



AMP-activated protein kinase: A remarkable contributor to preserve a healthy heart against ROS injury

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ABSTRACT

Heart failure is one of the leading causes of death and disability worldwide. Left ventricle remodeling, fibrosis, and ischemia/reperfusion injury all contribute to the deterioration of cardiac function and predispose to the onset of heart failure. Adenosine monophosphate-activated protein kinase (AMPK) is the universally recognized energy sensor which responds to low ATP levels and restores cellular metabolism. AMPK activation controls numerous cellular processes and, in the heart, it plays a pivotal role in preventing onset and progression of disease. Excessive reactive oxygen species (ROS) generation, known as oxidative stress, can activate AMPK, conferring an additional role of AMPK as a redox-sensor. In this review, we discuss recent insights into the crosstalk between ROS and AMPK. We describe the molecular mechanisms by which ROS activate AMPK and how AMPK signaling can further prevent heart failure progression. Ultimately, we review the potential therapeutic approaches to target AMPK for the treatment of cardiovascular disease and prevention of heart failure.

1. Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity in Europe and worldwide [1]. Abnormal production of reactive oxygen species (ROS) is a hallmark of various diseases, including cardiovascular diseases. Indeed, an imbalanced regulation between synthesis and removal of ROS plays a key role in heart disease pathogenesis. Adenosine monophosphate-activated protein kinase (AMPK), the key metabolic sensor and regulator of energy levels, has recently emerged as a redox sensor that contributes to the maintenance of cardiovascular physiology and the prevention of disease progression. This review will discuss the most recent discoveries regarding how AMPK impacts on cardiac health and protects against ischemia, myocardial infarction and cardiac hypertrophy in the presence of ROS and reactive species, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl

(HO^{\bullet}) and peroxynitrite ($ONOO^-$). Given the role that AMPK plays in ROS-related cardiovascular disease, we discuss how this multifunctional protein could represent a potential therapeutic target for preventing the onset and progression of cardiac diseases.

1.1. AMPK structure, function and activation

AMPK is described as an energy sensor, since it finely orchestrates the regulation of energy production and consumption pathways in the organism [2]. Conditions of cellular stress or limited energy supplies activate AMPK. In a situation of energy deficit (such as low glucose levels, hypoxia and oxidative stress) corresponding to low ATP levels, AMPK activation favors catabolic pathways that increase ATP production, and simultaneously inhibit biosynthetic and/or non-essential processes that consume ATP. However, the role of AMPK goes beyond the ordinary regulation of cell energy and metabolism, making it a major

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Abbreviation

ACC	acetyl-CoA carboxylase
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
CAMKK β	Ca ²⁺ /calmodulin-activated protein kinase kinase- β
CAT	Catalase
eEF	Eukaryotic elongation factor 2
eNOS	endothelial nitric oxide synthase
GSK-3 β	Glycogen synthase kinase-3 β
I/R	Ischemia/reperfusion
iNOS	inducible nitrtric oxide synthase
LKB1	Liver kinase B1
mPTP	Mitochondrial permeability transition pore
PGC1 α	Peroxisome proliferator-activated receptor- γ (PPAR γ) co-activator 1 α
PI3K	Phosphoinositide 3-kinase
SIRT1	Sirtuin 1
SOD	Superoxide dismutase

β -CTD, C-terminal domain; γ subunits: CBS1, CBS2, CBS3, CBS4, cystathionine- β -synthase repeats.

1.1.2. Function and regulation

The kinase activity depends on phosphorylation levels of the conserved threonine residue (Thr172) [9]. When Thr172 is phosphorylated by an upstream kinase, the auto-inhibitory domain is released. The β subunit is the scaffolding subunit necessary to form the heterotrimeric complex of AMPK. Moreover, β subunit also presents a glycogen binding domain proposed to modulate the kinase AMPK activity, accordingly to glycogen levels [10,11]. Mutations in the *PRKAG3* gene are indeed accompanied with elevated glycogen content in pig skeletal muscles [13]. The β subunit displays specific domains for binding pharmacological activators such as A769662 and 991 [14]. The main trigger for AMPK activation is the binding of AMP to the γ subunit, provoking a conformational change that promotes the phosphorylation on Thr172 from α subunit. This conformational change also protects Thr172 from being dephosphorylated by protein phosphatases (including PP2A) [15] and ensures a 10-fold increased activity [16].

1.1.3. Activation of AMPK

Almost 25 years ago, liver kinase B1 (LKB1) was identified as the

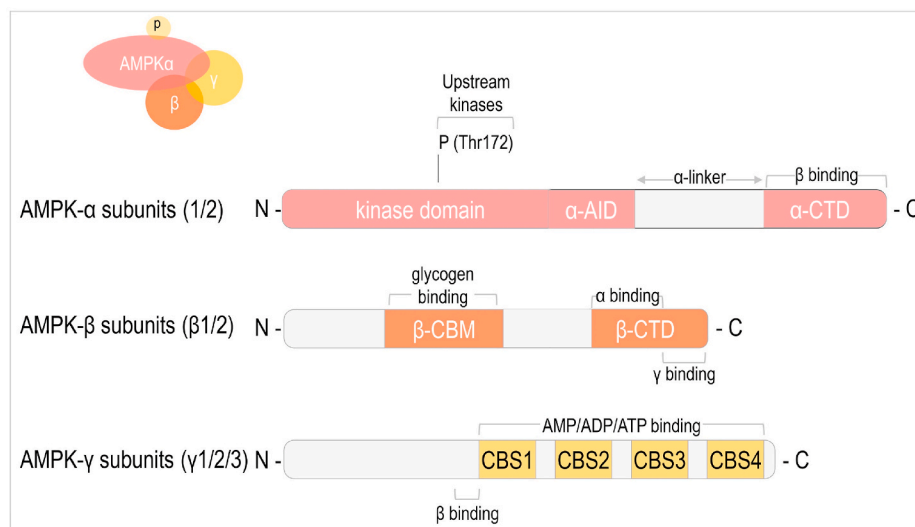


Fig. 1. Structure of the heterotrimeric AMPK complex.

player for the preservation of several cardiovascular processes. Indeed, in the heart, AMPK regulates cell growth and proliferation [3,4], stimulates angiogenesis, endothelial cell proliferation and function [5], and regulates blood pressure and vascular tone [6], all of which are important for preserving the physiological state of the heart.

1.1.1. Structure

AMPK is a heterotrimeric complex, comprising one catalytic α -subunit and two regulatory β - and γ -subunits [7,8] (Fig. 1). The α -subunit contains a kinase domain on the N-terminus, an auto-inhibitory domain that inhibits its kinase activity, and β -subunit-binding domain on the C-terminus [9]. The β subunit contains a glycogen binding domain (GBD) and a C-terminal domain that interacts with the α - and γ -subunits [10,11]. The γ -subunit contains four cystathionine- β -synthase (CBS) motifs that, when packed together, generate an adenyl binding site, that binds AMP, and to a lesser extent ADP and ATP [12].

Schematic representation of the linear domain structure of the α , β , and γ subunits of AMPK. α subunit: Kinase domain, containing the phosphorylation site Thr172; α -AID, auto-inhibitory domain; α -CTD, C-terminal domain; β subunits: β -CBM, carbohydrate-binding module;

major upstream kinase of AMPK that phosphorylates Thr172 on the α subunit and is responsible for increasing the AMP to ATP ratio [17,18]. Interestingly, the LKB1 pathway seems to be specific for activation of the $\alpha 2$ isoform. In fact, AMPK $\alpha 2$, but not AMPK $\alpha 1$, activation was abrogated in mice lacking LKB1 specifically in skeletal and cardiac muscles [19]. In this study, the authors also demonstrated that the $\alpha 2$ isoform is crucial for protecting cardiac muscle from damage during ischemia [19]. Later studies highlighted other important upstream kinases, including Ca²⁺/calmodulin-activated protein kinase kinase- β (CAMKK β) [18], which is triggered by increased intracellular Ca²⁺ concentration, and lastly transforming growth factor- β (TGF- β)-activated kinase1 (TAK1) [20], which activates AMPK by phosphorylation of Thr172 [21], probably by modulating LKB1/AMPK signaling axis, rather than a direct phosphorylation of AMPK [22]. Beyond conventional activation depending on AMP, ADP, and Ca²⁺, AMPK can be activated by unconventional pathways, including ischemia [19,23], hypoxia [24], oxidant signaling [25,26], hormones [27,28], DNA damage [29], and cytokines [30,31].

