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Ageing, sex and cardioprotection

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Abstract

Translation of cardioprotective interventions aimed at reducing myocardial injury during ischaemia-reperfusion from experimental studies to clinical practice is an important yet unmet need in cardiovascular medicine. One particular challenge facing translation is the existence of demographic and clinical factors that influence the pathophysiology of ischaemia-reperfusion injury of the heart and the effects of treatments aimed at preventing it. Among these factors, age and sex are prominent and have a recognised role in the susceptibility and outcome of ischaemic heart disease. Remarkably, some of the most powerful cardioprotective strategies proven to be effective in young animals become ineffective during ageing. This article reviews the mechanisms and implications of the modulatory effects of ageing and sex on myocardial ischaemia-reperfusion injury and their potential effects on cardioprotective interventions.

List of abbreviations:

AK: adenylate kinase
Akt: protein kinase B
AMI: acute myocardial infarction
ANT: adenine nucleotide translocator
CAD: coronary artery disease
ciRNA: circular RNA
CK: creatine kinase
ECM: extracellular matrix
ERK1/2: extracellular signal-regulated kinase 1/2
GSK3β: glycogen synthase kinase 3 beta
GWAS: genome-wide association studies
IHD: ischaemic heart disease
IPC: ischaemic preconditioning
IPostC: ischaemic postconditioning
IR: ischaemia-reperfusion
IS: infarct size
lncRNA: long noncoding RNA
LV: left ventricle
MI: myocardial infarction
MIM: mitochondrial inner membrane
MOM: mitochondrial outer membrane
mPTP: mitochondrial permeability transition pore
MtCK: mitochondrial creatine kinase
OXPHOS: oxidative phosphorylation
PCr: phosphocreatine
PiC: phosphate carrier
PI3-K: phosphoinositide 3-kinase
RCS: respiratory chain supercomplexes
RIPC: remote ischaemic preconditioning
RISK: reperfusion injury salvage kinase
RyR: ryanodine receptor
SR: sarcoplasmic reticulum
STAT3: signal transducer and activator of transcription 3
STEMI: ST-segment elevation myocardial infarction
TIMPs: tissue inhibitors of MMPs
VDAC: voltage-dependent anion channel

Drug and molecular target nomenclature used in the present article conforms to BJP's Concise Guide to Pharmacology Citation (Alexander *et al.*, 2015)

Akt:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=285
ANT:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=206#1063
Connexin43:
https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=connexin43&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database
ERK1/2:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=514
GSK3b:
https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=GSK3b&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database
Hexokinase:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=890
MMP:
https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=MMP&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database
PKA:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=284
PKC:
ttps://www.guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=ENZYME&familyId=286#node286
PKC epsilon:
https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1486
PKG:
https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=287
RyR:
https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=748
STAT3:
https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=STAT3&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database
TIMPs:
https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=TIMP&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database

1. Introduction

Key consequences of ischaemic heart disease (IHD) are contractile dysfunction, irreversible myocardial injury, heart failure, arrhythmia and death. Their magnitude depends mainly on the extension, severity and duration of the ischaemic insult. Although myocardial ischaemia may occur under different clinical conditions, including elevated oxygen demand, cardiac arrest or cardiac therapeutic interventions such as surgery, the most important clinical condition is acute coronary occlusion leading to severe transmural ischaemia and development of myocardial infarction (MI). In this case, the mainstay of treatment is limitation of the duration of ischaemia through emergency re-opening of the occluded vessel, generally through percutaneous coronary intervention, with pharmacological treatments to prevent re-occlusion. Even after a successful recanalisation of the culprit artery, restoration of blood flow is accompanied by a paradoxical worsening of the functional changes associated with ischaemia ("reversible reperfusion injury"), particularly arrhythmias and stunning, and in more severe cases by reperfusion-induced cell death ("lethal reperfusion injury"). This comes in addition to potential microvascular dysfunction or injury when establishing reflow. The contribution of lethal reperfusion injury to the final extent of MI has been irrefutably proven in the experimental setting (Piper *et al.*, 1998) and in patients with STEMI, although here the efficacy of the therapeutic interventions has been less consistent (Hausenloy *et al.*, 2017a). The time window to effectively prevent or limit myocardial injury in patients with STEMI is very short (few hours from the onset of chest pain), and the development of ways to reduce the myocardial consequences of ischaemia of a given severity and duration by interfering with the molecular mechanisms of ischaemic injury is a priority (Ibanez *et al.*, 2017).

Preclinical studies have identified a variety of molecular pathways potentially involved in cell death during ischaemia-reperfusion (IR) that are targetable by a therapeutic intervention (Hausenloy *et al.*, 2017b). Nevertheless, and despite promising outcomes in the experimental setting, the translation of such cardioprotective interventions to the clinical practice for the benefit of patients with IHD has repeatedly failed (Hausenloy *et al.*, 2017b;Heusch, 2017). The causes for this failure are multiple and have been reviewed elsewhere (Heusch, 2017;Heusch, 2018;Botker *et al.*, 2018;Davidson *et al.*, 2019;Kleinbongard *et al.*, 2019). The

inadequate simulation of the clinical conditions of patients (lack of confounders present in “real life”) has been the object of intense debate, as the patients with IHD recruited into cardioprotection clinical trials normally have an advanced age and several confounders, including one or more comorbidities (hyperlipidaemia, hypertension, metabolic disorders) and comedications, conditions that have not been properly recapitulated in the young and healthy animals used for research purposes. Among confounders, ageing and sex and their interplay (loss of hormonal influence is part of normal ageing) have been increasingly recognized as main determinants of the outcomes of IR injury (Davidson *et al.*, 2019). Neglecting their role is proposed as a fundamental contributor of the translational gap and may explain the suboptimal and sometimes inconsistent effects of cardioprotective strategies (Ferdinandy *et al.*, 2014). Here, we review the current knowledge about the role of ageing and sex as (patho)physiological modulators of IR injury development, cardioprotection signalling and efficacy of the therapeutic interventions.

2. Age and tolerance to ischaemia-reperfusion injury

Ageing of the human population is a global demographic phenomenon with profound effects on the epidemiology, severity and mortality rates of IHD (Moran *et al.*, 2014). About two thirds of myocardial infarctions occur in patients older than 65 years and one third occurs in patients older than 75 years (Engberding and Wenger, 2017). Despite this, randomised clinical trials preferentially include patients younger than 75 years, consistently underestimating the real number of elderly patients that cardiologists encounter in clinical practice (Lee *et al.*, 2001). Therefore, most treatments applied to elderly patients have been extrapolated from the results obtained in studies performed with younger patients and are not based on specific evidences that supports their efficacy and safety in this age segment.

