**INVITED REVIEW** 



# Ca<sup>2+</sup> mishandling and mitochondrial dysfunction: a converging road to prediabetic and diabetic cardiomyopathy

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#### Abstract

Diabetic cardiomyopathy is defined as the myocardial dysfunction that suffers patients with diabetes mellitus (DM) in the absence of hypertension and structural heart diseases such as valvular or coronary artery dysfunctions. Since the impact of DM on cardiac function is rather silent and slow, early stages of diabetic cardiomyopathy, known as prediabetes, are poorly recognized, and, on many occasions, cardiac illness is diagnosed only after a severe degree of dysfunction was reached. Therefore, exploration and recognition of the initial pathophysiological mechanisms that lead to cardiac dysfunction in diabetic cardiomyopathy are of vital importance for an on-time diagnosis and treatment of the malady. Among the complex and intricate mechanisms involved in diabetic cardiomyopathy,  $Ca^{2+}$  mishandling and mitochondrial dysfunction have been described as pivotal early processes. In the present review, we will focus on these two processes and the molecular pathway that relates these two alterations to the earlier stages and the development of diabetic cardiomyopathy.

Keywords Diabetic cardiomyopathy · Calcium mishandling · Mitochondrial dysfunction

### Introduction

Metabolic diseases (MetD) involve a cluster of illnesses with disrupted normal metabolism, among which are prediabetes, metabolic syndrome, and diabetes mellitus (DM). Diabetes mellitus (DM) is a chronic metabolic disorder that affects millions of people globally, with an exponential increase. Indeed, it is expected that this pandemic disease will affect more than 690 million people by 2045 [66]. These huge numbers are alarming, because this chronic illness is one of the major contributors, together with aging and obesity, to the increased rate of heart failure (HF) and related morbidity and mortality, worldwide [74, 206]. Moreover, DM is also a risk factor for many infectious diseases, i.e., tuberculosis, melioidosis, dengue, virus infection [279], and the actual pandemic of COVID-19 [283].

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According to the American Diabetes Association, the majority of cases of DM belong to one of two broad etiopathogenetic categories: type 1 DM (T1DM) and type 2 DM (T2DM). However, in many cases, diabetic individuals do not easily fit into a single class (see for review, [5, 6]). The usually described T1DM, previously known as juvenileonset diabetes or insulin-dependent diabetes, is an autoimmune disease that results from the destruction of insulinproducing  $\beta$ -cells in the pancreas, which leads to insulin deficiency. This autoimmune disease has been associated with different genetic predispositions and environmental factors. However, the underlying initial mechanisms are still poorly defined. T2DM accounts for 90-95% of all cases of DM [5] and constitutes a broad-spectrum syndrome, with a frequent late diagnosis; thus, target organs may be severely affected well before the symptoms appear. The cause of T2DM is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response.

In the heart, DM produces diabetic cardiomyopathy, which finally culminates in HF [112, 141]. Diabetic cardiomyopathy can be defined as the myocardial dysfunction that suffers patients with DM in the absence of hypertension and structural heart diseases such as valvular or coronary artery dysfunctions. Heart disease may initiate with diastolic dysfunction, mainly in T2DM (HF with preserved Fig. 1 EC coupling. AP induces Ca<sup>2+</sup> entry through the LTCC channels that induces RyR2 opening and Ca<sup>2+</sup> release from the SR.  $Ca^{2+}$  binds to the contractile machinery producing myocyte contraction. Ca2+ reenters the SR trough the SERCA2a and exits the cell trough the NCX, leading to the decrease of Ca<sup>2+</sup> transient and mechanical relaxation. Inset: Imbalanced ion currents can result in membrane potential alterations known as early and delayed afterdepolarizations, EAD, and DAD, respectively, according to the moment that they occur relative to the regular AP. EADs arise before the completion of AP, whereas DADs occur after AP completion. Possible ectopic beats are in red and blue. Below: An ECG with the ectopic beat produced by a DAD



ejection fraction, HFpEF,  $EF \ge 50\%$ ) that is later associated with systolic dysfunction, finally leading to systolic HF with EF < 40% [225].

Similar to many other insidious diseases, like hypertension, obesity, or cancer, T2DM starts several decades before reaching an overt and complete disease. Particularly, it may begin as a metabolic syndrome or as prediabetes. Indeed, metabolic defects precede overt clinical disease in most cases of T2DM. These metabolic derangements include impaired glucose tolerance and impaired fasting glucose. The presence of these alterations defines the prediabetic state [6, 253]. Because metabolic syndrome and prediabetes are usually silent conditions, the most studied entity is T2DM. Moreover, although T1DM and T2DM have different etiologies, both diseases share similar systemic metabolic imbalances [125, 225] that may be at the origin of the multiorgan alterations of DM, including diabetic cardiomyopathy [136].

Identification of the tight association between DM and cardiac disease occurred in 1972 when Rubler et al. (1972) described a new type of cardiomyopathy in patients with DM called diabetic cardiomyopathy [235]. Two years later, the Framingham study recognized the importance of DM in HF [141]. Subsequent studies showed that HF occurs at rates three to five times higher in DM patients than in the general population [112].

The underlying pathophysiology of the prolonged process that culminates in diabetic cardiomyopathy and HF is rather complex. However, it is known that impaired  $Ca^{2+}$  handling and mitochondrial dysfunction associated with enhanced ROS production and  $Ca^{2+}$ -calmodulin-dependent protein kinase

(CaMKII) activity (two crucial players in HF of different origins, [182]) appeared altered even at the prediabetic stages. In this early phase of the illness, diabetic cardiomyopathy and even DM symptoms are absent or unnoticed [98], making the knowledge of prediabetic prevalence, rather uncertain. However, the bad prognosis of prediabetic individuals underpins the decisive importance of studying the underlying mechanisms of the illness at the very early stages. Unfortunately, these earlier periods have been seldom assessed at the cellular and molecular level.

In the present review, we will focus on the molecular mechanisms mediating cytosolic, sarcoplasmic reticulum (SR), and mitochondrial  $Ca^{2+}$  mishandling in diabetic cardiomyopathy and how these alterations may be involved in the decrease in contractility, arrhythmias, and apoptosis observed that may finally end in overt HF. In the last part of the review, we will depict what is known about  $Ca^{2+}$  mishandling and mitochondria dysfunction in the early stages of the illness.

## Excitation-contraction coupling and cardiac mitochondria function

 $Ca^{2+}$  is a critical second messenger and  $Ca^{2+}$  homeostasis is crucial for maintaining cardiac function. Indeed, alterations in  $Ca^{2+}$  handling are associated with major cardiac disorders [152].

In each heartbeat, the  $Ca^{2+}$  entered through the L-type  $Ca^{2+}$  channels (LTCC) during the action potential (AP) binds to the ryanodine receptors (RyR2) and triggers a release of  $Ca^{2+}$  from the SR by a mechanism known as

Ca<sup>2+</sup>-induced-Ca<sup>2+</sup>-release [95] (Fig. 1). The rise in Ca<sup>2+</sup> allows actin-myosin interaction and muscle contraction (known as excitation–contraction coupling, ECC), but also increases the transport rate of mechanisms that remove Ca<sup>2+</sup> from the cytosol. These mechanisms are mainly the SR Ca<sup>2+</sup>-ATPase (SERCA2a) and the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX), causing the fall of Ca<sup>2+</sup> transient and mechanical relaxation. The enhanced activity of SERCA2a allows refilling of SR Ca<sup>2+</sup> store, whereas the NCX activity extrudes the Ca<sup>2+</sup> that entered the cell through the LTCC [22].

RyR2 are macromolecular complexes highly controlled by different interacting proteins that regulate their activity, like FK506-binding protein-12.6 (FKBP12.6), calmodulin (CaM), calsequestrin-2 (CASQ2), junctin (JCTN), triadin (TRDN), and *βII-spectrin*. Other proteins in the RyR2 complex regulate the level of RyR2 post-translational modifications, like protein kinase A (PKA), Ca<sup>2+</sup> calmodulin dependent protein kinase (CaMKII), SPEG, and protein phosphatase type-1 and type-2A (PP1 and PP2A, respectively). The RyR2 channel is also regulated by S-nitrosylation and oxidation ([22, 23, 160], and see below). SERCA2a activity is mainly regulated by phospholamban (PLN) [264]. PLN phosphorylation by PKA or CaMKII increases SERCA2a pump activity [99]. The PLN-SERCA2a duet is regulated by additional proteins, which either enhance or decrease SERCA2a activity and the rate of SR  $Ca^{2+}$  reuptake (see for reviews [153, 188]).

The heart has one of the highest energy demands in the organism, to support metabolism, contraction, and ion homeostasis. In healthy myocytes, the strategic positioning and abundance of mitochondria ensure the necessary ATP delivery to rapidly face this high energy demand. Under physiological Ca<sup>2+</sup> concentrations, local transfer of Ca<sup>2+</sup> to the mitochondria stimulates energy production by positively regulating the tricarboxylic acid (TCA) cycle enzymes and indirectly the pyruvate dehydrogenase complex (PDH), increasing NADH and FADH2 production, and enhancing electron transport chain (ETC) activity [200]. This SR-mitochondria Ca<sup>2+</sup> trafficking provides a fundamental link between Ca<sup>2+</sup>-dependent contraction and mitochondria metabolic output. Indeed, a fraction of cardiac mitochondria resides in close proximity to the junctional SR. This immediacy plays a functional role in excitation-metabolism coupling (EMC) and programmed cell death (Fig. 2B) ([58, 111] and see below). Additionally, ETC is the main cellular process that generates reactive oxygen species (ROS) under both physiological and pathological conditions [9].