AMPK presents multiple isoforms of each subunit, encoded by different genes. For example, $\alpha 1$ and $\alpha 2$ are encoded by *PRKAA1* and

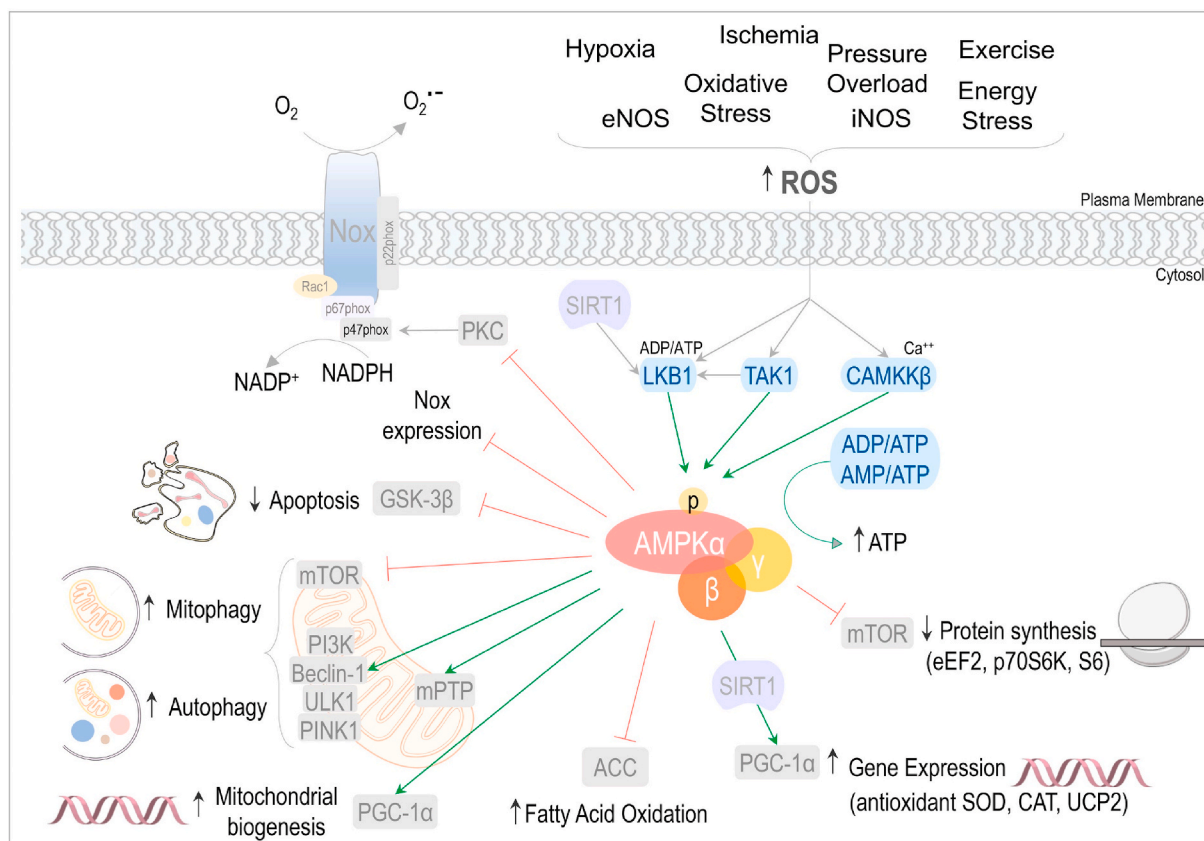


Fig. 2. Interactions between AMPK and ROS

PRKAA2 [32]; the two subunits $\beta 1$ and $\beta 2$ are encoded by *PRKAB1* and *PRKAB2* [33]; and the three γ subunits, $\gamma 1$, $\gamma 2$, and $\gamma 3$, are encoded by *PRKAG1*, *PRKAG2* and *PRKAG3*, respectively [34]. Each AMPK complex is formed by one α -subunit, one β -subunit and one γ -subunit, with a total possible combination of 12 distinct AMPK complexes [35]. Each cell type expresses a specific subset of these combinations, which raises some important questions: “Are these different combinations endowed with diverse functions?”, “Do they present different substrate specificities?”, “Do they have distinct subcellular localizations?”. AMPK expression is typically cytosolic, although it can move among different subcellular compartments in response to physiological stimuli, where it exerts distinct functions [36]. For example, after activation, AMPK can accumulate in the nucleus, where it promotes phosphorylation of peroxisome proliferator-activated-response- γ -coactivator 1 α (PGC-1 α) [37]. In addition, AMPK activation can occur at specific membrane domains, where it exerts an important role in controlling membrane protein signaling. In the cardiovascular system, AMPK isoforms may vary depending on cell types. For instance, AMPK $\alpha 1$ is the predominant isoform in endothelial cells [38], vascular smooth muscle cells [39], fibroblasts [40] and hematopoietic lineage [41], while AMPK $\alpha 2$ is mainly expressed in cardiomyocytes (as it is the case in skeletal muscles and liver) [3,32]. The heart expresses all isoforms except $\gamma 3$, although $\alpha 2/\beta 2/\gamma 1$ is the predominant subunit complex in the adult normal heart [33]. In human skeletal muscle, it was found that exercise activates the $\alpha 2/\beta 2/\gamma 3$ AMPK heterotrimer rather than $\alpha 2/\beta 2/\gamma 1$, indicating that the expression of AMPK subunits could be, not only tissue dependent, but could also respond to different types of stimuli [42]. With the help of genetic manipulations and clinical data, it was possible to understand the importance of AMPK subunits in the development of cardiovascular disease, such as mutations in the AMPK $\gamma 2$ subunit that leads to human hypertrophic cardiomyopathy and Wolf Parkinson White syndrome [43]. In addition to tissue-specific expression of AMPK, a recent study

demonstrated that specific AMPK ligands (e.g., AMP and A769662), could activate distinct AMPK isoforms based on their AMPK subunit composition [44], suggesting that different complex compositions can affect AMPK activation and its downstream targets.

Following activation, AMPK targets a multitude of proteins to exert its beneficial actions [45], by modifying gene expression and protein level regulation to restore cellular metabolism [46,47]. Moreover, it has been demonstrated that AMPK promotes mitochondrial biogenesis, ameliorating cellular energetic status by intensifying energy substrates [48].

In this review, we will focus on the targets of AMPK following activation induced by ROS and how under circumstances of oxidative stress, AMPK maintains redox homeostasis. First, we provide an overview of the mechanisms by which AMPK exerts its effects under oxidative stress, both in cardiac and non-cardiac cells. Then, we discuss the impact of the interplay between AMPK and ROS specifically in the heart and cardiovascular system, including during ischemia/reperfusion (I/R) induced injury and left ventricular hypertrophy. Finally, we will discuss the implication of AMPK in the transition to ROS-induced heart failure and the clinical treatments targeting AMPK, which show beneficial effects in preventing the heart from failing.

2. AMPK targets in ROS signaling

ROS are unstable and chemically reactive molecules derived from oxygen [49], known for their potential damage to DNA, RNA, proteins and lipids. Under physiological conditions they are continuously produced as by-products of mitochondrial metabolism and their levels are balanced to maintain biological functions, such as cell survival, proliferation, migration, and apoptosis [49]. However, situations of environmental stress induce a dramatic increase in ROS production, a process known as oxidative stress, ultimately leading to tissue damage

and injury [49,50]. The primary cellular source of ROS derives from the mitochondrial electron transport chain, although in the cardiovascular system they are also highly produced by nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases (Nox) and monoamine oxidases (MAOs) [51]. Cells also dispose of protective antioxidant mechanisms against ROS to guarantee their homeostasis, by activating the specific scavenger enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [52]. An altered balance between their bioavailability is tightly correlated with the development of several cardio-metabolic disorders, including diabetes, hypertension, atherosclerosis, cardiac hypertrophy and remodeling, myocardial infarction, cardiac arrhythmias, and ultimately heart failure [49,53–56].

Many studies have shown that AMPK is activated in response to an imbalanced redox status, suggesting a strong and direct crosstalk between AMPK and ROS [57]. AMPK seems to intervene in both directions, given its capability to suppress oxidative stress and control major contributors of ROS - the Nox family [58,59].

2.1. ROS activate AMPK

Several studies have investigated the effect on AMPK activation of specific ROS and reactive nitrogen species, such as hydrogen peroxide (H₂O₂), and peroxynitrite (ONOO⁻), respectively. In Fig. 2, we have summarized the most important and recent advances in the field of ROS and AMPK, and how the interplay between these two is regulated.

In response to ischemia, pressure overload, hypoxia, oxidative stress, energy stress and exercise, reactive oxygen species (ROS) increase and activate AMP-activated protein kinase (AMPK) complex. The upstream targets of AMPK are indicated in blue, while the downstream targets following AMPK activation are indicated in gray. The arrows indicate activation, and the bars indicate inhibition of the designated pathways. Activation of AMPK leads to reduced protein synthesis, increased gene expression, increased fatty acid oxidation, increased mitochondrial biogenesis, increased autophagy, reduced apoptosis, inhibition of Nox activity and expression. AMPK, adenosine monophosphate-activated protein kinase; ACC, acetyl-CoA carboxylase; CAMKKβ, Ca²⁺/calmodulin-activated protein kinase kinase-β; CAT, catalase; eEF, eukaryotic elongation factor 2; eNOS, endothelial nitric oxide synthase; GSK-3β, glycogen synthase kinase-3β; iNOS, inducible nitric oxide synthase; LKB1, liver kinase B1; mPTP, mitochondrial permeability transition pore; PGC1α, peroxisome proliferator-activated receptor-γ (PPARγ) co-activator 1α; PI3K, phosphoinositide 3-kinase; SIRT1, sirtuin 1; SOD, superoxide dismutase.