Advanced age not only increases the incidence of IHD due to progressive arterial disease among other factors, but also exacerbates its clinical manifestations and impairs the prognosis and survival of the patients (Shih *et al.*, 2011). Elderly patients are more likely to die after MI due to an increased rate of mechanical complications (papillary muscle rupture, left ventricular free wall rupture and ventricular septal defects), in-hospital mortality and long-term mortality after discharge (Shih *et al.*, 2011;Mehta *et al.*, 2001) According to the GISSI-1 study, each year of age is associated with a 6% increase in the relative risk of mortality

secondary to an acute coronary syndrome (Maggioni *et al.*, 1993). This is in part due to the fact that age is a main determinant of the extension of necrosis within the myocardial segment exposed to ischaemia (Shih *et al.*, 2011), which in turn, is critically associated with the future occurrence of arrhythmias and development of heart failure. Indeed, being old significantly increases the likelihood of developing adverse cardiac remodelling and heart failure secondary to MI (Ezekowitz *et al.*, 2009).

The impact of ageing on IS results from multiple factors including compromised functional reserve of the myocardium in elderly patients and increased burden of comorbidities (Ekerstad *et al.*, 2011;Barnett *et al.*, 2012). The heart develops some age-dependent structural and physiological adaptations, like changes in cardiomyocyte number, size and function (Bernhard and Laufer, 2008) and altered extracellular matrix (ECM) composition (Horn and Trafford, 2016) that may influence the severity of myocardial dysfunction after an ischaemic episode. Remarkably, the increased vulnerability of the aged heart to develop cardiomyocyte dysfunction during IR has been reproduced in a myriad of experimental models, including isolated cardiomyocytes, in which the contribution of cell-to-cell interaction, extracellular components, vascular factors and comorbidities is absent (Fernandez-Sanz *et al.*, 2015;Willems *et al.*, 2005). This evidence supports the concept that beyond other factors, aged cardiomyocytes develop endogenous changes that reduce their ability to tolerate and survive from an IR insult (Willems *et al.*, 2005;Strait and Lakatta, 2012).

Efficient therapeutic strategies specifically addressed to treat IHD in elderly patients are challenged by the fact that some well characterised endogenous cardioprotective pathways and targets are attenuated in aged cardiomyocytes (see section 4.1.), rendering them more resistant to otherwise powerful therapeutic interventions, i.e. ischaemic pre- and postconditioning. To what extent this feature affects other drug-induced cardioprotective strategies is a matter of research. Alterations in the inflammatory response induced by ischaemia, together with a gradual deterioration of the reparative fibrosis by mechanisms that involve the up-regulation of beta-galactosidase and other key senescent markers, such as p53 (Zhu *et al.*, 2013), have been shown to contribute to the higher prevalence of adverse remodeling and heart failure after MI in elderly (Westman *et al.*, 2016;Bujak *et al.*, 2008).

3. Mechanisms of reduced tolerance to ischaemia-reperfusion injury

3.1. Intracellular changes involved in IR susceptibility during ageing

Aged cardiomyocytes develop a multiplicity of abnormalities in mitochondrial function, calcium handling, metabolism and signalling pathways that can affect their susceptibility to IR (Jahangir *et al.*, 2007) (Figure 1). Some of the most prevalent pathophysiological traits of aged cardiomyocytes, i.e. energy demand/supply mismatch and excessive oxidative damage, resemble those of failing cardiomyocytes (Morita and Komuro, 2018). Regular arrangement of mitochondria and adequate mitochondria-sarcoplasmic reticulum (SR) communication are essential for metabolic plasticity, playing an important role in cell death or cardioprotection (Anmann *et al.*, 2014;Ruiz-Meana *et al.*, 2010). Calcium transferred from SR to mitochondria adjusts adenosine triphosphate (ATP) production with cell energy demand, and maintains the pool of the antioxidant glutathione through the regeneration of Krebs-coupled nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) (Kohlhaas *et al.*, 2017). Importantly, efficient inter-organelle calcium exchange strongly depends on the tight alignment (50-100nm distance) between ryanodine receptor (RyR https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=748) at the SR and the voltage-dependent anion channel (VDAC) at the mitochondria (Garcia-Perez *et al.*, 2008). Previous studies indicate that the anatomical proximity between SR and mitochondria is partially disrupted in aged hearts, altering calcium exchange and favouring oxidative stress at the interface between both organelles (Fernandez-Sanz *et al.*, 2014). RyR has been recently demonstrated to be a target of glycative damage during ageing in human and murine myocardium (Ruiz-Meana *et al.*, 2019). Therefore, glycation of RyR could be responsible for the disruption of the RyR-VDAC bridges, with detrimental functional consequences. Indeed, RyR glycation induces a SR-dependent calcium leak; in intact cells, chronic exposure of mitochondria to dysfunctional SR favours the concomitant increase of calcium precipitation in their matrix (Ruiz-Meana *et al.*, 2019). Excess of reactive oxygen species (ROS) and mitochondrial calcium are important triggers of mitochondrial permeability transition pore (mPTP) opening (Ong *et al.*, 2015), therefore mitochondria from aged cardiomyocytes meet the conditions necessary to be more

susceptible to undergo mitochondrial permeabilization during IR. As predicted, the contribution of mPTP in cell death has been consistently demonstrated to be more relevant in the aged cardiomyocytes exposed to IR than in young cells (Fernandez-Sanz *et al.*, 2015;Escobales *et al.*, 2014). Moreover, the endogenous increase in mitochondrial calcium prevents mitochondria from aged cardiomyocytes to actively buffer cytosolic calcium during IR, a feature that leads to a more severe cytosolic calcium overload, hypercontracture and cell death by mechanisms unrelated to mPTP (Fernandez-Sanz *et al.*, 2015;Ruiz-Meana *et al.*, 2011).

In addition to calcium handling, alterations in the VDAC channel (at the mitochondrial outer membrane [MOM]), the electron transport chain and the activity/expression of mitochondrial kinases have all been involved in ageing and cardioprotection. These components are part of the large protein supercomplex named mitochondrial interactosome (Figure 1), located in the contact sites between inner and outer mitochondrial membranes, which is responsible to deliver ATP more efficiently to the sites where energy demands are high (Timohhina *et al.*, 2009). The restriction of ATP and adenosine diphosphate (ADP) diffusion at the level of the MOM is an essential element of compartmentalized energy transfer. In adult cardiomyocytes, the MOM permeability to ADP is regulated by interaction of VDAC with cytoskeletal proteins, particularly with β tubulin II (Bagur *et al.*, 2016). The permeability of the MOM increases in middle-aged female rats, but not in male rats; this difference disappears during ageing and increased VDAC permeability becomes characteristic for both sexes. The IR itself can induce similar change in the VDAC permeability, therefore enhancing the contribution of hexokinase https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=890 to the regulation of cellular energy fluxes (Halestrap *et al.*, 2015).

