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Targeting mitochondrial impairment for the treatment of cardiovascular diseases: from hypertension to ischemia reperfusion injury, in search of new pharmacological targets.

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Abstract:	In the search of new molecular targets and drugs to counteract the onset of hypertension and more in general cardiovascular diseases, mitochondrial proteins represent promising pharmacological-target candidates. Indeed, several mitochondrial pathways result impaired in CVDs and it appears that ATP depletion and ROS production are a common treat of cardiac tissue degeneration. Thus, targeting mitochondrial dysfunction in cardiomyocytes can represent a successful strategy to prevent heart failure. In this context the identification of new pharmacological targets among mitochondrial proteins, is propaedeutic to design new selective drugs. Thanks to the recent advances in omics approaches, to a greater availability of mitochondrial crystallized protein structures and to the development of new computational approaches for the protein 3D-modelling and drug-design, it is now possible to investigate in greater detail impaired mitochondrial pathways in CVDs, in order to draw new powerful drugs able to target in a highly selective way a selected pharmacological-target to rescue mitochondrial dysfunction and prevent cardiac tissue degeneration.

	While the role of mitochondrial dysfunction in the onset of CVDs appears more and more evident, as reflected by the impairment of proteins involved in crucial mitochondrial metabolic pathways in CVD patients, we still need to gain new insights about proteins responsible for the cross-talk between mitochondria and cytoplasm in cardiomyocytes. In this context the mitochondrial transporters of the SLC25A family play a key role by leading metabolic pathways crucial for maintaining healthy cardiomyocytes, making mitochondrial carriers an interesting class of new possible pharmacological targets for CVD treatments.
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Title

Targeting mitochondrial impairment for the treatment of cardiovascular diseases: from hypertension to ischemia reperfusion injury, in search of new pharmacological targets.

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Abstract

Mitochondria and mitochondrial proteins represent a group of promising pharmacological-target candidates in the search of new molecular targets and drugs to counteract the onset of hypertension and more in general cardiovascular diseases (CVDs). Indeed, several mitochondrial pathways result impaired in CVDs and ATP depletion and ROS production resulted as being common traits of cardiac tissue degeneration. Thus, targeting mitochondrial dysfunction in cardiomyocytes can represent a successful strategy to prevent heart failure. In this context, the identification of new pharmacological targets among mitochondrial proteins paves the way to the design of new selective drugs. Thanks to the advances in omics approaches, to a greater availability of mitochondrial crystallized protein structures and to the development of new computational approaches for protein 3D-modelling and drug-design, it is now possible to investigate in detail impaired mitochondrial pathways in CVDs, to draw new powerful drugs able to target the selected pharmacological target in a highly selective way to rescue mitochondrial dysfunction and prevent cardiac tissue degeneration. While the role of mitochondrial dysfunction in the onset of CVDs appears more and more evident, as reflected by the impairment of proteins involved in lipid peroxidation, mitochondrial dynamics, respiratory chain complexes, and membrane polarization maintenance in CVD patients, little is known about proteins responsible for the cross-talk between mitochondria and cytoplasm in cardiomyocytes. Mitochondrial transporters of the SLC25A family, in particular, are responsible for the translocation of nucleotides (e.g. ATP), amino acids (e.g. aspartate, glutamate, ornithine), organic acids (e.g. malate and 2-oxoglutarate), and other cofactors (inorganic phosphate, NAD+, FAD, carnitine, CoA derivatives) between the mitochondrial and cytosolic compartments. Thus, mitochondrial transporters play a key role in the mitochondria-cytosol cross-talk by leading metabolic pathways such as the malate/aspartate shuttle, the carnitine shuttle, the ATP export from mitochondria, and the regulation of permeability transition pore opening. Since all those pathways are crucial for maintaining healthy cardiomyocytes, mitochondrial carriers emerge as an interesting class of new possible pharmacological targets for CVD treatments.

Keywords

Hypertension; ischemia reperfusion injury; cardiovascular diseases; mitochondrial impairment; mitochondrial dysfunction; mitochondrial diseases; phospholipids; cardiolipin; peptide-based treatments; mitochondrial dynamics; respiratory chain; voltage dependent anion channels; mitochondrial permeability transition pore; mitochondrial pyruvate carrier; aquaporin; genomics; transcriptomics; metabolomics; mitochondrial carriers; mitochondrial metabolite transport system; mitochondrial pharmacological targets; drug-repurposing; molecular modelling of mitochondrial proteins.

Abbreviations

CVD: cardiovascular diseases; IRI: ischemia reperfusion injury; CHD: coronary heart disease; MI: myocardial infarction; DCM: Dilated cardiomyopathy; HF: Heart Failure; SCDA: Short-Chain Dicarboxylacylcarnitine; HFrEF: reduced ejection fraction; HFpEF: heart failure with preserved ejection fraction; NGS: next generation sequencing; PAB: pulmonary artery banding; PAH: pulmonary arterial hypertension; T2D: type 2 diabete; RV: right ventricle; BP: blood pressure; NMR: Nuclear Magnetic Resonance; MS: Mass Spectrometry;LC: liquid chromatography; GC: gas chromatography; BCAA: branched chain amino acids; AAA: aromatic amino acids; TPP^+: triphenylalkylphosphonium cation; AOA: aminooxyacetic acid; MIM: mitochondrial inner membrane; MOM, mitochondrial outer membrane; IMS, intermembrane space; AAC, ADP/ATP carrier, coded in H. sapiens by SLC25A4, SLC25A5, SLC25A6, SLC25A31; TPC, thiamine pyrophosphate carrier, coded by SLC25A19; CAC, carnitine/acyl-carnitine carrier, coded by SLC25A20; ORC, ornithine carrier, coded by SLC25A15 (or SLC25A2); AGC, aspartate/glutamate carrier, coded by SLC25A12 and SLC25A13; DIC, dicarboxylate carrier, coded by SLC25A10; NDT, assumed to be the NAD+ carrier, coded by SLC25A51; MFT, assumed to be the FAD (folate/riboflavin) carrier, coded by SLC25A32; OGC, malate/2-oxoglutarate carrier, coded by SLC25A11; CIC, citrate carrier, coded by SLC25A1; PiC, phosphate carrier, coded by SLC25A3; MAS, malate/aspartate shuttle; TCA, tricarboxylic acid cycle; Bax, Bcl-2 associated X protein; Bak, Bcl-2 antagonist/killer-1; Bcl-2, B-cell lymphoma-2; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; UCP, uncoupling protein, coded by SLC25A7, SLC25A8, SLC25A9, SLC25A14, SLC25A27 and SLC25A30; CypD, cyclophilin D; CytC, cytochrome C; VDAC, voltage-dependent anion channel; AIF, apoptosis-inducing factor; PNC, pyrimidine nucleotide carrier, coded in H. sapiens by SLC25A33 and SLC25A36; ROS: reactive oxygen species; WGS: whole genome sequencing; PPARs: peroxisome

proliferator-activated receptors; OXPHOS: oxidative phosphorylation; AQP: aquaporins; PUFA: polyunsaturated fatty acids; ETC: electron transport chain; mPTP: mitochondrial permeability transition pore; ETF: electron-transferring flavoprotein.

Contents

Introduction

Heart failure is a complex multifactorial syndrome resulting in the chronic and progressive loss of ventricular function leading to both cardiac and systemic perturbations. Several causes can lead to heart failure, such as ischemic conditions (coronary heart disease, myocardial infarction) increased or altered workload (hypertension, heart valve abnormalities), cardiomyopathy (dilated, hypertrophic, both secondary and idiopathic), drug adverse effects or alcohol abuse. Despite the considerable etiopathogenetic differences, all these clinical situations show a remarkable common pathogenetic substrate. The evidences that heart failure relates to the impairment of mitochondrial function are remarkable, indeed the impairment of mitochondrial function can represent the main cause of increased oxidative stress in cardiomyocytes of patients affected by cardiovascular diseases (CVDs) and mitochondrial impairment can be both cause and effect of tissue damage in organs exposed to the effects of hypertension, like heart and kidneys [1]. It is retained that the increased oxidative stress at the level of blood vessels plays a key role in the pathophysiology of hypertension that can lead to vascular inflammation, heart hypertrophy and contractile dysfunction, till to myocardial infarction [2–4].

It is also known that myocardial infarction is the main cause of chronic heart failure and cardiovascular-related mortality [5,6]. Reperfusion strategies are the current standard therapy for treating myocardial infarction [7,8]. However, they may result in paradoxical cardiomyocyte dysfunction, known as ischemic reperfusion injury (IRI) [7,9]. Different forms of IRI are recognized, of which only the first two are reversible: reperfusion-induced arrhythmias, myocardial stunning, microvascular obstruction, and lethal myocardial reperfusion injury [7,9].

The molecular mechanisms of IRI are not fully known [7,9]. However, it is observed that the deprivation of blood flow to the heart causes an imbalance between oxygen demand and supply, resulting in a sufferance for the cardiac tissue [8]. Notably, also the abrupt availability of oxygen during reperfusion of the hypoxic cardiac tissue is itself considered responsible for ischemic tissue damage and/or dysfunction of the cardiac tissue [10,11].
Cardiomyocytes are indeed enriched in mitochondria and hypoxia conditions can impair the function of the respiratory chain slowing-down ATP production along oxidative phosphorylation.

The impairment of respiratory chain causes ATP depletion that can be responsible for serious damages to the cardiac tissue and reactive oxygen species (ROS) production due to the impaired mitochondrial respiration [12]. On the other hand the massive reoxygenation of ischemic tissues, along reperfusion, is retained the primary cause of IR injury, due to the abnormal production of ROS

following the massive reoxygenation after hypoxia [10–12]. Notably, studies in animal models of acute myocardial infarction suggest that the only myocardial IR injury accounts for up to 50% of the final size of a myocardial infarct [8].

More in general ROS increase and ATP depletion appear to be linked either to the impaired homeostasis of several metabolites and cofactors useful for the correct function of mitochondria or to the impairment of mitochondrial dynamics, the anomalous structural re-organization of mitochondria and mitochondrial membranes as a consequence of an altered fusion and fission cycle, and finally an alteration of the apoptosis/necrosis mechanisms leading to an early cell death [13,14]. In the following chapters we shall briefly present the role of different protein complexes, enzymes and transporters in cardiomyocyte mitochondria and we shall describe how their impairment can be responsible for the onset of hypertension and CVDs.

Role of the phospholipids in mitochondrial membranes and associations with CVDs: the case of cardiolipin.

Mitochondria are organelles delimited by two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Phospholipids are the main building blocks of each mitochondrial membrane in which they play structural and functional roles. In fact, physiological phospholipid composition provides correct mitochondrial membrane permeability and fluidity, and offers the proper environment for optimal structural conformation and functional activity of membrane proteins and enzymes. Phospholipids play a critical role in the membranes architecture and modulate mitochondrial function, dynamics, apoptosis and transport of substrates and proteins. Due to their key role in maintaining mitochondrial structure and function, alterations in phospholipid composition could negatively impact the structure, the permeability and fluidity of mitochondrial membrane, and thus the stability and activity of several membrane-associated proteins.

As mentioned above, heart ischemia, and particularly reperfusion, are characterized by a sudden and massive increase in ROS production [15].

Mitochondrial membrane phospholipids are rich in polyunsaturated fatty acids (PUFAs) [16]. The sensitivity of fatty acid molecules to ROS-production and oxidation damage increases as a function of the number of unsaturated bonds. Therefore, the high concentration of PUFAs in mitochondrial phospholipids and their location near the site of mitochondrial ROS production, makes these membrane components main targets of oxidizing agents. Thus, ROS attack to phospholipids can trigger lipid peroxidation, that can generate hydroperoxides and endoperoxides which are themselves

harmful to cellular structures. Among phospholipids, cardiolipin is commonly referred to as the signature phospholipid of the mitochondria being localized almost exclusively at the level of IMM. Cardiolipin presents interesting chemical structure consisting of two phosphatidyl residues linked by a glycerol bridge. Thus, this acidic phospholipid has a very specific ultrastructure with four acyl chains (rather than two) that gives to cardiolipin a dimeric nature, which is unique among membrane phospholipids and results in high specific conical shape [17]. Indeed, cardiolipin molecules are particularly rich in unsaturated fatty acids. Due to their location in the IMM near to the locus of ROS production and their high content of unsaturated acyl chains, cardiolipin molecules may readily undergo oxidative attack, particularly by ROS produced by mitochondrial electron transport chain (ETC). It has been reported that cardiolipin interacts with a number of proteins of the inner mitochondrial membrane, including the respiratory chain complexes and metabolite transporters [18], whose activity appears to be cardiolipin-dependent [18–20]. This phospholipid is also directly involved in different stages of the mitochondrial apoptosis process as well as in mitochondrial membrane stability and dynamics [19]. Therefore, ROS-induced oxidation of this phospholipid may affect several cardiolipin-dependent reactions involved in mitochondrial bioenergetics. Moreover, alterations in cardiolipin structure, content, and acyl chain composition have been associated with mitochondrial dysfunction in multiple tissues and in several physiopathological situations [21] including heart ischemia/reperfusion [22].