2.1.1. Upstream activation of AMPK by ROS

The principal stimulus for AMPK activation is characterized by changes in the ADP/ATP and AMP/ATP ratio: index of energy disequilibrium, often associated with metabolic stress and hypoxia. Contrary to the conventional AMP-/ADP-dependent activation of AMPK, it has been shown that AMPK can be activated by secondary mechanisms that do not require changes in ADP/ATP. Indeed, Toyoda and colleagues showed a direct activation of AMPK by ROS, particularly by H₂O₂, independently from intracellular changes in ADP/ATP ratio in rat skeletal muscles [60]. After acute exogenous administration of H₂O₂, the authors detected AMPK phosphorylation of Thr172, suggesting the involvement of one of the upstream kinases [60]. In another study performed in HEK293 cells, H₂O₂ induced oxidative modifications of Cys299 and Cys304 of the AMPKα subunit, leading to its activation in the absence of changes in ADP/ATP ratio [61]. However, these results were recently questioned [62]. In mouse myotubes, a model of skeletal muscle fibers, oxidative stress increases cellular AMP/ATP, suggesting that changes in adenine nucleotides, rather than direct oxidative modification, are the major drivers of AMPK activation during oxidative stress [63]. Conclusively, the main changes in mitochondrial ATP production, induced by oxidative stress, are responsible for AMPK activity and activation [62]. The discrepancies in adenine

nucleotides-dependent AMPK activation proposed by Toyoda and Zmijewski could arise from methodological differences, including alternative ways of measuring adenine nucleotides or different cell types used.

Hypoxic conditions (e.g., cellular deprivation of oxygen supply) lead to mitochondrial ROS accrual and increase in the enzymatic activity of AMPK [64,65]. LKB1 and CAMKKβ are the major upstream activators of AMPK, and both are activated by hypoxic conditions. In mouse embryonic fibroblasts isolated from AMPK WT and AMPKα1^{-/-}α2^{-/-} mice, ATP, ADP and AMP levels were not different under normoxia or hypoxic conditions. However, an increase in hypoxia-induced mitochondrial ROS resulted in AMPK activation via the upstream kinase LKB1 [66]. Accordingly, another group also found that in non-excitable cells, hypoxia-dependent ROS production did not alter ADP/ATP ratio [67]. In their experimental settings, the authors attributed AMPK activation to a ROS-dependent accumulation of intracellular calcium, responsible for CAMKKβ activation, rather than the LKB1 pathway. Indeed, in calcium-free hypoxic cells, AMPK activation was abrogated. Moreover, by using *in vitro* models of hypoxia in mouse embryonic fibroblasts isolated from LKB1^{-/-} mice, absence of LKB1 did not affect AMPK activation, whereas transfection with siRNA for CAMKKβ completely abolished its phosphorylation [67]. Similar conclusions were reached by Gusarova's group in alveolar epithelial cells, where hypoxia was responsible for AMPK activation via CAMKKβ [68,69]. Thus, we can speculate that AMPK activation pathways depend on the intensity of hypoxia: AMPK would be activated by LKB1 under 30 min of hypoxia [66], whereas a longer hypoxic insult would activate AMPK via CAMKKβ [67]. These results could be explained by the fact that after a period of hypoxia, intracellular calcium levels increase according to the duration of the event, reaching an excessive of calcium overload and consequent myocardial injury. Collectively, these results suggest not only that ischemia/hypoxia stimuli and reperfusion/reoxygenation duration highly modulate the response of AMPK in cells, but also that differences in experimental models (cell types and severity of the insult) could lead to contrasting results.

Among ROS, reactive nitrogen species also interact with AMPK to regulate cellular metabolism and function. Zou et al. focused their study on the interaction between AMPK and ONOO⁻ in endothelial cells. They demonstrated that low concentration (10 μM) of ONOO⁻ enhanced AMPK activity in bovine aortic endothelial cells. Mechanistically, they found that ONOO⁻ significantly increases phosphorylation of Thr172 of AMPK and Ser79 of ACC, a downstream target of AMPK [70]. Moreover, they found that AMPK activation induces increased enzymatic activity of endothelial nitric oxide (NO) synthase (eNOS), resulting in augmented NO production and bioactivity [70].

2.1.2. AMPK induced antioxidant enzyme expression in response to increased ROS

In order to mitigate ROS levels after oxidative stress, ROS themselves can trigger a feedback mechanism that stimulates the expression of antioxidant enzymes. The major player in this process is the transcriptional co-activator PGC-1α, a regulator of mitochondrial biogenesis and an important inducer of antioxidant gene expression during oxidative stress [71]. Interestingly, in the failing heart, PGC-1α activity is low and its limited expression contributes to mitochondrial dysfunction [72]. When ROS metabolism is disrupted, AMPK is activated and stimulates the expression of antioxidant enzymes to limit ROS production. Indeed, AMPK could directly phosphorylate PGC-1α in skeletal muscles, triggering the activation of its own transcription via a positive feedback loop [29]. Additionally, AMPK could also enhance antioxidant responses by stimulating sirtuin 1 (SIRT1)-dependent deacetylation of PGC-1α and FoxO1 increasing NAD⁺ levels [73]. Hyperglycemia is an inducer of mitochondrial ROS [74]. In HUVEC, AMPKα1 attenuates hyperglycemia-induced ROS production by enhancing expression of PGC-1α, ultimately limiting ROS production by stimulating SOD scavenger enzyme [74]. In this study, pharmacological treatment with AICAR and metformin, well-known AMPK activators, induces

AMPK-dependent increase in mRNA levels of PGC-1 α and SOD, an effect that was prevented by using an AMPK α 1 dominant negative form [74]. Rabinovitch and colleagues recently demonstrated that increase in mitochondrial ROS physiologically activates AMPK, which initiates an antioxidant program depending on PGC-1 α signal to protect cells from metabolic imbalances. Indeed, mouse embryonic fibroblasts stimulated with AMPK activator show a significant increase in mRNA levels of antioxidant genes, such as catalase, Sod1, Sod2, and Ucp2, which were drastically reduced in mouse embryonic fibroblasts isolated from AMPK α -deficient mice [75].

These data highlight the significance of the adaptive response induced by AMPK to protect cells subjected to nutrient and oxygen deprivation, altered metabolism, and energy stress.

2.1.3. AMPK prevents protein synthesis in response to increase ROS

To reestablish cellular energy homeostasis disturbed by oxidative stress, AMPK signaling can prevent protein synthesis and apoptosis depending on intracellular ATP concentrations [76–80]. Because oxidative stress alters ATP production, several studies aimed to investigate the crosstalk between AMPK and protein synthesis under an oxidative stress scenario. In cell culture of rat neonatal cardiomyocytes, high concentration of H₂O₂ (1 mM, 3–5 min) induced AMPK activation and subsequent inhibitory phosphorylation of raptor, the key regulatory subunit of the protein synthesis regulator mTORC1 [77,78]. Similar results were observed in mouse embryonic fibroblasts isolated from LKB1^{-/-} mice [78], rat skeletal muscles [81], and HUVEC [79], suggesting that AMPK prevents protein synthesis and attenuates energy stress by suppressing mTORC1 signaling in several tissues [82,83]. Thromboxane A2 receptor (TPR) also stimulates AMPK and limits protein synthesis in vascular smooth muscle cells (VSMC). Mechanistically, it has been shown that the increase in H₂O₂ after TPR activation or the exogenous administration of H₂O₂ to vascular smooth muscle cells induces Thr172 phosphorylation of AMPK and Ser79 of ACC, via an increased phosphorylation on Ser428 and Ser307 of LKB1 [80]. Together, these findings uncover AMPK as coordinator between cell growth and the energetic status of the cells.

2.2. Downstream AMPK signaling induced by ROS: Nox activation

2.2.1. Nox enzymes as major source of ROS in the cardiovascular system

Nox are the major contributors of ROS in the cardiovascular system [58]. The Nox enzymes are constituted of transmembrane proteins responsible for electron transport across the membranes. Particularly, they allow the reduction of oxygen to superoxide anion (O₂⁻) [84], which is generated by immune cells, such as macrophages, neutrophils, eosinophils, phagocytes and B-lymphocytes. Indeed, Nox was originally believed to be expressed in phagocytes for the only purpose of host defense [85]. However, a great deal has been learned about this multimeric enzyme, which is now recognized to be expressed in several tissues and cell types in the human body. To date, seven Nox isoforms have been identified: Nox1 to Nox5 and the dual oxidases Duox1 and Duox2 [86]. In the cardiovascular system, Nox1, Nox2, Nox4 and Nox5, are the most abundant. All these isoforms are expressed in the vascular smooth muscles [87,88]. Endothelial and epithelial cells specifically express Nox1 [88–90]. Nox2 and Nox4 are predominant in endothelial cells, fibroblasts, and cardiomyocytes [86,87,91–93]; while Nox5 is present in endothelial cells [94]. Structurally, all Nox isoforms present a cytoplasmic N terminus, six transmembrane domains containing four highly conserved histidine residues that form two heme binding sites, and a long cytosolic C terminus containing the flavin adenine dinucleotide (FAD)-binding and NAD(P)H-binding regions. In addition, Nox5, Duox1 and Duox2 have unique Ca²⁺-binding EF hand motifs, that allow Ca²⁺ to regulate their activity without requiring protein-protein interactions [95], while Duox1 and Duox2 contain an extra peroxidase domain [96]. To permit electron transport, Nox1 to Nox4 require the interaction with a small protein p22phox. While for Nox4 the presence of p22phox is

sufficient for its activity, Nox1, Nox2 and Nox3 activation depend on other regulatory subunits: (i) a cytosolic activator subunit (p67phox), which induces conformational changes to facilitate electron transport, (ii) a chaperone protein (p47phox) that brings the p67phox to the membrane after its phosphorylation by protein kinase C (PKC), and (iii) a small GTPase (Rac1 or Rac2), that anchors p67phox to the membrane and that is dissociated in the resting state.