Restriction of ATP/ADP diffusion through VDAC in cardiac cells is overcome by intracellular phosphoryl transfer through the phosphocreatine (PCr)/creatine kinase (CK) shuttle (Tepp *et al.*, 2016). IR injury compromises mitochondrial oxidative phosphorylation (OXPHOS) and compartmentalized intracellular energy transfer via the PCr/CK network (Bagur *et al.*, 2016). Similarly, for middle aged and old rats OXPHOS activity is much lower than for young rats (Tepp *et al.*, 2017). The efficiency of the CK shuttle, which is the main energy provider for ATPases in adult cardiomyocytes, decreases remarkably as a result of ageing, but reperfusion does not alter MtCK activity or expression or the ratio dimeric:octameric isoforms of MtCK

(Bagur, Tanguy et al. 2016). The decline in adenylate kinase (AK) system is very low with no sex-related differences in its activity or in mitochondrial isoform contribution. The contribution of glycolysis to ATP production is increased during ageing while the hexokinase activity doesn't change during healthy ageing (Tepp *et al.*, 2017). It remains unknown to what extent glycolysis replaces OXPHOS in the aged patients and whether that switch has cardioprotective effects.

The third possible players in ageing and cardioprotection are respiratory chain supercomplexes (RCS). In complexes I, III and IV, the ubiquinone and cytochrome c binding sites are facing each other favouring substrate channelling together with association of ATP synthase, adenine nucleotide translocator (ANT https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=206#1063) and phosphate carrier (Vonck and Schafer, 2009). RSCs disruption and lack of complex IV may be an important factor for the impairment of mitochondrial function in ageing (Ramirez-Camacho *et al.*, 2019), although data obtained in aged rats show that changes in cardiac bioenergetics are not caused by the functional impairment of RCS (Tepp *et al.*, 2017). Nevertheless, pre-existing deficiencies in OXPHOS during ageing may compromise the plasticity of ETC activity (Agarwal *et al.*, 2014). Indeed, during preconditioning changes in RCS may occur as a consequence of alterations in the lipid composition of mitochondrial membranes, as suggested in aged animals (Kuznetsov *et al.*, 2019).

3.2. Changes in extracellular matrix

ECM remodelling secondary to ageing may affect the response to IR injury in at least 3 different ways (Meschiari *et al.*, 2017;Mendes *et al.*, 2012;Frangogiannis, 2019): 1) it increases the distance for oxygen diffusion from the capillaries to the mitochondria of cardiomyocytes; 2) it disturbs integrin signalling and T-tubule function in cardiomyocytes, hampering post-ischaemic recovery of electromechanical function; 3) it induces stiffening of the vessel wall, which in turn reduces the ability of the vessels to adequately adjust coronary blood flow to oxygen demand. Oxygen extraction is already high at rest in the heart. Limited dilatation of conductance vessels, arterioles and precapillary sphincters results in low flow ischaemia.

The components of ECM are characterised by a slow turnover. In addition, they have a high degree of posttranslational modifications, both spontaneously and enzymatically. The products of partial degradation, fragmentation, advanced

glycation end-products (AGE) and other modifications accumulate in the interstitial space of the heart, in the vessel wall and other organs with increasing age (Birch, 2018). As a consequence, extracellular deposits and matrix stiffness increase, and mechano-transduction and normal integrin-related cell signalling deteriorate.

The most abundant component of the ECM of the heart is collagen, mainly synthesized by fibroblasts. Collagen I, which controls stiffness, and collagen III, which adds elasticity, are the subtypes that dominate in the myocardium. Proteoglycans, consisting of glycosaminoglycan attached to a protein core, provide the tissue turgor. An ageing heart is characterised by ECM changes in parallel with loss of cardiomyocytes (and eventually slight hypertrophy of remaining cells). Microscopic measurements of collagen content revealed that collagen type I fibres were increased in number and thickness in hearts of old male individuals without structurally visible heart disease (Mendes *et al.*, 2012). In healthy ageing, ECM changes are subtle, but since ECM matrix remodelling takes place both as a reparative and as a reactive process, reported changes in the ECM in the ageing heart are often due to interaction between age and subclinical or manifest age-related diseases (Meschiari *et al.*, 2017;Frangogiannis, 2019;Trial *et al.*, 2016); hypertension in combination with the decline in gonadal hormone influence being one example (Ludvigsen *et al.*, 2018).

The basement membrane of cardiomyocytes is part of the ECM and consists of a tiny laminin A layer and non-fibrillar-forming collagen type IV among other components. Examining the heart-tube of *Drosophila melanogaster* at different ages it was observed that age-dependent thickening of the basement membrane led to restrictions in contractility and that contractility loss could be reversed by reducing laminin A expression (Sessions *et al.*, 2017). Using super-resolution microscopy several collagen subtypes were detected in the T-tubules potentially rendering T-tubules structure and function sensitive to ageing, an hypothesis confirmed in male mice (Crossman *et al.*, 2017;Kong *et al.*, 2018).

ECM composition is dependent on the balance between synthesis and degradation of the ECM elements and network of macromolecular fibres. Matrix metalloproteinases (MMPs https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=MMP&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database) and tissue inhibitors of MMPs (TIMPs

https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=TIMP&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database) regulate degradation in a cascade of interrelated processes (Lindsey, 2018;Wang *et al.*, 2012).

A role for MMP-9 activation in ageing has evoked particular interest due to its multiple substrates. MMP-9 promotes replacement of elastin with collagen thereby increasing stiffening of the vessel wall, since elastin has a particularly important role in the vessel wall in providing reversible passive extensibility. MMP9 expression increases with age, and MMP-9 null male and female mice showed delayed development of age-dependent diastolic dysfunction, reversal of age-dependent increase in vascular permeability and inflammation markers (Yabluchanskiy *et al.*, 2014).

3.3. Change in function of endothelial cells, inflammatory cells and platelets

Other mechanisms might contribute to reduced IR tolerance in ageing. In both sexes, ageing is associated with enhanced platelet activation, aggregation, and secretion, reduced bleeding time and alterations in coagulation factors that are stored, synthesized, and/or released by platelets. In addition to platelet abnormalities, monocyte phenotype and function are altered in older adults, resulting in enhanced platelet-monocyte interaction and activation of thrombo-inflammatory pathways. Thus, platelets in older adults may be “primed” for exaggerated responses, enhancing susceptibility to adverse clinical outcomes in settings of IR (Mohebali *et al.*, 2014).