It has been reported that cardiolipin molecules bind cytochrome c to outer surface of the inner mitochondrial membrane [23] and that oxidative damage to cardiolipin results in the detachment of cytochrome c from mitochondrial membrane. This event is considered the initial step in the release of cytochrome c from mitochondria [24]. Results reported in literature demonstrated that peroxidized cardiolipin promotes Ca2+-induced mitochondrial permeability transition pore (mPTP) opening and cytochrome c release from isolated rat heart mitochondria [25].

mPTP opening is an important event in cardiomyocyte cell death occurring during I/R and therefore a possible target for cardioprotection. It has been proposed that during heart ischemia/reperfusion increased levels of peroxidised cardiolipin, due to ROS attack, may negatively affect ETC activity and increase the probability of the mPTP opening.

Thus, mitochondrial cardiolipin alterations seem to be intimately implicated in mitochondrial dysfunction during myocardial IRI, by affecting mitochondrial dynamics, oxidative phosphorylation processes at the level of the respiratory chain complexes, membrane depolarization, and mPTP opening. These observations point to cardiolipin as a potential target for cardioprotective interventions.

Mitochondrial dynamics in CVDs

A proper composition of phospholipids is also important for mitochondrial dynamics whose correct function influence mitochondrial morphology, number, and shape of mitochondria, and determines the maintenance of a balance between mitochondrial biogenesis and turnover [26]. As a consequence all the processes ensuring the mitochondrial homeostasis depends on a fine balance between fission and fusion processes [27].

Mitochondrial fission is a multi-step process that involves the recruitment, oligomerization and mitochondrial constriction regulated by the GTPase dynamin-related protein 1 (Drp1) and by other proteins (in mammalian Dynamin2 (Dnm2), fission protein 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamic proteins of 49 and 51 kDa (MiD49/51) [28]), to determine the cleavage of mitochondrial membranes allowing the division of one mitochondrion into two separate mitochondrial organelles.

Mitochondrial fusion, instead, is a process in which two neighboring mitochondria fuse their membranes to generate a larger daughter mitochondrion, which includes all the structures and characteristics of the two original mitochondria [27].

Mitochondrial fusion is coordinated by the mitofusins 1 and 2 (Mfn1 and Mfn2) on the outer mitochondrial membranes and by optic atrophy 1 (OPA1), a dynamin-like GTPase, on the inner mitochondrial membranes [29] (Fig. 1).

The fusion process is considered primarily a system of protection in which partially deficient ineffective and/or malfunctioning mitochondria seek to recover normal physiological activities by merging with new mitochondria formed by mitochondrial biogenesis processes. The fusion process is essential for embryonic development ensuring that regional losses of membrane potential are always transient [30]. In some situations, fusion proteins are used to connect mitochondria with other cellular structures, i.e.,, endoplasmic reticulum – mitochondria tethering [31].

Incorrect mitochondrial homeostasis linked to an unbalance of mitochondrial dynamics and more in general an altered regulation of Mfn1, Mfn2 and Opa1 [32] can contribute to the onset and/or progression of cardiovascular diseases [33–36]. It has been reported that also the disruption of Drp1 induces mitochondrial elongation, most likely due to the impaired mitochondrial dynamics that needs a functional Drp1 protein, causing mitochondrial dysfunction and accelerating mitochondrial senescence in heart mice [32,37,38]. On the other hand, it was observed that the simultaneous abrogation of mitochondrial fission and fusion in adult mouse hearts kept mitochondrial dynamics balanced with mild mitochondrial functional impairment [38] . Alterations in both mitochondrial dynamics and ROS production have been associated with endothelial dysfunction, development of

hypertension, and cardiac hypertrophy [39] and the protective role of mitochondria fusion in the vasculature during hypertension by limiting mitochondria ROS production has been reported [2].

Respiratory chain and oxidative phosphorylation in hypertension and CVDs

Oxidative phosphorylation is the process by which mitochondrial reducing equivalents are used to generate an electrochemical proton gradient across the inner mitochondrial membrane (protonmotive force, PMF). Four protein complexes, complexes I–IV, together with two electron shuttles (the membrane quinone coenzyme Q (CoQ) and the small protein cytochrome c (Cyt c)), forming the respiratory chain, are responsible of PMF buildup (REF). PMF thermodynamically drives the synthesis of ATP operated by complex V (F1 Fo ATP synthase). The main substrates for oxidative phosphorylation are mitochondrial NADH, which provides electrons to complex I (also known as NADH dehydrogenase) and succinate, which reduces a FAD molecule in the complex II (the Kreb's cycle succinate dehydrogenase).

The reduction of FAD to FADH2 is also linked to fatty acids catabolism, whose most important steps are within mitochondria along the β-oxidation, depending on the carnitine shuttle. β-oxidation of fatty acyl-CoA to trans-2-enoyl-CoA by acyl-CoA dehydrogenases is linked to the reduction of FAD to FADH2, which in turn transfers its electrons to the CoQ pool via the electron-transferring flavoprotein (ETF) localized at the matrix side of the inner mitochondrial membrane. It should be noted that, unlike the oxidation of succinate, the oxidation of fatty acids is completely dependent on the activity of complex I due to the step catalyzed by 3-hydroxy-acyl-CoA dehydrogenases in which NADH is produced.

All metabolic intermediates entering the tricarboxylic acid cycle, such as the glucose-derived or amino acid-derived pyruvate, and branched-chain amino acid-derived and fatty acid-derived acetyl-CoA and succinyl-CoA, can drive the reduction of NAD+ and FAD in the mitochondrial matrix, and hence can support the oxidative phosphorylation process.

The branched upper part of the mitochondrial respiratory chain then transfers reducing equivalents to the pool of CoQ, which is then oxidized by complex III, also known as CoQ:Cyt c oxidoreductase. Complex III transfers electrons to complex IV (also known as Cyt c oxidase) via Cyt c. Finally, it is in the binuclear center of complex IV that the chemistry of the reduction of molecular oxygen to water occurs. Oxygen is therefore the terminal acceptor of electrons coming from oxidative metabolisms and this is why oxygen is essential for the functioning of the organism.

Complex I, complex III and complex IV are proton pumps and contribute to the generation of the mitochondrial PMF. This is used for the synthesis of ATP, a process that requires the coordinated action of the complex V and membrane transporters ADP/ATP carrier (AAC) and phosphate carrier (PiC coded by SLC25A3) [40–42]. AAC and PiC (which are also members or regulators of the mitochondrial permeability transition pore [41,43–45]) provide the necessary ADP and inorganic phosphate for the ATP synthesis, a reaction catalyzed by F1 Fo ATP synthase. Note that this last reaction is reversible: in fact, in ischemic conditions the electron transport chain is not able to maintain the transmembrane mitochondrial potential and this function is performed by the ATP synthase complex, which hydrolyzes ATP to pump protons from the matrix to the cytosolic side of the inner mitochondrial membrane.

To support its energy-demand the heart needs a daily turnover of ATP in the order of kilograms [46–48] largely produced by mitochondrial oxidative phosphorylation, and only in small part (about 5% of the total) from glycolysis, and the Krebs cycle directly. In the healthy heart, the oxidation of fatty acids is the main metabolic pathway that supplies the mitochondrial respiratory chain with reducing equivalents, and minor contributions from carbohydrates, lactate and ketone

The inability of mitochondria to cope with the ATP needs of cardiomyocyte leads to energy depletion [49–51], which is central to the loss of contractile function. Moreover, respiratory activity is also required to compensate for proton leak and cation cycling [52].

Most studies that have directly examined the energetics of heart failure in humans demonstrate some form of impairment of respiratory chain capacity, resulting in a decrease in cellular ATP, phosphocreatine (PCr), or the PCr/ATP ratio (with a few exceptions; see [52] and references therein). Altered bioenergetics affects patients with heart failure with reduced ejection fraction (HFrEF) and those with heart failure with preserved ejection fraction (HFpEF) [52]. In ischemia and advanced stages heart failure, myocardial ATP levels can decrease up to 40%, but they remain very close to those observed in normal subjects in the early stages of heart failure, around 10 mmol per liter [53–55]. However, phosphocreatine and total creatine levels dramatically decrease at earlier stages up to 70%, profoundly altering the PCr/ATP ratio. This ratio correlates with mortality more tightly than left ventricular ejection fraction in patients with dilated cardiomyopathy [56].

In failing human heart, mitochondrial defects include decreased activities of all the complexes of the respiratory chain, particularly complex I and IV, and ATP synthase [48,57]. Electron transport proteins in the inner mitochondrial membrane aggregate into functional supercomplexes whose assembly is finely regulated [58–61]. It has been suggested that supercomplexes can reduce the ROS

production and finely tune the respiratory chain activity to the metabolic demands of cells [62,63]. Defects in supercomplex assembly have been related to heart failure [64]. It should be noted that despite the large number of available data, actually it is not possible to establish an unambiguous relationship between a particular defect in the respiratory chain and its severity from one hand, and the patient's clinical presentation on the other.

The voltage-dependent anion channels (VDACs) in CVDs

A crucial role in membrane depolarization is surely played by VDACs, also known as mitochondrial porins, representing the main protein channels of the OMM allowing the passage of nucleotides and metabolites playing a crucial role in cell metabolisms [65–67].

In human, there are three VDACs paraolgs of about 30 kDa, VDAC1, VDAC2 and VDAC3, encoded by three separate genes [66,68,69]. All paralogs are expressed in heart, kidney, skeletal muscle and brain, showing VDAC1 as the most abundant ubiquitously expressed paralog (i.e., 10 times more than VDAC2 and 100 times more than VDAC3 in HeLa cells), whereas VDAC2 and VDAC3 are abundantly expressed in testis [66,70].

Although the specific functions of the VDAC paralogs are not fully known, all VDACs act during membrane depolarization in the voltage range of approximately −40 to +40 mV [71–75] and show two states, open and closed state, for the selective passage of metabolites and ions . In the open state, VDACs are permeable to organic anions, ATP, ADP, Pi, and to metabolites [70], whereas in closed state, they transport K+, Na+, and Ca2+ [71].

In addition, VDACs are involved in mitochondrial apoptosis, in particular VDAC1 is considered a pro-apoptotic protein, VDAC2 has an anti-apoptotic function. whereas the role of VDAC3 in apoptosis regulation has not been fully clarified yet [71–76].

At molecular level it is retained that VDACs participate in the assembly of the mPTP together with the mitochondrial AAC and cyclophilin D [41,71–77]. It was also observed that VDAC participate in the mitochondrial stress response and in the permeabilization of OMM contributing also to regulate and trigger the opening of the mPTP [71].

In the IRI, the upregulation of VDAC1 was related to an increase in cardiomyocyte damage, as oxidative stress damage or harmful overload of mitochondrial Ca2+ [78], but the role of VDAC1 in the pathogenesis of cardiac alterations is not known [79].

Notably, a KO-mice for VDAC2 highlighted the involvement of VDAC2 in intracellular and mitochondrial calcium homeostasis by affecting the cardiomyocyte life. In fact, the loss of VADC2

leads to mitochondrial disorganization with a decrease of mitochondrial calcium uptake suggesting the critical role of this channel in cardiomyocytes [80].

The controversial role of aquaporins (AQP) in cardiac tissue degeneration related to mitochondrial function.

Aquaporins (AQPs) are a family of integral membrane proteins, constituted by six transmembrane domains interconnected by 5 loops, with both N- and C-termini placed in the cytosol, with a molecular weight between 20 kDa and 40 kDa. To date, 13 different AQPs (AQP0 – AQP12) have been identified in mammals, and they show tissue-specific expression patterns. AQPs are permeable mainly to water, but also to other small solutes, such as glycerol, urea, ammonia, CO_2, H_2O_2, depending on the pore selectivity, which is used to classify the aquaporins in 3 main groups: water-selective AQPs, aquaglyceroporins and unorthodox aquaporins [81,82].

Two aquaporins have been proposed to be located in the inner mitochondrial membrane, AQP8 and AQP9 [83,84], where they might be involved in the volume regulation of the mitochondrial matrix. A change in the mitochondrial volume that leads to mitochondrial swelling is the main factor which may trigger the mitochondria-mediated cell death, through both apoptosis and necrosis [85]. Despite what reported by Yang et al. (2006) [86], who tested both AQP8 and AQP9 and their role in the water movement pathway across inner mitochondrial membranes, providing evidence against aquaporin-facilitated water transport in mitochondria, later studies suggested that AQPs are indeed relevant for normal mitochondrial function [87], as well as they can transport other solutes.

For example, a rat recombinant mitochondrial AQP8 showed an ammonia transport even higher compared to the commonly recognized water transport activity [88]. In addition, it was reported that human AQP8 could transport H2O2 [89]. Ikaga et al. (2015) confirmed the presence of AQP8 in mitochondria of mouse adipose tissue, where it seems to contribute to the correct respiratory chain function. Furthermore, experiments with AQP8-knocked down cells suggested tissue-specific roles for AQP8 [90]. AQP9 is reported to be present in brain mitochondria [84] and in placenta mitochondria [91] and it is a glyceroaquaporin also permeable to the protonated lactic acid form, suggesting the involvement of AQP9 in the mitochondria lactate income that come out in the increased ROS-scavenging ability of mitochondria [91,92].