Nox enzymes have crucial roles in physiological and pathological processes. They physiologically generate ROS necessary to maintain cardiovascular homeostasis, whereas aberrant production of ROS can be fatal [97]. Nox have been associated with several cardiovascular diseases, including hypertension [98], atherosclerosis [99], endothelial dysfunction [100], vascular inflammation [98] and remodeling [88, 101]. Whether Nox enzymes are beneficial or not in the cardiovascular system has been debated since both positive and negative effects have been reported. For example, cardiac contractile dysfunction and interstitial fibrosis are reported following Nox2 activation in a model of pressure overload [102], while elevated endothelial Nox4-derived ROS promotes endothelial cell migration and angiogenesis during ischemia in an eNOS-dependent manner [103]. A plausible explanation for these different responses could depend on the isoform activated: Nox4 has been described for its beneficial effects, while Nox2 appears to be mostly detrimental. Over the years, a direct link between AMPK and Nox has emerged, highlighting the beneficial AMPK-dependent decrease in ROS production due to Nox inhibition.

It has been demonstrated that pharmacological activation of AMPK modulates Nox2 activity and reduces superoxide production by attenuating p47phox phosphorylation and translocation to the cell membrane in several cell types, including neutrophils [104], hyperglycemic cardiomyocytes [59,79] and endothelial cells [100]. A major distinction in the role of Nox within cardiac cell types (endothelium vs cardiomyocytes) needs to be clarified. An elegant recent study had employed mice in which Nox2 expression, thus ROS production, could be manipulated specifically in the endothelium upon tetracycline withdrawal from drinking water [105]. Endothelial Nox2 can activate AMPK, which consequently inhibits Nox enzymatic activity and reduces ROS production [105]. On the other hand, in cardiomyocytes, Nox2 is a target rather than the trigger of AMPK activation, which protects cells from oxidative stress working upstream of Nox2 [106].

2.2.2. Endothelial AMPK suppresses Nox expression and Nox-induced ROS production

In endothelial cells, AMPK activation improves endothelial function by suppressing Nox expression, attenuating ROS production and oxidative stress [105]. As mentioned above, the predominant isoform of AMPK in endothelial cells is AMPK α 1 β 1 γ 1, although the catalytic α 2 subunit is also present and has been reported to play an important role. Interestingly, endothelial cells isolated from murine aorta lacking AMPK α 2 present high levels of Nox2 activity and expression, as well as an excess of superoxide production. Pre-incubation with the ROS scavenger enzyme SOD, in AMPK α 2 deficient aortas, improved acetylcholine-induced endothelium-dependent relaxation, indicating that endothelial dysfunction was dependent on oxidative stress. Treatment with the AMPK activator AICAR suppressed superoxide production and Nox subunit expression, p47phox, p67phox, and gp91phox [107]. Moreover, genetic deletion of AMPK α 2 in LDLR^{-/-} mice enhanced 26S proteasome activity, nuclear translocation of NF- κ B, endothelial dysfunction, inflammation and oxidative stress due to Nox increased activity [107]. These studies suggest that AMPK α 2 is involved in the preservation of endothelial function in response to increased Nox-dependent ROS production.

Angiotensin II (Ang-II) increases vascular ROS production and causes endothelial dysfunction. AMPK α 1 deletion in mice treated with Ang-II infusion for 7 days (0.1 mg/kg/d), showed increased inflammation, pronounced endothelial dysfunction and enhanced Nox2 activity compared to WT littermates [101]. Also, in *in vitro* models of endothelial

cells, AMPK plays a role in the regulation of Nox2 expression, since the lack of AMPK α 1 increases Nox2 expression in response to Ang-II [101]. In agreement with these findings, another study demonstrated that AMPK α 1 reduces vascular oxidative damage and preserves endothelial function during chronic Ang-II treatment (7 days, 0.5 mg/kg/d) by limiting Nox2 activation and the following infiltration of inflammatory cells to the vessel wall [100]. Batchuluun and colleagues demonstrated that in human aorta endothelial cells subjected to high glucose, metformin and liraglutide (a glucagon-like peptide-1 receptor agonist) ameliorate the high glucose-induced oxidative stress by inhibiting PKC β 2 phosphorylation and following Nox activation. Moreover, both metformin and liraglutide prevent p47phox translocation to the membrane and Nox activation by restoring the phosphorylation levels of AMPK in endothelial cells, either alone or in combination [108]. Whatever the AMPK isoform expressed (α 1 or α 2), these data highlight the critical role of AMPK activity in maintaining redox homeostasis, by suppressing Nox expression and activity via inhibiting p47phox phosphorylation and translocation, ultimately protecting from endothelial dysfunction and vascular inflammation, two major contributors of cardiovascular diseases [101].

2.2.3. AMPK and ROS production: an interplay with Nox in cardiomyocytes

While the interaction between AMPK and Nox is widely described in endothelial cells, in the heart this axis has been investigated only recently and underlies a new key element to promote cardioprotection. Data from our laboratory demonstrated that administration of the AMPK α 2 activator A769662 protects the heart from ROS production and cell death [109]. Isolated adult rat cardiomyocytes and perfused rat hearts subjected to conditions of ischemia, showed increased AMPK phosphorylation, increased glucose uptake and increased cell survival with reduced ROS production after treatment with A769662 [109], suggesting that pharmacological AMPK activation diminished ROS production. Moreover, in hyperglycemic cardiomyocytes, the glucagon peptide-1 (GLP-1) activated AMPK subunit α 2 and reduced hyperglycemia-induced Nox2 activation and ROS production. Mechanistically, AMPK α 2 activation by GLP-1 limited PKC phosphorylation, a key element for p47phox translocation to the caveolae, and the further Nox2-induced ROS production [59]. Activation of AMPK by AICAR also confers cardioprotection in diabetic hearts by reducing oxidative stress and apoptosis [106]. A recent study underlines the importance of the AMPK/Nox2 pathway in preventing the cardiac injury in diabetic hearts subjected to I/R. Indeed, both in diabetic rat ischemic hearts and in cultured rat cardiac myoblasts (H9c2 cells) subjected to hypoxia/reoxygenation, AMPK phosphorylation was reduced, while Nox2 was highly expressed. By pharmacological activation and genetic manipulation of AMPK, Wang et al. demonstrated that AMPK works upstream of Nox2 and this interaction reduces oxidative stress and prevents cell death in I/R injured diabetic hearts [106]. AMPK activators, such as metformin, have been employed to limit infarct size and protect from cardiac fibrosis after myocardial infarction [110,111]. As mentioned above, contrarily to Nox2 isoform, the constitutive Nox4 mainly induces protective effects in the heart. Indeed, Nox4 has extensively been shown to protect against ischemic and pressure overload-induced cardiac injury [103,112,113]. However, some controversial data showed that high levels of Nox4-dependent ROS could have detrimental effects in the development of cardiac fibrosis, remodeling and heart failure [111, 114–116]. Future investigations are required to better clarify the interplay between ROS production and Nox4 in the setting of heart failure.

In summary, these findings underlie the multi facets role of AMPK activation as a guardian of metabolism and regulator of ROS production, ultimately affording cardioprotection in complex pathologies such as metabolic diseases, ischemia and heart failure. Moreover, given the tight interplay between AMPK and Nox, a direct target on AMPK would provide a strategical approach for the treatment of cardiovascular

diseases.

2.3. Mitochondrial ROS production and AMPK regulation

In cardiomyocytes, more than 95% of ATP is produced within mitochondria by oxidative phosphorylation, making them the primary source of cellular energy production. Since AMPK senses energetic balance (particularly AMP/ATP ratio), it is not surprising that any damage or alteration in mitochondrial function can modulate AMPK activity. It is widely established that impairments in mitochondrial functionality result in cardiovascular disease development and heart failure. Since the heart has a limited regenerative capability, it is of primary importance that mitochondrial functions, such as mitophagy and biogenesis, are constantly kept under control [117]. As mentioned before, AMPK activation induces mitochondrial biosynthesis, to maintain myocardial homeostasis against I/R injury by increasing the expression of PGC-1 α [74]. The proposed mechanism of interaction between AMPK and PGC-1 α involves a direct interaction at Thr177 and Ser538 sites of PGC-1 α [29]. However, AMPK also indirectly promotes expression of PGC-1 α by controlling p38MAPK activation and SIRT1-induced deacetylation [118,119].

During I/R, hypoxia, cardiac inflammation and hypertrophy, ROS production results in altered homeostasis, leading to oxidative stress and mitochondrial dysfunction. Under this scenario, oxidative stress could induce autophagy as a protective mechanism to promote degradation of oxidized substances and cellular survival [120,121]. In addition to mitochondrial biogenesis, AMPK is invested in mitochondrial turnover, supporting its critical involvement in regulating autophagy of cardiomyocytes. AMPK regulates autophagy by two major pathways, involving either a direct phosphorylation of ULK1, Beclin-1, and phosphatidylinositol 3-kinase catalytic subunit type 3 [122–124], or exerting an AMPK-dependent brake on mTOR activity, which induces ULK1 phosphorylation [124]. Mitochondrial ROS also induce the expression of ataxia-telangiectasia mutated serine/threonine kinase (ATM), an upstream enzyme of LKB1, which has been demonstrated to phosphorylate LKB1 and thus AMPK in response to DNA damage [125]. ROS production during I/R increases LKB1 as well as AMPK phosphorylation, in accordance with mTOR repression [126]. These data underline the importance of ATM/LKB1/AMPK axis in repressing mTOR and increasing autophagy in response to ROS production.