Ageing is also associated with chronic, low-grade inflammation, and characterised by increases in circulating pro-inflammatory cytokines and ROS, that can both be produced within endothelial cells or by neighbouring immune cells (Chung *et al.*, 2019). A vicious cycle activated by inflammation and oxidative stress, both of which exacerbate one another, impair nitric oxide (NO) bioavailability and endothelial function in aged arteries (Chung *et al.*, 2019). Age-associated dysfunction in cardiac microvascular endothelial cells and impaired induction of cardioprotective pathways may contribute to the increased severity of IR in older patients (Edelberg *et al.*, 2002). Moreover, ageing hearts have impaired angiogenic function, and following acute arterial obstruction, a decline in collateral number, diameter, and remodelling can be observed in aged mice, associated to impaired angiogenesis and resulting in more severe ischaemic tissue injury (Faber *et al.*,

2011). This age-dependent impairment in ischaemia-induced neovascularization might be due, at least in part, to oxidative stress-related dysfunction of endothelial progenitor cells (Lam, 2015).

4. Effect of age on cardioprotection treatments

4.1. Ischaemic conditioning

The conditioning strategies by which IR-induced damage of the myocardium is reduced include brief non-lethal periods of IR, which are performed either 1) before the sustained phase of IR directly to the heart (preconditioning, IPC) or to distant organs or tissues (remote ischaemic preconditioning, RIPC) or 2) at the onset of reperfusion (postconditioning, IPostC). Studies analysing myocardial conditioning in middle-aged or old animals are reviewed here or elsewhere (Boengler *et al.*, 2009;Calabrese, 2016;Ferdinandy *et al.*, 2014).

The effectiveness of IPC to protect against myocardial IR injury declines with age as shown in aged (18 months) mouse hearts *in vitro* (Peart *et al.*, 2014) and in mice older than 13 months *in vivo* (Boengler *et al.*, 2007). Also, the loss of IS reduction by IPC is demonstrated in 12-18 months (Adam *et al.*, 2013) or in 18 months old rat hearts *in vitro* (Schulman *et al.*, 2001). Whereas the majority of studies describes a loss of IPC-induced cardioprotection with age, some recent investigations also indicate preserved myocardial protection by IPC in 17 months old rat hearts *in vitro* (Webster *et al.*, 2017) or in 20 months old rat hearts *in vivo* (Dong *et al.*, 2017b).

Data on the effectiveness of the cardioprotection by IPostC are also controversial and seem to depend on the postconditioning algorithm as well as on the age of the animals. In aged cardiomyocytes, hypoxic postconditioning fails to increase cell survival (Zhang et al., 2018). In middle-aged mouse hearts *in vivo* (older 13 months), the effectiveness of IPostC depends on the strength of the postconditioning stimulus (Boengler et al., 2008), whereas the IS reduction by IPostC is lost in both middle-aged (12 months) and aged (20 months) mice *in vitro* (Perez et al., 2016). The abolished cardioprotection by IPostC in mice seen with advancing age is also evident in rats where IPostC is cardioprotective in aged (16-18 months) hearts *in vivo* (Yin et al., 2009), but not in old (24 months) hearts *in vitro* (Chen et al., 2016).

The cardioprotection by RIPC is present in young rat hearts *in vivo* but is abolished in aged (22-24 months) animals (Behmenburg *et al.*, 2017).

The loss of cardioprotection with ageing originates from reduced expression/phosphorylation and subcellular localization of proteins involved in the protective signalling cascades, like mitochondrial connexin43 https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=connexin43&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database, RISK pathway (including the prosurvival Akt https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=285 and ERK1/2 https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=514 cascades) , protein kinase C (PKC https://www.guidetopharmacology.org/GRAC/ReceptorFamiliesForward?type=ENZYME&familyId=286#node286) and signal transducer and activator of transcription 3 (STAT3 https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=STAT3&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database) (Boengler *et al.*, 2009;Kang *et al.*, 2017;Whittington *et al.*, 2013) as well as alterations of mitochondrial function (Figure 1). The activation of STAT3 is also attenuated in the presence of other cardiovascular risk factors (Pipicz *et al.*, 2018), conditions in which IPC and IPostC often are ineffective (Xia *et al.*, 2016). Therefore, for the cardioprotection of aged and diseased myocardium the development of strategies to preserve signal transduction and mitochondrial function are needed.

4.2. Pharmacological treatments

Age is known to confound the cardioprotective efficacy of pharmacological treatments which protect the heart against acute IR injury by targeting protective signalling pathways (Boengler *et al.*, 2009) (see table for summary). Pharmacological preconditioning with volatile anaesthetic agents such as isoflurane and sevoflurane was attenuated in aged rat hearts (20-24 months), and this effect was attributed to a failure to activate the Akt pathway to generate signalling ROS and to inhibit mPTP opening (Sniecinski and Liu, 2004;Nguyen *et al.*, 2008;Zhu *et al.*, 2010). Furthermore, isoflurane preconditioning was also attenuated in human atrial

cardiomyocytes harvested from aged (74 ± 6 years) compared to middle-aged (54 ±7 years) patients undergoing cardiac surgery, subjected to simulated IR injury (Mio *et al.*, 2008).

Pharmacologic postconditioning with the anaesthetic agents sevoflurane or isoflurane reduced IS in the myocardium of young (3-5 months age) but not aged (20-24 months age) rats, and this was attributed to the failure to activate the pro-survival Reperfusion Injury Salvage Kinase (RISK) signalling pathway, comprising Akt and extracellular signal-regulated kinase 1/2 (Erk1/2), and lack of inhibition of the mPTP opening (Li *et al.*, 2013;Chang *et al.*, 2012). Furthermore, pharmacological inhibition of glycogen synthase kinase 3 beta (GSK3β https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=GSK3b&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database), using SB-216763, reduced IS and inhibited mPTP opening in young (3-5 months age) but not aged (20-24 months age) rats (Zhu *et al.*, 2011). Interestingly, signalling through the protein kinase G (PKG https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=287) or protein kinase A (PKA https://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=284) pathway by sphingosine was preserved in aged rat hearts suggesting that this signalling cascade may not be affected (Vessey *et al.*, 2008;Vessey *et al.*, 2009).

5. Sex and gender

Several reports have shown that sex and gender play a major role in the development, perception, therapy, response to treatment and outcome of various diseases, including cardiovascular diseases (Regitz-Zagrosek and Kararigas, 2017). Sex and gender are often mistakenly used in the literature and there is a generalised lack of prospective design to properly study their effect. Sex is a biological attribute based on sex-chromosome assignment and defined by the resulting sex hormones, as well as genetic and epigenetic factors that lead to sex differences. Gender is a construct that includes socio-cultural and behavioral aspects, which give rise to gender identity, norms and relations. There are close interactions between sex and gender that affect human physiology and pathology, as sex hormones may influence behaviour and lifestyle, while gender may modify a patient's adherence to treatment,

for example. However, for the purpose of the present review article, we consider here the biological aspects that affect disease and lead to pronounced sex differences. In particular, we discuss the hormonal, genetic and epigenetic mechanisms that contribute to different types of IHD developing between men and women.