To reach the mitochondrial matrix,  $Ca^{2+}$  needs to cross two lipid bilayers, the outer and the inner mitochondrial membranes. In the outer mitochondrial membrane,  $Ca^{2+}$ crosses through the voltage-dependent anion channel (VDAC), which allows the transport of ions and small proteins (Fig. 2B). The inner mitochondrial membrane is completely ion impermeable, and the driving force for  $Ca^{2+}$  entry is the mitochondrial membrane potential ( $\Delta \Psi_m$ around - 150 to - 180 mV) [159]. Mitochondrial matrix Ca<sup>2+</sup> concentration balance is achieved by two main mechanisms; Ca<sup>2+</sup> enters mostly through the mitochondrial Ca<sup>2+</sup> uniporter complex (MCU) and exits through the  $Na^+ Ca^{2+}$  $Li^+$  exchanger (NCLX) or through the H<sup>+</sup>/Ca<sup>2+</sup> exchanger (HCX) in tissues where mitochondrial NCLX activity is low [266]. Heart inducible MCU deleted mice present normal resting mitochondrial Ca<sup>2+</sup> levels indicating alternative Ca<sup>2+</sup> entry mechanisms [156]. The MCU is a large complex with multiple regulatory proteins. It is formed by the inner membrane components, MCU, a dominant-negative MCUb and EMRE (essential MCU regulator), and the intermembrane space regulators: the EF-hand proteins MICU1, MICU2, and MICU3. Additionally, MCUR1 seems to be involved in MCU-EMRE interaction [79]. On the other hand, recent evidence suggests that besides its well-established role in mitochondria-mediated cell death, the mitochondrial permeability transition pore (mPTP) participates in the maintenance of mitochondria Ca<sup>2+</sup> homeostasis through the extrusion of mitochondrial  $Ca^{2+}$  [148, 180]. The identity of the mPTP is still under discussion, but one of the most accepted conformations is that it is formed by VDAC in the outer membrane and the adenine nucleotide translocase (ANT) in the inner membrane and the regulatory protein cyclophilin D (CyD) in the matrix side [17] (Fig. 2B). The mPTP opening can be induced by Ca<sup>2+</sup> and ROS. ADP, H<sup>+</sup>, Mg<sup>2+</sup>, and NADH increase the  $Ca^{2+}$  threshold for mPTP opening [17].

### SR-Ca<sup>2+</sup> leak and SR-mitochondria interaction

SR Ca<sup>2+</sup> release in cardiac myocytes occurs via local events referred to as Ca<sup>2+</sup> sparks [64]. The normal twitch Ca<sup>2+</sup> transient in ventricular myocytes results from the temporal and spatial summation of thousands of Ca<sup>2+</sup> spark events which are synchronized by the AP and L-type Ca<sup>2+</sup> current (I<sub>Ca</sub>) via Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release [49, 50, 95, 177, 178]. Ca<sup>2+</sup> sparks also occur in a stochastic manner during rest, even in the absence of Ca<sup>2+</sup> influx, although at very low frequency in cardiac cells [64, 241]. Diastolic SR Ca<sup>2+</sup> leak may be augmented by an increase in SR Ca<sup>2+</sup> load (above a certain threshold, small changes in SR Ca<sup>2+</sup> load result in far greater increases in Ca<sup>2+</sup> release) [247] and by factors that alter RyR2 regulation [25, 191] (Fig. 2A).

Three different sites have been found to change RyR2 activity: Ser2808 (2809 in human heart), Ser2814 (2815 in humans), and Ser2030 [288, 291, 298] (Fig. 2A). Activation of CaMKII and the subsequent phosphorylation of S2814 are generally considered the critical post-translational modifications that enhance SR Ca<sup>2+</sup> leak in HF [87, 233], ischemia–reperfusion injury [78, 237], and several other



Fig. 2 A RyR2 receptor regulation. RyR2 interacts with different proteins that regulates its function either directly or through RyR2 phosphorylation at different sites (FKBP12.6, juctin, triadin, calsequestrin, PP1, PP2A, CaM, CaMKII, and PKA. RyR2 is phosphorylated at Ser2814/15 site by CaMKII, at Ser2808/9 site by PKA and CaMKII and at Ser2030 by PKA. B MAMs. Mitochondria and ER/SR are linked by several proteins resulting in optimized Ca<sup>2+</sup> communication between these two organelles. Fundc1, GRP75, and Mfn-2 serve as anchoring proteins, narrowing the space between IP3R2/RyR2 and VDAC. The mPTP structure is not clear and it's opening can be induced by increases in ROS, Ca<sup>2+</sup>, decreased  $\Delta \Psi m$ , resulting in release of the mitochondrial content including proapoptotic proteins. C SR-nuclear cross-talk. The outer membrane of the nuclear envelope extends into the cytoplasm to form a continuum with the SR. The inner membrane deeply invaginates into the nucleus forming what is called the nucleoplasmic reticulum. Ca2+ inside the nucleus can bind calmodulin (CaM) and activate gene transcription. Additionally, CaM-Ca<sup>2+</sup> can activate CaMKII that will in turn phosphorylate and activate other transcription factors. Finally, CaM-Ca<sup>2+</sup> activates calcineurin resulting in additionally gene transcription activation

diseases, including diabetic cardiomyopathy [93, 256, 261]. Importantly, CaMKII may be also persistently over-activated (autonomous activation of the kinase) by posttranslational modifications including autophosphorylation [129], oxidation [91], S-nitrosylation [57, 92], and O-GlcNAcylation (OGN) [91] as well as by reactive carbonyl species [26, 301]). These modifications occur in several cardiac diseases, including diabetic cardiomyopathy, a disease characterized by the increase in oxidative stress. CaMKII can phosphorylate different substrates, including RyR2 and PLN, which are associated with increased SR Ca<sup>2+</sup> leak and enhanced propensity to arrhythmias and cell death (see below).

RyR2 may also suffer redox modifications. Although the current understanding of redox-mediated RyR2 activity remains incomplete, its impact on RyR2 function and dysfunction is supported by a variety of experimental results (see for review [55, 85, 171]). Oxidation of RyR2 SH residues increases the activity of RyR2 channels and the rate of calcium fluxes in isolated SR vesicles [124] and enhances SR Ca<sup>2+</sup> leak and arrhythmias [18, 268]. S-nitrosylation and S-glutathionylation have been also shown to increase the activity of RyR2 channels in vitro in subcellular cardiac muscle fractions and in isolated cardiomyocytes [86, 171, 239] (Fig. 2A).

Mitochondria and endoplasmic reticulum (ER) or SR are in close contact (approximately 15-50 nm apart) at multiple sites called mitochondria-ER contact sites (MERC) [58, 111]. The fraction of membranes comprised in these interactions is defined as the mitochondrial associated membranes (MAMs). MERCs are involved in multiple critical functions among which is ER/SR-mitochondria Ca<sup>2+</sup> shuttling [149, 229, 230, 234]. These narrow spaces constitute high concentration domains of Ca<sup>2+</sup> released by IP3R or RyR2 that affect mitochondrial Ca<sup>2+</sup> homeostasis, energetics, metabolism, and mitochondrial dynamics [300]. Several protein-like structures acting as physical tethers link both organelles. Among these proteins are mitofusin 2 (Mfn-2) [62, 75, 201] and GRP75, which link the Ca<sup>2+</sup> channel IP3R, isoform 2 (IP3R2), to VDAC [263]. Similarly, IP3R2 has been described to bind with the FUN14 domain containing 1 (FUNDC1) to modulate SR Ca<sup>2+</sup> release [295]. Seidlmayer et al., 2019, recently showed the crucial role of physical tethering of SR and mitochondria by Mfn-2 for metabolic feedback induced by IP3 (Fig. 2B) [245]. VDAC has also physical interactions with the RyR2, which, coupled with MCU co-localization with the RyR2, helps to explain how ER/SR-mitochondrial Ca<sup>2+</sup> transport is possible [146]. Studies by De La Fuente et al., 2016, showed that Ca<sup>2+</sup> signaling activity promotes MCU recruitment to dyad (RyR2) areas [76]. In this way, MCU "hot spots" can be formed at the mitochondria-SR/ER associations favoring local Ca<sup>2+</sup> signaling and the excitation-energetics coupling (Fig. 2B).

 $Ca^{2+}$  leak from the RyR2 has been related to either reduced or increased mitochondrial  $Ca^{2+}$  content, both

resulting in mitochondrial dysfunction and enhanced ROS production [119, 240]. Recent experiments indicated that the increase in RyR2 activity induces mitochondria depolarization, altering mitochondria Ca<sup>2+</sup> homeostasis and elevating mitochondrial ROS efflux. Thus, the increase in SR Ca<sup>2+</sup> leak would trigger a vicious circle that further activates RyR2, SR Ca<sup>2+</sup> leak, and mitochondrial dysfunction [119].

### Cytosolic Ca<sup>2+</sup> and nucleus cross talk

There is a close contact as well as an intense crosstalk between the SR, the nucleus envelope, and the nucleus itself. The outer membrane of the nuclear envelope extends into the cytoplasm to form a continuum with the SR. The nucleus membrane expresses different Ca<sup>2+</sup>-handling proteins like RyR type 1 and IP3 receptors, SERCA2a, PLN, and NCX [61, 94, 110, 163, 217, 293, 314]. In addition, the inner membrane deeply invaginates into the nucleus forming what is called the nucleoplasmic reticulum (see for review [186]). The relation between nuclear and cytosolic  $Ca^{2+}$ movements and the role played by the membrane nuclear Ca<sup>2+</sup> handling proteins are not yet completely understood. However, it is known that cytoplasmic  $Ca^{2+}$  transients are associated to nucleoplasmic Ca2+ transients, which are main players in the regulation of gene expression, a process termed excitation-transcription coupling [77, 297] Fig. 2C. Cytosolic Ca<sup>2+</sup> would passively diffuse to the nucleus and then extruded via nuclear pores (NP) distributed along the nuclear envelope and then taken up by either the SERCA2a at the nuclear membrane or the SR, the neighboring mitochondria, or extruded by the NCX in the nearby T tubules (see for review [62]). Shanin et al. 2001 showed that NP conductance is controlled by the levels of Ca<sup>2+</sup> and ATP close to the nuclear envelope and by the stimulation of different receptors, particularly IP3R, located at the nuclear envelope [246]. Other receptors identified in the nuclear envelope are  $\alpha$ -adrenergic, endothelin-1, and angiotensin II [30, 265, 296] — indicating that IP3 may also originate at the nuclear level for a local control of  $Ca^{2+}$  [62] — as well as  $\beta$ 1 and  $\beta$ 3-adrenergic receptors [31], which may be involved in the  $\beta$  regulation of gene expression [281]. Thus, as stated by Dewenter et al., 2017, in a relatively recent review " ... cytosolic and nuclear Ca<sup>2+</sup> cycling are part of a highly sophisticated system which controls  $Ca^{2+}$  -dependent signaling by modifications of global Ca<sup>2+</sup> oscillations as well as Ca<sup>2+</sup> regulation within microdomains" [65, 77, 195].