Although the studies of AQP expression in cardiac mitochondria are lacking, the cited literature let us suppose that more dedicated investigations are necessary to clarify the possible role of AQPs in cardiac mitochondria and in the CVDs development.

Mitochondrial pyruvate carrier (MPC)

A metabolite playing a key role in the crosstalk between cytoplasm and mitochondria is surely pyruvate coming from the oxidation of glucose along glycolysis, thanks to the reduction of NAD+ to NADH. For the correct function of glycolysis, cells need to re-oxidize NADH to NAD+ through the transfer of reducing equivalents to mitochondria thanks to the malate/aspartate shuttle, whose function is also important in the context of tricarboxylic acid cycle responsible for pyruvate oxidation, fueling respiratory chain complexes and oxidative phosphorylation (Fig. 1). In this context a mitochondrial transporter recently proposed to be involved in the onset of cardiomyopathies is the mitochondrial pyruvate carrier that in *H.sapiens* consists of a heterodimer protein complex formed by the monomer MPC1, encoded by SLC54A1, and the monomer MPC2, encoded by SLC54A2 [93–95], MPC1 is expressed at high levels in heart, muscle and tongue, whereas MPC2 is mainly expressed in the liver.

MPC catalyze the transport of pyruvate, derived from glycolysis, in the mitochondria where it is oxidized to acetyl-CoA by pyruvate dehydrogenase complex (PDH) or carboxylates to oxalacetate by pyruvate carboxylase. Both acetyl-CoA and oxalacetate take part in TCA cycle to generate reducing equivalent, NADH and FADH2, which contribute to the synthesis of ATP in the oxidative phosphorylation process. In the heart, the fate of pyruvate in the mitochondria reflects the metabolic flexibility between glucose and fatty acid oxidation by shifting towards the carboxylation to oxalacetate due to the increased fatty acids oxidation leading to the accumulation of acetyl CoA that is an inhibitor of PDH [96].

The expression levels of MPC are related to pathological cardiac hypertrophy and downregulation of MPC was observed in failing human heart and in the cardiac hypertrophy mouse model [97]. In cardiomyocyte knockout for MPC1/2 in mice models, cardiac hypertrophy is characterized by increased levels of anabolic metabolites as well as amino acids, and intermediate of pentose phosphate pathway and a decreased levels of TCA cycle intermediates [98]. The metabolic remodeling, associated with the decreased expression of MPC that causes cardiac alteration and dysfunction, have different potential mechanisms.

The first explanation could be linked to the inability of mitochondria to supply pyruvate for replenishing TCA cycle to generate reducing equivalents for oxidative phosphorylation. However, ATP levels are unchanged in the hearts of murine models deleted for MPC [98].

Interestingly, hypoxia causes MPC downregulation that causes post-translational protein modifications, which are associated with myocardial hypertrophy responses [99].

The accumulation of TCA cycle intermediates, as fumarate and succinate, causes post-translational modifications as protein succinylation, that are in general associated with heart failure, although the biochemical link between metabolic re-modelling events led by MPC and cardiac tissue degeneration remain to be clarified in a more detailed way [100].

Mitochondrial carriers of the SLC25A family as new pharmacological targets for counteracting cardiac tissue degeneration in CVDs, from hypertension to IRI.

While it is evident that uncontrolled hypertension increases risk of all-cause and CVD mortality with a growing trend [101], it appears crucial to find a class of pharmacological targets that can regulate the correct function of proteins involved in oxidative phosphorylation, fission, fusion, biogenesis, mitochondrial dynamics and all the known metabolic pathways depending on healthy mitochondria. In this regard a new class of pharmacological targets that may act as a master regulator of the above cited processes is represented by the mitochondrial transporters of the SLC25A family.

Mitochondrial transporters are present in all eukaryote organisms with i.e., 35 members in *S. cerevisiae*, 58 in *A. thaliana* and 53 members in *H. sapiens* [18,77,102,103]. The human mitochondrial carriers, members of the SLC25A family, are involved in the shuttle of several metabolites (such as nucleotides, organic acids and amino acids) and cofactors across the largely impermeable inner mitochondrial membrane and show a different tissue expression pattern [18,41,104,105]. Based on their variegated substrate specificity it is expected that they may play a crucial role in counteracting tissue degeneration in CVDs.

Indeed, mitochondrial carriers play a key role in different moments of the cell-cycle. The human ADP/ATP carriers (AACs) [41,42,106], involved in the export of ATP synthesized in the mitochondrial matrix, are also responsible for the regulation of the permeability transition pore opening, and missense mutations of ANT1 were linked to cardiomyopathies [107,108]. The Asp/Glu carrier (AGC) and the 2-oxoglutarate/malate (OGC) carrier or dicarboxylate carrier (DIC) [109–114], main actors of the malate/aspartate shuttle (MAS), ensure the exchange of reducing equivalents across the inner mitochondrial membrane. Notably, AGC is also involved in the supply of aspartate to the cytoplasm for the biosynthesis on *N*-acetyl-aspartate in the brain [110], but the alteration of cytoplasmic aspartate in the cardiac tissue was also associated with cardiac hypertrophy [115]. The carnitine carrier (CAC) [116,117], plays a crucial role in the carnitine shuttle allowing the fatty acid oxidation, but reduced plasma carnitine levels can cause benign cardiac hypertrophy [118]. The citrate carrier (CIC), plays a pivotal role in inflammation and involved in the supply of acetyl moieties to

the cytoplasm to be used for protein acetylation and also for histone acetylation [119–121]. Missense mutations of the citrate carrier were retained responsible for the cardiac hypertrophy observed in model organisms or patients affected by citrate carrier deficiency [122]. The ornithine carrier (ORC) [123,124], main actor of the urea cycle, is responsible for the catabolism of amino acids and atherosclerosis appears to be linked to aberrant amino acid metabolism [125]

Thus, the knowledge about the role played by all mitochondrial carriers in metabolic pathways may be used for the design of new drugs able to target mitochondrial carriers aiming to limit cardiac tissue degeneration to counteract IRI, by reducing ROS production and by limiting the excess of apoptosis observed after massive reoxygenation of ischemic tissues [10–12].

More in general the impairment of mitochondrial activity is often observed in patients with cardiovascular diseases (CVDs) [46,126,127] and it is retained that several mitochondrial proteins, i.e., respiratory chain protein complexes, together with mitochondrial dehydrogenases, transferases and transporters, leading metabolic pathways and playing a key role in the modulation of mitochondrial dysfunction and/or mitochondrial apoptosis [18,77,128–132], may represent a group of new molecular targets for developing new drugs, to counteract the mitochondrial abnormalities observed in patients affected by hypertension and vascular inflammation, before myocardial infarction.

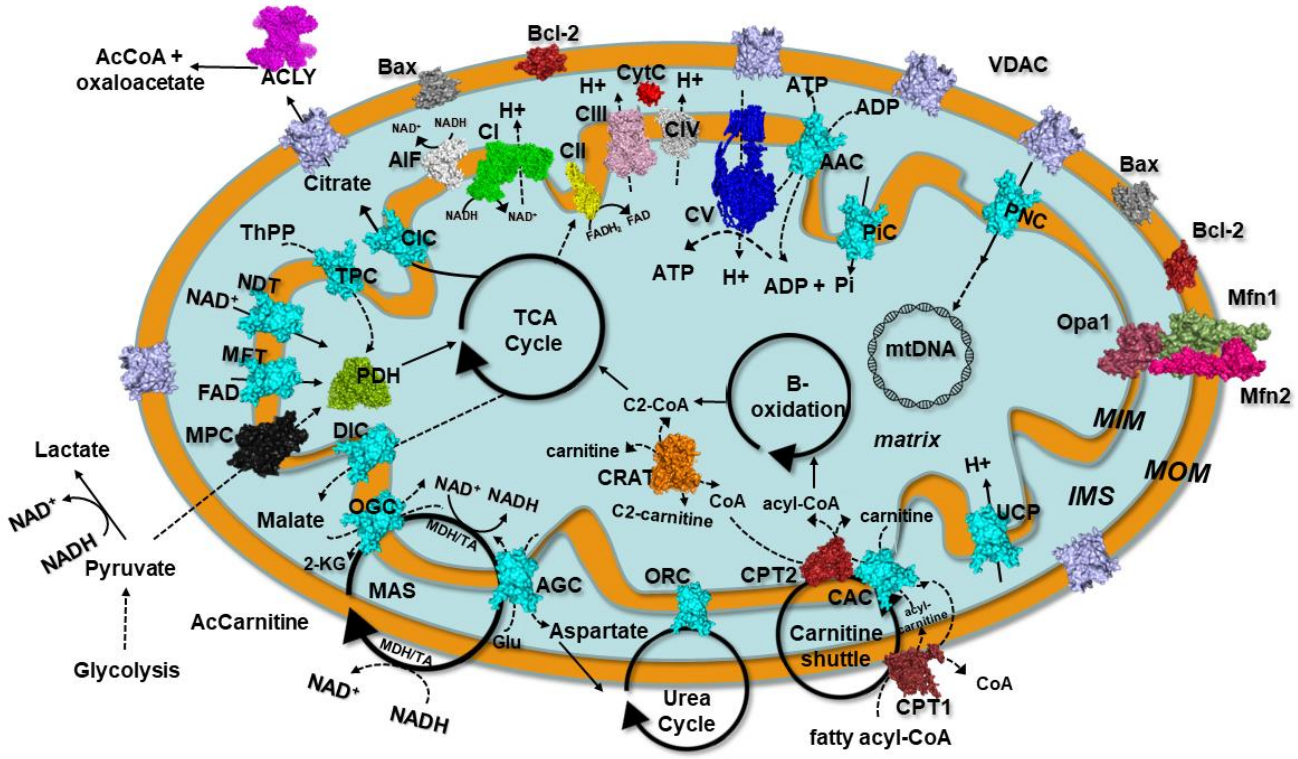

Figure 1 Scheme of a mitochondrion with a set of representative proteins, pathways and cycles. Respiratory chain complexes, mitochondrial transporters and other proteins are reported in surf representation and labeled. ATP synthase (CV) is reported in blue. Mitochondrial carriers are reported in cyan. VDAC is reported in pink. Bax and Bak/Bcl-2 are reported in dark grey and firebrick, respectively. MPC is reported in black; PDH in light green; CRAT in orange; CPT1 and CPT2 in dark violet; AIF in white; CytC in red. Complex I (CI), complex II (CII), complex III (CIII) and complex IV (CIV) are reported in green, yellow, magenta and grey, respectively, Opa1 is reported in dark magenta; Mfn1 in dark green; Mfn2 in hot-pink, according to PyMOL colors. Black circular arrows indicate cyclic pathways. Red arrows indicate impaired pathways or reactions. Black solid/dashed lines indicate the possible direction of the reported reactions. Abbreviations: MIM: mitochondrial inner membrane; MOM, mitochondrial outer membrane; IMS, intermembrane space; AAC, ADP/ATP carrier, coded in H. sapiens by SLC25A4, SLC25A5, SLC25A6, SLC25A31; TPC, thiamine pyrophosphate carrier, coded by SLC25A19; CAC, carnitine/acyl-carnitine carrier, coded by SLC25A20; ORC, ornithine carrier, coded by SLC25A15 (or SLC25A2); AGC, aspartate/glutamate carrier, coded by SLC25A12 and SLC25A13; DIC, dicarboxylate carrier, coded by SLC25A10; NDT, assumed to be the NAD+ carrier, coded by SLC25A51; MFT, assumed to be the FAD (folate/riboflavin) carrier, coded by SLC25A32; OGC, malate/2-oxoglutarate carrier, coded by SLC25A11; CIC, citrate carrier, coded by SLC25A1; PiC, phosphate carrier, coded by SLC25A3; MAS, malate/aspartate shuttle; TCA, tricarboxylic acid cycle; Bax, Bcl-2 associated X protein; Bak, Bcl-2 antagonist/killer-1; Bcl-2, B-cell lymphoma-2; MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; UCP, uncoupling protein, coded by SLC25A7, SLC25A8, SLC25A9, SLC25A14, SLC25A27 and SLC25A30; CypD, cyclophilin D; CytC, cytochrome C; VDAC, voltage-dependent anion channel; AIF, apoptosis-inducing factor; PNC, pyrimidine nucleotide carrier, coded in H. sapiens by SLC25A33 and SLC25A36.

Molecular details about metabolic pathways led by mitochondrial carriers involved in the onset of cardiomyopathies or myocardial degeneration.

Under physiological conditions, in the cardiac tissue, the main substrates for energy metabolism are free fatty acids (FFAs) and glucose with FFA oxidation being the main process to obtain energy. FFA oxidation is directly linked to oxidative phosphorylation, through the tricarboxylic acid cycle (TCA). While TCA is used to oxidate the acetyl-CoA produced by FFA, oxidative phosphorylation produces ATP, by restoring NAD+ and FAD useful both for the TCA, respiratory chain complexes and for the same FFA. Similarly, the correct function of FFA is linked to the correct function of the carnitine shuttle, allowing the entry of long chain fatty acids or the correct function of the pyruvate carrier, directly linked to glycolysis.