5.1. Sex differences in tolerance to IR injury in preclinical and clinical studies

It is well-established that sex impacts on all aspects of cardiovascular health and disease. Sex-specific differences exist in the epidemiology, clinical presentation, underlying pathophysiological mechanisms, treatment, and clinical outcomes in patients with acute MI (AMI) (Mehta *et al.*, 2016). However, experimental and clinical studies taking sex differences into account are scarce. Therefore, it is essential to include both sexes when undertaking experimental and clinical studies investigating AMI and consider the possible interactions between sex and comorbidities.

Women are often older than men when they present with their first AMI (mean age of 72 years compared to 65 years) (Benjamin *et al.*, 2019), and this has been attributed to the protective role of circulating oestrogens against endothelial dysfunction and lipid deposition in the vasculature (Chakrabarti *et al.*, 2014). Consistent with this assumption, the incidence of AMI increases substantially in postmenopausal women (Benjamin *et al.*, 2019). In terms of clinical presentation of AMI, women most often present with atypical chest pain and angina equivalent symptoms (such as dyspnea, weakness, fatigue, and indigestion), when compared to men who present with typical chest pain (Rubini *et al.*, 2014). This difference impacts on the management and mortality of AMI, as many female patients are misdiagnosed. A number of studies have shown that women with AMI are less likely to be managed using guideline-recommended medical therapies (Jneid *et al.*, 2008); undergo cardiac catheterisation (Jneid *et al.*, 2008); and to receive timely reperfusion (Mehilli *et al.*, 2005). In terms of pathophysiology underlying AMI, there are important sex differences. Plaque rupture, the main cause of AMI, is less common in women, when compared to men (55% vs. 76%, respectively) (Falk *et al.*, 2013), which is of major relevance given that AMI without obstructive coronary artery disease is more common among women (Chokshi *et al.*, 2010).

In experimental animal studies of AMI there are sex differences in terms of susceptibility to acute IR injury [reviewed in (Blenck *et al.*, 2016)]. In aged female animals, reduced tolerance to IR has been largely attributed to oestradiol deficiency.

Preclinical studies have demonstrated the beneficial effects of chronic or acute exogenous oestradiol administration in adult male and female, gonad-intact and gonadectomised animals (Korzick and Lancaster, 2013). However, conflicting evidence exists regarding the efficacy of menopausal hormone therapy in cardioprotection of postmenopausal women (Korzick and Lancaster, 2013). Similarly, in older men, the decline in testosterone levels with advancing age is associated with a number of symptoms and adverse cardiovascular outcomes. Yet, the advantages of testosterone therapy in older hypogonadal men have not been adequately resolved (Orwoll, 2017). Despite some inconsistencies observed using rodent models of AMI, in general, female mice and rats appear to be protected against acute myocardial IR injury compared to males (Dow *et al.*, 2015), specifically after more prolonged ischemic time (Penna *et al.*, 2009;Dow *et al.*, 2015). Neutrophil infiltration, post-AMI adverse left-ventricular (LV) remodelling, cardiac rupture, and mortality were lower in female when compared to male mice, irrespective of IS (Cavasin *et al.*, 2004;Fang *et al.*, 2007;Shioura *et al.*, 2008). The mechanisms underlying sex-dependent differences in the susceptibility to acute myocardial IR injury include the presence of oestrogen, lower calcium levels and enhanced NO signalling (Murphy and Steenbergen, 2007), less apoptosis (Wang *et al.*, 2010), more efficient ROS-generated aldehydes detoxification (Lagranha *et al.*, 2010) and more favourable interplay between ROS and the expression/activation of some kinase-dependent survival pathways (Ciocci *et al.*, 2018;Wang *et al.*, 2010;Pagliaro and Penna, 2017) in females than in males.

5.2. Biological mechanisms

5.2.1 Effect of hormones on IR injury

All the steroid sex hormones, oestrogens, progesterone and androgens, modify IR injury, especially when given at pharmacological concentrations. These hormones exert a wide variety of responses via their nuclear receptors and have additional effects via non-genomic cellular responses in the cardiovascular system (Lucas-Herald *et al.*, 2017). The non-genomic effects are particularly well described for oestradiol in the context of acute cardioprotection (Deschamps and Murphy, 2009;Sovershaev *et al.*, 2006). Non-nuclear receptors consist of a G-protein coupled oestrogen receptor and extra-nuclear oestrogen receptors subtypes α and β (Deschamps *et al.*, 2010). Both male and female hearts harbor oestrogen receptors.

Oestradiol is a cardioprotective agent both in male and female hearts when given as acute pretreatment, although a slightly weaker effect was observed in male rat hearts (Sovershaev *et al.*, 2006). The cardioprotection is due to the activation of phosphoinositide 3-kinase (PI3-K) pathway converging on GSK3β and eventually leading to mitochondrial protection. Another cardioprotective mechanism stimulated by oestradiol is the upregulation of NO-signalling and S-nitrosylated proteins (Deschamps *et al.*, 2010). Interestingly, antiblastic-induced cardiotoxicity, wich shares some mechanisms of cardiomyocyte damage with those of IR injury (excessive ROS production, mitochondrial dysfunction) has been shown to be ameliorated by oestrogens (Cadeddu *et al.*, 2019). The effect of acute administration of progesterone is variable and clearly dependent on the model. Progesterone reduces calcium sensitivity, contractility and action potential duration in female hearts but not in male hearts (Feridooni *et al.*, 2017). Acute administration of the main androgen subtype, testosterone, might also provide cardioprotection. The heart of both sexes contains aromatase, the enzyme responsible for converting androgens to oestrogens. Some of the reported effects of testosterone might therefore be oestrogen-mediated. Interestingly, increasing aromatase in male mice leads to upregulation of Akt phosphorylation, reduction in ischaemic contracture but reduced recovery of systolic function at reperfusion in isolated hearts (Bell *et al.*, 2014).

The results of studies investigating the role of these hormones on IR injury in a chronic (more physiological) setting are variable. Some authors report female hearts to be less vulnerable, others do not find significant differences. These discrepancies may be due to differences in the endpoint-selection and the experimental models. In addition to age, several pathophysiological changes might abrogate the female advantage (Bell *et al.*, 2008). Exposure to angiotensin II leads to marked up-regulation of collagen and microscopic fibrosis and substantial impairment of post-ischaemic function after IR compared to sham rats. In this setting, sex-dependent differences in functional recovery were not significant despite differences in angiotensin II-induced molecular remodelling. When IR injury was compared among rats of the same sex with and without intact gonads, loss of gonadal function was associated with increased IR injury in box sexes (Ross and Howlett, 2012) These results might differ somewhat depending on the timing of the gonadectomy (i.e. young or fully mature animals) and the duration of the study.

However, experiments using specific oestrogen receptor knockout mice confirm the beneficial role of oestrogen in IR injury (Deschamps *et al.*, 2010).