As will be discussed below, different proteins involved in  $Ca^{2+}$  handling and the interactions and cross-talks SRmitochondria and nucleus are modified and/or dysregulated in different models of diabetic cardiomyopathy and even in the prediabetes state. These changes are part of the cardiac maladaptive remodeling and constitute main players in diabetic cardiomyopathy development.

# Ca<sup>2+</sup> mishandling in diabetic cardiomyopathy

As described above,  $Ca^{2+}$  is a key element in ECC, EMC and excitation-transcription coupling, but also in triggering apoptosis and genetic adaptive (maladaptive) responses, like hypertrophy [65, 195]. Therefore, it is critical to establish the alterations in  $Ca^{2+}$  handling as well as the more relevant changes (expression, function, and regulation) of proteins involved in  $Ca^{2+}$  handling in the evolution of diabetic cardiomyopathy to delineate strategic actions to avoid the progression of the disease.  $Ca^{2+}$  mishandling in diabetic cardiomyopathy is the main cause of depressed contractility, slow relaxation, triggered arrhythmias, and altered cellular processes, as apoptosis or mitophagy.

#### a. Main triggers of diabetic cardiomyopathy

When diabetic cardiomyopathy evolves to HF, it shares most of the alterations observed in HF from different etiologies, i.e., left ventricular hypertrophy, interstitial fibrosis, cell death, diastolic and systolic dysfunction, impaired contractility, Ca<sup>2+</sup> mishandling, altered substrate utilization, myocardial lipotoxicity, inflammation, impaired autophagy, endoplasmic reticulum stress, and oxidative stress [136]. Additionally, all these mechanisms are linked and can be coordinated. Although the triggers for these changes include hyperglycemia, hyperlipidemia, and hyperinsulinemia, the beginning of the different cardiac abnormalities is not yet clearly determined. Belke et al., 2004, suggested hyperglycemia as the main factor responsible for diabetic cardiomyopathy in both, type 1 and 2 of DM [20]. The authors argue that despite insulin levels differ between the two models, both exhibit important hyperglycemia and progressive cardiomyopathy with increasing plasma glucose levels and advanced glycation end products (AGEs) [1]. In contrast, other insulin-resistant models with modest hyperglycemia show only discreet cardiomyopathy [19, 223]. Indeed, hyperglycemia induces oxidative-stress, producing tissue/cell damage in several target organs including the heart [40]. Hyperglycemia leads to the formation of AGEs, forming irreversible cross-links in many large proteins (such as collagen), generating myocardial fibrosis and increased stiffness [10, 13, 48, 56, 120, 184]. Probably more important in the context of this review, cross-linked AGEs were demonstrated in RyR2, associated with a decrease in the activity of RyR2 [27], and associated with a decreased expression of SERCA2a [28], in part through their action on nuclear gene expression (see below). These changes have been related to diastolic and systolic dysfunction in diabetic cardiomyopathy [154]. Experimental evidence also demonstrated a crucial role of hyperglycemia on CaMKII activation and arrhythmogenesis in diabetic cardiomyopathy [93], although additional pathological factors exacerbated the risk for arrhythmias in this cardiomyopathy [122].

#### Animal models

There are a wide variety of preclinical animal models of DM with specific characteristics that may be relevant to studying type 1 or type 2 DM. Among the animal models used as experimental tools to study T1DM are the streptozotocin (STZ) model, [32], which is the most frequently model used to mimic T1DM, and the spontaneous type 1 diabetes models, among which are the non-obese diabetic (NOD) mouse [185], possibly the most used of spontaneous models, the OVE26 mouse model [90] and the heterozygous Ins2+/- Akita diabetic mouse [306] (Table 1 and Supplemental Table 1a and b).

To study T2DM, the most used models are the ob/ob [311] and db/db [54] mutant mice, the Zucker diabetic fatty (ZDF) rat [52], and the spontaneously diabetic model Otsuka Long-Evans Tokushima Fatty (OLETF) rat model [142]. There are also models of diet-induced diabetes, for instance, low STZ and high fat diet (HFD), high fat and high sucrose diet (HFHS), or the fructose-rich diet (FRD), all of which can result in insulin resistance and T2DM [220]. HIP rats are obese rats expressing the human isoform of amylin, a pancreatic hormone co-secreted with insulin in the pancreatic  $\beta$ -cells, used as a model of late-diabetic cardiomyopathy [47]. Non-mammals' models, like zebra fish, C. elegans, and Drosophila melanogaster have been also used as DM models [29, 80] (Table 2 and Supplemental Table 2a and b). These models may be very useful, because of their shorter cycle life, the whole-genome interference RNAi library available, and the low maintenance cost [144].

All these models were valuable experimental tools for studying the underlying mechanisms of T1DM and T2DM in the human being, and in all cases, their use present different types of advantages and disadvantages (for review about this issue, see [42, 143]). In all cases, extrapolation of the results to humans has to be cautious, because, in addition to some unique characteristics that the models may have, the studies may reflect different stages and severities of the illness, different predispositions to cardiomyopathies, or even confounding effects produced by the toxicity of drugs or the genetic mutation used to induce DM [42].

#### b. Low contractility and slow cardiac relaxation

Despite the model diversity, contractile depression, delay relaxation associated with a diminished transient  $Ca^{2+}$  amplitude, and prolongation of intracellular  $Ca^{2+}$  decay are hall mark characteristics of diabetic cardiomyopathy in the hearts of T1DM and T2DM, as shown in Tables 1 and 2 [20,

248, 249]. In several diabetic cardiomyopathy models, of either T1DM or T2DM, altered relaxation appears before a significant decrease in contractility occurs. Moreover, when intracellular Ca<sup>2+</sup> transients were measured, changes in contractility and relaxation are associated to similar changes in  $Ca^{2+}$  transient's amplitude and relaxation, underpinning the importance of Ca<sup>2+</sup> mishandling in diabetic cardiomyopathy (see supplementary Tables 1 and 2). A reduced SR Ca<sup>2+</sup> content may be due to a reduced SERCA2a pump activity, an increased SR Ca<sup>2+</sup> leak via RyR2, an enhanced Ca<sup>2+</sup> extrusion via NCX, and/or a decrease in I<sub>Ca</sub>. Tables 1 and 2 of supplementary materials display some results of different aspects of Ca<sup>2+</sup> handling and mitochondrial alterations in different models of diabetic cardiomyopathy. Tables 1 and 2 are a summary of most common alterations observed in different models.

A decrease in SERCA2a expression or activity is a common finding in the different models in which this protein has been explored [20, 28, 67, 203]. In some diabetic models, for instance, in STZ treated rats or the Akita model in T1DM or the db/db mouse model in T2DM, there is an increase in PLN expression and a decrease in PLN phosphorylation, which should lead to SERCA2a inhibition [216], and may well explain the decrease in  $Ca^{2+}$  transient and contractility as well as the slow relaxation typical of this disease [20]. Indeed, it has been shown that restoration of SERCA2a expression with an adenoviral vector improves contractile function and reverts the increased cardiomyocyte size in T2DM hearts [236]. However, although SERCA2a depression appears as a main mechanism for the decrease in contractility and relaxation observed in DCM, some T2DM diabetic cardiomyopathy (T2DC) models show decreased contractility and even relaxation, without changes in SERCA2a expression or PLN alterations (Supplementary Table 2). Interestingly, studies by Kralik et al., 2005, indicated a greater depression of Ca<sup>2+</sup> transient decay in OVE26 than in db/db myocytes, in association with a significant decrease in SERCA2a expression in the OVE26 but without changes in db/db myocytes, despite similar degrees of diminished contractility [151]. These somewhat unexpected findings may indicate different stages of evolution of the illness, different species and preparations, but also that other mechanisms may be playing a role in the decrease in contractility observed in T2DC. For instance, Pereira et al., 2006, described a decrease in SR Ca<sup>2+</sup> content associated with a decrease in RyR2 expression, L-type Ca<sup>2+</sup> current, and an increase in NCX expression, all of which may contribute to decrease SR Ca<sup>2+</sup> load [177]. Unfortunately no measurements of SERCA2a activity or expression were performed in this study, to explain the impairment of relaxation described. Chou et al., 2017, found similar expression of SERCA2a but slower Ca<sup>2+</sup> transient decay, attributed to prolonged AP duration due to CaMKII-dependent slowing of Ca<sup>2+</sup> current

