8) for the high conductance state, while slight cationic selectivity (PK^{+}/PCl^{-} = 2.8) for low conductance state was observed.

It was known that Cyclosporin-A, the mPTP inhibitor binds CypD, as described above. However, it was not clear whether CypD was a component of the pore itself. To solve this question, properties of MMC channel from liver mitochondria from wild-type and CypD-lacking mice were compared in detail (De Marchi *et al.*, 2006). The pores observed in the two cases were indistinguishable. The only clear difference was their sensitivity to Cyclosporin-A. It was therefore concluded that CypD is a modulatory component of the PTP and does not constitute MMC channel pore (De Marchi *et al.*, 2006).

In 2013, Bernardi group finally published a paper in which electrical mPTP activity was related to dimers of ATP synthase (Giorgio *et al.*, 2013). First, they have identified the in-gel activity of monomers and dimers of ATP synthase after separation of mitochondrial proteins by blue native electrophoresis. Second, the gel-purified ATP synthase monomers or dimers, devoid of ANT, VDAC, and CypD were incorporated into planar lipid bilayers made of purified soybean azolectin and channel activity was recorded. When the experimental medium consisted of 50 mM KCl, 1 mM Pi, and 0.3 mM Ca^{2+} no channel activity was observed when either monomer or dimer of ATP

synthase were added to the recording chamber. However, the addition of Bz-423, a proapoptotic agent (Boitano *et al.*, 2003) that was previously shown to target ATP synthase (Johnson *et al.*, 2005), elicited channel activity only when dimers but not monomers of ATP synthase were added to the bilayer. A similar activity could be elicited by Bz-423 in the presence of phenylarsine oxide (PhAsO), a sensitizer of the mPTP to Ca^{2+} (Krauskopf *et al.*, 2006). This channel activity could be inhibited by γ-imino ATP (AMP-PNP), a nonhydrolyzable ATP analog and ADP in the presence of Mg^{2+} ions, which also exhibited a partial inhibitory effect when present alone. Curiously, channel activity was not inhibited by Cyclosporin-A in agreement with the lack of CyPD in the preparation and the fact that ATP synthase dimer was extracted in the presence of 10 mM Pi, which sensitizes the mPTP even in the absence of CyPD. Channel openings were still observed in the presence of bongkrekic acid and could not be elicited by atractyloside, selective inhibitors of ANT. These results have been further confirmed by recording the megachannel activity of highly purified preparation of bovine ATP synthase dimers (Urbani *et al.*, 2019).

Further evidence for the role of ATP synthase dimers in the formation of mPTP pore came from the planar bilayer recordings of the BN-PAGE purified yeast F_1/F_O-ATP synthase enzyme (Carraro *et al.*, 2014). The ATP synthase dimers did not elicit any currents unless Ca^{2+}, PhAsO, and $Cu(OP)_2$ were added. Moreover, the channel activity was inhibited by Mg^{2+}-ADP similarly to that found for mammalian ATP synthase dimer. The unitary conductance of the channels formed by ATP synthase dimers ranged from 250 to 300 pS thus was lower than this one observed for mammalian MMC. It should be noted that the dimer preparation did not contain Tom20 or Tim54 and therefore that channel activity could not be due to the twin-pore translocase (Rehling *et al.*, 2003). Using the same approach Bernardi's team also showed that dimers of Drosophila F-ATPase form channels opened by Ca^{2+}, Bz-423, PhAsO, and $Cu(OP)_2$ with a single-channel conductance of only 53 pS (in 100 mM KCl) (von Stockum *et al.*, 2015).

This sequence of papers from Paolo Bernardi group pointed to ATP synthase dimers as the molecular entity responsible for mPTP activity. In contrast, Alavian et al. (Alavian *et al.*, 2014) from Elizabeth Jonas group, showed that purified human Fo subunit of ATP synthase alone, which is formed by an octameric ring of c subunits reconstituted into liposomes, exhibited channel activity. This channel had a high conductance of around 100–300 pS, 500–750 pS, 1,500 pS, and 1,800–2,000 pS and

resembled the activity of MMC. It was unselective with permeability ratio $Na^+/K^+ = 1.5$. The c-subunit activity was inhibited by AMP, ADP, and ATP, and for ATP the EC50 concentration was found to be 660 μM. In parallel, Alavian et al. determined the EC50 concentration for ATP applied on MMC activity recorded in SMV and found to be lower at least by order of magnitude (50 μM). The activity of c-subunit ring was inhibited by anti–pan-c-subunit antibody. This antibody was also capable of inhibiting MMC activity induced by Ca^{2+} in SMVs. The activity of the c-subunit channel was, in contrast to purified ATP synthase monomers, not affected by Ca^{2+}. To further demonstrate that the c-subunit ring could create a pore, Alavian et al. substituted with valines four highly conserved glycines within the first alpha-helical region of the c-subunit with the assumption that this will interfere with the tight packing of the c-subunit molecules within the ring structure. When reconstituted into liposomes, all mutants demonstrated increased single-channel conductance compared to that of the WT c subunit and the conductance of quadruple mutant was the largest. This channel was also insensitive to block by ATP. Then, Jonas group hypothesized that F_1 binding to the c-subunit ring would inhibit the activity of the channel. To this end, they applied purified individual F_1 proteins to reconstituted active c-subunit channels. Curiously, it turned out that β subunit but not γ, δ, or ε subunits of ATP synthase had an inhibitory effect. To further associate the activity of Fo - complex of ATP synthase with the mPTP, Alavian et al. tested associated channel activity and regulation of following preparations: i) purified recombinant c subunit lacking CypD and OSCP reconstituted into proteoliposomes - here neither Ca^{2+} nor Cyclosporin-A had an effect on the channel activity; ii) purified ATP synthase monomers containing OSCP but lacking CypD reconstituted into proteoliposomes - here infrequent channel activity strongly enhanced by the addition of recombinant CypD protein either in the presence or absence of Ca^{2+} was observed; this activity was inhibited by Cyclosporin-A; iii) mitochondria and SMV containing endogenous CypD and OSCP and SMV exposed to urea what denatured and removed extramembrane proteins, including F_1 components such as OSCP, β subunit, and CypD - here the activity of mitochondria and SMVs was regulated by Ca^{2+} and Cyclosporin-A, what was completely absent from the urea-exposed SMVs; in this case 1 mM ATP still was able to inhibit the activity of the channel. In jet another study Mnatsakanyan et al. (Mnatsakanyan *et al.*, 2019), from Jonas group, confirmed their initial conclusions by recording megachannel activity of purified porcine monomeric ATP synthase. Altogether, these patch-clamp experiments in which channel activity was

recorded from reconstituted highly purified protein complexes indicated in a compelling way that ATP synthase either as a dimer or as a monomer is responsible for MMC activity.