Other hormones with sex-dependent concentration differences in a lifetime perspective are oxytocin and the insulin-like peptide relaxin, both produced in increasing amounts during pregnancy. Under experimental conditions, both have been shown to be cardioprotective during IR (Sarwar *et al.*, 2017;Gonzalez-Reyes *et al.*, 2015).

5.2.2. Genetic, epigenetic mechanisms

Genetic mechanisms strongly affect susceptibility to IR insult. Surprisingly, the number of X chromosomes negatively affects cardiac IR injury in mice, suggesting that one X is better than two, irrespective of gonadal sex (Li *et al.*, 2014). Higher susceptibility of XX mice is due to the number of X chromosomes rather than the absence of a Y chromosome. Interestingly, most X chromosome genes do not show large sex differences in their levels of expression, since one X chromosome is randomly transcriptionally silenced in XX female adult somatic cells. However, some genes “escape” inactivation and are expressed from each X chromosome, so that they have higher gene transcript levels in XX cells compared to XY cells (Arnold *et al.*, 2017).

After completion of the human genome project, several candidate gene studies have been conducted to estimate the effects of specific gene variants on coronary artery disease (CAD) risk in both sexes (Khera *et al.*, 2016). Several sex-specific genetic CAD risk predictors have been identified, and while some variants conferred risk in both sexes, others showed significant effects only in males (Yamada *et al.*, 2002;Silander *et al.*, 2008;Khera *et al.*, 2016). However, the majority of common CAD variants identified so far only cause modest increases in CAD risk by unknown mechanisms, and whether sex-specific effects exist has been rarely tested. Since mitochondria are mainly derived from the mother (Byars and Inouye, 2018), variations in mtDNA might also account, at least in part, for sex differences. In addition, gene variants on the Y chromosome might also contribute to cardiovascular phenotypes in men. The Y chromosome is routinely excluded from large-scale GWAS and it is therefore the most underexplored portion of the human genome to date.

In addition to gene variants, a number of epigenetic modifications can alter gene expression and IHD risk in a sex-specific manner (Hartman *et al.*, 2018).

Firstly, epigenetic mechanisms control sex-specific gene expression during development, and such early epigenetic changes can alter the phenotype much later and even affect subsequent generations (Hartman *et al.*, 2018). Prenatal hypoxia has been shown to modulate DNA methylation and repression of cardiac PKC epsilon https://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1486 gene in a sex-dependent manner, linking fetal hypoxia and pathophysiological consequences in the hearts of adult offspring (Patterson *et al.*, 2010). Consistent with these preclinical results, the risk of MI in women is associated with enhanced DNA methylation at specific loci sensitive to prenatal conditions (Talens *et al.*, 2012). In post-natal life, oestrogens have been shown to protect the myocardium against IR through epigenetic regulation of urocortin receptor type 2 levels in female rats (Cong *et al.*, 2013). Unfortunately, the majority of studies investigating the role of epigenetics in cardioprotection during post-natal life did not stratify data by sex (Hartman *et al.*, 2018). Therefore, future studies will be needed to precisely determine the role of sex on epigenetic regulatory processes in IR injury.

5.2.3. Sex differences in cardiovascular ageing

The heart exhibits sex-biased distinct structural and functional features at all ages that contribute to differences in the pattern of biological ageing between males and females. Morphometric analysis using autopsied human hearts has revealed that men experience a more pronounced age-dependent decline in the number of ventricular cardiomyocytes (and compensatory increase in cardiomyocyte volume) than women (Olivetti *et al.*, 1995). Consistent with this, it has been shown that cardiomyocytes from aged male rats develop a more significant reduction in peak calcium transients and systolic function than aged females (Howlett, 2010). Whereas some prevalent pathophysiological changes present in ageing, like atrial fibrosis, do not clearly differ between the sexes, others may differ in their magnitude and prevalence, such as aortic valve calcification (men >women), reduced sensitivity to beta-adrenergic stimulation (men >women), increased LV wall thickness (women >men), concentric remodeling and diastolic dysfunction (women> men) (Keller and Howlett, 2016). Particularly the latter might be due to higher levels of ECM components in left ventricular samples of older women compared with those of younger women or younger and older men (Dworatzek *et al.*, 2016). However, the

molecular mechanisms are poorly understood. Collectively, the contribution of biological sex to cardiovascular ageing deserves further investigation.

5.3. Sex-specific pharmacology in the heart

Basic anatomical and physiological features, such as body and organ weight, body composition and fat content and distribution, liver metabolism and renal function, influence the effects of drugs. Given that these features differ between males and females, efficacy and safety of pharmacological treatments may also be sex-specific. Consequently, the risk of experiencing adverse drug reactions and their severity are different between male and female patients. Sex-specific drug therapeutic and deleterious effects are expected to stem from sex differences in pharmacokinetic and pharmacodynamic properties of drugs, which, however, are under-investigated (Gaignebet and Kararigas, 2017). Nevertheless, it is well known that there are pronounced sex differences in the levels of the cytochrome P450 enzymes and their metabolism of drugs (Schwartz, 2007;Freire *et al.*, 2011). Notably, protection from pre- and post-conditioning by nitrite depends on *ALDH2https://www.guidetopharmacology.org/GRAC/DatabaseSearchForward?searchString=ALDH2&searchCategories=all&species=none&type=all&comments=includeComments&order=rank&submit=Search+Database* genotype (Ormerod *et al.*, 2017). A major contradiction, though, is the fact that these differences are usually not considered when defining drug dosing. Similarly, it is well established that women are underrepresented in the design and development of novel therapeutics and technologies. As a result, assessment of drug efficacy and safety is primarily performed in one sex and it is taken for granted that they should be the same in the other sex, which, in many situations, is not the case. Collectively, this practice leads to non-effectively-treated patients contributing to further suffering and important socioeconomic implications.

6. Summary and recommendations for future research

Biological age is the strongest predictor of cardiovascular health. Due to the progressive ageing of human population, heart-related diseases have become the leading cause of death and disability worldwide; among them, IHD accounts for the majority of this medical impact (Moran *et al.*, 2014;Benjamin *et al.*, 2019).

Importantly, being old has been identified to be an independent determinant of the extension of MI and the outcome of cardioprotective strategies after an IR episode. During ageing, a plethora of molecular changes occur (Figure 2). Aged cardiomyocytes display excessive mitochondrial calcium accumulation and ROS production that play a fundamental role in the susceptibility to mPTP opening and cell death upon IR injury in multiple experimental models (Briston *et al.*, 2019). Nevertheless, the exact molecular nature of mPTP remains elusive and attempts to translate experimental strategies aimed at preventing cell death secondary to mPTP opening to the clinical context have failed (Trankle *et al.*, 2016). Moreover, ageing induces a variable degree of cell energy demand/supply mismatch secondary to altered cytoarchitecture (deficient SR-mitochondria calcium exchange and defective supramolecular assembly of respiratory chain supercomplexes) but also to inadequate transfer of intracellular energy and progressive metabolic remodelling (i.e., partial replacement of OXPHOS by glycolysis) (Fernandez-Sanz *et al.*, 2014;Ruiz-Meana *et al.*, 2019;Tepp *et al.*, 2017;Ramirez-Camacho *et al.*, 2019). Whether these mechanisms impact on the outcome of CAD in elderly patients remains unclear.