Table 1 Su	mmary of exc	itation-contra	action couplia	ng parameter c	characteristics	in myocardic	opathy of type 1	diabetes					
Model	Experi- mental preparation	Contractil- ity /relaxation	Systolic /diastolic function	Ca <sup>2+</sup> transient amplitude/ decay time	SERCA2a/ PLN ratio and SR Ca <sup>2+</sup> load	RyR2	LTCC $(I_{Ca})$	NCX (for- ward)	Ca <sup>2+</sup> SpF or waves/ arrhyth- mias	PKA	CaMKII	Observations	References
STZ treated rats	Whole animal (ECC), isolated heart, myocytes and mito- chondria, sarco- lemma prepara- tions	$\downarrow$ or=/ $\downarrow$ or= / $\downarrow$	↓ or=/↓	$\downarrow$ or $\uparrow$ $\downarrow$ or $\uparrow$ diastolic Ca <sup>2+</sup> Non- uniform Ca <sup>2+</sup> release	$\downarrow$ SR Ca <sup>2+</sup> $\downarrow$ SER- $\downarrow$ SER- $\downarrow$ SER- $\downarrow$ CA2a expres- sion but $\uparrow$ phos $\uparrow$ or = PLN expres- sion $\downarrow$ , $\uparrow$ or = PLN	↓ RyR2 expres- sion ↓ [3H]Ry binding [3H]Ry affinity ↑ RyR2 phosp at \$2808 & \$2814	↓ or = expres- sion ↑ affinity AP prolonga- tion	→	←	↑ or ↓	<i>←</i>	Effects partially reverted with exercise. Increased myocytes necrosis and apoptosis. Reduced mitochondrial $Ca^2 + uptake, = NCLX$ activity. Reduced mito- chondrial function	[27, 39, 67, 104, 157, 158, 164, 203, 248, 249, 256, 304, 309]
STZ treated mice	Whole animal (ECC), isolated myocytes		↓ or=/↓	(us) ↓	↑ PLN expres- sion (ns) and OGN	S2814A mice were protected from AF			←	$\rightarrow$	←	Mitochondrial dis- ruption. Increased inflammation and apoptosis. Increased oxidative stress and OGN. Increased AF reverted by CaMKII inhibition of CaMKII inhibition of CaMKII oxidation (and oxida- tive stress generation) protected from AF. Diastolic dysfunction less severe in mice protected from CaM- KII OGN or oxidation. Increased arrhythmias frequency. CaMKII deletion protected from arrhythmias. Increased SR Ca <sup>2+</sup> Heak	316J 316J

	References	[169, 250, 251, 299, . 305]	[84, 213, 244]	[16, 44, 161, 179, 215]	ed pressure was
	Observations	Disorganized mitochon- dria in heart tissue and mitochondrial damage Increased mitochon- drial biogenesis. Mitochondrial dys- function and increased oxidative stress in high glucose treated OV26 myocytes	Reduced LV weight. Higher respiratory ratio, decreased com- plex IV expression. Increased arrhythmia frequency	Conserved or reduced ventricular dimensiom: Increased palmitate and reduced glucose oxidation. No mito- chondrial uncoupling but increased UCP2 and UCP3 expression. Reduced mitochondria function and ETC gene expression. Reduced and increased oxidativ stress. Reduced cristae density and mitochon- drial disruption	is included when develop
	CaMKII				relaxation
	PKA				ractility/
	Ca <sup>2+</sup> SpF or waves/ arrhyth- mias				control. Cont
	NCX (for- ward)			1 expres- sion = activity	ifference vs c
	LTCC $(I_{Ca})$			↓ reduced cell surfaces LTCCs	's control. =, no d
	RyR2				decreased v
	SERCA2a/ PLN ratio and SR Ca <sup>2+</sup> load	↓SERCA2a expres- sion = PLN expres- sion		$\$ SERCA2a expression expression = PLN expression = or $\downarrow$ PLN phosp $\$ $\$ SSR Ca <sup>2+</sup> content	s control. U
	Ca <sup>2+</sup> transient amplitude/ decay time	¢7 dN	ND/↑	↓ /=	↑, increased v
	Systolic /diastolic function	$\rightarrow$ / $\rightarrow$	↓ or=/ ↓	$\downarrow$ or=/ $\downarrow$	eptozotocin.
	Contractil- ity /relaxation	† / (su) †	↑ / ↑	Modest↓ or=/ND	llitus, STZ str
(tinued)	Experi- mental preparation	Whole animal (ECC), isolated heart, myocytes and mito- chondria	Whole ani- mal ECC, isolated hearts, and mito- chondria	Whole animal ECC, isolated hearts, myo- cytes, and mito- chondria	diabetes mel
Table 1 (con	Model	OV 26 mice	NOD mice	Akita	TIDM type 1

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NCX sodium/calcium exchanger.  $Ca^2 + SpF$  calcium sparks frequency. PKA and CaMKII are referring to their activity, measured directly or by canonical targets phosphorylation. *Phosp* phosphorylation. *LTCC* L type calcium channel current. *AGEs* advanced glycation end products. *ND* not determined. (*ns*) not significant difference. *AF* atrial fibrallation

Table 2 Sumr	nary of excitatic	on-contraction c	coupling parame	ter characteristi	ics in myocardic	pathy of type 2	c diabetes						
Model	Experimental preparation	Contractility /relaxation	Systolic /diastolic func- tion	Ca <sup>2+</sup> transient amplitude/ decay	SERCA2a/ PLN ratio and SR Ca <sup>2+</sup> load	RyR2	$(I_{Ca})$	NCX	Ca <sup>2+</sup> SpF or waves/arrhyth- mias	PKA	CaM- KII	Observations	References
db/db mice	Whole animal (ECC), isolated heart, and myo- cytes	$\uparrow$ $\uparrow$ $\uparrow$	↑ / ↑ / ↑	↓ /↑	↓ SERCA 2a expression expression iny = $PLN$ iny = $PLN$ phos- phorylation ↑ $PRCa^{2+}$ load	$= or \downarrow RyR2$ expression. RyR2 phosp (S2814) (S2814) (S2814) $\downarrow$ Synchrony of $\downarrow$ Synchrony of $\downarrow$ Cartratease Cartratease Cart leak	= or J reduced cell surfaces LTCCs	<i>←</i>	↑ or ↓	11	←	Initial decrease in SERCA expres- sion associated with increased relaxation prevented by, prevented by, diastolic and systolic Ca, ing normalizes or reduces micochondrial mem- by and micochon- drial function. Micochondrial mem- price potential and micochon- drial function. Micochondrial mem- drial function. Micochondrial mem- trate and solution and solution and solution.	216, 261]
ob/ob mice	Whole animal (ECC), isolated heart, and myocytes	†/†	↑ /=	↓ or=/↑	= SERCA2a but increased oxidation and reduced activity							Increased cardio- mycoyte length. Mitochondria swelling, disorganization of cristica and loss of integrity. Reduced mito- tion. Increased oxidative stress. Slower mito- thondrial Ca <sup>2+</sup> uptake	[37, 71, 83, 97, 167]

Table 2 (conti	nued)											
Model	Experimental preparation	Contractility /relaxation	Systolic /diastolic func- tion	Ca <sup>2+</sup> transient amplitude/ decay	SERCA2a/ PLN ratio and SR Ca <sup>2+</sup> load	RyR2	LTCC [( <i>I</i> <sub>Ca</sub> )	NCX Ca <sup>2+</sup> SpF waves/arrh mias	or PKA lyth-	CaM- KII	Observations	References
Lauker diabetic fatty (ZDF) rats	Whole animal (ECC). ECC). cytes, and myo- cytes, and mitochondria mitochondria	↓ or =/↓ Altered time course	=/=or ↓	$\downarrow$ or = /= or $\uparrow$	= SERCA2a and PLN expression = SR Ca <sup>+</sup> = SR = SR Ca2 + load		→			←	No changes in mydfilament Ca <sup>3+</sup> sensitivity. Left ventritivity. Left ventritivity. CaMKIB hypertrophy- day and propering ity and men- ity and men- metodorial (2a) is and oxidative stress. Reduced mitodorial (2a) is and oxidati	[73, 127, 227]
OLETF 30% Sucrose feeded	Isolated hearts, and mito- chondria		↑ <i> </i> =	ND/↑	↓ SERCA2a expression						Effects age dependent	[2]
HFHS diet treated mice	Whole animal (ECC), iso- lated heart	↑/=	<i>`</i> /=								Increased oxida- tive stress and posttranslational protein modifi- cations	[220]
High fat diet +low dose of STZ treated mice	Whole animal					S2814A mice protected from AF					Increased oxida- tive stress and OGN. Increased AF reverted by CaMKII inhibi- tion (AC3-1). Inhibition of CaMKII oxida- tion but not OGN protected from AF	[192]
High fat diet drosophila melanogaster	Whole animal (ECC)	<i>↑/=</i>	CIN /↑								↓ IP <sub>3</sub> Rs expres- sion	[29]
T2DM type 2 c	fiabetes mellitu: ion is included v	s.↑, increased v vhen echocardio	vs control. ↓, dec ographic (ECC)	creased vs conti data is available	rol. =, no diffe e. <i>SERCA2a</i> sa	rence vs control. rcoplasmic retic	Contractility/re ulum ATPase 2.	laxation is inclu PLN phosphola	ded when d nban. <i>RyR</i> 1	eveloped syanodin	l pressure was mea e receptor 2. <i>NCX</i>	sodium/calcium

exchanger. Ca2a+SpF calcium sparks frequency. PKA and CaMKII are referring to their activity, measured directly or by canonical targets phosphorylation. Phosp phosphorylation. LTCC L type calcium channel current. OGN O-GlcNAcylation. AF atrial fibrillation. OLETF Otsuka Long-Evans Tokushima Fatty rat model of diabetes mellitus, HFHS high fat and sugar, HFD high fat diet. ND not determined

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inactivation and an increase in late Na<sup>+</sup> current ([69] and see Supplementary Table 2). Other authors showed mitochondrial dysfunction associated to either decrease contractility or relaxation [97, 134, 227]. Indeed, impaired energy metabolism has been also associated to hyperglycemia cardiac contractile deficiency [150]. In the Zucker diabetic fatty (ZDF) rats, a well-known model of T2DM, the decrease in contractility and relaxation observed without significant alterations in SR Ca<sup>2+</sup> load was attributed to an increase in SR Ca<sup>2+</sup> sparks or a diabetic-induced alteration at the myofilament level [73]. The first possibility seems unlikely, since the increase in spontaneous Ca<sup>2+</sup> sparks would lead to a decrease in SR Ca<sup>2+</sup> content, unless a concomitant increase in SR  $Ca^{2+}$  uptake occurs, which seems not to be the case. In contrast, changes at the contractile protein level, including depressed sensitivity of myofilament to Ca<sup>2+</sup>, have been described by others in STZ-treated rats [3, 187], ZDF rats [127] and ob/ob mice [167] (Supplemental Table 2). This finding is very interesting since studies in human cardiac tissue report alterations at the myofilament level in T2DC [21, 107, 140].