In this context, oxygen consumption represents a crucial step given that at the end of the respiratory chain O2 is reduced to water and in hypoxic conditions al the cited metabolic pathways can undergo serious damages or cause metabolic re-modeling, as observed in some types of cancers.

As a consequence of what above reported, the crucial role of mitochondrial function in the cardiac muscle appears evident. At the same time, the expression and the function of mitochondrial carriers allowing the exchange of crucial metabolites across the IMM are pivotal for maintaining healthy cardiomyocytes, although this pivotal role becomes evident in the case of missense mutations characterized in the related mitochondrial rare diseases

Here, we review the expression and the function of human mitochondrial carriers playing a major role in the cardiac mitochondrial metabolism, whose main proteins and pathways can represent an important target of new drugs for counteracting cardiac tissue degeneration and CVDs.

ADP/ATP carrier or Adenine Nucleotide Transporter (AAC or ANT)

The nucleotide transport is critical for mitochondria to ensure many metabolic processes crucial for cell viability such as mitochondrial DNA and RNA synthesis, ATP transport to the cytosol, and regulation of intrinsic mitochondrial apoptosis [41,42,72,106,133].

Among nucleotide transporters, the mitochondrial ADP/ATP carrier (AAC), also named adenine nucleotide translocator (ANT), occupies a relevant place.

In humans, four paralogous genes code for AACs; AAC1, encoded by SLC25A4 gene, AAC2, encoded by SLC25A5 gene, AAC3, encoded by SLC25A6 gene, and AAC4, encoded by SLC25A31 gene.

AAC1 is the predominant paralog of heart and skeletal muscle, whereas AAC2 is expressed in all human tissues particularly in the liver and kidney, AAC3 is ubiquitously expressed and AAC4 is selectively expressed in testis [41,106,134] .

The main function of AACs is to transport ATP from the mitochondrial matrix to the cytosol in exchange with ADP. The kinetic characterization of AACs underline that the apparent Km is in the micromolar range for all four paralogous carriers [42,135,136] . AAC3 shows a K_m for ADP of 2-3 times higher than that of AAC1 and AAC2, and AAC4 a K_m for ADP of about 10-20 times higher than that of AAC1-3. These different kinetic characteristics correlate with different functions of these paralogs in mammalian tissues.

AACs participate in the regulation of the mitochondrial permeability transition pore (mtPTP), whose opening is initiated by a variety of factors, i.e., from the reduction of mitochondrial inner membrane potential, to the increase of ADP and ROS [72,137]. It was proposed that AAC1 and AAC3 have pro-apoptotic properties, whereas AAC2 and AAC4 would show anti-apoptotic properties. The different behaviors of the highly similar paralogs appear to be due to their different abilities in participating in the formation and regulation of the mPTP [72,137–140].

Notably, the deletion of AAC1 or AAC2 shows severe effects on cardiomyocyte function, also related to their apparent different role in mPTP opening regulation. AAC1 deficiency leads to dilated cardiomyopathy with defects in myocardial mechanics, histopathological alterations, and activation of apoptosis [141], whereas AAC2 deficiency is associated with cardiac noncompaction [137].

These different effects are attributed to the different roles of the two AACs in cardiomyocytes and cardiac tissue function. Indeed, while AAC1 null mutant permits cardiomyocyte maturation and normal cardiac tissue development, but results in adult hypertrophic cardiomyopathy, AAC2 null mutant is embryonic lethal being associated with cardiac hypertrabeculation/noncompaction [137,141]. Considering that mitochondrial alterations (damages), such as restricted oxidative phosphorylation, β-oxidation, and impaired production of energy, contribute to cardiac hypertrophy, the overexpression in the rat of AAC1 showed a positive effect on preserving mitochondrial and cardiac function in hypertension-conditions [142]. The increase of ADP/ATP transport across the inner mitochondrial membrane ameliorates mitochondrial structure and function by preventing cardiac damages [142].

In addition, it was observed that AAC1 plays a crucial role in cardiomyocyte apoptosis during left ventricular hypertrophy (LVH) that developed in response to pressure overload [143]. It was also observed that individuals SLC25A31 (coding for AAC4) rs201279313 deletion versus wild-type genotype had better diastolic blood pressure response to beta-blocker therapies [144].

Mitochondrial phosphate carrier (PiC)

The mitochondrial phosphate carrier (PiC) has a main role in oxidative phosphorylation by transporting inorganic phosphate (Pi) from the cytosol to the mitochondrial matrix to guarantee ATP synthesis [18,145–148]. PiC function appears also critical for Ca^{2+} chelation in the mitochondrial matrix [149]. Mutations of PiC gene were associated with hypertrophic cardiomyopathy, muscular hypotonia, growth retardation and death in the first year of life [150].

This gene encodes two alternative splicing isoforms, A and B, that are localized differently in human tissues, with PiC-A abundantly expressed only in the heart and muscle, and PiC-B ubiquitous [18,151–153].

The kinetic constants are different between two isoforms, the K_m of PiC-A for Pi on the external side is higher than that of PiC-B (about 2.2 and 0.78 mM, respectively), whereas K_m of Pi on the internal side is very high for both isoforms (about 8.5 and 6.5 mM, respectively), by confirming a main transport of Pi from cytosol to mithocondrial matrix [154]. By considering tissue distribution and K_m, PiC-B ensures the energy demand of all tissues, whereas PiC-A operates in the presence of increased energy requirements [154].

PiC deficiency is associated with hypotonia, hypertrophic cardiomyopathy and lactic acidosis, a disease caused by defects of PiC-A-mediated transport, which leads to a decrease in mitochondrial ATP synthesis [155]. Besides its role in mitochondrial energy metabolism, PiC is also a modulator and a component of the mPTP [41,72,77,140,156,157]. In particular, PiC regulates mPTP function via Pi, which works as an allosteric regulator of mPTP assembly. In relation with PiC role in the regulation of mPTP assembly, it was observed that the genetic inhibition of PiC desensitizes the mPTP and is responsible for the onset of cardiomyopathy [158]. Notably, cardiomyocytes deleted for PiC show an impaired mitochondrial ATP synthesis, resulting in the upregulation of genes of glucose metabolism and the switch to anaerobic ATP synthesis, altering the dynamic of myofilaments by triggering cardiac hypertrophy [159,160]. Notably, an abnormal transport of inorganic phosphate in left ventricular mitochondria was observed in mitochondria from spontaneously hypertensive rats [161].

ATP-Mg/Pi carrier (APC)

The APC function is important to respond to fluctuations of matrix ATP concentration. In human, there are three paralogs of APC, namely APC1, encoded by SLC25A24, APC2, encoded by SLC25A25, and APC3, encoded by SLC25A23. In addition, a fourth paralogous gene, SLC25A41

encodes a protein very similar to APC1-3, called APC4, showing a shorter N-terminal region compared to the other three APCs [18,103,162]

These carriers catalyze a reversible electroneutral counter-exchange of divalent ATP-Mg^{2+} for HPO_4^{2-} in both directions [18,103,162,163].

The tissue distribution of APCs shows that APC1 is most expressed in the testis. A low expression of APC1 was also found in the small intestine, pancreas, kidney, spleen, liver, skeletal muscle, and heart, whereas it is absent in the brain and the lung. APC2 is expressed in the kidney, lung, small intestine, pancreas, liver, and heart and especially in the brain and skeletal muscle [18,103,162,163], whereas APC3 is expressed at high levels in the brain, heart testis, skeletal muscle, liver and lung and at lower levels in all other tissues [18,103,162–164]

The K_m for ATP-Mg is about 0.2 mM for APC1 and APC2, whereas K_m for APC3 and APC4 was not estimated. APC1 shows a higher V_{max} compared to APC2 [163].

Interestingly, the APC1-3 activity can be regulated by Ca^{2+} for the presence of three EF-hand Ca^{2+} binding motifs in the N-terminal region of APCs, which lacks in the N-terminal region of APC4. It is retained that all three paralogs may contribute to the uptake of Ca^{2+} in the mitochondria, participating in the regulation of mPTP opening, and thus to the regulation of cell death pathways. In this regard it was observed that SLC25A23 interacts with Ca^{2+} uniporter (MCU) in the regulation of Ca^{2+} mitochondrial uptake [165]. The differential expression of SLC25A25 mediated by the peroxisome-proliferator-activated receptor γ coactivator-1α (PGC-1α) transcription factor, involved in mitochondrial biogenesis regulation and the remodeling of muscle tissue [166,167], was proposed to affect myofibrillar structure and oxygen consumption of cardiomyocytes [168].

Carnitine/acylcarnitine carrier (CAC)

For energy needs, the cardiomyocytes use free fatty acids (FFAs) as the main energy source, thanks to beta oxidation and the following AcetylCoA oxidation, catalyzed by enzymes of the tricarboxylic acid cycle into the mitochondria. The mitochondrial transport of FFAs happens via the carnitine/acyl-carnitine (CAC) shuttle [116,117].

In humans, CAC, encoded by SLC25A20 gene, is expressed in different human tissues following different tissue-specific expression patterns. High expression of CAC was found in the liver, heart and skeletal muscle, whereas lower expression was found in the brain, kidney, pancreas and lung [117].

The mammalian CAC can transport acylcarnitine with short and long acyl-chains, besides carnitine. Depending on the concentration of substrates in the different mitochondrial compartments, CAC can

catalyze an exchange or a uniport of substrates. The exchange mode guarantees the import of acylcarnitine from the cytosol to the mitochondrial matrix for performing the β-oxidation, in exchange with the inner mitochondrial carnitine. Notably, the K_m for acylcarnitine on the external side is in the micromolar range, much lower than K_m of carnitine (K_m =0.5-1 mM) [117]. The uniport transport of carnitine guarantees to equilibrate the carnitine levels between mitochondrial matrix and cytosol by considering that the Km of carnitine for CAC is about 10 mM from the matrix side and the intramitochondrial carnitine concentration is about from 2 to 5 mM. The concentration of mitochondrial carnitine and acyl-carnitines can play a crucial role in the regulation of β-oxidation and in the switching from a glycolytic to an oxidative metabolism [117] .

The deficiency of CAC causes a disorder characterized by the accumulation of acyl-carnitines with long acyl-chains occurring in all tissues that use fatty acid oxidation for energy requires [117],

CAC deficiency commonly causes cardiomyopathy, skeletal muscle myopathy, liver abnormalities, and is usually fatal, if not recognized in time and treated [117,169]. Notably, impaired L-carnitine uptake correlates with higher blood pressure in adult men, and L-carnitine restores endothelial function in aortic rings from spontaneously hypertensive rat [170]. In postmortem analysis, the myocardium, liver and kidney present a marked steatosis resulting from a defect of β-oxidation and a local accumulation of triglyceride. This accumulation disrupts cell function and causes arrhythmias due to the presence of metabolites of fatty acids that lead to electrophysiological anomalies in the cardiac tissue [171].

Aspartate/glutamate carrier (AGC)

L-glutamate levels play a crucial role in the mitochondrial metabolism of cardiac tissue. In humans L-glutamate is imported into the mitochondria by the aspartate/glutamate carriers (AGC) and the glutamate carriers (GC) widely distributed in all eukaryote organisms in multiple paralogs, showing tissue-specific expression patterns [18,109–111,172,173].

The AGC takes part in the MAS, important to transfer the reducing equivalents of NADH plus H+ from the cytosol to the mitochondria in order to re-oxidize NADH to be used in glycolysis [111].

In humans there are two AGC paralogs, namely AGC1, encoded by SLC25A12, and AGC2 encoded by SLC25A13, which catalyze a 1:1 exchange of L-aspartate for L-glutamate plus a H^+. GC paralogs observed in *H. sapiens* are also two, namely GC1, encoded by SLC25A22, and GC2, encoded by SLC25A18, which catalyze a net uniport of L-glutamate plus a H^+ [18,109–111,172].

Structurally, AGC proteins are constituted of about 675 amino-acids (aa) organized in a C-terminal domain of about 340 aa, which presents all the structural features typically observed in mitochondrial

carriers, and a N-terminal domain of about 330 aa, which contains 8 EF-hand Ca^{2+} binding motifs [111], despite of what observed in GCs lacking EF-hands.

AGC1 and AGC2 different tissue expression patterns also appear related to the status of cell proliferation and specific energy requirement of tissues. AGC1 is expressed at high levels in the brain, heart, and skeletal muscle and is absent in the liver where AGC2 is very abundant [111,173].

The high expression of AGC1 in the heart guarantees metabolic flexibility with a performant MAS [174] responsible for the maintenance of redox homeostasis in support of glycolysis and ATP synthesis both at the mitochondrial level and along glycolytic pathways.

AGC1 and AGC2 catalyze an electrogenic transport across the inner mitochondrial matrix and the K_m for L-aspartate is about 50 μM for both proteins, but differences are shown in Vmax parameters; the Vmax are about 50 μmol/min/g protein and about 200 μmol/min/g protein for AGC1 and AGC2, respectively [111].