A large controversy arose as a result of studies by Walker group, in which it was shown that HAP1-A12 cells incapable of producing the c-subunit preserve the PTP activity as measured by Ca^{2+} retention capacity of mitochondria (He *et al.*, 2017b). Recently, mitochondria derived from this cell line were further investigated by the patch-clamp technique (Neginskaya *et al.*, 2019). In contrast to the mitochondria of the wild-type HAP1 cells in which the classic MMC activity of 1.3 ± 0.2 nS with a subconductance state of 0.4 ± 0.04 nS was detected, the mitochondria of HAP1-A12 cells contained channel of a much smaller conductance of 0.3 ± 0.07 nS with a subconductance state of 0.13 ± 0.03 nS. Curiously, this channel was blocked by Cyclosporin-A but also partially by ADP and bongkrekic acid. Similar features, including conductance and sensitivity to ADP and bongkrekic acid, were previously described for a purified ANT converted to a channel by Ca^{2+} treatment (Brustovetsky & Klingenberg, 1996) indicating that in the absence of mPTP its function could be substituted by ANT.

Doubts about the c-subunit involvement in the mPTP channel activity were not the only ones; indeed, Walker group excluded from this phenomenon the contribution of both subunits of the peripheral stalk of the ATP synthase and the whole enzyme once assembled. To achieve these unexpected conclusions, clones were generated from HAP1-A12 cells via the disruption of ATP5F1 and ATP5O genes encoding respectively for subunits b and OSCP (28784775). In both cases, mPTP showed unaltered its properties by opening following stress stimulation and being inhibited by CsA, which refuted OSCP protein as interactor between mPTP and CypD as consequence. With the same approach and in the same cell line, two years later also subunits e, f, g, 6.8PL and DAPIT were removed (31213546) leading to similar conclusions. In addition, disruption of these proteins inevitably entails defects in ATP synthase assembly putting aside the Bernardi group's idea about the involvement of dimers in the mPTP activity.

Nevertheless, an even stronger case for the ATP synthase being responsible for MMC activity came from a study by Antoniel et al. (Antoniel *et al.*, 2018). This is the first report in which the activity of a single amino acid mutant of ATP synthase was studied by patch-clamp. As mentioned earlier, it has been known from earlier studies that the activity of MMC was blocked by protons (Szabo *et al.*, 1992). In this study,

Antoniel et al. checked whether unique histidine in OSCP subunit of the ATP synthase is important for MMC block by acidic pH. The activity of MMC from the wild type and from the OSCP H112Q mutant cells was recorded in a standard symmetrical solution of 150 mM KCl, 0.1 or 0.2 mM $CaCl_2$, 10 mM Hepes, pH 7.3. Both channels exhibited similar maximal conductance of 1 nS and several subconductance states with a prevalent substate of around 500 pS in agreement with previous observations on the MMC activity. As observed previously acidification of the bath to pH 6.5 resulted in almost complete inhibition of the MMC activity. In contrast to that, in mitoplasts from OSCP H112Q cells a decrease in pH to 6.5 did not cause considerable changes of the open probability but MMC was still sensitive to classical mPTP inhibitor Ba^{2+} (Szabo *et al.*, 1992).

Further and detailed information about electrophysiological properties of the mPTP have been recently reviewed by Neginskaya MA et al. (33359307). This seminal paper provides all evidence to claim that patch-clamp investigations, in the field of electrophysiological studies, are able to i) discern among different mPTP pathways occurring in a given genetic or biochemical condition and ii) understand the contribution of proteins or drugs in a highly controlled biophysical system.

V. The mPTP - from mitochondrial fractions to intact cells

Since the 1990s, a decade of very active mPTP research, several methods were also developed to study the opening of the mPTP in intact cells. The majority of methods are based on cell imaging, fluorescent dyes and pharmacological inhibition of mPTP (Bonora *et al.*, 2016; Petronilli *et al.*, 1998). Depolarization of the mitochondrial transmembrane potential has been recognized a consequence of the mPTP (Petronilli *et al.*, 1993; Zamzami *et al.*, 1996). Several studies have measured variations of the mitochondrial transmembrane potential as an indicator of the opening of the mPTP (Huser & Blatter, 1999; Rama Rao, Jayakumar & Norenberg, 2003; Briston *et al.*, 2017). However, since many other events besides the mPT can induce alterations in the mitochondrial transmembrane potential (including the mitochondrial metabolism per se, lipid peroxidation, ion cycles or activity of uncoupling proteins), it is logical to assume that variations of the mitochondrial transmembrane potential are not a good method to follow mPTP opening. The peroxidation of lipids that are a part of mitochondrial membranes can induce configurational changes that will lead to alterations in membrane properties, including a progressive increase in membrane permeability, and consequently, depolarization of the membrane potential (Stark, 1991; Wong-Ekkabut *et*

al., 2007). When inducing the mPTP with oxidant agents, one must eliminate artifacts caused by non-specific alterations in mitochondrial membrane permeability. Furthermore, transient openings of mPTP are very difficult to follow by measuring the mitochondrial membrane potential using fluorescent dyes. The collapse of the mitochondrial membrane potential is not systematic and to detect measurable changes in the distribution of the fluorescent dyes, longer periods of the PTP openings are usually required (Dumas *et al.*, 2009; Petronilli *et al.*, 2001). Also, the use of Cyclosporin-A as a potential inhibitor of mPTP-induced mitochondrial depolarization is not a reliable methodology. In fact, Cyclosporin-A inhibits calcineurin in cells as well, which can cause artifacts. The use of FK-506 (tacrolimus), which inhibits calcineurin but not the mPTP (Rodrigues-Diez *et al.*, 2016), or mitochondrial-directed Cyclosporin-A (Malouitre *et al.*, 2009) are often useful strategies to account for Cyclosporin-A lack of specificity in intact cells. For this reason, other approaches to measure mPTP *in situ* should be used to complement the information obtained by the measurement of mitochondrial membrane potential.

The 2-deoxyglucose method has been used since 1995 to follow mPTP opening. Until this date, there was only indirect evidence that pore opening occurred during the reperfusion of hearts previously submitted to ischemia. Facing this lack of proper methodology, there was a need for a method that would provide direct evidence of mPTP in critical time points during the reperfusion phase. Griffiths and Halestrap developed an elegant methodology that follows the opening of the mPTP (Griffiths & Halestrap, 1995). The authors used 2-deoxy[^{3}H]glucose, since it enters the cell through the glucose transporter and is phosphorylated to 2-deoxy[^{3}H]glucose-6-phosphate. This metabolite cannot be further metabolized and is trapped in the cell. However, it does not cross the mitochondrial inner membrane unless the mPTP is in an open state. Once the mPTP opens, 2-deoxy[^{3}H]glucose-6-phosphate enters the matrix of mitochondria. Later, treatment of isolated mitochondria with EGTA will chelate $Ca^{2+}$and therefore the mPTP will re-close, entrapping 2-deoxy[^{3}H]glucose-6-phosphate in the matrix. The measurement of the disintegrations per minute (d.p.m.) in the isolated mitochondrial fractions allows the calculation of the uptake of 2-deoxy[^{3}H]glucose-6-phosphate in the mitochondria and, therefore, demonstrate that the pore opened during that period. Since this method was developed, several authors have used in different settings (Kerr, Suleiman & Halestrap, 1999; Rama Rao *et al.*, 2003; Ayoub, Radhakrishnan & Gazmuri, 2017).