The molecular underlying mechanisms of the decrease in SERCA2a expression and/or activity are not completely clear, when no changes in PLN expression or phosphorylation are found. Yet, experimental studies indicate different and possibly nonexclusive mechanisms. For instance, it was shown that the impairment of relaxation observed in diabetic cardiomyopathy in association with a decrease in the activity of SERCA2a was due to enhancing oxidative stress and SERCA2a oxidation [167, 267]. Experiments by Bidasee et al., 2004, showed that SERCA2a peptides were modified by cross- and no crosslinking AGEs, decreasing SERCA2a activity [28] (see also Supplementary Table 1). Indeed, diabetic cardiomyopathy is associated with significantly enhanced cardiac AGE and AGE receptors (RAGE) levels, which colocalize in cardiomyocytes [184]. The decrease in SERCA2a expression in myocytes subjected to hyperglycemia was coupled with a significant reduction in SERCA2a promoter activity and, in turn, associated with overall levels of nuclear O-GlcNAcylation (OGN), greatly suggesting the Sp1 OGN, one of the main transcriptional regulators of SERCA2a (ATP2A2 gene) expression [53]. O-GlcNAcylation of the transcription factor Sp1 was also described in, associated to diastolic dysfunction and SERCa2a decreased expression [106].

Alterations of RyR2 function were also described in several models of diabetic cardiomyopathy ([20, 26, 67, 116, 216, 248, 249, 256, 304, 309] and see Tables 1 and 2 of supplementary material). A common finding, mainly in T1DC, is a reduced expression and activity of RyR2 [26, 67, 248, 303, 304, 308], resulting in reduced contractility, by decreasing SR Ca<sup>2+</sup> gain [22]. In some of the studies, RyR2 alterations were associated with an increase in SR Ca<sup>2+</sup> leak either in STZ-induced T1DC [[20, 248, 249, 304] or in db/ db T2DC [13] (Tables 1 and 2 of supplemental material). which would either produce or exacerbate the decrease in SR Ca<sup>2+</sup> content and contractility observed in diabetic cardiomyopathy. Of note, the greater activity of RyR2, produced, for instance, by PKA or CaMKII phosphorylation, would not only favor SR Ca<sup>2+</sup> leak and the decrease in contractility, but also systolic Ca<sup>2+</sup> release, counteracting the effect of SR Ca<sup>2+</sup> leak. Indeed, it was shown that fractional Ca<sup>2+</sup> release is enhanced by CaMKII-dependent phosphorylation of RyR2 [190, 280], for a given SR Ca<sup>2+</sup> load [162]. Moreover, the increase in SR Ca<sup>2+</sup> leak can also contribute to slowing twitch relaxation. For instance, Shao et al., 2009, showed in STZ-induced T1DC a delayed relaxation and Ca<sup>2+</sup> transient decrease in the absence of significant changes in SERCA2a expression or PLN phosphorylation [249]. These results emphasize the putative role of SR Ca<sup>2+</sup> leak on the depressed contractility and delayed relaxation observed in diabetic cardiomyopathy [24].

The increase in SR Ca<sup>2+</sup> leak in diabetic cardiomyopathy has been associated with different mechanisms. In STZ animals, RyR2 has been shown to be phosphorylated at S2808/9 [248, 304], a residue mainly phosphorylated by PKA, but also by CaMKII [99], and at the CaMKII site S2814/5, in association with a decrease in PKA and an increase in CaMKII activities, indicating that the increase in S2808/9 phosphorylation should be therefore attributed to CaMKII [249] (Fig. 2A). As previously mentioned, the activity of CaMKII has been shown to be increased in different models of either T1 or T2 diabetic cardiomyopathy [69, 73, 122, 261]. The increase in SR Ca<sup>2+</sup> leak has been also associated to a decrease in the RyR2 regulatory protein FKB12.6 [20, 248, 304]. FKBP12.6, a peptidyl-prolyl cis-trans isomerase, tightly associates with RyR2, stabilizing its closed conformational state and facilitating channel closure [35] (Fig. 2A). In T1DC, Yaras et al., 2007, showed a decreased expression of FKB12.6 and RyR2, RyR2 phosphorylation and SR Ca<sup>2+</sup> leak, with an increased activity of PKA and PKC, that could be blocked by inhibition of PKC and the angiotensin 1 receptors [303]. These results linked Ca<sup>2+</sup> mishandling in diabetic cardiomyopathy with the increased activity of the renin-angiotensin system observed [221]. The increase in ROS production is a main mechanism of RyR2 alterations in STZ-induced diabetic cardiomyopathy that may be reversed by increasing of antioxidant protection [276]. Bidasee et al., 2004, showed a decrease in RyR2 activity with no changes in RyR2 expression in STZ rat myocytes, attributed, as in the case of SERCA2a, to AGEs [249]. In STZ rat myocytes, Tian et al., 2011, further showed an increase in the leakness of RyR2, independent of RyR2 phosphorylation and/ or FKB12.6 downregulation. This effect was attributed to the enhanced cyclic adenosine diphosphate ribose levels

observed in these myocytes [269]. Moreover, and as discussed for SERCA2a, the underlying mechanisms of the decrease in RyR2 expression described in different models of diabetic cardiomyopathy are not clear. In STZ-induced diabetic cardiomyopathy, it has been suggested that insulin deficiency may play a role, due to its influence on gene expression [249].

The results regarding NCX function and I<sub>Ca</sub> in diabetic cardiomyopathy are controversial (Tables 1 and 2 and Tables 1 and 2 of supplemental material). In db/db myocytes, NCX function was reported to be normal [20, 69]. On the contrary, Stolen et al., 2009, and Pereira et al., 2006, reported an enhanced NCX activity in db/db mice [216, 261], while in T1DM, it was found to be depressed due to a decrease in the NCX expression or activity [34, 67]. As discussed above, in the cases of enhanced activity, it may contribute to SR Ca<sup>2+</sup> unloading and depressed contractility of diabetic cardiomyopathy. The results on I<sub>Ca</sub> are also mixed; whereas several authors reported that I<sub>Ca</sub> was not affected in diabetic cardiomyopathy [67, 138, 157, 166, 304], other authors describe a decrease in  $I_{Ca}$  suggesting that this reduction may be also involved in the reduced SR  $Ca^{2+}$  content observed in diabetic cardiomyopathy [34, 165, 216, 261, 287]. As noted above, these conflicting results might arise from the diabetes model used and/or possibly more important, from the degree of evolution of diabetic cardiomyopathy.

In summary, SERCA2a and RyR2 appeared to be the most affected ECC proteins in diabetic cardiomyopathy (although not unique) and in turn the responsible for the slowing of relaxation, the decrease in SR  $Ca^{2+}$  load, and the decrease in the amplitude of  $Ca^{2+}$  transients and contractility observed in most diabetic cardiomyopathy models [20, 67, 158, 216, 248, 304].

#### c. Cardiac arrhythmias

Beyond the alterations in contractility, cardiac arrhythmias propensity is also a hallmark in diabetic cardiomyopathy, and diabetic patients are at an increased risk of cardiac arrhythmias and sudden death, arrhythmia's most fearsome consequence [199, 259, 274]. Reentry is the common arrhythmogenic mechanism. Reentry occurs when an AP did not extinguish being able to reactivate a region already recovered from refractoriness. Reentry can arise from abnormalities in conduction, repolarization, or both (Tse et al., 2016). In diabetic cardiomyopathy both abnormalities are present, establishing a powerful arrhythmogenic substrate, further strengthened by systemic factors, like autonomic neuropathy or inflammation [139, 196] and associated heart diseases like coronary artery disease [69, 126, 198, 260, 312]. Moreover, altered ionic currents and Ca<sup>2+</sup> mishandling favor EADs, DADs, and spontaneous APs and constitute the arrhythmogenic trigger. In the advanced diabetic cardiomyopathy, these mechanisms coexist making the dissection of each one difficult (see for review [274]). A detailed review of cardiac arrhythmias in diabetic cardiomyopathy has been recently published [122]. In this review, we will concentrate on triggered arrhythmias that occur at the cellular level due to  $Ca^{2+}$  mishandling.

Abnormal Ca<sup>2+</sup> cycling is linked to triggered activity which may occur due to an imbalance in ionic currents favoring a depolarizing net inward current [51]. This imbalance originates membrane potential alterations called early and delayed afterdepolarizations, EADs and DADs, respectively, according to the moment that they occur relative to the regular AP. EADs arise before the completion of AP, whereas DADs occur after AP completion (Fig. 1, inset). These mechanisms may produce sustained arrhythmias by reentry circuits [174]. EADs occur usually in the presence of prolonged repolarization and are attributed to reactivation of  $I_{Ca}$  [131], although the NCX current ( $I_{NCX}$ ) may also be involved [289]. DADs are caused by spontaneous Ca<sup>2+</sup> releases from the SR [22]. In the context of SR Ca<sup>2+</sup> overload and RyR2 sensitization, the  $Ca^{2+}$  released by a group of RyR2 activates neighboring RyR2. This activation may propagate in a regenerative way along the myocytes, as Ca<sup>2+</sup> waves [63]. Ca<sup>2+</sup> waves are potentially arrhythmogenic. since the extrusion of  $Ca^{2+}$  through the NCX may trigger a transient inward current (I<sub>ti</sub>) that depolarizes the cell membrane. If the magnitude of depolarization attains the membrane potential threshold, a spontaneous AP and contraction occur [22, 190, 258] (Fig. 1, inset).

Abnormal RyR2 gating and SR  $Ca^{2+}$  leak have been critically linked with RyR2 mutations or posttranslational modifications of RyR2 or associated regulatory proteins [23, 81, 99, 118, 292]. As discussed above, an increase in SR  $Ca^{2+}$  leak and arrhythmogenesis was associated with different types of RyR2 alterations in diabetic cardiomyopathy models of T1DM and T2DM [20, 93, 248, 249, 304] (see Tables 1 and 2 of supplemental materials).