The deficiency of AGC1 or AGC2 is associated with the impairment of MAS activity. AGC1 knockout leads to developmental arrest, epilepsy, seizures and hypomyelination and these alterations mainly affect the brain, in particular neurons where the function of AGC1 is critical for the MAS pathway [110,175]. Similarly, AGC2 mutations are responsible for the onset of neonatal intrahepatic cholestasis (NiCCD) or type 2 citrullinemia (CTLN2), which can cause liver failure [109]. It was observed that both paralogs are expressed in the cardiac tissue, with AGC1 being predominantly expressed in atria [173].

Concerning the role of mitochondria in cardiomyocytes, it was proposed that heart damage associated with myocardial infarction suffers a set of sequential biochemical and metabolic modifications within cardiomyocytes, where mitochondrial activity, controlling the redox state through the MAS, could play a relevant role [176]. More in detail, it was proposed that the downregulation of MAS activity, tightly linked to the flux of the TCA, the electron transport chain, to the uptake of the amino acid L-glutamate and the regulation of cytosolic and mitochondrial calcium homeostasis, can be crucial for preserving mitochondrial function and cell survival in ischemia events [174,177].

In murine models established for studying the role of AGC and MAS in cardiac tissue damage, the murine models deleted for the two paralogs showed in the cardiac tissue a different response at the physiological level to variations of Ca^{2+} concentration. While the deletion of AGC2 shows the same MAS activation observed in control murine models in presence of different concentrations of Ca^{2+} the deletion of AGC1 leads to a desensitization of the MAS activation in presence of Ca^{2+} [178].

It was proposed that the different behaviors can be related a different distribution of both paralogs in the mitochondria of specialized heart compartments showing a different response to Ca^{2+} concentration variation [178].

Citrate carrier (CIC)

Citrate is a crossroad metabolite in the intermediary metabolism of mammalian cells, becoming the starting substrate of TCA cycle, to provide higher ATP levels, and fatty acid biosynthesis. Citrate is synthesized in mitochondria from acetyl-CoA and oxaloacetate (OAA) by citrate synthase, the first enzyme of TCA cycle, and is transported from the mitochondria to the cytosol via the mitochondrial carrier of citrate/isocitrate (CIC), encoded by the nuclear gene of SLC25A1. CIC mediates the transport (export) of mitochondrial citrate/isocitrate in exchange with cytosolic malate [18,120].

In the cytosol, citrate exerts a well-known key regulatory function of energy production by inhibiting glycolysis at the level of phosphofructokinase 1 (PFK1) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatases (PFK2) [179], the bifunctional enzyme which synthesizes fructose 2,6-bisphosphate (F2,6P), the mast allosteric activator of glycolysis. At the same time, citrate indirectly also inhibits piruvate kinase (PK), leading a decrease in the levels of fructose-1,6-biphosphate (F1,6P), an activator of PK [180]. It was also reported that citrate is an activator of acetyl-CoA carboxylase (ACC), which synthesyzes malonyl-CoA, the substrate for fatty acids biosynthesis and, the inhibitor of carnitine palmitoyltransferase 1 (CPT-1) [181], consequently playing a key role also for fatty acid β-oxidation. Furthermore, citrate inhibits pyruvate dehydrogenase (PDH) and succinate dehydrogenase (SDH), leading the regulation of TCA cycle and many other biological processes, including inflammation, insulin secretion, histone acetylation, and cancer development, taking part in the so-called citrate pathway [120,182].

In the citrate pathway, the citrate, transported in the cytosol via CIC carrier, is also the substrate of ATP-citrate lyase (ACLY), providing acetyl-coenzyme-A and OAA [119,120].

Typically, cytosolic malate dehydrogenase (MDHc) converts OAA to malate, which is then transported into the mitochondria in exchange for citrate by the CIC carrier. Alternatively, OAA is converted into pyruvate by the malic enzyme (ME), by generating NADPH, utilized for fatty acid biosynthesis, to protect against the excess of oxidative stress, or, in some conditions, to produce ROS and NO through the NADPH oxidase, and inducible NO synthase (iNOS), respectively [120,182,183]. Acetyl-coenzyme A provides unit to lipid biosynthesis, including arachidonic acid, the precursor of prostaglandins, which play a pivotal role in inflammation [120,182,183].

Citrate also causes post-translational modifications of proteins, in particular acetylation and malonylation of histones, highlighting the main role of the citrate pathway in addressing to metabolic needs of cells [120,121,182,183].

In the citrate pathway, the mitochondrial CIC represents one of the main actors. CIC is encoded by the human gene SLC25A1, located on chromosome 22q11, and consisting of eight exons which encode a 311 amino acid protein. SLC25A1 is expressed at high levels in the liver, kidney, pancreas

and brain and at a low level in muscle tissue. The apparent K_m for citrate transport is in the 5-50 μM range, also depending on the pH used in kinetics assays [184–186].

Pathogenic mutations in the SLC25A1 gene were associated with the agenesis of the corpus callosum, and impaired neuromuscular transmission, observed in CIC deficiency affected patients [122,187–189]. In addition, an abnormal heart development was observed in zebrafish knockdown embryos with oedema of the hindbrain, heart, yolk sac and tail, and a reduced blood flow to the tail [122]. It was also proposed that SLC25A1 and citrate exert cardioprotective effects in ventricles by maintaining fatty acid synthesis, also in hyperoxia conditions, which cause the inhibition of fatty acid synthesis in atrial cardiomyocytes, showing a lower expression of SLC25A1 and other genes involved in citrate-dependent pathways, compared to what observed in ventricular cardiomyocytes [190].

Clues about the involvement of other mitochondrial carriers in CVDs

Beyond the above cited mitochondrial carriers already clearly associated with cardiac dysfunction, as observed in patients affected by the corresponding mitochondrial carrier deficiencies, other mitochondrial carriers appear to play a role in the onset of CVDs or the prevention of cardiac tissue degeneration and/or in the prevention of ischemic injury. Indeed, it was proposed that the ornithine carrier and other basic amino acid carriers (coded by the nuclear genes SLC25A2, SLC25A15 and SLC25A29), can be involved in the import of basic amino acids into mitochondria for mitochondrial protein synthesis and amino acid degradation [18,123,124,191], but it was also proposed that the above cited amino acid carriers (with specific reference to SLC25A2) can play a key role also in dimethyl arginine, asymmetric dimethylarginine (ADMA) metabolism and nitric oxide (NO)-dependent pathways crucial for vascular homeostasis [192,193].

Similarly, the altered expression (or missense mutations) of mitochondrial carriers coded by SLC25A32 (folate/FAD carrier) and SLC25A34 (proposed to be the most similar carrier to the yeast isopropyl-malate carrier [18,194]) were associated with the impairment of fatty acid biosynthesis of cardiomyocytes [195,196].

Another group of mitochondrial carriers, namely the so called mitoferrin-1 and mitoferrin-2, coded by SLC25A28 and SLC25A37, together with carriers coded by SLC25A38 (proposed as a transporter of glycine and serine by comparison with its yeast ortholog [197]) and SLC25A39 (recently proposed as a carrier of glutathione [198,199]) proposed to be important for heme biosynthesis and iron homeostasis [18,162,200,201] were associated to abnormal iron regulation and accumulation and retained responsible for triggering pathological processes typical of cardiovascular diseases [202,203].

Notably, the last group of cited carriers still lack a complete characterization by transport assays on the purified recombinant proteins reconstituted in proteoliposomes, as done for other members of the mitochondrial carrier family [42]. Transport assays of radiolabelled substrates on proteoliposomes still represent the golden standard technique for the characterization of these proteins,

In addition the impairment of the mitochondrial carrier coded by SLC25A34 [18,194] together with dicarboxylate/Pi carrier (coded by SLC25A10, [18,114]), 2-oxoglutarate/malate carrier (coded by SLC25A11, [111–113,204]), pyrimidine carrier (coded by SLC25A33, [18,205]) and the not yet characterized carriers MTCH1 and MTCH2 (coded by SLC25A49 and SLC25A50, respectively [18]) was associated to diabetic cardiomyopathy [206–209]. Notably, SLC25A11 downregulation was associated to high blood pressure in a murine model used to study the mechanism for the regulation of water balance in kidney [210].

There are also clues about the involvement of other mitochondrial carriers coded by SLC25A26, SLC25A44 and SLC25A46 in altered metabolic pathways involved in the onset of CVDs. More in detail, the *S*-adenosylmethionine (SAM) carrier, coded by SLC25A26, appears to play a crucial role in providing methyl group for DNA methylation [18,211]. Missense mutations of the SAM carrier were associated with cardiopulmonary failures [212]. It is expected that the mitochondrial carrier coded by SLC25A44, proposed to be involved in the translocation of branched chain amino acids (although transport assays on proteoliposomes reconstituted with the purified SLC25A44 recombinant protein still lack) [213], can play a crucial role in cardiovascular health, which depends on the correct homeostasis of branched chain amino acids [214].In addition, a recent transcriptome-wide association study (TWAS) revealed an association between lacunar stroke, high blood pressure and the expression of SLC25A44 [215]. The uncharacterized mitochondrial carrier coded by SLC25A46 was proposed to be involved in mitochondrial cristae maintenance and fission promotion, by mediating Mfn1/Mfn2 oligomerization, thus it is SLC25A46 is retained a promising molecular target for mitochondrial dynamics maintenance/rescue in CVDs [216–218].

The last group of metabolite transporters of the mitochondrial carrier SLC25A family proposed to be involved in the setting of heart failure consists of uncoupling proteins (UCPs coded by SLC25A7, SLC25A8, SLC25A9, SLC25A14, SLC25A27 and SLC25A30 [18,162]. The downregulation of UCP was associated with the onset of cardiomyopathies mainly due to their involvement in C4 metabolites export from mitochondria and in the regulation of mitochondrial membrane potential, important for mitochondrial energy metabolism maintenance and mitochondria ROS production [219–223]. Recent studies provided also some clues about a possible relationship existing between genetic polymorphisms observed at the uncoupling protein 1 (UCP1) [224,225] and intronic single nucleotide

polymorphisms in SLC25A42 [226,227] with variations of systolic and diastolic blood pressure [224,225,228].

Concerning SLC25A42, involved in the metabolism of coenzyme A [226,227], it should be noticed that mutations of SLC25A42 are associated to the onset of mitochondrial myopathy [229], although its involvement in cardiac mitochondrial dysfunction remains questionable. Notably, it was proposed that association between UCP polymorphisms and blood pressure increase was independent of obesity phenotypes [224,225]. It was also proposed that apoptosis of cardiomyocytes is an important regulatory mechanism that is involved in the cardiac adaptive response to pressure overload and the apoptosis of cardiomyocytes may be suppressed, in part, by UCP2 overexpression [230].

Interestingly, UCP2 participates to the regulation of blood pressure, as supported by the fact that its expression in skeletal muscle was found lower in hypertensive rats compared to normotensive rats [231], Ucp2−/− mice shows an increase of salt diet-induced hypertension [232] an increase of the superoxide level and a decrease nitric oxide-dependent dilatation. In addition, UCP2 was found downregulated in rat model treated with high salt levels, leading to an increase of oxidative stress and renal damage [233]. So, UCP2 seems to function against oxidative stress by controlling blood pressure and hypertension damage [234].

Structural analyses

From a structural point of view, it is known that mitochondrial transporters of the SLC25A family are difficult to be crystallized. Indeed, the first member of mitochondrial carriers, the bovine ADP/ATP carrier (AAC) paralog 1 was crystallized in 2003 [235] thanks to the employment of carboxyatractyloside, an AAC powerful/highly selective inhibitor [42,236,237]. Carboxyatractyloside has pro-apoptotic properties and binds/inhibits the AAC from the intermembrane space, stabilizing the carrier in its cytoplasmic conformation (open towards the intermembrane space) [18,42,103,104,236,238,239]. Starting from that structure, two other AAC from yeast were crystallized in a complex with carboxyatractyloside in the same cytosolic conformation in 2014 [240]. But only a few years ago, in 2019, an AAC was crystallized in the matrix conformation (open towards the mitochondrial matrix) thanks to the employment of a nanobody and the bongkrekic acid, a second AAC selective inhibitor [42,236,237]. Bongkrekic acid has anti-apoptotic properties and binds/inhibits AAC from the matrix face, stabilizing the carrier in its matrix conformation (open towards the matrix space). Thanks to the existence of these crystallized structures, it is possible to create trustable 3D comparative models of all the members of the SLC25A mitochondrial carrier family, to perform virtual screening of chemical libraries to identify new small

molecules with high affinity for the investigated mitochondrial carriers [42,241] involved in the onset of CVDs. The existence of the two conformations crystallized in complex with two selective inhibitors with opposite properties for apoptosis regulation, will help in searching for pro-apoptotic or anti-apoptotic small molecules, structurally related to the known inhibitors [42], aiming to regulate mPTP opening as well as to stimulate impaired mitochondrial carriers involved in the regulation of respiratory chain activity, fusion/fission events, ROS homeostasis and the other related CVD-impaired mitochondrial metabolic pathways [18], trying to counteract ischemia reperfusion injury, cardiac tissue degeneration and/or cardiomyocyte (mitochondrial) dysfunction.