Advanced age may also aggravate adverse remodelling and the occurrence of arrhythmias secondary to MI due to changes in the composition of ECM and altered inflammatory response (Mohebali *et al.*, 2014;Meschiari *et al.*, 2017). Platelet abnormalities and enhanced platelet-monocyte interactions present in ageing modify the thrombo-inflammatory profile of elderly patients, and might require therapeutic adjustments and/or differential approaches, the efficacy of which has not been specifically addressed in clinical trials. Some canonical intracellular signalling pathways involved in the endogenous cardioprotection become hampered in the aged heart, rendering conditioning strategies less -or non- effective (Boengler *et al.*, 2009). This is partly explained by reduced expression/phosphorylation and altered trafficking and localization of intracellular proteins that participate in protective signalling cascades, but also by mitochondrial dysfunction during ageing, increased production of circulating cytokines and ROS within endothelial cells, reduced NO bioavailability and less neovascularization, factors that increase the threshold necessary to trigger cardioprotection (Edelberg *et al.*, 2002). Cardioprotection includes multitarget strategies (Figure 3). Further studies are needed to determine whether the confounding effect of age can be overcome by increasing the

cardioprotective stimulus (such as combining agents), and to elucidate the mechanisms through which ages negatively impacts on cardioprotective signalling pathways.

The intersection between ageing and sex has pathophysiological consequences in terms of cardio protection. Ageing leads to a decline in the oestrogen and testosterone levels. It is well established that the oestrogen status of females modulates the susceptibility of the heart to IR injury. Similarly, reduction in testosterone levels in older men worsens cardiovascular outcomes. Yet, the benefit of menopausal hormone therapy (either oestradiol or testosterone) is controversial (Korzick and Lancaster, 2013;Orwoll, 2017). Sex also has specific genetic and epigenetic implications, the contribution of which to the susceptibility to IR injury has not been fully unraveled. The efficacy and safety profiles of therapeutic interventions differ among the sexes. With some exceptions, the risk of experiencing adverse drug reactions is higher in women than in men. Nevertheless, these differences are not considered when defining the dose and timing of treatments and women tend to be underrepresented in clinical trials. Therefore, a better understanding of the effects of sex in pathophysiology and pharmacology would lead to the identification of targets and the development of pharmacotherapies applied in a sex-specific manner, thereby contributing towards a more appropriate and individualised medical care for both men and women (Kararigas *et al.*, 2016). To this extent, artificial intelligence by means of *in silico* models may prove helpful in predicting sex-specific drug responses in large scales, which could be translated into clinical practice (Huang *et al.*, 2018;Cui *et al.*, 2018).

Current evidence argues for more research conducted in appropriate experimental models, where advanced age and biological sex are taken into consideration, as well as cardioprotective clinical trials carefully designed to integrate the basic scientific knowledge on these confounders to the patients.

Nomenclature of Targets and Ligands:

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

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Table: Summary of major studies investigating the confounding effect of age on the cardioprotective efficacy of pharmacological conditioning agents

Pharmacological conditioning agent	**Model of IRI**	**Confounded by age**	**Mechanism**	**References**
Preconditioning with adenosine A1 receptor agonist (CCPA1), PKC activator (DAG), and mitochondrial K^{ATP} channel activator (diazoxide)	Rat	Yes	Failure to activate known mediators of IPC - adenosine A1 receptor, PKC, and mitochondrial K^{ATP} channel	(Schulman *et al.*, 2001)
Volatile anaesthetic preconditioning (isoflurane/sevoflurane)	Rat Human atrial cardiomyocytes	Yes	Failure to activate Akt, produce signalling ROS and inhibit MPTP opening	(Sniecinski and Liu, 2004;Nguyen *et al.*, 2008;Zhu *et al.*, 2010;Mio *et al.*, 2008)
Helium preconditioning	Rat	Yes	Failure to activate Ca^{2+}-sensitive potassium channels and uncouple mitochondria	(Heinen *et al.*, 2008)
Dexmedetomidine preconditioning	Rat	Yes	Failure to have antioxidant effect	(Dong *et al.*, 2017a)
High-dose folic acid preconditioning	Rat	Yes	Failure to maintain dimerization of eNOS	(Zuurbier *et al.*, 2014)
Volatile anaesthetic postconditioning (isoflurane/sevoflurane)	Rat	Yes	Failure to activate Akt and Erk1/2 and inhibit MPTP opening	(Li *et al.*, 2013;Chang *et al.*, 2012)
SB-216763 postconditioning (GSK-3β inhibitor)	Mouse	Yes	Failure to inhibit MPTP opening	(Zhu *et al.*, 2011)
Sphingosine	Rat	No	Persisting cardioprotection via PKG and PKA	(Vessey *et al.*, 2009;Vessey *et al.*, 2008)
Amobarbital postconditioning (reversible mitochondrial inhibitor)	Rat	No		(Chen *et al.*, 2012)