3. ROS-mediated vascular inflammation: AMPK activation prevents the development and progression of endothelial dysfunction and the onset of atherothrombosis

Inflammatory processes and enhanced oxidative stress are the major causes for endothelial dysfunction. This pathological condition is highly associated with cardiovascular and metabolic diseases, such as hypertension, atherosclerosis and type 2 diabetes [127–129]. Endothelial dysfunction is characterized by particularly reduced levels of NO, which cause loss of endothelium-dependent vasodilatation due to eNOS uncoupling, which produces superoxide anions instead of NO [70], providing a substantial source of ROS. Under pathological conditions, such as inflammation or oxidative stress, levels of NO can also be regulated by endothelial inducible NOS (iNOS), whose expression is increased in the damaged endothelium. The detrimental effects of iNOS consist in an uncontrolled production of NO which causes an overproduction of ONOO $^-$ and a parallel reduction of eNOS activity [130]. It has been shown that AMPK activation, in response to ONOO $^-$, enhances eNOS and NO production to restore endothelial functions [70,131]. AMPK also improves vascular function by increasing hsp90-mediated eNOS activation and consequent NO availability [132]. Later evidence showed that AMPK activation counteracts oxidative stress-induced endothelial dysfunction by promoting mitochondrial biogenesis via AMPK/PGC-1 α dependent mechanism [133]. Collectively, these studies highlight the critical role of AMPK in preserving ROS-induced

endothelial dysfunction, suggesting that it could represent a new target for the prevention of cardiovascular diseases such as atherosclerosis.

Atherosclerosis is a chronic inflammatory vascular disease affecting medium and large arteries promoted by oxidative stress. It develops progressively after several key events, including oxidation of lipoproteins, endothelial cell activation, and macrophage infiltration, leading to the formation of complex atherosclerotic plaques [134]. Given the properties of AMPK in regulating the redox status, one may speculate that it could be a player in the pathogenesis of atherosclerosis. Kohlstedt et al. demonstrated that the endothelial AMPK α 2 isoform, not AMPK α 1, attenuates angiotensin converting enzyme (ACE) expression via phosphorylation of p53 and upregulation of miR-143/145 [135], indicating a novel role for AMPK α 2 isoform in preventing Ang-II formation. In addition, it has been demonstrated that AMPK also affects biological functions of macrophages, which play a key role in plaques formation, progression and rupture, critical steps for cardio-vascular acute events [134]. Indeed, AMPK α 1 activation suppresses inflammation and favors the switch towards anti-inflammatory phenotype of monocytes [136–138]. Accordingly, tissue-specific deletion of AMPK α 1 in myelomonocytic cells induces a pro-inflammatory endothelial phenotype with higher aortic infiltration of macrophages and cytokines (TNF- α , INF- γ and IL-6) [139]. Similar results were obtained in LDLR $^{-/-}$ mice where AMPK α 1 deletion in myeloid cells exacerbates atherosclerosis, increases chemokine expression in aortas and macrophage infiltration in atherosclerotic plaques [140]. In addition, several results reported the role of AMPK in platelet aggregation, and thrombus formation and stability. Although concerns have been raised regarding which catalytic α isoform is expressed in human platelets [141], it has been demonstrated, including by our group, that the AMPK α 1 is the sole isoform expressed in human platelets, whereas both AMPK α 1 and AMPK α 2 can be expressed in murine platelets [142–144], suggesting that signaling events could diverge between mouse versus human in these cells. In platelets, AMPK-induced ACC phosphorylation is involved in the control of lipid metabolism, limiting platelets arachidonic acid-containing phosphatidylethanolamine plasmalogen content, thromboxane release upon platelet activation and subsequently arterial thrombosis [145].

Altogether, these results give insights into the role of AMPK under pathological contexts of endothelial dysfunction, inflammation and atherothrombosis.

4. ROS-dependent regulation of AMPK in ischemia and I/R injury

Myocardial ischemia occurs in response to an insufficient oxygen supply, typically after a coronary artery occlusion. The prolonged absence of blood supply induces mitochondrial dysfunction and increased oxidative stress resulting in irreversible injury and tissue necrosis, known as myocardial infarction. Paradoxically, restoration of oxygen and nutrients to the tissue (reperfusion) exacerbates the cell death induced by ischemia. Indeed, while mitochondrial oxidative stress production is limited during ischemia, the onset of early reperfusion induces a burst of ROS, which further enhances cardiac injury [146]. Among the mechanisms proposed to be involved in I/R injury, inhibition of the electron transport chain and related ATP depletion, opening of the mitochondrial permeability transition pore (mPTP), endothelial dysfunction, severe mitochondrial oxidative stress, apoptosis, autophagy, and reduced NO production are critical steps that contribute to cardiac damage [147–149].

During myocardial ischemia, AMPK is rapidly activated and stimulates glucose uptake and glycolysis to promote anaerobic ATP production. Such adaptive mechanisms are capable to restore cellular energy conditions, playing an important role in protecting and limiting the damage in the heart during the acute phase of ischemia [38]. Isolated hearts from mice overexpressing a dominant negative form of AMPK subjected to reduced coronary flow show exacerbation of left ventricle contractile dysfunction, cardiac necrosis and endoplasmic reticulum

stress-mediated apoptosis [38]. Similarly, in a perfused mouse heart model of no-flow ischemia, the lack of AMPK α 2 subunit reduced the ability to store glycogen, reduced glucose uptake as well as anaerobic glycolytic flux and impaired ATP production [150]. The lack of AMPK α 2 aggravated the ischemic contracture and delayed post-ischemic functional recovery. The substantial difference in the contractile function of the heart between these studies could be explained by the distinctive level of AMPK deficiency. Indeed, mouse overexpressing a dominant negative form of AMPK, by titrating both AMPK α 1 and α 2 isoforms almost completely lacks AMPK activity/activation, whereas the animals lacking AMPK α 2 subunit still express AMPK α 1 subunit which remains activated by ischemia [150]. In the past, it was postulated that AMPK activation could be, in contrast, detrimental during the first period of reperfusion where AMPK potentially promotes the stimulation of fatty acid oxidation in parallel to glycolysis, inducing the uncoupling between a high rate of glycolysis and a low rate of glucose oxidation [151]. Such uncoupling increasing lactate production and acidosis was proposed to lower post-ischemic function recovery. However, it was shown that acidosis may be cardioprotective at reperfusion [150,152,153]. In support of this, another study showed that the activation of AMPK during early reperfusion was associated with a reduction in infarct size due to the inhibition of mPTP opening [154]. A crucial difficulty in revealing the role of AMPK during reperfusion could be explained by the lack of understanding of optimal timing to activate AMPK to protect the heart from injury and failure.

At reperfusion, AMPK stimulates myocardial fatty acid oxidation via the inhibitory phosphorylation of ACC, to restore energy homeostasis. However, in an ACC double knock-in (DKI) mouse model of myocardial ischemia, in which AMPK is functional but unable to phosphorylate ACC, fatty acid oxidation rates and cardiac function were not different from WT animals [155]. This unexpected result clearly suggests that additional mechanisms independent of the AMPK/ACC axis are involved in the maintenance of myocardial fatty acid oxidation [155]. Accordingly, in ACC DKI mice, levels of uncoupling protein 3 (UCP3), responsible for increasing fatty acid oxidation rates [156], were significantly higher compared to WT [155]. Interestingly, MacLellan et al. reported that the increased fatty acid oxidation mediated by UCP3 was accompanied by a reduction in ROS production in skeletal muscles [156]. Although in MacLellan's study the impact of AMPK has not been investigated, it has been demonstrated by others that AMPK activation increases UCP3 in rat muscles and reduces ROS production [157]. In the light of these results, we hypothesize that AMPK might control ROS production via myocardial fatty acid oxidation during pathological states independently from ACC, suggesting an additional protective mechanism to prevent oxidative stress in the ischemia/reperfused heart.

Myocardial ischemia results into a significant inflammatory response characterized by a massive infiltration of immune cells, such as neutrophils and macrophages. This inflammatory process is triggered to heal the myocardium. In fact, inflammatory cells are a source of oxidants, cytokines, and growth factors, which support fibroblast proliferation and angiogenesis to repair the injured myocardium [158]. However, their recruitment to the ischemic area is exacerbated during reperfusion, leading to a further increase in oxidative species, constituting an additional layer to mitochondrial oxidative stress and participating to myocardial damages, remodeling and heart failure [159]. Since AMPK activation attenuates ROS production, we could speculate that it protects the heart from I/R injury by blocking the excessive ROS cascade generated by immune cells during the initial phase of reperfusion. In line with this hypothesis, it has been shown that pharmacological activation of AMPK with metformin protects against I/R injury by alleviating ROS production in macrophages [160]. Similarly, neutrophils are a great source of ROS in the ischemic heart. It was shown that in primary isolated neutrophils, H₂O₂-induced AMPK/p38-MAPK signaling promotes neutrophils extracellular trap formation to prevent bacterial infection [161], however, to our knowledge, such data does not exist in the context of ischemic heart.