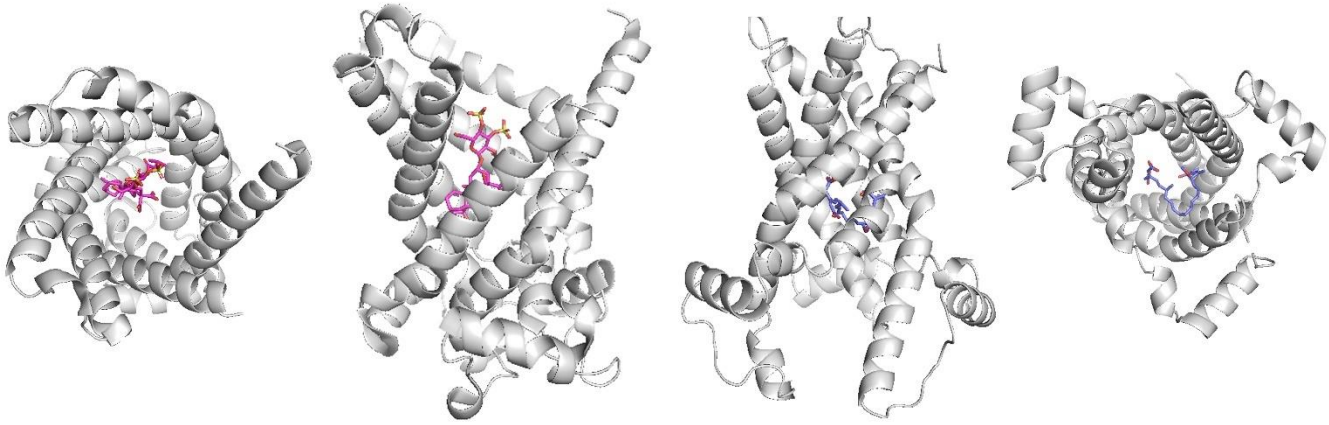

Figure 2 From the left, top view and lateral view of the 3D comparative model of the human AAC2 in cytosolic conformation, based on the bovine ADP/ATP carrier paralog 1 crystallized structure (1okc.pdb[235]*), and lateral view and bottom view of the human AAC2 in matrix conformation, based on the Thermothelomyces thermophilus (6gci.pdb) AAC structure (6gci.pdb* [242]*) are reported in white cartoon. Carboxyatractyloside was docked in the human AAC2 by superimposition with the bovine crystallized AAC1 and it is reported in magenta sticks. Bongkrekic acid was docked in the human AAC2 by superimposition with T.thermophilus AAC and it is reported in blue sticks. Superimposition operations were performed by using PyMOL. 3D comparative models were built according to protocols previously described* [42,116,162,172,226,243]

In addition, if the small molecules with high affinity for the generated 3D comparative models of mitochondrial carriers, will be chosen through the virtual screening of drug libraries in the context of the drug repurposing approaches, the newly identified small molecules with high affinity for mitochondrial transporters involved in CVDs, might be translated very quickly from the bench side to the clinics [244–246].

Recent advances in OMICS based approaches for the study of mitochondrial dysfunction in hypertension and CVDs

Cardiovascular disease encompasses a range of conditions extending from myocardial infarction to congenital heart disease most of which are heritable. However, the general late onset of CVDs and the clinical heterogeneity of CVDs is so extensive that the overwhelming majority of patients suspected of CVDs have no genetic diagnosis, which in turn is an obstacle for establishing a clear pathogenic understanding [247,248].

Limits in the whole genome sequencing (WGS) based approaches in the diagnosis of hypertension and CVDs.

For all the above mentioned aspects, next generation sequencing (NGS) technologies [249–257] have emerged in the last 20 years as a fundamental instrument to detect possibly causal mutations in genetically undiagnosed CVDs, given the unique opportunity to sequence thousands protein coding genes in once [258]. To date the available diagnostic NGS studies of CVDs differ in the number and type of regions targeted for sequencing, including mitochondrial genome [250–252] or nuclear genes encoding proteins with predicted mitochondrial location [253–256]. Although all these studies have added novel mutations and genes to the list of CVD candidate genes, the success rate in gene discovery related to CVD causing genes is still further low. Several reasons may account for this poor result, including technical and genetic considerations. The former is based on the possibility that causal mutations are located in non-targeted regions that are therefore excluded from sequencing. Further, the genome of each individual host on average ~2,500 nonsynonymous sequence variants compared to the genome reference sequence, only including evolutionary conserved positions [259]. Bioinformatics approaches are therefore necessary but also limiting in predicting truly deleterious mutations, so that benign mutations can be mistaken for deleterious ones and vice versa [260]. An additional limit in the identification of causal mutations is the general lack of knowledge about the CVD mode of inheritance in a given patient. In particular, de novo CVD mutations have no chance to be detected, unless parental DNA is not simultaneously sequenced [261]. More recent NGS analyses allowed to associated mitochondrial DNA mutations to inherited essential hypertension and CVDs [262–264].

Transcriptomics analyses in CVDs related to mitochondrial dysfunction

Lot of information was obtained in the last 20 years by the employment of NGS based approaches in the diagnosis and more in general in the study of CVDs. All the available information can to date be integrated with the information deriving from studies focused on other "omics" approaches based on more recent applications of transcriptomics and metabolomics analyses that can help in the study of an impaired metabolic pathway/network consisting of several genes encoding proteins involved in the metabolism of several small molecules.

On this concern, the analysis of gene expression by an RNA-sequencing approach can help in the understanding of the complex processes of energy generation in the onset of hypertension and CVDs, trying to increase our knwoledge related to the molecular basis of metabolic changes often responsible for the onset and development of CVDs. In this perspective, a recent study reported about the association between the impairment of mitochondrial bioenergetics and the development of the right ventricle (RV) failure, the major risk factor for mortality in patients with complex congenital heart diseases such as hypoplastic left heart syndrome or tetralogy of Fallot [265]. Using a murine model of pulmonary artery banding (PAB) [55], a RV pressure overload was induced and all the transcriptional changes were analysed both in the right and in the left ventricle. In addition, the activity of electron transport chain complexes was also estimated. Data obtained by RNA-sequencing of RV myocardium from RV pressure overload-induced RV failure, showed almost 2000 genes differentially expressed, that clustered within several dysregulated pathways that are downregulated or upregulated in several pathological conditions. In particular RV failure showed on the one hand a decreased expression of electron transport chain genes and mitochondrial antioxidant genes, on the other an increased expression of oxidant stress markers. On the contrary the transcription of electron transport chain genes increased in the left ventricle of RV failure, probably in order to preserve the hyperdynamic left ventricular function. A deep understanding of the transcriptional changes together with all the complex energetic processes of energy generation could be strategically useful in developing novel diagnostic tools and novel therapeutic targets. In this perspective an in vivo phenotypic evaluation and transcriptomic analysis on the hearth of two strains, hypertensive and normotensive, of rats was performed [266]. As well known, several antihypertensive drugs exhibit blood pressure (BP)-independent protective effects on different pathology, such as left ventricular hypertrophy. 6 principal classes of antihypertensive drug (an angiotensin-converting enzyme inhibitor, an angiotensin receptor blocker, hydrochlorothiazide as diuretic, a calcium-channel blocker, a vasodilating β-blocker, and hydralazine) were administered and examined in two inbred rat strains: SHRSP (stroke-prone spontaneously hypertensive rat) and WKY (Wistar Kyoto rats) as control. The

obtained microarray data showed a BP-dependent and BP-independent gene expression changes in the hearth of SHRSP rats. Notably different levels of mRNA expression were induced particularly by RAS blockade, such as for the Nppb and Acta 1 genes that are known to be hypertrophic markers.

Metabolomics analyses in CVDs related to mitochondrial dysfunction

Concerning the more recent applications of metabolomics analyses, it is known that metabolomics is the study of small molecules, commonly known as metabolites, within cells, biofluids, tissues, or organisms [267]. Generally, the metabolome of a given biological system is defined as the set of end products deriving from physiological and environmental stimuli. Thus, studying the metabolite composition of living entities provides deep insights into the chemical pathways occurring continuously within cells as a result of the combination of genetic cues and external factors. The metabolome is inherently very dynamic as small molecules are continuously absorbed, synthesized, and degraded; the product of a reaction becomes the substrate for the consecutive or parallel reactions, inducing a change in the final composition and concentration of the metabolites [268]. In other words, metabolomics offers the opportunity to gain information on the metabolites that can be affected by factors such as diet, intestinal microbiota, physical activity, environmental exposures, etc.

A desired result of metabolomics studies is the identification and the quantification of possible biomarkers involved into metabolic processes that are perturbed during metabolic diseases.

Nowadays, metabolomics benefits from two main analytical approaches, namely the targeted and the non-targeted analytical approaches [269]. The first approach focuses on a well-defined set of metabolites within a biological sample, thus requiring *a priori* knowledge of them. The identification and quantification of these metabolites are made possible by the use of reference compounds and the addition of internal standards. Changes in observed concentrations can help detect any alterations in a metabolic pathway or reveal the presence of an unknown metabolic process. One of the main advantages of the targeted approach is a greater sensitivity and selectivity since the extraction of metabolites and the optimization of instrumental parameters allow to obtain a reliable quantitative result [270,271]. The non-targeted approach, on the other hand, aims to reveal any specific patterns or signatures of metabolites associated with a genotype, drug treatment, clinical subpopulation, or a comparative group, such as a control group. The main goal of a non-targeted analysis is to detect and identify as many metabolites as possible within a biological sample. This set of metabolites is specific to each biological sample and constitutes the metabolic fingerprint of that sample. In this way, the non-targeted method, compared to the targeted approach, does not know *a priori* the metabolites of interest. The metabolites identified and semi-quantified during the analysis are then generally

exploited to rationalize the differences between the metabolic fingerprints of two or more groups of biological samples. The main advantage of the non-targeted approach, providing an overview of the metabolite composition of the biological sample, lies in the possibility of correlating certain compounds to the alteration of a metabolic pathway and, therefore, allowing the identification of new disease biomarkers and/or helping to discover new metabolic pathways [272,273] Among the analytical techniques suitable for metabolomics studies, Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) are gaining great attention in metabolomics, thanks to their high selectivity and reproducibility in both targeted and non-targeted methods.

The use of NMR to identify and quantify the set of metabolites in a sample is based on the interaction between magnetically active nuclei contained in a given sample and an external magnetic field. In biomedical studies, ^{1}H, ^{13}C, ^{15}N, and ^{31}P are the most frequently considered nuclei. Different resonance frequencies are associated to the detected nuclei depending on their chemical and magnetic environment in the metabolite. Thus, more information can be gathered to build the metabolic fingerprint of the sample under examination [274–278]. Once a single resonance frequency or a set of frequencies are associated with a metabolite, a quantitative analysis can be performed without the need for a reference compound for those metabolites with concentrations down to the micromolar range [279,280].

One of the main advantages of MS over NMR is its inherent increased sensitivity [281]. This makes MS a widely used technique in the context of targeted analyses, allowing the identification and quantification of metabolites even contained in small quantities (femtomolar range) within a biological sample. The metabolites, after an ionization phase, are identified based on their mass/charge ratio (*m/z*). The resulting ions are then separated in the mass separation unit through different techniques (time-of-flight, quadrupole, and ion trap mass analyzers), which are chosen based on the desired resolution and accuracy. Tandem MS (MS/MS) is generally performed to improve accuracy during identification (or annotation) of the metabolite, thanks to the information provided by the further fragmentation of the ion from the first separation unit [282]. Most often an embedded system is used, consisting of a mass spectrometer coupled with liquid chromatography (LC) [283–286], gas chromatography (GC) [287–289], or their combination [290]. In this case, through chromatography, a separation of the metabolites is carried out before their ionization. The integration of the area below the peak relative to each metabolite and the comparison with the calibration curve previously built ad hoc allows to estimate the concentration within the sample.

Table 1 contains a selection of CVD studies performed by applying metabolomics to biological samples. Good results have been obtained in correlating metabolic profiles in the case of different pathologies, such as type 2 diabete (T2D) or pulmonary arterial hypertension (PAH). Furthermore,

the identification of specific biomarkers was exploited to establish alterations in the metabolic pathways involved in CVD reflecting mitochondrial impairment.

In this context, metabolomics analyses highlighted an altered ratio of BCAA/AAA in T2D patients suffering coronary heart disease (CHD) (Tab. 1). Decrease in lactate, medium/long-chain acyl-carnitine, and succinate was observed either in physiological or pathological hypertrophy (Tab. 1). Increased levels of circulating C16 and C18: 1 acyl-carnitines were also observed in patients with end-stage heart failure compared to those with chronic systolic failure (Tab. 1). For a list of representative alterations of metabolites in plasma/tissues from CVDs affected patients, detected by metabolomics analyses, see Tab. 1.

Interestingly, recent studies have applied metabolomics to studying biological samples to identify alterations in metabolic profiles and predict the risk of related heart failure [291–295].

Both targeted and non-targeted approaches have proved useful in obtaining information related to cardiovascular disease, where NMR and MS are finding more and more applications in this field of science.