Nieminen and co-workers (Nieminen *et al.*, 1995) developed another fluorescent method using the fluorescent dyes calcein-AM and tetramethylrhodamine methyl ester

(TMRM) to monitor the mPT in intact cells. Calcein is a hydrophilic fluorescein derivative, that when esterified with an acetoxymethyl (AM) group, acquires the ability to cross the cellular membrane and to become entrapped in a non-fluorescence form. Once in the cytosol, intracellular esterases hydrolyze the AM group and the now-fluorescent calcein becomes entrapped inside the cell. Cleavage of AM moieties is in fact a widely used strategy to entrap fluorescent probes inside cells. TMRM is a cell-permeant fluorescent compound positively charged that once inside the cell is sequestered by mitochondria, depending on the mitochondrial membrane potential. Nieminen and co-workers (Nieminen *et al.*, 1995) demonstrated that when cells are loaded with calcein at 37ºC, the fluorescent dyes accumulate predominantly in the cytosol, while mitochondria appear as dark spots when using confocal microscopy to image calcein fluorescence. In opposition, due to its positive charge, TMRM accumulates in active mitochondria; hence the calcein-unlabeled dark spots appear now labeled with TMRM fluorescence. Once the mPTP opens, mitochondria loose TMRM fluorescence and the dark spots become filled with fluorescent calcein. Using laser-scanning confocal microscopy, this method allowed the authors to monitor the mPTP in intact hepatocytes after exposure to t-butylhydroperoxide (Nieminen *et al.*, 1995). However, this method was challenged by Petronilli and colleagues who pointed out some caveats and advised caution in interpreting the results obtained (Petronilli *et al.*, 1998). Since the esterified form of calcein (Calcein-AM) can cross intracellular membranes, its diffusion between the different organelles can also occur and it may label other cell spaces beside the cytosol (Petronilli *et al.*, 1999). The esterified form of calcein can also be cleaved by mitochondrial esterases and the fluorescent calcein may become entrapped inside the mitochondrial matrix. Regarding the dark spots observed by Nieminen et al (Nieminen *et al.*, 1995) in confocal microscopy images of calcein fluorescence, Petronilli et al. (Petronilli *et al.*, 1998) suggested that TMRM could quench calcein fluorescence, or that the concentration of calcein inside the mitochondrial matrix could reach values high enough to cause calcein self-quenching. Thus, and to overcome the flaws of the method developed by Nieminen and co-workers (Nieminen *et al.*, 1995), Petronilli et al. (Petronilli *et al.*, 1998; Petronilli *et al.*, 1999) suggested loading cells with Calcein-AM in the presence of 1mM of $CoCl_2$. In the presence of Co^{2+}, calcein fluorescence in the cytosol and nucleus is quenched, and because Co^{2+} does not cross the mitochondrial inner membrane, mitochondria appear as green-fluorescent bodies against a dark background. Under a condition promoting mPTP opening, calcein can exit mitochondria and Co^{2+} can flow to the mitochondrial matrix. A decrease of calcein fluorescence intensity in the mitochondrial matrix can be

measured by fluorescence microscopy or in a regular multi-plate-based fluorescence assay. This method proved to be a useful tool for the *in situ* study of mPTP modulation and it has been used often under the form of commercial kits.

As described before (section 3), mitochondrial swelling is a valuable method to study mPTP opening in isolated mitochondria. However, the observation of mitochondrial swelling in intact cells after mPTP opening is still somewhat controversial. Regarding this subject, some authors defend that morphological alterations observed in mitochondria in intact cells result from mitochondrial swelling (Minamikawa *et al.*, 1999); on the other hand, others defend that, in intact cells, mitochondrial swelling does not occur immediately after mPTP opening because of the presence of proteins in cytosol which may inhibit osmotic water entry in mitochondria (Dumas *et al.*, 2009). Because of this, mitochondrial swelling is not a uniformly accepted end-point for measuring mPTP opening in intact cells.

As conclusion, the evaluation of mPT in intact cells is not a straightforward process and subject to confounding factors and artifacts. Until now, there is no single reliable method to measure in situ the dynamic of mPT (see Figure 5 for the techniques referred in this section). More difficulties arise when the interest is to measure the low-conductance state of the mPTP, because most of the available methods are designed to evaluate its high-conductance form. Thus, to be more reliable, a combination of different methods is required to evaluate mPTP dynamics in intact cells.

VI. Not always a bad guy: the physiological roles of the mPTP

Advances in the understanding of the mitochondrial permeability transition phenomenon have determined the physiological and pathological roles of mPTP opening. Recent studies have shown that opening of the mPTP not only induces mitochondrial dysfunction and apoptosis, but it is also a physiological mechanism involved in different cellular regular function and development. While the pathological changes induced by the mPTP opening are well established, the evidence of the involvement of the mPTP during physiological conditions has been remained unproven for a long time. In the context of physiological role of mPTP, two possible stages for its open state were proposed: a full-conductance irreversible opening for permanent permeability, or an alternative variant – transient and flickering short-term opening of mPTP (Hou *et al.*, 2014; Wang *et al.*, 2008; Li *et al.*, 2020). Full-conductance open state is leading predominantly to apoptosis and cell death, while transient short-term

open state with smaller and more variable conductance more likely is prevalent during physiological conditions (Perez & Quintanilla, 2017).

Evidence for the physiological opening of the mPTP has been described including a flickering opening (Hausenloy *et al.*, 2004; Hausenloy *et al.*, 2010; Korge *et al.*, 2011; Crompton, 1999); furthermore, the association between transient mPTP opening and "superoxide flashes" have been observed in striated muscle mitochondria (Wang *et al.*, 2008). Based on the suggestion that this transient opening of the mPTP may release mitochondrial matrix Ca^{2+} to maintain mitochondrial homeostasis, the model for the physiologic function of the mPTP in addition to its well-regarded role in cell death was proposed (Elrod *et al.*, 2010). The most prominent physiological role of the mPTP in mitochondrial and cellular Ca^{2+} homeostasis apparently is its capacity to act as a Ca^{2+} efflux mechanism (Biasutto *et al.*, 2016; Hurst *et al.*, 2017; Takeuchi *et al.*, 2015; Krebs, 2017; Altschuld *et al.*, 1992; Li *et al.*, 2020; Xu *et al.*, 2020). The evidence for the transient opening of mPTP as a physiological Ca^{2+} efflux pathway included early demonstrations of the inhibition of Ca^{2+} release in the presence of mPTP inhibitor Cyclosporin-A in isolated adult rat ventricular cardiomyocytes (Altschuld *et al.*, 1992). Transient or low conductance opening of the mPTP has been proposed to serve as an additional mode of Ca^{2+} efflux that mitigates sustained matrix Ca^{2+} overload (Bernardi & von Stockum, 2012; Ichas & Mazat, 1998). Numerous studies supported this hypothesis of the physiological role of the mPTP pore as Ca^{2+} efflux mechanism (Gainutdinov *et al.*, 2015; Elrod & Molkentin, 2013; Bernardi & von Stockum, 2012; Korge *et al.*, 2011; Elrod *et al.*, 2010). Nevertheless, further investigations of mPTP participation in mitochondrial Ca^{2+} efflux are still needed. This is important in the light of recent divergent studies, where mitochondrial Ca^{2+} efflux rates measured in intact HeLa cells were completely unaffected by mPTP inhibition either by Cyclosporin A or by siRNA-mediated reduction of the ATP synthase c subunit (De Marchi *et al.*, 2014a), suggesting that the mPTP may not play a role in Ca^{2+} efflux under physiological conditions. However, most experiments addressing the role of the mPTP in Ca^{2+} homeostasis are based on using Cyclosporin-A, which requires some refinements and additional precautions, considering the specificities of Cyclosporin-A and ambivalence of pore selective inhibitors. Notably, the inhibitory effect of Cyclosporin-A depends on the expression level of CyPD, which is rarely assessed (Bernardi *et al.*, 2015). The variation of Cyclosporin-A inhibitory ability could range greatly with a matching difference in sensitivity to Cyclosporin-A, which, for example, was absent in NIH3T3