Cardiac mitochondria being the primary source of ROS, highly contribute to mitochondrial dysfunction, cardiomyocyte damage, and heart failure. AMPK activation plays a remarkable role in protecting the heart against I/R by regulating autophagy to eliminate malfunctioning intracellular components, such as dangerous oxidative species. Therefore, meticulous mitochondrial quality control has a critical relevance in maintaining healthy cells and cardiac functions. In human and murine failing hearts, where ROS production was higher than in normal hearts, the cardiac predominant isoform AMPK α 2 undergoes a switch towards AMPK α 1 [162,163]. Despite the presence of α 1 isoform, AMPK α 2 contribution was indispensable to protect the heart from failing, by reducing ROS production, improving mitochondrial function and increasing the protective autophagy via PINK1/Parkin/SQSTM1 pathway [162]. Pharmacological activation of AMPK with AICAR showed a beneficial increase in mitophagy in hypoxic H9c2 cardiomyoblasts [164]. Other *in vivo* and *in vitro* studies have elucidated the capacity of AMPK to protect the heart against hypoxia by inducing SIRT1/AMPK-mediated autophagy in hypoxic H9c2 cells [165], or against myocardial infarction by activating the AMPK/mTOR/ULK1 pathway in isoproterenol-induced myocardial fibrosis [166]. Ding et al. recently demonstrated that PCSK9, a protein expressed in the murine heart, plays a crucial role in AMPK-mediated autophagy in ischemic heart. Indeed, PCSK9 protects from ROS production, damaged mitochondrial DNA, reduces infarct size and increases contractile function in mice with permanent left anterior descending (LAD) coronary artery ligation [167]. The mechanism proposed in hypoxic primary mouse cardiomyocytes involves ROS-induced phosphorylation of ATM/LKB1/AMPK, ultimately responsible for enhanced protective autophagy [167]. Matsui and colleague elegantly elucidated a distinct role of AMPK activation during ischemia and I/R. Indeed, ischemia stimulates autophagy by AMPK-dependent mTOR inhibition, whereas reperfusion after ischemia stimulates autophagy through a Beclin1-dependent, but AMPK-independent, mechanism, suggesting that autophagy during the reperfusion phase may be detrimental [168]. During hypoxia, in which the production of ROS is elevated, AMPK can afford cardioprotection not only by inducing autophagy, but also by the inhibition of protein synthesis, either phosphorylating eukaryotic elongation factor 2 (eEF2) kinase and consequently inactivating eEF2 [169–171], or inducing inhibition of protein synthesis regulatory mTOR [168]. Indeed, glucose deprivation in cultured cardiac myocytes induces AMPK activation, mTOR inhibition, enhanced eEF2 phosphorylation and decreased protein synthesis [168].

Pharmacological agents, such as metformin, A769662, and AICAR are all AMPK activators that protect the heart from increased myocardial I/R injury-induced oxidative stress in diabetes. Metformin activates AMPK in myocardial I/R and keeps the mPTP closed, thus improving survival rates after myocardial infarction [172]. Similarly, in diabetic rat hearts subjected to I/R (35 min of ischemia, 120 min of reperfusion), pharmacological activation of AMPK with A769662 inhibited the oxidative stress-induced opening of the mPTP, reducing myocardial infarction size in both diabetic and non-diabetic rat hearts [173]. Moreover, AMPK afforded cardioprotection against myocardial infarction via GSK-3 β (glycogen synthase kinase-3 β) phosphorylation at Ser9, a kinase that can induce cell death [173]. Relatedly, in streptozotocin-induced diabetic rat model, an antioxidant treatment protects the heart against I/R-induced ROS apoptosis by reducing infarct size via the AMPK/Akt/GSK-3 β pathway [174]. Myocardial infarction size was also reduced in rats subjected to LAD ligation (30 min) and reperfusion (4 h) after administration of exendin-4, a synthetic analog of GLP-1, by activating SIRT1/PGC-1 α /AMPK axis [175]. Metformin has further been reported to restore PGC-1 α levels and induce mitochondrial biogenesis by phosphorylating eNOS on Ser1177 in *db/db* mice subjected to ischemia (LAD permanent ligation) and to I/R [110,176].

In summary, enhanced ROS production due to defective mitochondria in chronic hypoxia and I/R can be limited by activation of AMPK to promote cell survival in the ischemic heart, conferring AMPK the role of

key regulator in mitochondrial autophagy. Moreover, all these results indicate that AMPK is a critical regulator of myocardial ischemia, ensuring protection by reducing myocardial oxidative stress, apoptosis, and myocardial infarction.

5. AMPK-ROS and cardiac hypertrophy

Cardiac hypertrophy is a physiological adaptive response to elevated blood pressure and pressure overload, which ultimately leads to heart failure. It is characterized by abnormal enlargement and thickening of the left ventricle and it is a distinctive attribute in hypertension, myocardial infarction and hypertrophic cardiomyopathy.

AMPK regulates protein synthesis via eEF2 kinase [170,171] and cell growth via phosphorylation of the tuberous sclerosis complex 2 (TSC2) [177], which further activates mTOR and protein synthesis by phosphorylating p70S6K and ribosomal protein S6 [81]. Several groups tested the hypothesis that AMPK activation could attenuate the development of hypertrophy, given the inhibitory effects on protein synthesis. Once activated, AMPK inhibits mTOR signaling and regress cardiac hypertrophy induced by hemodynamic stress [178]. Lack of AMPK α 2 in mice exacerbates pressure overload-induced left ventricle hypertrophy, by increasing the phosphorylation of p70S6K, ribosomal protein S6, and eukaryotic initiation factor 4E [179,180]. Other results showed that LKB1, the upstream kinase of AMPK, was reduced in mice with pathological cardiac hypertrophy, while its expression in cardiomyocytes reduces protein synthesis and cardiac hypertrophy induced by phenylephrine [181]. In agreement with these results, transverse aortic constriction (TAC) operated mice revealed a decreased AMPK and ACC phosphorylation [182]. Accordingly, others demonstrated that during TAC-induced left ventricle hypertrophy, AMPK α 2 isoform expression was decreased of 30% when compared to healthy hearts [183]. Moreover, pharmacological activation of AMPK by AICAR or resveratrol in cultured rat neonatal cardiomyocytes also regulates phosphorylation of p70S6K and eEF2, diminishing protein synthesis and protecting from phenylephrine-induced hypertrophy [184,185]. In *in vivo* experiments, AICAR, metformin and adiponectin have been effective in reducing pressure overload-, Ang-II- and isoproterenol-induced hypertrophy in murine hearts [182,186–189]. Taken together, these results emphasize the critical position of AMPK, and in detail the AMPK α 2 isoform, in protecting the heart against hypertrophy by hampering protein synthesis.

In the ischemic heart, excessive ROS-induced oxidative stress is known to impair myocardial function [190–193]. Detrimental ROS accumulation similarly occurs in cardiac hypertrophy, where this elevation triggers hypertrophic signaling [194–196]. In this section, we show whether AMPK-mediated protection against hypertrophy occurs in response to a direct interplay with oxidative stress. Dolinsky et al. observed in cardiac hypertrophic models (Ang-II infused mice and spontaneously hypertensive rats) that the LKB1/AMPK axis inhibits oxidative stress and the consequent mTOR/p70S6K cascade, preventing a permissive environment for abnormal cardiomyocyte growth [197]. Additionally, resveratrol also activates the LKB1/AMPK signal transduction pathway and leads to cardioprotection by limiting oxidative stress via increased eNOS phosphorylation, improving vascular function and attenuating cardiac hypertrophy [196]. In a more recent study on right ventricle cardiac hypertrophy, AMPK afforded cardioprotection via activation of the antioxidant enzymes thioredoxin-1 and SOD2, inhibiting ROS production [198]. Activation of AMPK was induced by exogenous administration of c1q/tumor necrosis factor- α -related protein 9 (CTRP9), which is normally secreted by cardiac cells and function as a “cardiokine” through paracrine and autocrine effects [199]. Furthermore, AMPK protects against pressure overload-induced hypertrophy by activating protease-activated receptor 1 (PAR1) on cardiomyocytes, diminishing ROS production, and increasing ACC phosphorylation and ULK1 pathway [200]. Beyond mitochondrial dysfunction and ROS production, autophagy impairment may aggravate

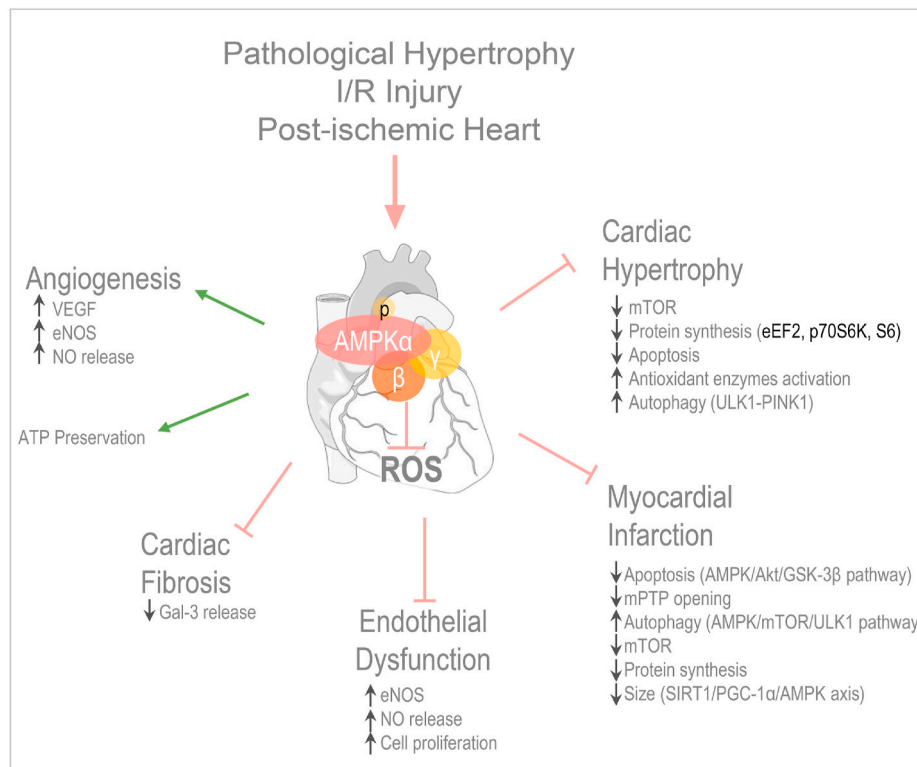


Fig. 3. AMPK prevents the transition to heart failure.

cardiac hypertrophy. A recent study performed on a model of pressure overload-induced cardiac hypertrophy identified a new AMPK-dependent protective mechanism via direct phosphorylation of PINK1 on ser495 [162]. PINK1 phosphorylation promoted parkin recruitment to the phagosome and initiates mitophagy in TAC-operated mice, with a final reduction of ROS.