As discussed above, the importance of CaMKII-dependent phosphorylation of RyR2 on SR Ca<sup>2+</sup> leak and triggered arrhythmias in diabetic cardiomyopathy has been emphasized in several studies [261]. Indeed, CaMKII may be activated by different mechanisms, all of which are present in diabetic cardiomyopathy [282], i.e., T287-phosphorylated CaMKII, M281/282-oxidized CaMKII, and S280 O-linked Glycosylated CaMKII are increased in both, animal diabetic cardiomyopathy models and diabetic human heart samples [73, 93, 183, 207]. The first associations of Ser2814 phosphorylation (the CaMKII site) with SR Ca<sup>2+</sup> leak in diabetic cardiomyopathy were almost simultaneously made by Shao et al. [249], who also described an increase in RyR2 phosphorylation at Ser2808/9 site (mainly phosphorylated by PKA), and Stolen et al. [261], who described an increase in Ser2814 site phosphorylation and in CaMKII-dependent phosphorylation of Thr17 site of PLN. In contrast, they did not find any significant increase in RyR2-Ser2808/9 site phosphorylation. Erickson et al. [93] also associated the increase in CaM-KII activity with the enhanced SR Ca<sup>2+</sup> leak and ventricular arrhythmias produced by hyperglycemia. An increased CaMKII-dependent phosphorylation of RyR2 and in Thr17 site of PLN, associated with an increased SR Ca<sup>2+</sup> leak, was also described in a prediabetes model ([255] and see below).

An association between redox modifications of RyR2 and arrhythmias was observed in mice treated with HFD. These mice present ventricular arrhythmias, an increased expression of NADPH oxidase, isoform 2 (NOX2) at the heart level, and enhanced activity of RyR2 associated to a decrease in free thiol residues compared to control. Ventricular arrhythmias were prevented by treatment of the animals with apocynin, a ROS scavenger and potent NOX2 inhibitor [238]. In a HIP rat model of late-onset T2DC, an increase in DADs was observed, associated to enhanced CaMKII and PKA phosphorylation and oxidation of RyR2 [219].

The mechanism of the increased SR Ca<sup>2+</sup> leak produced by the sole increase in  $Ca^{2+}$  RyR2 sensitivity is difficult to explain. An increase in SR Ca<sup>2+</sup> leak cannot be sustained by increasing RyR2 activity, unless there is a simultaneous enhancement of SR Ca<sup>2+</sup> uptake that continuously refills the SR, to maintain the necessary SR Ca<sup>2+</sup> level to activate RyR2 [273]. In consonance with this premise, Pereira et al. [216], working in db/db mice, described a decrease in SR Ca<sup>2+</sup> load associated with a diminished frequency of  $Ca^{2+}$  sparks. Similar results were obtained in STZ-treated rats by Lacombe et al., 2007, [157] (see Tables 1 and 2 of supplemental material). Therefore, it is possible that the increase in SR  $Ca^{2+}$ leak described in db/db mice [261] and HIP rats [219], and in STZ-treated rats by several authors [122, 192, 219, 248, 249, 304], could be associated not only with RyR2 phosphorylation or activation, but also with mechanisms that enhance SERCA2a activity (like PLN phosphorylation or decreased PLN expression), able to maintain the SR Ca<sup>2+</sup> threshold for a persistent SR Ca2+ leak. An increase in CaMKII-dependent PLN phosphorylation was indeed observed by Stolen et al., 2009, in db/db mouse myocytes [261], and by us, in prediabetic mice and rats [98, 255]. In contrast, the increase in SR Ca<sup>2+</sup> leak observed by Shao et al., 2009, is more difficult to explain, because no increase in PLN phosphorylation or decrease in PLN expression were observed [249].

Earlier experiments by Nordin et al., 1985, described that ventricular muscles from diabetic rats were more prone than normal myocardium to develop delayed after depolarizations and triggered activity under conditions believed to cause myoplasmic Ca<sup>2+</sup> overload, like increasing extracellular Ca<sup>2+</sup> [208]. More recently Chou et al., 2017, showed in db/ db mice an increased propensity to ventricular arrhythmias and alternans [69]. These experiments were performed

in vivo or in ex vivo preparations, and the propensity to ventricular arrhythmias was induced by a specific pacing protocol that allows SR Ca<sup>2+</sup> loading. Besides, the authors described a significant increase in total PLN phosphorylation. This increase may be the result of sympathetic overactivity, which is common in diabetic patients [172] and may contribute to increasing PLN phosphorylation levels. In line with this concept, the paradoxical triggered arrhythmias observed in HF [218] that occur despite the decreased SR Ca<sup>2+</sup> load could be explained by the preserved  $\beta$ -adrenergic responsiveness in animals with HF. Indeed, it was found that  $\beta$ -adrenergic stimulation resulted in a greater increase in SR Ca<sup>2+</sup> load in HF than in control rabbit myocytes [218].

In summary, triggered arrhythmias due to Ca<sup>2+</sup> mishandling constitute a very common finding in different models of diabetic cardiomyopathy and several studies point to CaMKII activation and RyR2 phosphorylation as a main player in this type of arrhythmias in diabetic cardiomyopathy.

Although the above results referred to ventricular triggered arrhythmias, it is important to mention that diabetes increases the risk of atrial fibrillation [286]. Although the pathogenesis of atrial fibrillation is not well clarified, the atrial structural and electromechanical remodeling described in diabetic cardiomyopathy constitute a more than suitable substrate for triggered arrhythmias, and several of the underlying mechanisms described for ventricular arrhythmias may be shared by atrial fibrillation [173]. As it will be discussed below, changes in myofilament responsiveness to Ca were described in ventricular and atrial tissue of patients with diabetic cardiomyopathy [140].

Finally, most of the results described above were obtained in ventricular myocytes of rodents. However, as with any animal model, there are limitations in extrapolating the results to human disease, which include its rapid intrinsic resting heart rate and variations in ion channel distribution and kinetics underlying depolarization and repolarization. Moreover, most of the studies in isolated myocytes are usually performed at an artificially low stimulation frequency and temperature, which further complicate interpretations and extrapolations to human being. However, it is also true that rodents have proved to be an excellent tool for exploring underlying mechanisms of arrhythmias (see for review [70]). Besides, several studies in the intact heart performed at higher frequencies and more physiological temperatures support conclusions obtained in isolated myocytes [11, 278] In any case, it should be cautious when extrapolating rodents results to human disease.

# Nuclear Ca<sup>2+</sup> and gene transcription in diabetic cardiomyopathy

As stated above, there is close interaction between cytosolic  $Ca^{2+}$  and nuclear envelope and nuclear  $Ca^{2+}$ . Besides, it is also known that the nucleus is equipped to produce InsP3 and to release and take up free Ca<sup>2+</sup>, independently of cytosolic InsP3 or Ca<sup>2+</sup> [89]. Among the Ca<sup>2+</sup>-dependent pathways mediating transcriptional regulation in cardiomyocytes are the ones mediated by calmodulin, CaMKII, and calcineurin, which are overactive in several cardiac diseases, including diabetic cardiomyopathy. These pathways would be involved in re-expression of a fetal gene program, inducing maladaptive hypertrophy and remodeling of ion channels and transporters, ultimately impairing cardiac function [77, 82]. Indeed, it has been shown that changes in nuclear  $Ca^{2+}$ occur before cytosolic Ca<sup>2+</sup> alterations in disease development, supporting the crucial role of nuclear Ca<sup>2+</sup> in the activation of maladaptive gene programs [176]. The effect of enhanced CaMKII activity on nuclear Ca<sup>2+</sup> and ventricular remodeling has been recently reviewed [122, 155] and may have also a role in diabetic cardiomyopathy remodeling. Interestingly, a transcriptomic analysis of cardiac left ventricle of STZ-treated rats revealed perturbations in the expression of genes corresponding to proteins expressed in mitochondria and in genes regulating cardiac fatty acid metabolism [113].

# Mitochondria dysfunction in diabetic cardiomyopathy

In the context of DM, reduced glucose uptake as a result of the lack of insulin or the insulin resistance induces a substrate shift toward increased free fatty acid internalization and oxidation [257]. Additionally, increased levels of mitochondrial acetyl CoA and cytosolic citrate inhibit PDH and glycolysis, reducing glucose oxidation (Randle cycle) [222]. This metabolic shift that results in reduced ATP production, increased ROS generation, and impaired mitochondrial respiration capacity is also observed in human diabetic cardiomyopathy [7, 226]. Interestingly, reduced mitochondrial energetics and increased ROS production were observed in mice with a cardiomyocyte-specific deletion of the insulin receptors, suggesting that lack of insulin signaling might also be the cause for the phenotype observed in T2DM hearts [33]. However, increasing glucose uptake to control levels in a model of T1DM worsened mitochondrial function and diabetic cardiomyopathy [290].

Although the exact pathophysiologic mechanisms of diabetic cardiomyopathy are not completely understood (see above), mitochondrial dysfunction plays a central role [43, 88, 114, 136]. In this context, mitochondria quality control, mitochondrial fission and fusion, mitochondrial biogenesis, and mitophagy are central players in mitochondria dynamics and in the evolution of heart disease [242]. Mitophagy, a type of selective autophagy that clears damaged or unwanted mitochondria [109] is important for cardiovascular homeostasis and protection of the myocardium in cardiovascular diseases (for a review on the role of mitophagy in CD. see [197]). In diabetic cardiomyopathy, clearance of damaged mitochondria could reduce oxidative stress and apoptosis [168]. In HFD-treated mice, cardiac-specific deletion of the autophagy-related 7 (atg7) gene resulted in reduced autophagy and mitophagy and exacerbated mitochondrial dysfunction, fibrosis, and cell death by apoptosis [271]. Moreover, T1DM OVE26 mice presented increased cardiac mitochondrial area and number associated with impaired function and increased oxidative stress [250]. These results emphasize the importance that clearance of dysfunctional mitochondria needs to be balanced by new mitochondria biogenesis. As described below, similar dynamic alterations were found in the prediabetic heart [98]. Mitochondrial biogenesis seems to be reduced in skeletal muscles in patients with T2DM [228] and in adipocytes from ob/ ob mice [68], while in HFD-treated rats, mitochondrial biogenesis is increased as a potential protective mechanism to increase fatty acids consumption and reduce ROS production [130].