fibroblasts and HL60 cells (Li *et al.*, 2012). The relative expression of CypD and of Fo-ATP synthase may also be crucial, in consideration of the cross-linking experiments in beef heart mitochondria, which indicate that there is much less CypD than b, d, and OSCP subunits and many Fo-ATP synthase channels will be insensitive to Cyclosporin-A even if CypD is expressed (Bernardi *et al.*, 2015). In this regard, the negative effect of Cyclosporin-A sensitive/insensitive mPTP functioning, in particular, as the mechanism of mitochondrial Ca^{2+} efflux, should not be undoubtedly taken into consideration. Despite the continued discussions, majority of research suggests that the mPTP appears to act as a normal Ca^{2+}-release mechanism that is required for proper metabolic regulation (Bernardi *et al.*, 2015).

Apparently, Ca^{2+} remains the important regulator and inductor of pore opening, given its numerous indirect roles regulating and modulation the mPTP (Biasutto *et al.*, 2016; Hurst *et al.*, 2017). At physiological levels, Ca^{2+} could stimulate the transient opening of the pore, while Ca^{2+} overload changes the balance from physiology to pathology, leading to the sustained mPTP opening with the subsequent mitochondrial and cellular dysfunction (Perez & Quintanilla, 2017; Mnatsakanyan *et al.*, 2017; Hurst *et al.*, 2017; Nesci *et al.*, 2018; Lamb, 2020). Recent studies also highlighted a crucial role of mPTP not only in cardiac, neurodegenerative and other pathologies, but also its involvement in cardiac and brain development (Folmes *et al.*, 2012; Perez & Quintanilla, 2017). The importance of the mPTP in health and disease is evidenced since it also plays a role in protecting against different brain and cardiac disorders in the elderly population, besides the fact that transient opening of this structure could be the principal mediator of heart and brain development (Perez & Quintanilla, 2017) and be involved in neutrophil activation and ROS release (Vorobjeva *et al.*, 2020). In this context mPTP is highlighted as a gating mechanism underlying differentiation in the developing heart and brain, implicating cross-talk between genetic and metabolic signaling (Folmes *et al.*, 2012). Transient mPTP opening directly regulates cellular energy metabolism as it uncouples oxidative metabolism from ATP synthesis, a mechanism that operates in concert with ROS flashes to promote cardiomyocyte differentiation (Folmes *et al.*, 2012). Knockout of the mPTP component cyclophilin D results in elevated mitochondrial matrix Ca^{2+}, enhancing the activation of Ca^{2+}-dependent dehydrogenases reducing metabolic flexibility (Elrod *et al.*, 2010). It was demonstrated that mPTP opening in the early heart is a physiological event for the development of the organ. In the fetal heart, myocytes exhibit low mitochondrial

membrane potential, high levels of ROS production, and opening of the mPTP. Inhibition of the mPTP with Cyclosporin-A led to serious maturation of mitochondrial structure and function, decreasing intracellular ROS levels and increasing mitochondrial membrane potential, which accelerated myocyte differentiation (Hom *et al.*, 2011).

Concerning the participation of the mPTP not only in cardiac but also in neural development, it was reported that cultured embryonic mouse cortical neural progenitor cells demonstrate intermittent spontaneous bursts of mitochondrial superoxide generation that require a transient opening of mPTP (Hou *et al.*, 2014). It was shown that both mitochondrial ROS scavengers and mPTP inhibitors such as Cyclosporin A reduced the frequency of mitoflashes and enhanced proliferation of cortical neural progenitor cells, whereas prolonged mPTP opening and superoxide generation increased the incidence of mitoflashes and promoted differentiation of these neuronal cells (Hou *et al.*, 2012). The evidence of mPTP participation in the cardiac and brain development is practically unquestionable; however there is significant complexity and specificity in superoxide generation and opening of the mPTP in other cell types (concerning the studies of hematopoietic progenitor cells and vascular progenitor cells (Arnold *et al.*, 2000; Davies *et al.*, 2005). Generally, the physiological participation of the mPTP in cell development and differentiation is dependent on the specific cell type, the subcellular localization of mitochondria and the temporal occurrence within the developmental stage (Mnatsakanyan *et al.*, 2017; Perez & Quintanilla, 2017). The transient pore opening has been shown to be associated with a transient depolarization of mitochondrial membrane potential ΔΨm (Jou, 2011; Hurst *et al.*, 2017). While the exact mechanism responsible for these transient openings of the mPTP and the mitochondrial membrane potential dissipation is still unclear, the physiological roles are beginning to be elucidated. Indeed, the frequency of these transient openings has been associated with metabolism, aging, wound healing, and playing an essential role in cell differentiation (Shen *et al.*, 2014; Vega-Naredo *et al.*, 2014; Ding *et al.*, 2015).

VII. Future perspectives in mPTP regulation

Sharing a dual role as generator of life and key participant in cell death mechanisms, the mPTP has attracting a lot of attention in the field of drug discovery. In the past, it has been extensively studied for a long time for therapies aimed in blocking ATP synthesis in cells, especially those of pathogens cause of infective pathologies in

humans (15591164). By binding subunit c of F_O ATP synthase, as well as mycobacterial subunit ε, antimicrobials were able to selectively and effectively eliminate strains of microorganisms becoming resistant to drugs. Another classical example of F_1F_O-ATP synthase as pharmacological target, comes from the 1,4-benzodiazepine (Bz-423), a drug belonging to benzodiazepine family able to induce a selective, ROS- and mPTP-dependent apoptotic cell death in lymphocytes B by interacting with OSCP subunit (15850986), the identical site used by CypD to modulate mPTP; the discovery of Bz-423 permitted to treat autoimmune disorders like lupus erythematous.

Subunit c, together to CypD, has been defined as one important player of the mPTP. But both targets own conceptual and technical difficulties in the screening of mPTP opening inhibitors: CypD is definitively recognized as regulator and not a pore component, thus its targeting may only desensitize the pore activity; subunit c would be part of the membrane-bound F_O portion and as such is involved in proton translocation for ATP generation. Therefore, its inhibition may led to not negligible side effects, as it happens for Oligomycin, an antibiotic from which F_O part takes the name and *N*,*N* -dicyclohexylcarbodiimide (DCCD), just to mention the most common. Oligomycin is a natural macrolide acting as potent inhibitor of both synthesis and hydrolysis of mitochondrial ATP and chromatophores. It is produced from Streptomyces and exists in six different isoforms from A to F on the basis of the R group attached to the macrolide. Oligomycin binds side chains of amino acids located on two consecutive subunits c and is able to inhibit mPTP opening in living cells by 50% at a concentration of 10 μM (28566520). Otherwise, DCCD is a lipid soluble carbodiimide with strong inhibitory properties of both portions of F_1F_O-ATP synthase, depending on the use. At low concentrations (it is estimated up to 50 μM) it covalently interacts with the c-ring through an essential carboxyl amino acid (Asp61) of subunit c and inhibit mPTP opening by 45% if used in the range 7.5-15 μM; at higher concentrations, it interacts also with F_1 portion through a Glu residue in the ß subunit. Given to the toxic nature of these drugs, they are not intended for use in therapy for diseases involving an abrupt opening of the mPTP. Indeed, despite they significantly inhibit mPTP activity *in vitro*, they also would deplete mitochondrial ATP causing an additional injury in more complex models of disease.