Table 1. List of selected studies of metabolomics in CVD, the analytical methodology, and their principal findings.

Case study	Metabolic information	Analytical technique	Analytical approach	Ref
The pathological link between coronary heart disease (CHD) and T2D	T2D has important effects on BCAA and (aromatic amino acid) AAA metabolism in CHD patients, indicating impaired cardiac energy metabolism and mitochondrial impairment.	^{1}H NMR	Targeted	[296]
The pathological link between CHD and PAH.	Lactate and threonine are significantly correlated with PAH, pulmonary vascular resistance, and N-terminal pro-B type natriuretic peptide.	^{1}H NMR	Non-targeted	[297]
Heart hypertrophies in response to chronic exercise training (physiological hypertrophy) and hemodynamic stresses (pathological hypertrophy)	Decreases in medium- and long-chain acylcarnitines, succinate, and lactate suggest an increase in carbon flux through oxidative metabolic pathways	LC-MS/MS	Targeted	[298]
Dilated cardiomyopathy (DCM)	Reduced levels of metabolites in glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle, together with large decreases in triacylglycerol levels in myocardial tissues, suggest that reduced energy	LC-MS/MS	Non-targeted	[299]

	production leads to cardiac contractile dysfunction in the symptomatic phase. A mild reduction in glutathione and a compensatory increase in ophthalmotherapy levels suggest an increase in oxidative stress in diseased tissues			
Heart failure and myocardial infarction (MI)	Time-dependent decreases in purines, acylcarnitines, fatty acids, and sphingolipids suggest global effects in energy metabolism, with concomitant increases in oxidative stress markers. In addition, an accumulation of BCAAs in MI associated with myocardial insulin resistance was observed.	LC-MS/MS and GC-MS	Non-targeted	[300]
	Increased levels of circulating C16 and C18: 1 acylcarnitines are found in patients with end-stage heart failure compared to those with chronic systolic failure. A decrease is observed after long-term mechanical circulatory support in patients wit	LC-MS/MS	Targeted	[301]
	A set of metabolites, including histidine, phenylalanine, spermidine, and C34: 4 phosphatidylcholine show a similar value to BNP for the diagnosis and prognosis of heart failure (HF).	LC-MS	Non-targeted	[302]
Cardiovascular risk prediction	An SCDA-containing metabolite factor found in peripheral blood is associated with myocardial death/infarction.	LC-MS/MS	Targeted and non-targeted	[303]
	The level of TMAO, a metabolite derived from the intestinal microbiome and dietary phosphatidylcholine, is related both to the pathogenesis of coronary heart disease and to increased cardiovascular risks.	LC-MS	Targeted	[304]

Acronyms used in the table: Congenital Heart Disease (CHD); Pulmonary Arterial Hypertension (PAH); Type 2 diabetes (T2D); Branched-Chain Amino Acids (BCAA); myocardial infarction (MI); Heart Failure with reduced Ejection Fraction (HFrEF); B-type natriuretic peptide (BNP); Heart Failure (HF); Short-Chain Dicarboxylacylcarnitine (SCDA); Trimethylamine-N-oxide (TMAO); Dilated cardiomyopathy (DCM).

CVD treatments based on the targeting of mitochondria

A number of pharmacological approaches have been attempted to improve the functioning of the mitochondrial respiratory chain during heart failure [51,305] and mitochondrial uncouplers are were considered promising candidates being among the earliest known small molecule to be investigated as mitochondrial drugs for several biomedical indications [306]. Mitochondrial uncouplers have been mostly studied as weight-reducing agents even though numerous possible therapeutical applications are currently being investigated including cancer, both metabolic and neurodegenerative diseases, and ischemia−reperfusion injury [307]. Among mitochondrial uncouplers a recent promising candidate for the treatment of hypertension is represented by OPC-163493, a liver-localized/targeted mitochondrial uncoupler that ameliorates various complications of diabetes. OPC-163493 treatment lower blood pressure, extended survival, and improved renal function in the rat model of stroke/hypertension, possibly reducing mitochondrial ROS production by enhancing NO bioavailability in blood vessels [308].

The study of specific mitochondrion-targeted small molecules for the treatment of CVDs is still in its infancy [309–311] but among the most significant BGP-15, a 2-hydroxylamine derivative protecting against several oxidative stress-related diseases in animal models deserves to be mentioned. Indeed, BGP-15 has recently been evaluated as a possible drug for counteracting hypertension-induced lung damage in a murine model of pulmonary arterial hypertension. In particular, it modulates mitochondrial dynamics by enhancing mitochondrial fusion or preventing its fission [312]. Mdivi-1, a mitochondrial division/mitophagy inhibitor acting as a selective dynamin-related protein 1 (Drp1) inhibitor, reduced the expression of Drp1 in spontaneously hypertensive rats with consequent amelioration of the inflammatory responses and vessel protection [313]. Mitochonic acid [(MA)-5], a novel indole acetic acid derivative, modulates mitochondrial bioenergetics binding the mitochondrial protein mitofilin, thus promoting oligomerization of the ATP synthase and supercomplex formation, and favoring mitochondrial ATP synthesis. Mitochonic acid ameliorates cardiac myocyte damage in murine models of renal disease [314], but its involvement in mitochondrial respiration in hypertension has never been investigated.

Despite the widespread use of multivitamin supplements [77], several studies are ongoing for investigating the effects of dietary supplements on BP and the development of hypertension [315–317]. CoQ is among the small molecules used as dietary supplements with evidence of possible benefits in the handling of hypertension [318]. CoQ is a cofactor of the respiratory chain complexes. It is essential for the electron transfer in the mitochondrial inner membrane [319] and functions as a

powerful natural antioxidant that protects against oxidative stress. CoQ deficiency leads to electron transport chain dysfunction in humans that can be recoverd by oral supplementation [320]. Decreased circulating CoQ has been observed in heart failure and plasma concentration of CoQ is inversely related to mortality, with an inverse correlation observed between plasma CoQ and mortality [321,322]. Among the various clinical studies carried out (see [305]), one lasting two years has demonstrated the safety of prolonged administration of CoQ and its efficacy as an adjuvant in the treatment of heart failure [323].

In order to improve the targeting of mitochondria antioxidants able to regulate mitochondrial redox status were conjugated triphenylalkylphosphonium cation (TPP^+) for obtaining TPP^+-conjugated compounds (MitoQ, for example) that have demonstrated promising antioxidants properties in experimental models of hypertension [324,325] by attenuating oxidative damage in endothelial cells. Few quinone conjugates containing TPP moieties, such as MitoQ, SkQ, and other plastoquinones have shown encouraging results in preclinical models of heart failure [77,305,324,326–330]. Short-chain synthetic CoQ analogues are also considered in mitochondrial diseases, but still not in human heart failure [331]. It would be of interest to evaluate as potential CVDs drugs also some small molecules already proven to be able to target mitochondria at the dermal level [332], provided that suitable formulations may be found to address those compounds to the heart [333].

In the context of the mitigation of oxidative stress, nitroxides have also been proposed to counteract the increased mitochondrial superoxide production observed in several experimental hypertension models, in turn damaging several components of the ETC and impairing ATP synthesis. Moreover, superoxide activates mitochondria-dependent apoptosis and exacerbates cellular oxidative stress. The mitochondrial-targeted antioxidant mitoTEMPO, a piperidine nitroxide conjugated to a TPP^+, attenuated hypertension and vascular oxidative stress in angiotensin II-induced and DOCA-salt mice hypertension models by decreasing mitochondrial superoxide production and increasing vascular nitric oxide production [334,335]. Unfortunately, mitoTEMPO is readily reduced in mitochondria to the corresponding hydroxylamine [336] with reduced antioxidant potential than the parent nitroxide [334]. This is why the proxyl-based mitochondria-targeted nitroxides mCP1and mCP2 (i.e., nitroxides containing a pyrrolidine core) with greater bioreduction resistance and, hence, higher cellular accumulation have been proposed [337,338]. However, having reduced reactivity with $O_2^{-\bullet}$ compared with mitoTEMPO, the effects are offset and, overall, both proxyl- and TEMPO-based nitroxides show similar protection of respiration in H_2O_2-treated endothelial cells as well as similar *in vivo* antihypertensive and antioxidant effects in angiotensin II-infused mice.

Recently interesting experimental evidence has been obtained that nitrate is capable to significantly improve muscle mitochondrial functionality [339]. Nitrate exerts an oxygen-sparing effect without

changes in circulating lactate concentrations and with maintained or even increased work performance. It has been demonstrated that dietary nitrate reduces oxygen cost during physical exercise, improves the oxidative phosphorylation efficiency (P/O ratio) and decreases mitochondrial state 4 respiration with and without atractyloside and respiration without adenylates. Clinical trials have shown that a nitrate supplement can improve exercise capacity in heart failure patients [340]. From a nutritional point of view, these data represent an important step that leads to rationalize the beneficial effect on health of a diet rich in green leafy vegetables, foods particularly rich in nitrates. A nitrate-nitrite-NO pathway has been envisaged: circulating nitrate, derived both from endogenous NO production and from dietary intake, is excreted in saliva and reduced to nitrite by commensal bacteria in the oral cavity [341]. Further reduction of nitrite to NO and other reactive nitrogen intermediates have now been identified [342]. Although the mechanism of this effect of nitrate on mitochondrial efficiency is still under study, nitrate has been shown to reduce the expression of the ADP/ATP translocator. An obvious candidate for the effect of nitrate (and its derivatives) on the efficiency of the mitochondrial respiratory chain is cytochrome c oxidase (complex IV). The binuclear center of this enzyme is able to bind, in addition to oxygen, numerous molecules. Beside the classic ligand carbon monoxide, cyanide and azide, hydroxyl or water ligands coming from the catalytic cycle, chloride ions, nitrosyl-derivative or nitrite-derivative can be observed at the binuclear site [343–345]. Although the main target of nitrate and its derivatives remains to be identified, the supplement of this simple inorganic compound could be a precious ally for the therapy of heart failure, able to improve the thermodynamic efficiency of the oxidative phosphorylation system.

Tiron, a catechol-based benzenedisulfonic acid, is a mitochondrion-localized antioxidant that not only has superoxide scavenging properties but also acts as an iron chelator agent able to sequester the excess of mitochondrial iron responsible for the generation of ROS and oxidative damage. It decreased hydrogen peroxide-induced contractile responses in mesenteric resistance arteries [346], as well as improved baroreflex sensitivity and decreased oxidative stress in hypertensive rats [347].

While numerous antioxidants showed promising preclinical results as candidates for the therapy of mitochondrial diseases, no FDA-approved drug is currently available. The reasons why antioxidants failed in that respect have been reviewed [348] with the main one being related to the dr Jekill/mr Hyde behavior of ROS: they have essential roles (at physiological level) but may cause pathologies (at higher levels)[77,349]. The antioxidants studied so far cannot distinguish between physiological and pathological levels [350]. It would be of interest to study new formulations of carefully tailored mixtures of redox-active compounds to finely tune the redox properties of the mixtures and gain redox buffering power. As an example, one could exploit some recent outcomes obtained from reverse engineering studies with melanins and redox-active drugs (e. g., paracetamol and clozapine) where

the polypharmacological, antioxidant, and pro-oxidant activities of drug candidates may be studied at a basic level.

One molecule that has raised a lot of hope for the treatment of mitochondrial dysfunction is represented by the cell-permeable tetrapeptide Elamipretide (Bendavia, MTP-131, SS31), a water-soluble tetra peptide that by selectively binding to cardiolipin it is able to concentrate in the inner mitochondrial membrane [351,352]. By interacting with the cytochrome c / cardiolipin complex [353], as well as several mitochondrial proteins including complex III, complex IV and complex V [354] this peptide has been shown to improve electron transport and ATP synthesis, as well as reduce the production of ROS by the respiratory chain [355–358]. After giving very interesting results in preclinical models of heart failure, where improvements in ejection fraction were associated with normalization of cardiolipin levels and improved activity of mitochondrial complexes I, IV, and ATP synthase, [359–362], the results of clinical trials have unfortunately been much less encouraging. In fact, the molecule proved to be no superior to placebo in improving cardiac parameters in phase II trials [363,364].

The heart uses several substrates at the same time to produce energy. Mitochondrial fatty acid oxidation (FAO) is the predominant metabolic pathway used in the healthy adult human heart, being responsible for 60-80% of cardiac ATP production, followed by minor contributions from bodies of glucose, lactate and ketone. The heart can change the relative contribution of these substrates in order to adapt to varying physiological and pathological conditions. Fatty acid oxidation, which is unchanged or slightly increased in early heart failure, decreases in advanced heart failure [55,365,366]. The switch to greater glucose utilization appears to be a trait recognized by many authors in the advanced stages of heart failure, but the available data do not always agree on this. Probably the appearance of insulin resistance, frequent in patients with cardiovascular diseases, contributes to make the metabolic pattern in the late heart failure not exactly clear [55,365–367]. The metabolic significance of this change of preferentially used energy substrate is also not completely clear. Although the reduction in fatty acid metabolism can be interpreted as a mechanism to reduce oxygen consumption by the mitochondrial respiratory chain, which is scarcely available in heart failure, the real reason is still debated [55,368,369].