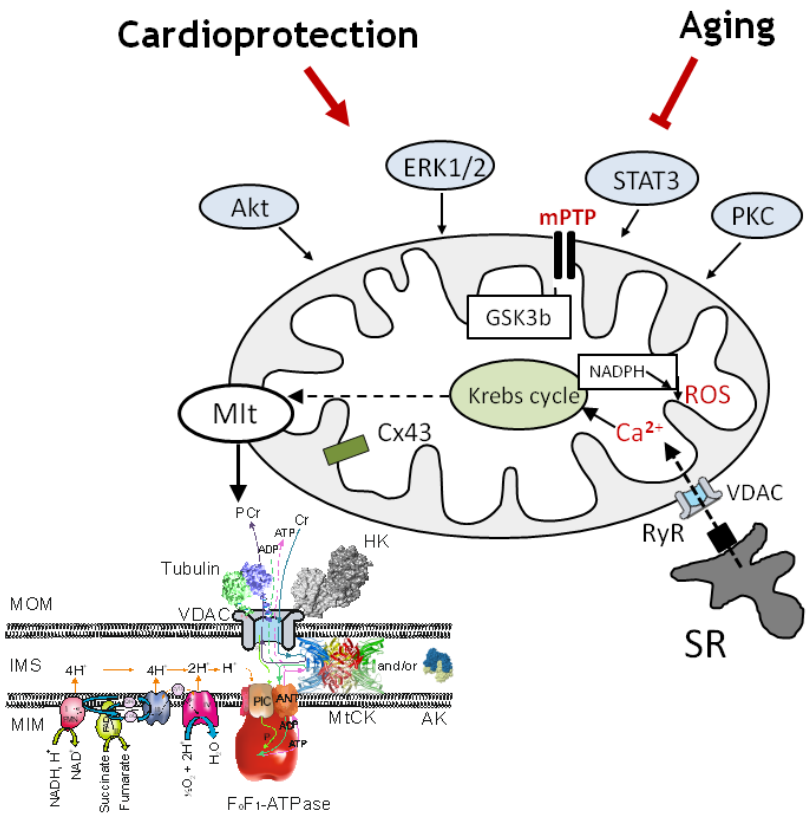

Figure 1

Figure 1: Molecular players involved in the mitochondrial cardioprotective response during IR that may be altered in the aged cardiomyocytes. Supercomplex mitochondrial interactosome (MIt), consisting of mitochondrial ATP synthase, phosphate carrier (PiC), voltage-dependent anion channel (VDAC), mitochondrial creatine kinase (MtCK), adenine nucleotide translocase (ANT) and respiratory chain complexes, is shown separately. MtCK: mitochondrial creatine kinase (MtCK); MIM: mitochondrial inner membrane; MOM: mitochondrial outer membranes; IMS: intermembrane space; HK: hexokinase; AK: adenylate kinase; SR: sarcoplasmic reticulum.

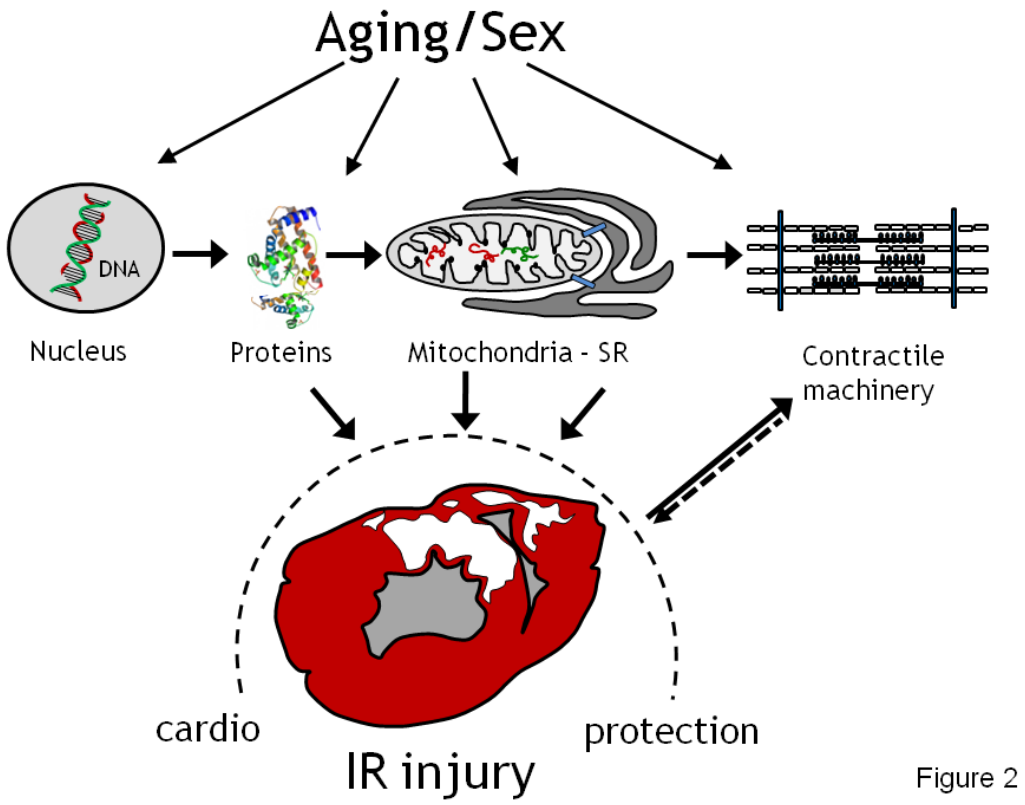

Figure 2: Intracellular targets altered by aging and sex that can be targeted by specific cardioprotective strategies.

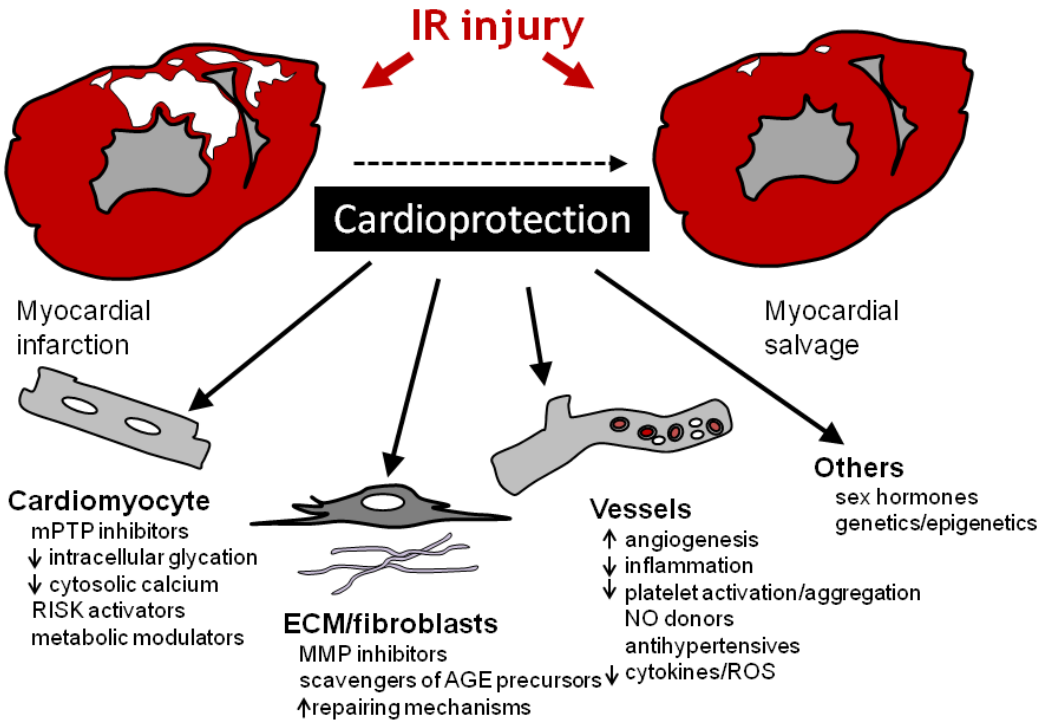

Figure 3

Figure 3: Multitarget strategies involved in cardioprotection. Multiple cardiac and extra-cardiac factors modulate IR injury in the heart and are potentially targetable by cardioprotective interventions.