In order to overcome this obstacle and considered last findings on the importance of c-ring in mPTP modulation, many efforts are ongoing in making less

toxic subunit c inhibitors. This goal can be achieved by identifying the minimum and essential core of drugs recognizing subunit c and adapting the surrounding chemical structure to minimize side effects, but maintaining the inhibitory potential. A small-molecule library of subunit c inhibitors has been obtained a couple of years ago by modifying the functional core of the Oligomycin leading to new compounds able to strongly reduce reperfusion damage in animal models of global ischemia without interfering with ATP production (30060655). In detail, compound 10 was able to inhibit mPTP opening *in vitro* by 40-50% at very low concentrations (0,5-1 μM) and to reduce apoptotic cell death in heart tissues by 40% when administered at reperfusion time for 10 minutes. Compound 10, together with 5c and 6g, showed low toxicity, probably thanks to the exclusively localization in mitochondria and their reversible binding upon the acute treatment. In a 2013 study, Danshensu (DSS), the main constituent of Salvia miltiorrhiza (Danshen) which represents a traditional Chinese herb, provided substantial cardioprotection against myocardial I/R injury in terms of IS, cell viability loss, and creatine kinase-MB, cardiac troponin and LDH dosages; these features were dependent from the modulation of subunit c protein (23200898). In reperfused rat hearts, DSS acts downregulating subunit c mRNA and protein levels and it is able to reduce mPTP opening and the consequent cardiac I/R injury ameliorating heart parameters and cardiomyocytes survival (29250127).

The importance of CypD in mPTP-mediated cardioprotection has been highlighted by genetic studies in animals by modifying the expression of its *Ppif* encoding gene. CypD can be druggable by a long list of drugs, almost all derived from CsA. CsA was isolated for the first time in 1971 from a fungus and entered the clinical practice only some years later; its binding with CypD is guaranteed by a tryptophan (Trp-121) within a short a-helical of the protein (20676357). Its excellent activity in inhibiting mPTP opening at low concentrations (from 0,2 to 1,2 μM) and the undoubted results *in vitro* and in preclinical models, have made CsA one of the most used positive controls for assessing mPTP function. However, CsA has multiple side effects both as mPTP inhibitor, probably due to a diffuse localization pattern inside cells where effects in the cytosol (27699266, 12453541) and in the nucleus (7474947) are described, and immunosuppressant. In the effort to counteract the first one, an incorporation of CsA into poly-lactic/glycolic acid (PLGA) nanoparticles (CsA-NP) has been proposed for a better mitochondrial localization; treatment with CsA-NP at the time of reperfusion

increased cardioprotection with significant reduction of IS using lower concentrations compared to CsA alone (26861678). To answer to the second issue, attempts have made to produce CsA derivatives consisting in a significant decrease (thousands fold less) of its immunosuppressant role; it is the case of N-methyl-isoleucine-4-cyclo-sporin (NIM-811) and N-methyl-D-alanine-3-N-ethyl-valine-4-cyclosporin (Debio025). NIM-811 and Debio025 are semisynthetic analogues of CsA in which cytosolic side effects were quite abolished by the elimination of the calcineurin-binding motif (15377880, 12065751). In details, when used as mPTP inhibitor, induced by Ca^{2+} overload and inorganic phosphates in living cells and isolated mitochondria, NIM-811 exerts the same potency of CsA as evaluated also by apoptosis detection. In our opinion, as well as the breakdown of immunosuppressant capabilities, it gains an additional advantage: while CsA owns a limited window of action in term of concentrations (0.2 μM-1.2 μM) outside of which is not useful and makes difficult its use in clinical trials, NIM-811 did not and lack of toxicity. As regards Debio025, a comparative study performed in brain- and heart-isolated mitochondria, classified Debio025 as 10-fold more powerful compared to CsA. It is characterized by an additional modification at the third amino acid compared to NIM-811 with whom it already shares a variation at the fourth amino acid of the polypeptide chain. Multiples additional studies have reported their beneficial effects in terms of reduced cell death, recovery of left ventricle (LV) contractile function and improved survival (17557911, 19221132, 20430770).

Despite the preclinical potential of these drugs, only CsA entered the trial procedure lasting for 15 years and ended with the failure at phase III in cardiac reperfusion injury. Indeed, CIRCUS (26321103) and CYCLE (26821623) consisting of a single intravenous bolus of CsA (2.5 mg/kg) before revascularization had no effect on ST-segment resolution or cardiac enzymes and did not improve clinical outcome. These findings have definitively put aside the study by Piot et al. (18669426) who had seen the hope in the use of CsA to treat reperfusion injury.

Another nonselective inhibitor (as they are NIM-811 and Debio025) of CypD, is Sanglifehrin A (SfA) described by Halestrap and co-workers in 2002 fully (12095984). SfA is recognized as a strong mPTP opening inhibitor in a time-dependent way and with a greater extend if compared to CsA; downstream events are recognized in the recovery of cardiac performance upon I/R and in a significant reduction of LDH release (14659807). Being SfA structurally different from CsA, this permitted to avoid the

formation of calcineurin-SfA complexes; but preserving the ability to bind CypA, although at a different site, it continues to exert discrete immunosuppressant activities. Like DCCD for subunit c, SfA seems to strongly bind CypD because of his failure to detach upon several wash out. This fact, together with the immunosuppressant activity, may discourage the attempts to its use in clinical practice, as a prolonged permanence inside cells may led to side effects.

Small-molecule inhibitors exist also for CypD; this is the case of C-9 and C-19. Initially identified as therapeutic opportunity to delay Alzheimer disease symptoms, thus by preventing the interaction between CypD and amyloid beta (Aβ) to contain mitochondrial-dependent neuronal stress (24555519), especially C-9 was also demonstrated useful in vitro for the treatment of many other diseases such as acute pancreatitis, damages caused by UV radiation and other neurodegenerative diseases, thanks to their properties to inhibit mPTP and mitochondrial impairment. However, the most potent inhibitor of the category currently known is C31 (31336123) able to restore mitochondria parameters also in hepatic injury.

Originally suggested as mPTP regulator and most commonly known as peripheral benzodiazepine receptor for its high affinity in binding benzodiazepines, the 18 kDa translocator protein (TSPO) has been proposed as potent inducer of mPTP once an interaction is established with Protoporphyrin IX (PPIX) (7983042). In 2010, TSPO targeting by 3,5-Seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303) showed promising cardioprotective effects in a rat model of cardiac ischemia; its administration prior reperfusion reduced IS by about 40% and concomitant cell death. But the desensitization of the mPTP opening seems to be secondary to the remarkable antioxidant properties (20215409).