The increased ROS generation leads to oxidative stress, inducing several cellular changes and activating cell death mechanisms that include autophagy, necrosis, and apoptosis and have distinctive roles. Autophagy acts as a homeostatic process that results in the lysosomal degradation of damaged organelles, protein aggregates, and is a critical step in tissular damage. The imbalance between autophagy and apoptosis may establish the progression of diabetes complications. In fact, apoptosis can be downregulated by autophagy [284]. Fatty acids accumulation results in ER stress [254] impairing autophagy and resulting in accumulation of dysfunctional organelles [115, 214]. Increasing autophagy and reducing myocyte apoptosis improve cardiac function in STZ mice [121].

ROS, including the superoxide anion, the hydroxyl radical, and hydrogen peroxide, are signaling molecules with important roles in both cardiac physiology and disease. Under physiological conditions, cardiac ROS are involved in heart development and cardiomyocyte maturation, Ca<sup>2+</sup> handling, ECC, and vascular tone (reviewed in [46]). Mitochondrial ROS production involves mainly complex I and III activity. Excessive ROS production can result in  $\Delta \Psi m$ dissipation and reduction in NADH production, resulting in the emission of H<sub>2</sub>O<sub>2</sub>, a mechanism known as ROS-induced ROS release [315]. Lipid accumulation in the ER results in ER stress that also induces ROS production, releasing Ca<sup>2+</sup> from the ER storage and further increasing mitochondrial ROS production [45, 272]. When ROS disbalance occurs, oxidative stress can damage mitochondrial DNA, inducing protein oxidative damage and impairing energetics and further increasing ROS production [102, 300]. Indeed, dysregulated ROS production and oxidative stress have been implicated in several cardiac diseases, including cardiac

hypertrophy, HF, cardiac ischemia–reperfusion injury, and diabetic cardiomyopathy (reviewed in [175, 189, 193, 275]).

Additionally, Ca<sup>2+</sup> can stimulate nitric oxide synthase (NOS), generating reactive nitrogen species (RNS) that can further enhance ROS production [36, 133]. Therefore,  $Ca^{2+}$ and ROS form a vicious cycle that can induce membrane depolarization, mitochondrial protein and DNA damage, mPTP opening, and cell death. Alterations in energy and ROS/Ca<sup>2+</sup> coupling have been observed in HF, supporting the importance of  $Ca^{2+}$  in modulating energy and ROS production [209]. Antioxidant daily injection in mice models of T1DM and T2DM resulted in reduced oxidative stress and myocyte apoptosis, and restoration of diastolic function [204]. Mice lacking p66shc, a protein involved in ROS production [108], are protected from STZ-induced diastolic and systolic dysfunction, reducing diabetes induced cell apoptosis and necrosis [232]. Furthermore, hyperglycemia induces mitochondrial oxidative stress and mitochondrial fragmentation, which can cause cellular injury and dysfunction [307].

It is known that when mitochondrial Ca<sup>2+</sup> overload occurs, the mPTP can open, resulting in the release of mitochondrial content (including Ca<sup>2+</sup>) and causing cell death [17]. Indeed, it was shown that FUNDC1 is upregulated in diabetic human hearts and Akita and STZ animal models of T1DM. The increased FUNDC1 is associated with increased MAM formation by binding to IP3R2, resulting in increased mitochondrial Ca2+ levels, mitochondrial dysfunction, increased ROS formation, and decreased mitochondrial membrane potential, while FUNDC1 deletion protected mice from cardiac dysfunction [294]. Mitochondrial Ca<sup>2+</sup> overload due to SR Ca2+ leak can cause mitochondria dysfunction, oxidative stress, and apoptosis as seen in HF [240]. As discussed above, CaMKII was overactivated in several models of diabetic cardiomyopathy producing RyR2 phosphorylation and increasing SR Ca<sup>2+</sup> leak [93, 248, 261]; additionally, CaMKII may directly favor mitochondria Ca<sup>2+</sup> uptake increasing MCU activity; however, this is a controversial issue that deserves further investigation [103, 137, 181, 205, 270]. Activated CaMKII phosphorylates Drp-1, a mitochondrial membrane protein, which results in increased mPTP opening, myocyte death, and heart hypertrophy after  $\beta$ -adrenergic stimulation [302]. This phosphorylation was also observed in ventricular samples from dilated cardiomyopathy and ischemic heart failure patients [302]. Increased Drp-1 acetylation has been also detected in obese Zucker and HFD fed mice, monkeys, and isolated myocytes treated with palmitate [128] resulting in heart dysfunction, mitochondrial fission, and cell death mediated by VDAC-1. Initiation of cell death by mitochondria in response to diverse stress signals, like hypoxia, oxidative stress, and  $Ca^{2+}$  overload, can be induced by opening of the mPTP, resulting in loss of  $\Delta \Psi m$  and mitochondrial swelling or by permeabilization of the mitochondrial outer membrane and release of proapoptotic proteins [117].

On the other hand, reduced SR Ca<sup>2+</sup> load and Ca<sup>2+</sup> transients result in decreased mitochondrial Ca<sup>2+</sup> uptake [145]. Reduced mitochondrial Ca<sup>2+</sup> content also has deleterious effects on mitochondrial function. It can further reduce PDH phosphatase activity, resulting in decreased glucose and increased fatty acid utilization, increasing lactate production, oxygen consumption, and eventually altering contractile function [79]. Suarez et al. [262] described that reduced mitochondrial Ca<sup>2+</sup> in diabetic cardiomyopathy due to reduced MCU expression results in reduced  $\Delta \Psi m$ , increased ROS, and apoptosis in STZ-treated mice. Additionally, reduced cytosolic Ca<sup>2+</sup> transients can reduce mitochondrial Ca<sup>2+</sup> content, affecting ATP generation as seen in T1DM hearts [158, 308]. Reduced Ca<sup>2+</sup> retention capacity as a result of increased sensitivity for mPTP opening can also lead to reduced mitochondrial Ca<sup>2+</sup> content and impaired energetics in T1DM rat model and human diabetic cardiomyopathy [8, 212]. Results are less conclusive from T2DM models; however, reduced Ca2+ transients are also observed [20, 83, 97, 216, 261] that could result in reduced mitochondrial Ca<sup>2+</sup> content. Levels of MCU complex proteins like EMRE, MICU1, MCU, and MCUb are also affected in both T1DM and T2DM, indicating that mitochondrial Ca<sup>2+</sup> entry could be affected in DM not only by the amount of cytosolic Ca<sup>2+</sup> or the magnitude of SR Ca<sup>2+</sup> leak but also by mechanisms inherent to mitochondria themselves [79]. In T1DM mice, there is a reduction in MCU and EMRE levels and an increase in the expression of MCUb, resulting in reduced cardiac mitochondrial Ca<sup>2+</sup> content. MCU adenoviral expression restored mitochondrial bioenergetics, reduced apoptosis, and improved cardiac function [262]. In db/db mice, reduced MICU1 expression is related to increased oxidative stress and myocyte apoptosis, while MICU1 restoration increased mitochondrial Ca<sup>2+</sup> content and inhibited the development of diabetic cardiomyopathy, observed as enhanced heart function and reduced cardiac hypertrophy and fibrosis [134]. Is worth to note that in the later, the authors only compared mitochondrial Ca<sup>2+</sup> content in db/db mice with or without MICU1 overexpression, without comparison with control mice.

Additionally, alterations in the expression and/or activity of the MCU complex proteins can be caused by transcription and/or protein expression regulation. For example, overexpression of microRNA (miR)-181c in DM results in COX-1 reduced expression. This in turn leads to increased ROS production, reduced MICU1 expression, and mitochondrial  $Ca^{2+}$  overload, which ultimately results in mitochondrial and heart dysfunction [14] (see [132] for an extensive review of the role of miR in regulating mitochondrial  $Ca^{2+}$ ).

Fig. 3 A. Fructose rich diet induces SR Ca<sup>2+</sup> leak and arrhythmias. Representative confocal images of isolated myocytes from control diet (CD) or fructose-rich diet (FRD) mice. While CD myocytes barely show spontaneous  $Ca^{2+}$  release events (SCaRE), FRD myocytes showed sparks, waves, and spontaneous contractions (SC and white arrows). B Arrhythmias in fructose reach diet animals. I to IV: FRD mice presented increased frequency of arrhythmias compared to CD mice. C SR-AIP mice expressing the CaMKII inhibitor at the SR did not show arrhythmogenic events in CD nor FRD. Adapted from ([98] and [255])



## Early alterations in diabetic cardiomyopathy: the prediabetic model

The prediabetic state can be define as a metabolic dysfunction with impaired glucose tolerance and impaired fasting glucose, usually asymptomatic [6, 207]. However, cardiac defects in DM have a long progression before attaining the stage of diabetic cardiomyopathy with evident diastolic and systolic symptoms. Knowledge of alterations occurring at the early stages of the disease is necessary and essential for setting the first steps in the evolution of this cardiac illness. Unfortunately, and despite the acknowledgment of the prolonged evolution of diabetic cardiomyopathy and the fact that the prevalence of prediabetes is rising [72], studies tracking the early stages of diabetic cardiomyopathy are scarce. Even with this limitation, there is evidence that subjects with impaired glucose tolerance have impaired diastolic function of the left ventricle [12] and prediabetes and borderline diabetes are associated with an increased risk of sudden cardiac death possibly due to cardiac arrhythmias [59, 277]. Moreover, there is evidence suggesting an increased propensity to arrhythmias in young people with DM even in the absence of detectable systolic dysfunction [243].