In the context of the cardiac lipid homeostasis and dyslipidemia and CVD [370,371], statins and fibrates have been employed for reducing triglycerides and increasing in high-density lipoprotein levels through their interactions with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and peroxisome proliferator-activated receptors (PPARs), respectively, aiming to prevent/counteract CVDs [372,373]. Notably, it was observed that the prolonged exposition to statins

cause a decrease of mitochondrial function and an increase in ROS production [374], despite of what observed in samples from individuals treated with fibrates showing an increased mitochondrial biogenesis [375,376] with the proposed direct involvement of the overexpression of the mitochondrial carnitine/acyl carnitine transporter [116,376].

Moreover, the level of peroxisome proliferator-activated receptor-α (PPARα), a transcription factor highly expressed in the heart and responsible for fatty acid transport into the mitochondria, has been reported to be downregulated during the heart failure. Also, the PPAR-γ co-activator PGC-1α is reduced in failing hearts. These data suggest that decreased PPARα and PGC-1α activity might represent the main cause of the reduced fatty acid oxidation observed in heart failure [55,377–379]. However, the inconclusive effects of the pharmacological approach on these transcription factors make the real etiopathogenetic importance of this metabolic switch frankly unclear [51,380–382].

Conclusions

In the search of new pharmacological targets and new small molecules to counteract the onset of hypertension and more in general CVDs, mitochondrial proteins, and above all the members of SLC25A mitochondrial carrier family, represent a promising group of candidate targets. Indeed, mitochondrial carrier family consists of metabolite transporters able to specifically translocate metabolites and cofactors, whose concentration allows the correct function of dehydrogenases, transferases, and other enzymes crucial for cardiomyocytes function (from the tricarboxylic acid cycle to beta-oxidation, to protein degradation, nucleotide biosynthesis, fission and fusion events, oxidative phosphorylation, to the regulation of permeability transition pore opening)[18,41,77]. The downregulation or missense mutations impairing mitochondrial transporters are already known to play a crucial role the related mitochondrial deficiencies showing degeneration of the cardiac tissue [137,383].

Recent advances in our knowledge about the 3D structural features of the mitochondrial targets, also with reference to mitochondrial carriers, involved in CVDs could feed both *de novo* design and virtual screening of chemical libraries for the rational identification of selective drugs. Where the crystal structure of the target is unknown, it might be profitable employing our knowledge about structural features shared by the proposed mitochondrial targets and proteins with known 3D structures in the context of comparative 3D modelling based approaches [128,162,241,384,385].

As an example, AGC and APC of the mitochondrial carrier family contain EF-hand Ca^{2+} binding motifs, thus sharing a pivotal domain with calmodulin (CaM), a well-known cytosolic Ca^{2+}-binding protein that serves as a control element for many enzymes [386]. It would be advisable to verify, both

in silico and *in vitro*, the activity of known CaM ligands [387] on the crystallized EF-hand Ca^{2+} enriched N-terminal regions of APC and AGC [388].

Concerning the cross-talk between mitochondria and cytoplasm, it is observed that cardiomyocyte dysfunction may depend on an excess of necrosis/apoptosis due to an unbalance of metabolites or reducing equivalents between the mitochondrial and matrix and the cytoplasm, as observed in presence of alterations of the MAS [111,176,177]. Indeed, it was observed that with the onset of ischemia, the MAS flux rapidly decreased to a new steady-state in proportion to the reduction in blood flow causing a redistribution of shuttle-associated metabolites in both cytoplasm and mitochondria. The new distribution of metabolites appears to be responsible for the dramatic acceleration in glycolysis and the switch to lactate production that occur immediately after the onset of ischemia as mediated by the reduced MAS [389]**.** Conversely, it was shown that a transient shut-down of the MAS by aminooxyacetate (AOA, able to target malate dehydrogenases of the MAS [111,390]), during ischemia, and/or a transient stimulation of the MAS by sildenafil (able to target again malate dehydrogenases of the MAS, with the final effect of transferring electrons and protons from glycolysis-derived cytosolic NADH into the matrix to be used along the electron transport chain, using malate as an electron carrier [391]) before the reperfusion (the shut-down) or along the reperfusion (the stimulation), can modulate IRI and induce cardioprotection [177].

Experimental studies performed on the immunosuppressive drug cyclosporine revealed its ability to protect the heart and kidneys from IRI [392] through the binding to the mPTP constituent cyclophilin-D, thus preventing the opening of the mPTP and cellular apoptosis. Unfortunately, the use of cyclosporine in patients with hypertension is limited by numerous adverse effects [393,394].

However, targeting mPTP opening still appears a promising strategy in order to counteract IRI. In this regard the existence of the crystallized AAC in both the cytosolic-/ matrix-conformations, in complex with two conformation-selective inhibitors (the pro-apoptotic carboxyatractiloside and the anti-apoptotic bongkrekic acid), may help in the set-up of virtual screening of chemical libraries for the identification of AAC highly selective molecules with antiapoptotic properties able to bind AAC from the matrix face, as the bongkrekic acid does, to counteract cardiomyocyte dysfunction, by preventing the opening of the mPTP [41,42,395,396].

On this regard, the regulation of mPTP opening can be crucial for containing the damage of myocardial ischemia and reperfusion injury. Indeed, IRI appears to be ascribed to the massive arrival of oxygen observed along reperfusion, causing a crazy increase in ROS production, which triggers necrosis/apoptosis events through the opening of mPTP [396–400].

More in general, thanks to the possibility to build good 3D comparative models of mitochondrial carrier of the SLC25A family, in both cytoplasmic or matrix conformations [162], it might be possible

to screen drug libraries [42,102] searching for already approved drugs (in the context of the drug-repurposing approaches), able to modulate mitochondrial pathways led by mitochondrial carriers involved in CVDs, according to specific needs to protect cardiomyocytes, to be translated quickly in clinics.

The screening of drug libraries in the context of the drug repurposing approaches is still considered the most straightforward approach to find drugs for unmet diseases and health emergencies. The pros and cons of finding new therapeutic uses for old drugs have been recently reviewed [401]. The above-reported case of paracetamol and clozapine as well-established drugs displaying redox activities is exemplary and the SOSA approach [402] might be focused on all currently used drugs displaying off-target activities on mitochondria. For example, some antihypertensives belonging to the renin-angiotensin system (RAS) blocker class have shown mitoprotective properties and, among them, the angiotensin receptor blocker valsartan alleviated left ventricular hypertrophy, ameliorated mitophagy, and improved myocardial mitochondrial biogenesis in swine renovascular hypertension [403], although the mechanism involving mitochondrial proteins is still unclear.

The new available diagnostic tools based on sequencing analysis (i.e., WGS) coupled with epidemiological studies can help to identify genetic polymorphisms associated to the risk/susceptibility that some individuals/populations have to develop CVDs related to blood pressure alterations. In addition, the recently improved omics approaches can provide detailed indications about the differential expression of specific genes and/or the altered concentration of specific metabolites and cofactors [77,295,296] involved in characterized metabolic pathways, already from specific signals detectable from patients biological samples (blood, saliva, urines). Based on these analyses, the early identification of an altered metabolic pathway can help in preparing a personalized therapy for rescuing the affected pathways led by specific mitochondrial transporters, by using drugs able to modulate the activity of the investigated carriers to re-establish the homeostasis of the altered metabolites and cofactors, for preventing or counteracting cardiac tissue degeneration.

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Author contribuitions

ST, VT and CLP presented data about mitochondrial carriers of the SLC25A family, mitochondrial pyruvate carrier, and voltage dependent anion channels as pharmacological targets for CVD; VP and RDL presented data about mitochondrial dynamics in CVDs; GLP, DM, and GP presented data about cardiolipin and lipid peroxidation in cardiac mitochondrial dysfunction; MNS; NS and LG presented data about the role of mitochondrial AQP in mitochondrial function; MV, LCB, LP, ADG presented data about NGS and transcriptomics analyses in CVDs; BM and VG presented data about metabolomics analyses in CVDs; LLP and CLP presented data about respiratory chain complex proteins in CVDs; MMC, LLP, CLP and GL presented data about pharmacology aspects of the cited proteins in CVDs; CLP, LLP and VT contributed to the structural biology analysis. All authors contributed to write, and edit the manuscript. All authors have read and agreed to the published version of the manuscript."

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Dear Biochemical Pharamcology Editorial office,

We are submitting here the invited review titled “**Targeting mitochondrial impairment for the treatment of cardiovascular diseases: from hypertension to ischemia reperfusion injury, in search of new pharmacological targets**” for the "**Emerging Mechanisms in the Pathogenesis of Hypertension**" special issue of **Biochemical Pharmacology**. In the submitted manuscript, we have briefly presented what is known about the role played by mitochondria in cardiomyocyte dysfunction, with the collaboration of several experts in the field. Furthermore, we have proposed a group of mitochondrial proteins as potential pharmacological targets of new drugs based on the role played by the discussed proteins in the cross-talk between mitochondria and cytoplasm in hypertension and more in general in cardiac tissue degeneration and cardiovascular diseases.

The authors hope that the editorial office and the reviewers will retain that our review can be of interest for Biochemical Pharmacology readers and will evaluate positively our review for publication on Biochemical Pharmacology.

Best regards,

Ciro Leonardo Pierri, PhD

Fig. 1

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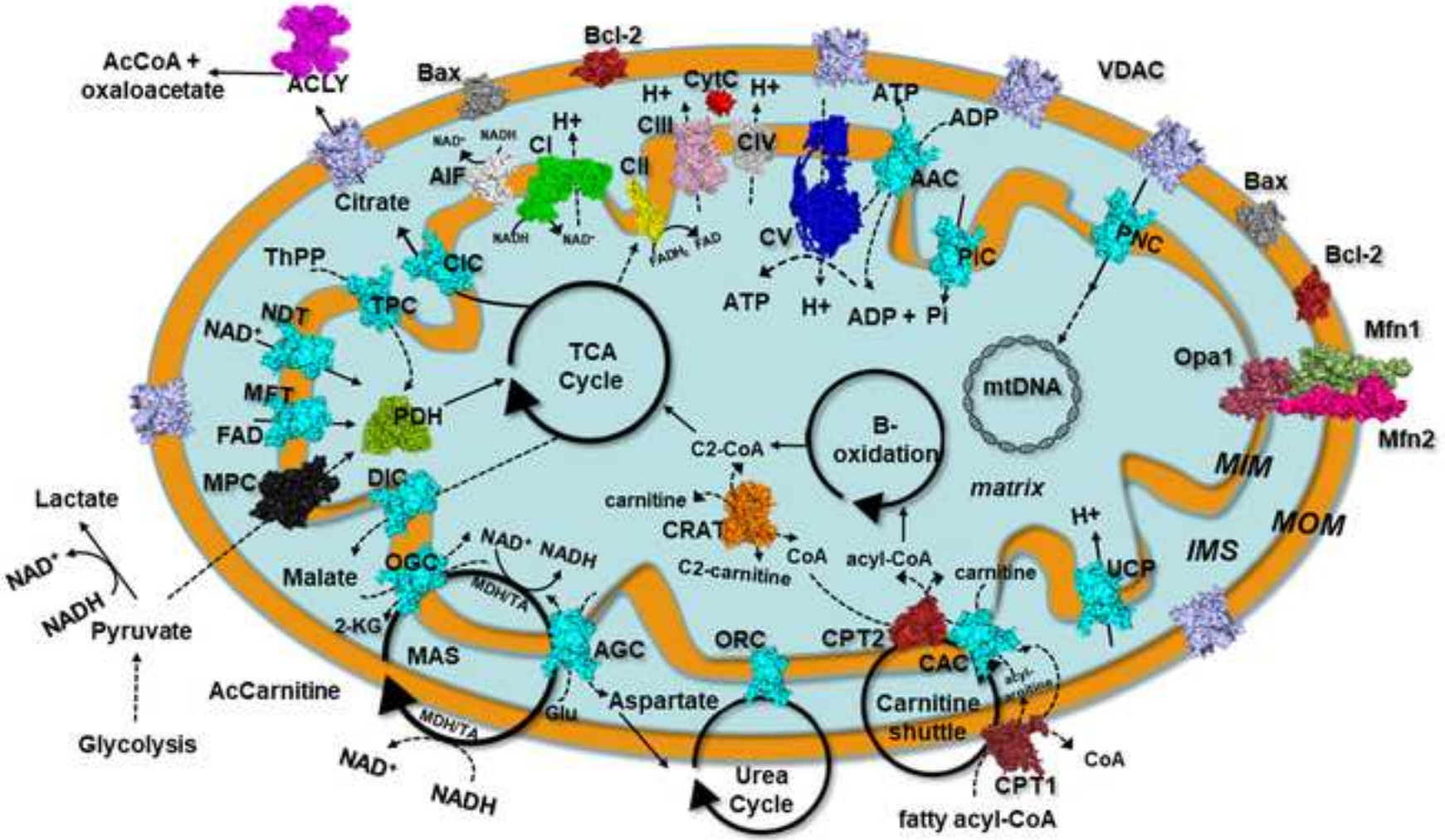

Fig. 2

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