Further supporting an indirect mechanism in the modulation of mPTP, in 2014, studies on TSPO KO mice have excluded the possibility that ligands of TSPO, and TSPO itself, may regulate mPTP activity by showing that the presence or the absence of the protein in hearts subjected to IRI was dispensable (24692541). However, safety and efficacy of this drug were evaluated few years later in 180 ST-elevation myocardial infarction (STEMI) patients undergoing percutaneous coronary intervention (PCI). This multicenter, double-blinded, phase II study named as MITOCARE, in which TRO40303 has been administered just before revascularization, showed the inefficacy of the

compound in reducing or limiting reperfusion injury (25179768). IS quantification, LV ejection fraction evaluation, creatine kinase and troponin I dosages did not differ between placebo and treated patients.

Drugable properties of mPTP have highlighted also by compounds derived from cinnamic anilide, such as GNX-4728 and GNX-4975. They modulate mPTP in a CypD- and subunit c-independent way showing beneficial effects in transgenic mouse model of ALS (25565966) with delayed onset of symptoms, increased lifespan and the inflammatory picture reduction. It has been hypothesized that GNX-4975 shares the same binding site of calcium in mPTP opening; once opened, ANT and PiC would be subjected to conformational changes to form an interface in the inner membrane where the compound would accumulate (26920024).

A screening performed in isolated mitochondria among thousands of compounds registered in the National Institute of Health repository identified many others small-molecule inhibitors, based on a isoxazole functional core. Of all, ML-404 selectively inhibited mPTP opening without side effects up to 100 μM; this compound increased CRC of mitochondria and was classified as 2 fold more potent than GNX-series (25834903). Having a synergistic effect with CsA, ML-404 and other isoxazole-based compounds (such as compound 60) do not act via CypD binding (26286375).

Another strategy to inhibit pores opening via CypD-independent interactions, are compounds based on the N-phenylbenzamide scaffold that have acquired pharmacological relevance being able for the full inhibition of the pore at low concentrations without interfering with ATP synthase production (26693836).

As for TRO40303, many compounds that promote mPTP desensitization act first as potent oxidant scavengers: the best case is represented by a gallic acid-derivative, the mitochondriotropic antioxidant ($AntiOxBEN_3$) (29513043) and Agomelatine (AGO), a melatonin receptor agonist. Antioxidants usually own low toxicity, especially AGO that protected the rat ischemic myocardium by acting on other upstream targets of the mPTP among which the enhanced phosphorylation of GSK-3B. Melatonin itself, as antioxidant is able to modulate mPTP, too; but new evidence and ongoing works are directed toward an additional and direct effect on the pore (30962427, 28398674). At the moment, melatonin is considered as the safest drug to be used as mPTP inhibitor.

Melatonin is a chronobiotic indolamine mainly synthesized and secreted in the pineal gland with a marked circadian rhythm. Given its high grade of lipophilicity, pineal melatonin can diffuse across cell membranes, allowing the distribution of this molecule throughout all cells of the body and influencing organs function (Tan *et al.*, 1999). However, increasing evidence supports that rather than this passive diffusion process, there are also more active or facilitated mechanisms that favor melatonin uptake and its cellular internalization, probably via members of the SLC2/GLUT and PEPT1 families (Hevia *et al.*, 2008; Mayo *et al.*, 2018; Huo *et al.*, 2017). Indeed, some cellular organelles, especially mitochondria, were found to accumulate high concentrations of melatonin (Leon *et al.*, 2004). A recent study has also uncovered that large amounts of melatonin are synthesized by mitochondria during oocytes maturation, being important for the maintenance of energy metabolism, mitochondrial function and the proper quality of oocytes (He *et al.*, 2016). Enzymes involved in the synthesis of melatonin were found in mitochondria and isolated mitochondria retain the capacity to produce melatonin (He *et al.*, 2016; Coelho *et al.*, 2015). These findings underline the importance of melatonin for the mitochondria and raise questions regarding the physiological and protective effects of this neurohormone on these organelles. In addition to its antioxidant role, melatonin has a protective effect on neural disorders and other diseases via modulating the activity of mPTP and apoptosis responses (Petrosillo *et al.*, 2009; Espino *et al.*, 2010). Likewise, melatonin stimulates uncoupling proteins (UCPs) what contributes to the dissipation of the electrochemical proton gradient across the IMM and the consequent reduction of the MMP (Pan *et al.*, 2018; Tan *et al.*, 2016).

As previously mentioned, mPTP modulation by melatonin is one of the novelty roles underlying the broad of protective actions of melatonin in diverse diseases, especially in neurological disorders. Early observations in rat brain astrocytes showed that melatonin seems not only to suppress mitochondrial ROS formation but also targets Ca^{2+} -mediated mPT to protects against cell death (Jou *et al.*, 2010). In a later study and with the use of fluorescence laser scanning imaging microscopy it was found that melatonin prevents mitochondrial depolarization and mPTP neurotoxicity under disturbed Ca^{+2} homeostasis by preserving the protective conformation of mPTP (Jou, 2011). Recently, Waseem and colleagues demonstrated that melatonin addition to isolated brain mitochondria inhibits the opening of mPTP, probably hindering the mPTP-mediated mitochondrial dysfunction (Waseem, Tabassum & Parvez, 2016). In

this study, cultivated isolated mitochondria were exposed to Ca^{2+} and 5-hydroxydecanoate to stimulate mitochondrial swelling and induce mPTP opening. Melatonin administration significantly restores mitochondrial swelling and MMP and improves mitochondrial respiration. Furthermore, the benefits of melatonin are also visible in the heart from aged mice, as well as, in frozen-thawed sperm, where melatonin administration inhibits mPTP opening and improves cellular respiration and ATP production (Fang *et al.*, 2019; Sahach *et al.*, 2008). However, the exact mechanism of action by which melatonin regulates mPTP remained unclear and only related molecular mechanisms have been proposed.

Interestingly, a promising publication provides some evidence of a potential mechanistic action of melatonin on the regulation of mPTP via inhibiting cyclosporin D (Zhou *et al.*, 2018). Zhou et al. transfected cyclophilin D mutants (mimicking permanent phosphorylation) into melatonin-treated endothelial cells and demonstrated that melatonin represses Ripk3-PGAM5-CypD cascade, attenuating necroptosis and cardiac ischemia-reperfusion injury. Besides this, Andrabi and co-workers recorded inner mitochondrial membrane by using a patch-clamp approach in liver mitoplast from rodents to evaluate the direct effect of melatonin on mPTP (Andrabi *et al.*, 2004). This work uncovered that melatonin inhibits mPTP opening in a dose-dependent manner; but there was no evidence about the target protein. Overall, these publications reveal that melatonin modulates mPTP activity and preserves the optimal MMP and mitochondrial integrity, contributing to the maintenance of cells functions and survival. ~~However, whether melatonin could directly interact or modulate mPTP is still evolving.~~

To summarize, it is important to note that the mPTP could be the principal mediator of mitochondrial physiological and pathological functions, acting as a significant contributor to cell functions in both development and cell death processes. Figure 6 shows the interplay between physiological low-conductance forms of the mPTP and the deleterious large-conductance forms. Despite major progress has been made in understanding the nature of the mPTP, further studies are required to clarify its physiological roles.