In a prediabetes model for T2DM, induced by a FRD [4, 101], it was described that as early as 3 weeks of FRD, there was an enhanced ROS production and an increase in

oxidized CaMKII activity in association with enhanced SR Ca<sup>2+</sup> leak due to RyR2 phosphorylation at the CaMKII site (Ser2814). The increased SR Ca<sup>2+</sup> leak occurs without significant changes in SERCA2a or PLN expression, but in association with an increase in phosphorylation Thr17 site of PLN and SERCA2a activity [98]. Surprisingly, SR Ca<sup>2+</sup> leak was high enough to trigger in vivo ventricular arrhythmias [255] (Fig. 3), at this early stage of the evolution of the disease. These alterations were prevented by the CaMKII inhibitor KN-93, by co-treatment with the reactive oxygen species scavenger Tempol, in SRAIP mice, in which the CaMKII inhibitor autocamtide inhibitory peptide (AIP) is targeted to the SR [135], or in S2814A animals in which the CaMKII site of the RyR2 is replaced to Ala and cannot be phosphorylated [60, 280] (Fig. 3). Interestingly, and despite the increase in SR Ca<sup>2+</sup> leak, SR Ca<sup>2+</sup> load did not change in FRD myocytes. Thus, the increase in SR Ca<sup>2+</sup> leak and consequent arrhythmogenic Ca<sup>2+</sup> waves and arrhythmias observed in FRD myocytes may result from the combination of a decrease in SR Ca<sup>2+</sup> threshold for Ca<sup>2+</sup> leak and an increase in SERCA2a activity, possibly by CaMKII-dependent PLN phosphorylation, able to maintain SR Ca<sup>2+</sup> content above Ca<sup>2+</sup> threshold for  $Ca^{2+}$  leak.

In prediabetes, the exacerbated SR Ca<sup>2+</sup> leak was also associated with augmented cardiac apoptosis, mitochondrial

swelling, and mitochondrial membrane depolarization in FRD mice [98]. These alterations did not occur in S2814A myocytes treated with FRD, supporting the concept that the increase in CaMKII-S2814 phosphorylation-induced SR Ca<sup>2+</sup> leak was associated with increased mitochondrial SR Ca<sup>2+</sup> load, dissipation of  $\Delta \psi$ m, opening of the mPTP, and release of apoptotic factors [211]. These experiments emphasize that apoptosis is already present at the early stages of the illness preceding diabetic cardiomyopathy, highlighting one of the possible mechanisms involved in cardiac damage. The experiments further indicate that ROS production is upstream the activation of CaMKII in the arrhythmogenic and apoptotic signaling cascade triggered by FRD and suggest an analogous signaling pathway for both phenomena [98, 255].

Both, the arrhythmia and the apoptosis observed in the prediabetes model were not only linked to increased CaMKII activity but also to ultra-structural changes that include increased proximity between SR and mitochondria, which could favor Ca<sup>2+</sup> trafficking from one organelle to the other, and an increased mitochondria density, that additionally were smaller and more spherical [98], suggesting dynamics changes, such as mitochondria fission, at this early stage. This remodeling was prevented in AC3I mice, with cardiac-targeted CaMKII inhibition [98]. A more recent paper in FRD mice also revealed an increase in several tethering proteins, like mitofusin-2 (Mfn2), Grp75, and VDAC, and confirmed the increased proximity of the SR-mitochondria microdomains, the decrease in mitochondria diameter, and the increase in mitochondria roundness and density previously described, associated to an enhancement of fission protein, Drp1 [98]. Again, all these changes did not occur in AC3-I transgenic mice [100].

Interestingly, very similar results were recently obtained in a different model of prediabetes, the sucroseinduced metabolic syndrome with obesity (MTs) in rats [231]. MTs cardiomyocytes exhibited increased CaMKII activity, RyR2 phosphorylation at Ser2814 and PLN phosphorylation at Thr17 site, enhanced SERCA2a activity, and spontaneous Ca<sup>2+</sup> waves that were inhibited by CaMKII inhibition. Moreover, the propensity to cardiac arrhythmias in obesity/hyperlipidemia, a main contributing factor in the development of metabolic syndromes, was also associated with increased CaMKII activity as well as PLN and RyR2 phosphorylation at the CaMKII sites [313]. In this study, there was also an increase in RyR2-Ser2808 phosphorylation which was significantly diminished by CaMKII inhibition, supporting the notion that phosphorylation of Ser2808 in diabetic cardiomyopathy was evoked by CaMKII activation.

Diastolic dysfunction was also observed at the prediabetic stage of Otsuka Long-Evans Tokushima Fatty [194], and in a prediabetic model of Long-Evans rats fed with HFD and treated with a single low dose of STZ, both models of T2DM. Although in this case no increase in CaMKII activity was found, there was an increased production of ROS in cardiac subsarcolemmal mitochondria and of Mfn2 expression [147].

Contractile decline and Ca<sup>2+</sup> mishandling were also observed at the earliest stages of the STZ model T1DM, associated to a reduction of Vmax of SERCA2a and RyR2 conductance, although without major changes in SERCA2a, RyR2, NCX, or PLN expression [170]. Unfortunately, no attempts to measure protein phosphorylation or CaMKII activity were performed, which might provide some clue to explain the results obtained.

It is worthwhile to mention here that in rats with high sucrose diet-induced metabolic syndrome, it was also observed increased RyR2 phosphorylation at Ser2808 associated with reduced FKBP12.6 expression, with significantly increased SR  $Ca^{2+}$  leak, depressed SR  $Ca^{2+}$  loading, and reduced  $Ca^{2+}$  transient amplitude vs. controls [210].

Taken together and despite the reduced amount of information, it is clear that prediabetes is a silent process that initiates detrimental molecular pathways before any other apparent alteration could be detected. However, being prediabetes a condition in which blood glucose levels are above normal but below the defined threshold of diabetes [6, 207], the results described above emphasize the need of routinely testing of prediabetes in the population. Such strategy, together with lifestyle modifications (diet and exercise habit), would help to prevent the arrival of serious and definitive heart alterations or delay the onset of this disease [202, 285]. Unfortunately, health organizations have not uniform criteria for screening prediabetes in individuals that do not present risk factors for T2DM (obesity, hypertension, familiar background, etc.) [15]. In addition, the metabolic conditions in diabetic cardiomyopathy are tightly linked to hypertension and obesity, as well as coronary artery disease. Indeed, hypertension and hyperlipidemia are considered accelerating factors of hyperglycemia and diabetes-induced organ damage [41]. It has long been known that he superimposition of hypertension on diabetes further aggravates microvascular and macrovascular complications through additive mechanisms that include arteriolar and capillary damage in retinal, renal, coronary, cerebral, and peripheral vascular territories. Patients with hypertension and concomitant DM compared to non-hypertensive diabetics were found to have higher rates of cardiovascular death, myocardial infarction, angina pectoris, amputation, and stroke independent of other risk factors (hypertension diabetes study),

emphasizing the need of blood pressure and obesity control in prediabetes patients.

# Results from patients with diabetic cardiomyopathy

In human diabetic cardiomyopathy, the results typically showed early alterations in diastolic function and even subclinical signs of systolic dysfunction before the appearance of any clinical symptoms in T1DM [243, 252, 310] as well as in T2DM [96, 105].

Unfortunately, only limited results were found at the level of Ca<sup>2+</sup> handling, cardiomyocyte proteins, or mitochondria function. In isolated human atrial trabeculae of T2DC asymptomatic patients, it was found a prolonged systolic Ca<sup>2+</sup> rise in association with decreased expression of RyR2 and enhanced phosphorylation of RyR2, associated with enhanced PKC and PKA activities. CaMKII expression or activity was not measured. In these trabeculae, no changes in relaxation nor in SERCA2a or PLN expression were found. These patients have normal ejection fraction but fasting glucose was significantly increased [224]. Interestingly, Yaras et al., 2007, showed that inhibition of PKC antagonized the hyperphosphorylation and restored the depleted protein levels of RyR2 in a rat model of T1DC [303].

The decreased contractility in human diabetic cardiomyopathy has been also associated with alterations at the myofilament levels. Jweied et al., 2005, described a decreased in Ca<sup>2+</sup> myofilament sensitivity in ventricular trabeculae of T2DC patents presenting diastolic dysfunction although increased ejection fraction. The decrease in Ca<sup>2+</sup> myofilament sensitivity was associated with an increased phosphorylation of troponin I and T [140]. A decrease in Ca<sup>2+</sup> myofilament sensitivity would increase the rate of intracellular Ca<sup>2+</sup> decay, favoring mechanical relaxation, and might be viewed as a compensatory mechanism for the decrease in SR Ca<sup>2+</sup> uptake, although the authors did not explore this point. Moreover, similar decrease in Ca<sup>2+</sup> myofilament responsiveness was recently observed in atrial tissue [21]. These findings do not exclude the participation of Ca<sup>2+</sup> handling alterations in human diabetic cardiomyopathy but emphasized the fact that an alteration at the contractile proteins may contribute to cardiac dysfunction at least in T2DC.

### **Concluding remarks**

DM is a complex syndrome and diabetic cardiomyopathy is the result of multiple alterations that finally evolve to HF. The insidious evolution of diabetic cardiomyopathy has precluded a clear knowledge of the underlying mechanisms of the illness. However, the increasing prevalence of the disease warrants a deep and better understanding of the mechanisms of diabetic cardiomyopathy at the molecular level, particularly at the early stages of the malady. Among these mechanisms, Ca<sup>2+</sup> mishandling and mitochondria dysfunction, as well as maladaptive gene programs, play a crucial role in the different types and models of diabetic cardiomyopathy and are responsible, at least in part, for the diminished contractility and slowed relaxation, the propensity to triggered arrhythmias, and the increase in cell death. Moreover, strong experimental evidence indicates that the increased activity of CaMKII is one of the prevailing mechanisms involved in Ca<sup>2+</sup> mishandling that may lead to triggered arrhythmias and mitochondrial dysfunction. Possibly more important, all these alterations can be detected at the very early stages in the progression of diabetic cardiomyopathy or in conditions with high risk to evolve to DM, like obesity or metabolic syndrome. These studies underpin the need for timely detection of the illness at the possibly unique stages at which reversal of Ca<sup>2+</sup> handling alterations and mitochondrial dysfunction could be achieved, and sudden cardiac death could be prevented.

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#### Declarations

Conflict of interest The authors declare no competing interests.

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