Increased ROS generation, oxidative stress and altered autophagy induce mitochondrial dysregulation, which exacerbate cardiac dysfunction and remodeling. AMPK activity is critical to preserve the heart function during pressure overload, and it re-establishes the compromised oxidative status, to protect from pathological hypertrophy.

O-linked attachment of the monosaccharide β -N-acetyl-glucosamine (O-GlcNAcylation) is a post-translational modification occurring on serine and threonine residues. O-GlcNAcylation participates in pathological hypertrophy development and it increases in response to increased workload. Our laboratory recently identified a novel molecular mechanism that elucidates how AMPK attenuates O-GlcNAcylation to prevent cardiac hypertrophy development [201]. Because O-GlcNAc can promote ROS generation in hyperglycemic models [202], we can postulate that activation of AMPK could reduce O-GlcNAc and O-GlcNAc-mediated ROS production, preventing the development of cardiac hypertrophy.

6. Targeting AMPK and ROS to prevent the transition to heart failure

Heart failure is a complex disorder characterized by an impaired capability of the heart to contract and relax and meet the body's energy demand without any increase in filling pressure [203]. Clinically, heart failure is a complex clinical syndrome that displays an array of characteristics depending on left ventricular EF. Over the past decade, there has been increasing awareness about phenotypic differences between heart failure with reduced (HFrEF) and preserved (HFpEF) ejection fraction. Ejection fraction is lower than 40% in HFrEF and above 50% in

HFpEF patients, reflecting predominant systolic or diastolic dysfunction, respectively. Despite extensive research, heart failure remains the prevalent cause of morbidity and mortality in developed countries [1]. Pathological changes in cardiac tissue, such as accumulation of cardiac fibroblasts, increased inflammation, endothelial dysfunction and apoptosis are all key elements contributing to adverse remodeling of the left ventricle observed in both heart failure phenotypes, i.e. HFrEF or HFpEF.

Interestingly, experimental and clinical data have demonstrated that abundance of myocardial ROS, generating oxidative stress, can lead to the progression and development of myocardial remodeling and heart failure [54,204]. Indeed, increased Nox activity, uncoupled eNOS, and dysfunctional mitochondria are all common features of the failing myocardium [205,206]. Whether AMPK plays a substantial role in protecting the heart is not under discussion. However, here we accentuate some aspects of AMPK-dependent ROS regulation involved in crucial processes for the transition to heart failure, including cardiac fibrosis, apoptosis, angiogenesis and endothelial dysfunction.

Ventricular remodeling can develop in response to pressure overload, where cardiomyocyte hypertrophy and interstitial fibrosis culminate in wall stiffness. ROS play a major contribution in remodeling, since they seem to promote pro-fibrotic phenotypes in the heart [207], whereas ROS scavenger treatments limit the progress of remodeling [208]. Several groups have focused on the AMPK-induced ROS reduction to attenuate the shift to heart failure [209–211]. In a model of isoproterenol-induced cardiac fibrosis, the lack of AMPK α 2 exacerbates ROS level production via increased expression of Nox enzymes (Nox4 and Nox 2, to a less extent), and decreased expression of antioxidant enzymes, such as CAT, SOD1, and SOD2 [209]. A more recent study associated the SIRT1/LKB1/AMPK axis to a beneficial decrease in ROS accumulation and consequent protection against hypertrophy, fibrosis, and apoptosis induced by Ang-II infusion [211]. Mechanistically, treatment with fibroblast growth factor 21 (FGF21), which had already showed cardioprotective results in isoproterenol-induced cardiac hypertrophy in mice [212], activates SIRT1/LKB1/AMPK pathway and

Table 1
Therapeutic strategies to activate AMPK.

Drugs	Mechanisms for AMPK activation	Downstream effects
Metformin	LKB1 Changes in ADP/ATP ratio	↓ Systolic dysfunction [186] ↓ Hypertrophy [188] ↑ eNOS phosphorylation [110, 176,220] ↑ PGC-1 α expression [221] ↑ Autophagy [222,223] ↑ Cardiac fibrosis (collagen synthesis) [224]
Resveratrol	LKB1	↓ Hypertrophy [225] ↓ Perivascular and interstitial fibrosis [225] ↑ PGC-1 α expression [226–228]
Statins	AMP/ATP independent Unclear	↓ Oxidative stress ↓ Apoptosis ↓ Inflammation [229] ↓ Endothelial dysfunction [208]
Thiazolidinediones	Inhibition of respiratory chain AMP/ATP ratio Hyperosmotic-induced Thr172 phosphorylation	↓ Fibrosis [230,231] ↓ ROS by inhibition of Nox [79] ↓ Hypertrophy [232] ↓ Diastolic dysfunction [232] ↑ Coronary vasodilation [233] ↑ Cardiac dysfunction [233]
SGLT2 inhibitors	?	↓ Oxidative stress [234] ↓ Endothelial permeability (Angé et al., unpublished)

alters the transcriptional activity of FoxO1 to stimulate CAT and SOD2 expression, reducing ROS accrual and eliciting cardioprotection [211].

In addition to cardiac hypertrophy and fibrosis, endothelial dysfunction and angiogenesis play a crucial part in the transition to heart failure [101,105,213]. AMPK exerts a pivotal role in angiogenesis: LKB1/AMPK signaling axis exerts a proangiogenic role in endothelial cells during ischemia [214], AMPK promotes the potent stimulator of angiogenesis VEGF [5], and promotes eNOS activation with reduction of cardiac hypertrophy, increase of capillary density, and decrease in fibrosis during pressure overload [215]. Moreover, TAK1 also phosphorylates AMPK α 1 to stimulate VEGF-induced and cytokine-induced angiogenesis by increasing SOD2 expression [216]. To elucidate whether Nox-derived ROS production might be beneficial or not, an elegant recent study had demonstrated that endogenous ROS exposure, by increased activity of Nox2 in endothelial cells, activates CaMKK β -AMPK axis to induce phosphorylation of eNOS and increase NO-dependent vasodilation [105]. Additionally, AMPK activation was able to induce autophagy, improve endothelial cell dysfunction and proliferation in response to Nox2-induced ROS [105].

Finally, ROS formation and myocardial oxidative stress is the widely-accepted mechanism of chronic anthracycline-induced cardiomyopathy, associated with poor prognosis [217]. Several compounds, such as adiponectin have been shown to activate AMPK and protect against doxorubicin-induced cardiomyopathy via an AMPK-dependent anti-apoptotic function [218]. The natural phenolic compound oleuropein, which is present in high concentration in olives and olive tree leaves prevents doxorubicin cardiomyopathy through activation of AMPK in parallel to eNOS phosphorylation and attenuation of nitro-oxidative stress [219].

Collectively, these findings suggest that not only AMPK regulates the cardiac redox state to protects against cardiac fibrosis and further heart failure, but also protects the heart from oxidant-induced cell death and failure by increasing defensive mechanisms such as autophagy, angiogenesis and endothelial cell proliferation (Fig. 3).

Schematic summary of the cardioprotective effects of AMPK in response to increased ROS induced by pathological hypertrophy and ischemia reperfusion (I/R) injury.

7. AMPK: a versatile target to protect the heart from failure

Pharmacological AMPK activation protects the heart by preventing or delaying the transition to failure. A large body of studies have demonstrated that AMPK protects the heart by acting on cellular metabolism, although, it is conceivable that cardioprotection may also be conferred through direct modulation of non-metabolic properties such as proliferation, fibrosis and angiogenesis. Several drugs have shown the positive effects of AMPK activation, such as metformin, statins, resveratrol, suggesting their potential clinical application for treatment of heart failure (Table 1).