~~As described before, mPTP opening has been linked to diverse acute pathological conditions, including neurodegenerative disorders, heart failure, and age-dependent diseases (Britti *et al.*, 2018; Morciano *et al.*, 2017; Kwong & Molkentin, 2015; Rottenberg & Hoek, 2017; Tarocco *et al.*, 2019). Hence, the mPTP can be~~

~~considered as a potential drug target. Several studies have addressed the importance of mPTP regulatory molecules to prevent the cell from abolishing mitochondrial functions and possibly cell death. Among the various molecules studied, the use of melatonin (N-acetyl-5-methoxytryptamine) has gained interest in the past few years because this natural and ubiquitous hormone has powerful antioxidant properties and effects on the MMP. The major aims of researchers to date have focused on exploring (a) whether melatonin exerts its inhibitory effects on mPTP by regulating intermediate signaling pathways and (b) whether melatonin can directly inhibit mPTP opening and confer protection.~~

Melatonin is a chronobiotic indolamine mainly synthesized and secreted in the pineal gland with a marked circadian rhythm. Given its high grade of lipophilicity, pineal melatonin can diffuse across cell membranes, allowing the distribution of this molecule throughout all cells of the body and influencing organs function (Tan *et al.*, 1999). However, increasing evidence supports that rather than this passive diffusion process, there are also more active or facilitated mechanisms that favor melatonin uptake and its cellular internalization, probably via members of the SLC2/GLUT and PEPT1 families (Hevia *et al.*, 2008; Mayo *et al.*, 2018; Huo *et al.*, 2017). Indeed, some cellular organelles, especially mitochondria, were found to accumulate high concentrations of melatonin (Leon *et al.*, 2004). A recent study has also uncovered that large amounts of melatonin are synthesized by mitochondria during oocytes maturation, being important for the maintenance of energy metabolism, mitochondrial function and the proper quality of oocytes (He *et al.*, 2016). Enzymes involved in the synthesis of melatonin were found in mitochondria and isolated mitochondria retain the capacity to produce melatonin (He *et al.*, 2016; Coelho *et al.*, 2015). These findings underline the importance of melatonin for the mitochondria and raise questions regarding the physiological and protective effects of this neurohormone on these organelles.

~~There is an accumulative recognition of the magnitude of melatonin in the regulation of mitochondrial function and signaling pathways. Several works during the last years have reported that melatonin protects mitochondria by directly detoxifying ROS and reactive nitrogen species (RNS) and by acting as a signaling molecule that upregulates the expression of genes encoding members of antioxidant defense system (Wang *et al.*, 2018; Zhai *et al.*, 2017; Song *et al.*, 2016).~~ In addition to its antioxidant role, melatonin has a protective effect on neural disorders and other diseases via

modulating the activity of mPTP and apoptosis responses (Petrosillo *et al.*, 2009; Espino *et al.*, 2010). Likewise, melatonin stimulates uncoupling proteins (UCPs) what contributes to the dissipation of the electrochemical proton gradient across the IMM and the consequent reduction of the MMP (Pan *et al.*, 2018; Tan *et al.*, 2016).

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Interestingly, a promising publication provides some evidence of a potential mechanistic action of melatonin on the regulation of mPTP via inhibiting cyclosporin D (Zhou *et al.*, 2018). Zhou et al. transfected cyclophilin D mutants (mimicking

permanent phosphorylation) into melatonin-treated endothelial cells and demonstrated that melatonin represses Ripk3-PGAM5-CypD cascade, attenuating necroptosis and cardiac ischemia-reperfusion injury. Besides this, Andrabi and co-workers recorded inner mitochondrial membrane by using a patch-clamp approach in liver mitoplast from rodents to evaluate the direct effect of melatonin on mPTP (Andrabi *et al.*, 2004). This work uncovered that melatonin inhibits mPTP opening in a dose-dependent manner; but there was no evidence about the target protein. Overall, these publications reveal that melatonin modulates mPTP activity and preserves the optimal MMP and mitochondrial integrity, contributing to the maintenance of cells functions and survival. However, whether melatonin could directly interact or modulate mPTP is still evolving.

To summarize, it is important to note that the mPTP could be the principal mediator of mitochondrial physiological and pathological functions, acting as a significant contributor to cell functions in both development and cell death processes. Figure 6 shows the interplay between physiological low-conductance forms of the mPTP and the deleterious large-conductance forms. Despite major progress has been made in understanding the nature of the mPTP, further studies are required to clarify its physiological roles.

VIII. Conclusions

(1) Advances in methodologies, including genetic manipulation, has accelerated mPTP research, converting it in a constantly evolving entity, which is present in different species, although with different forms of regulation.

(2) The mPTP plays crucial roles both in cell physiology and pathology; not only exceesive mPTP opening is involved in the pathophysiology of different human diseases, but regulated mPTP opening is critical for the resulation of cell and mitochondrial ionic and redox balance, and plays an apparent important role in fetal development.

(3) Opening of the mPTP increases mitochondrial membranes permeability to different solutes, switching from a lower to a higher conductance state according to different stress or physiological stimuli.

(4) The mPTP is notoriously activated by increased oxidative stress and Ca^{2+} concentrations, being finely regulated by the availability of several other metabolites.

(5) No single methodology can accurately measure mPTP opening rates.
(6) The more recent models suggest a role for the ATP synthase as a structural component of the mPTP, although doubts still exist on the subunit(s) responsible for the channel activity.

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Figure legends

Figure 1. Mitochondrial Ca^{2+} transport system. Ca^{2+} transport to mitochondria initiates through the outer mitochondrial membrane (OMM) by the large-conductance channel, the voltage-dependent anion channel (VDAC). For Ca^{2+} influx through the inner mitochondrial membrane (IMM), three main mechanisms are considered: mitochondrial Ca^{2+} uniporter (MCU), rapid mode of Ca^{2+} uptake (RaM), and through mitochondrial ryanodine receptor (mRyR). The MCU is a macromolecular complex composed of pore-forming and regulatory subunits: mitochondrial Ca^{2+} uniporter (MCU), MCU dominant negative beta subunit (MCUb), essential MCU regulator (EMRE), the family of mitochondrial Ca^{2+} uptake proteins (MICU 1-3), mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1), and SLC25A23. Ca^{2+} influx through MCU multi-protein complex is driven by the large electrochemical gradient (mitochondrial membrane potential ~−180 mV) for Ca^{2+} across the IMM, generated by proton pumps (Complexes I, III and IV) of the electron transport chain. For Ca^{2+} efflux through the IMM, three main mechanisms are available, namely: Na^{+}/Ca^{2+} exchanger (NCXm), H^{+}/Ca^{2+} exchanger (HCXm) and the mitochondrial permeability transition pore (mPTP). The current proposal about the mPTP is based on the formation of ATP synthase dimers, in which the adenine nucleotide translocator (ANT) and Cyclophilin D (CypD) are likely regulators of the mPTP complex. The phosphate carrier (PiC) may also be a pore regulator, while the pro-apoptotic proteins Bax/Bak function in the outer membrane to contribute to regulation of the mPTP opening and release of pro-apoptotic factors from mitochondria. Besides this, leucine zipper- EF-hand containing transmembrane protein (LETM1) was proposed as another Ca^{2+}-transport system. However, the role of LETM1 in the mitochondrial Ca^{2+} influx and efflux through the IMM is still under discussion.

Figure 2. Mitochondrial swelling as a classical hallmark of the mPTP *in vitro*. The upper part of the figure represents mitochondrial swelling associated with mPTP when induced *in vitro*. Isolated mitochondrial fractions (e.g. liver or heart) are normally suspended in a reaction buffer with sucrose or KCl as osmotic support (~200 mOsmolar), represented in the figure as orange hexagons. Mitochondrial membranes are particularly impermeable to sucrose and show regulated permeability to K^{+} and Cl^{-} ions.

Upon opening of the mPTP (e.g. by using a pro-oxidant agent in the presence of excess calcium), conformational alterations in IMM proteins (in red) leads to the formation of the mPTP complex through which molecules under 1,450 Da flow (including osmotic support molecules). Water follows by osmosis, leading to swelling of IMM and burst of the OMM due to the smaller surface area of the latter. Intra-cristal space also decreases. This effect leads to uncoupled mitochondrial respiration and mitochondrial depolarization, leading to calcium-induced calcium release. Mitochondrial swelling can be followed by following the pseudo-absorbance of the mitochondrial suspension at ~540 nm, the isosbestic point for mitochondrial cytochromes (the wavelength at which the total absorbance of the different cytochromes in mitochondria does not change during the event).

The lower panel shows electron microscopy images of liver mitochondria before (left) and after (right) undergoing calcium-induced mPT. Lower panel kindly provided by Drs. Sabine Schmitt and Hans Zischka, Helmholtz Center Munich, Germany.

Figure 3. The different models for the composition and structure of the mPTP. From left to right: a) the initial model, in which the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane had a structural role, together with the outer mitochondrial membrane voltage-dependent anion channel (VDAC). The mPTP structure and opening is regulated by the matrix chaperone cyclophilin D (CyD), while hexokinase (HK) and the peripheral benzodiazepine receptor (PBR) also have regulatory roles through the VDAC; b) knock-out experiments revealed that the VDAC and the ANT are dispensable for the mPTP complex. Instead, the phosphate transporter (PiC) was next proposed as the mPTP structural component, whose opening would be regulated by the ANT and CyD; c) at the same time, some proposals suggested that the mPTP would be not one but several possible entities composed by distinct proteins and which would be converted into the mPTP through the binding of matrix chaperones; d) the most recent proposal suggests that the structural component of the mPTP is the ATP synthase, from which two theories arise: the first one placing monomers of the enzyme at the basis of the mPTP opening, the second one, the dimers. Whereas the ANT and PiC, both components of the mitochondrial phosphorylative system would also have a regulatory role. Regardless of the model proposed, there are common motives, including that increased ROS and Ca^{2+} contribute to pore opening, together with a decrease in adenine nucleotides, namely ATP.

Figure 4. Mitochondrial permeability pore direct and indirect regulators (inducers and inhibitors).

Abbreviations:

ABT-737 - 4-[4-[[2-(4-chlorophenyl)phenyl]methyl]piperazin-1-yl]-N-[4-[[(2R)-4-(dimethylamino)-1-phenylsulfanylbutan-2-yl]amino]-3-nitrophenyl] sulfonylbenzamide

ADP Adenosine 5′ -diphosphate

ATP Adenosine 5′ -triphosphate

AUL-12 (AuIIIBr2(ESDT)), ESDT: ethylsarcosinedithiocarbamate)

B4G2 23-hydroxybetulinic acid derivative

DIDS 4,4′ -Diisothiocyanatostilbene-2,2′ -disulfonic acid

EM20-25 5-(6-chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione

GNX-4728 substituted cinnamic anilide

JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5]benzodiazepine

MitoQ 10-(6′ -ubiquinonyl)decyltriphenylphosphonium bromide

NADH Nicotinamide adenine dinucleotide, reduced form

NADPH Nicotinamide adenine dinucleotide phosphate, reduced form

$O_2^{\bullet-}$ Superoxide anion

PK11195 1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide

SfA Sanglifehrin A

TMD#7538 - N-phenethyl-6-phenyl-2, 3, 4, 9-tetrahydro-1H-carbazol-1-amine

TRO40303 3,5-Seco-4-nor-cholestan-5-one oxime-3-ol

UTP Uridine 5′ -triphosphate

ΔΨ – mitochondrial transmembrane potential

Figure 5. Some possible techniques for evaluating the mitochondrial permeability transition (MPT) in intact cells.

Top left: Evaluation of mPTP opening by using a ΔΨ-dependent dye such as TMRM is based on its accumulation in mitochondria with a closed mPTP, in which the electric gradient is maintained. Because mPTP opening is not the only mechanism for loss of

ΔΨ, proper controls need to be performed with Cyclosporin A (CyclA), a pore inhibitor. Because this compound is not specific for its effect on cyclophilin D since it also inhibits calcineurin, a proper control with other calcineurin inhibitors is needed (e.g. FK506). This method can be used with a fluorescent plate reader, by flow cytometry or by fluorescent microscopy.

Top right corner: a more specific method involves using 2-deoxy [^{3}H] glucose, which is retained in cells after phosphorylation, although it does not permeate intact mitochondria. Upon mPTP opening, that radioactive molecule permeates mitochondria. Isolation of mitochondria and quantification of radioactivity in a scintillation counter allows for measuring the extension of mPTP opening. Although specific, this is a complex and often time and equipment consuming methodology.

Lower left corner: One of the variants of the calcein-AM method involves loading cells with that dye under specific temperature conditions, in conjugation with TMRM. After cleavage of the AM moiety by esterases, green-fluorescent calcein labels the cytosol, while red-fluorescent TMRM labels polarized (intact) mitochondria. After mPTP opening, mitochondrial TMRM labeling is lost, while calcein now permeates mitochondria. Usually, the more reliable methodology to follow these events is by using fluorescent microscopy.

Lower right corner: An adaptation of the calcein-AM method in which cobalt chloride (CoCl) is used to quench calcein fluorescence, which in this protocol is spread throughout the cell, including in mitochondria. As cobalt does not permeate mitochondria, CoCl+calcein treated cells show dark cytosol and green mitochondria. Upon mPTP opening, cobalt permeates mitochondria, and the green mitochondrial fluorescence is lost. This method can be used with a fluorescent plate reader, by flow cytometry or by fluorescent microscopy.

In all methods, proper controls with mPTP inhibitors (as well as negative controls) must be performed to avoid artifacts.

Figure 6. Reversibility of the mPTP - not always a bad guy. The mPTP has been described as undergoing different conformational changes, leading to alternative fates for the cell. The figure shows how mild elevations in calcium and oxidative stress, as well as cytosolic signaling can trigger the formation of a reversible low conductance mPTP, which has been observed under normal mitochondrial functioning, and in particular occasions including during cell differentiation. Low-conductance forms of the

mPTP can be useful to decrease mitochondrial membrane potential to avoid membrane hyperpolarization and excessive ROS production, as well as to discharge mitochondria from accumulated calcium. On the other hand, an excessive matrix calcium accumulation, together with persistent and elevated oxidative stress due to increased mitochondrial ROS production, as well as pro-apoptotic signaling may lead to the formation of an irreversible large-conductance form of the mPTP, which can lead to bioenergetic collapse, and contribute to cell death. Represented are also some factors (small triangles and squares) which regulate mPTP opening. One of such protein factors in the matrix chaperon cyclophylin D (CypD), which has been previously shown to interact with putative pore components, including the ATP synthase.