7.1. Mechanisms of modulation of AMPK activity by pharmacological agents and downstream effects

7.1.1. Metformin

Metformin is the first-line drug in the treatment of type 2 diabetes, for its glucose-lowering effect, which results in decreased hepatic glucose production and increased glucose utilization. However, the mechanism of action remains unclear. It has been proposed a direct action of metformin on AMPK, involving LKB1 [235] and AMPK phosphorylation on Thr172 site [236], and inhibition of glycerophosphate dehydrogenase [237]. In addition to these direct pathways, metformin can indirectly activate AMPK by changes in the ADP/ATP ratio due to inhibition of the respiratory chain [238]. A growing body of evidence show that metformin alleviates the development and progression of heart failure. Beyond the effects of metformin on cardiac metabolism, preclinical studies have shown that chronic activation of AMPK with low-dose of metformin improves cardiac function and cell survival in murine models of heart failure [239]. Metformin has been proved a valid tool to activate AMPK and trigger signaling events to protect the heart from pressure overload-induced hypertrophy. Indeed, metformin reduces cardiac fibrosis and inhibits collagen synthesis in cardiac fibroblast via TGF β -1/SMAD3 signaling [224], inhibits mTOR response and activates autophagy [222,223] to alleviate cardiac hypertrophy. With different signaling pathways, metformin-mediated AMPK activation protects against I/R injury by phosphorylating eNOS at Ser1177 and activating PGC-1 α [110,176], and by inducing antioxidant enzymes expression [221]. Moreover, it has been shown as a valuable tool to limit infarct size and protect from cardiac fibrosis after myocardial infarction [110,111]. Even in a dog model of tachycardia-induced heart failure, metformin proved its efficacy in reducing cardiomyocyte apoptosis and improve cardiac function via AMPK/eNOS/NO pathway [220]. All these findings underlie metformin as a preferential AMPK activator to attenuate cardiac hypertrophy and protect against I/R injury. Concerning clinical studies, the UKPDS trial was the first to show that metformin significantly reduced mortality after a 10-year follow-up, due to decrease in myocardial infarction events [240]. More recently, a meta-analysis confirmed that metformin treatment was associated with reduced mortality, and guaranteed equal safety to other glucose-lowering agents in diabetic patients with heart failure [241]. The mechanisms involved in the protective effect of metformin in cardiovascular diseases are still partially unclear and may include AMPK dependent and independent targets [242].

7.1.2. Resveratrol

Resveratrol is a natural antioxidant found in several plants, including red grapes, more and more studied for its capacity in rescuing the heart from failure. Anti-hypertrophic effects of resveratrol have been demonstrated by direct activation of AMPK via LKB1 [184]. Additionally, resveratrol significantly attenuates the adverse pressure overload-induced cardiac remodeling, suppressing perivascular and interstitial fibrosis via inhibition of oxidative stress and inflammation [225]. Amelioration of endothelial dysfunction induced by resveratrol has been demonstrated to be mediated by AMPK/PGC-1 α signaling induced by Sirt1 and Sirt3 [226–228,243]. Collectively, the crosstalk

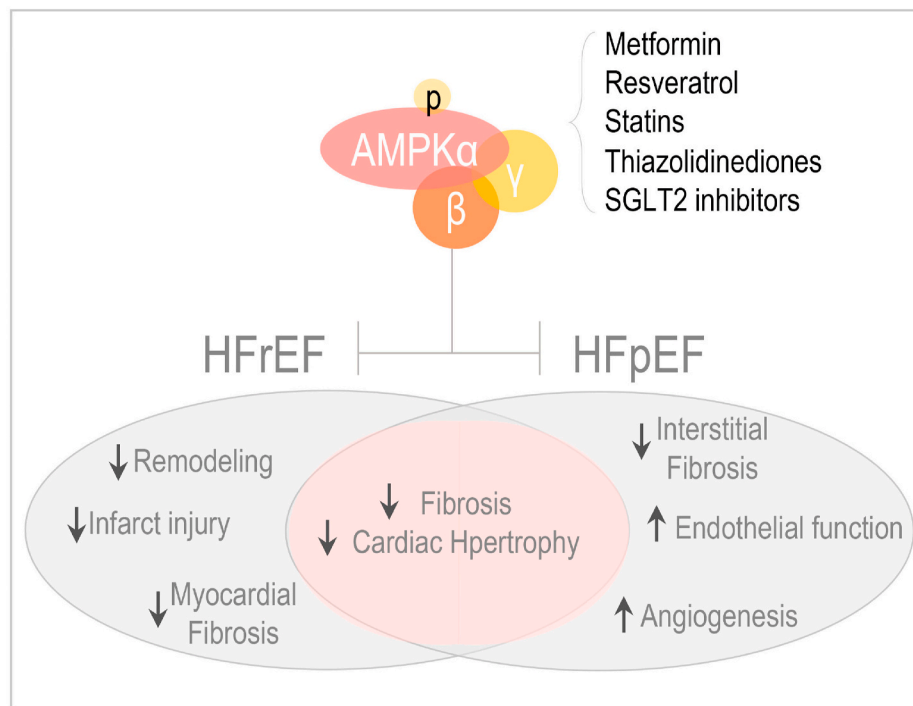


Fig. 4. AMPK protects against heart failure. After activation by pharmacological agents, AMPK protects the heart from failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF).

between AMPK and resveratrol, makes this natural polyphenol an interesting therapeutic tool for the prevention of heart failure.

7.1.3. Statins

Statins are a class of drugs with the capacity to lower cholesterol levels in blood. However, several studies have reported their cardioprotective effect in protecting the myocardium from remodeling and I/R injury, by attenuating fibrosis and hypertrophy in rats [208]. Interestingly, statins can activate AMPK via phosphorylation of Thr172 in the $\alpha 1$ subunit and induce an eNOS-dependent NO signaling, capable to restore endothelial function, increase angiogenesis and maintain cardiovascular homeostasis [131,244,245]. However, statin-induced AMPK phosphorylation may be independent of the cellular ATP to AMP ratio [236], although the exact mechanism is unclear. An extensive review reported the beneficial effect of statins in animal model of doxorubicin-induced cardiotoxicity, where they attenuate oxidative stress, inflammation and apoptosis [229]. Together, these results suggest that statins can represent interesting pharmacological tool to mitigate the progression of heart failure.

7.1.4. Thiazolidinediones

Thiazolidinediones are a class of drugs widely used for diabetic treatment, which have been demonstrated to activate AMPK by distinct mechanisms. Similarly to metformin, thiazolidinediones inhibit respiratory complex I of the mitochondrial respiratory chain, increasing AMP/ATP ratio, thus stimulating the phosphorylation and activation of AMPK [246]. Furthermore, under hyperosmotic stress, rosiglitazone, can increase phosphorylation of Thr172 within the α -subunit [247]. Rosiglitazone has divergent effects. It activates AMPK and hampers Nox activation, thus limiting oxidative stress in skeletal muscles [79]. Other studies demonstrated its beneficial effects in attenuating cardiac fibrosis in diabetic rats and Ang-II-induced fibrosis [230,231]. However, growing evidence from both basic and clinical studies indicate controversial and harmful effects of rosiglitazone in the heart by inducing pathological cardiac hypertrophy and consequent heart failure [248–250]. On the other hand, pioglitazone, another compound

belonging to thiazolidinediones, have been shown to attenuate cardiac fibrosis, hypertrophy, and diastolic dysfunction via AMPK-mediated signaling [232], suggesting that differences in binding affinity for PPAR- γ may prevent undesired effects in the heart.

7.1.5. SGLT2 inhibitors

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are the newest class of oral anti-hyperglycemic agents that have been approved by the FDA for the treatment of diabetes mellitus. Given the favorable cardiovascular outcomes in patients with diabetes treated with SGLT2 inhibitors, SGLT2 represents a new therapeutic target for type 2 diabetes and heart failure. The multiple positive effects in preventing cardiac failure and the proposed mechanisms of SGLT2 inhibitors have been widely discussed [251]. However, a clear explanation of the cardioprotective effects of SGLT2 still needs to be clarified. A recent study showed, using docking studies, that SGLT2 inhibitors have high affinity for the extracellular Na^+ -binding site of the Na^+/H^+ exchanger, which is responsible for coronary vasodilation in healthy murine hearts perfused with empagliflozin, dapagliflozin and canagliflozin [233]. Additionally, a more recent study demonstrated that empagliflozin ameliorates cardiac dysfunction and protects against oxidative stress damage via Sestn2/AMPK/mTOR signaling pathway in obese mice [234]. Additional evidence comes from unpublished data from our laboratory, which demonstrate that canagliflozin, an inhibitor of SGLT2, acts through $\alpha 1$ AMPK and protects the endothelium against vascular injury, such as sepsis. Because of the ongoing clinical trial, this class of compounds represents an interesting therapeutic approach to prevent cardiac dysfunction and the development of heart failure.

Put into perspective, AMPK activation by drugs cited above, represents a valuable target to counteract the development of heart failure. One may speculate that it prevents both HFpEF, by improving endothelial function, reducing fibrosis and inducing angiogenesis, and HFrEF, by attenuating infarct size injury and fibrosis, thus reducing LV remodeling and depressed systolic function (Fig. 4).

8. Conclusions

AMPK is a crucial energy sensor in the organism and minimal allusions to stress can activate it to re-establish cellular homeostasis. ROS-induced activation of AMPK and AMPK signaling protect the heart from oxidative stress. In endothelial cells, cardiomyocytes and cardiac fibroblasts, AMPK plays a crucial role in regulating the cardiac redox state to protect the heart from failure. By increasing defensive mechanisms such as autophagy, angiogenesis and endothelial cell proliferation and function, it essentially participates to delay the transition of cardiac pathological states to failure.

The extensive literature on the AMPK role in cardioprotection highlights important and innovative mechanisms that could be exploited to generate targeted therapies directed to the prevention of cardiac diseases and to attenuate or delay the degenerative conditions of the heart leading to failure.

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Declaration of competing interest

None.

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None.

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