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Mesenchymal stromal cells for improvement of cardiac function following acute myocardial infarction: a matter of timing

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1 **Mesenchymal stromal cells for improvement of cardiac function following**
2 **acute myocardial infarction: a matter of timing**

3

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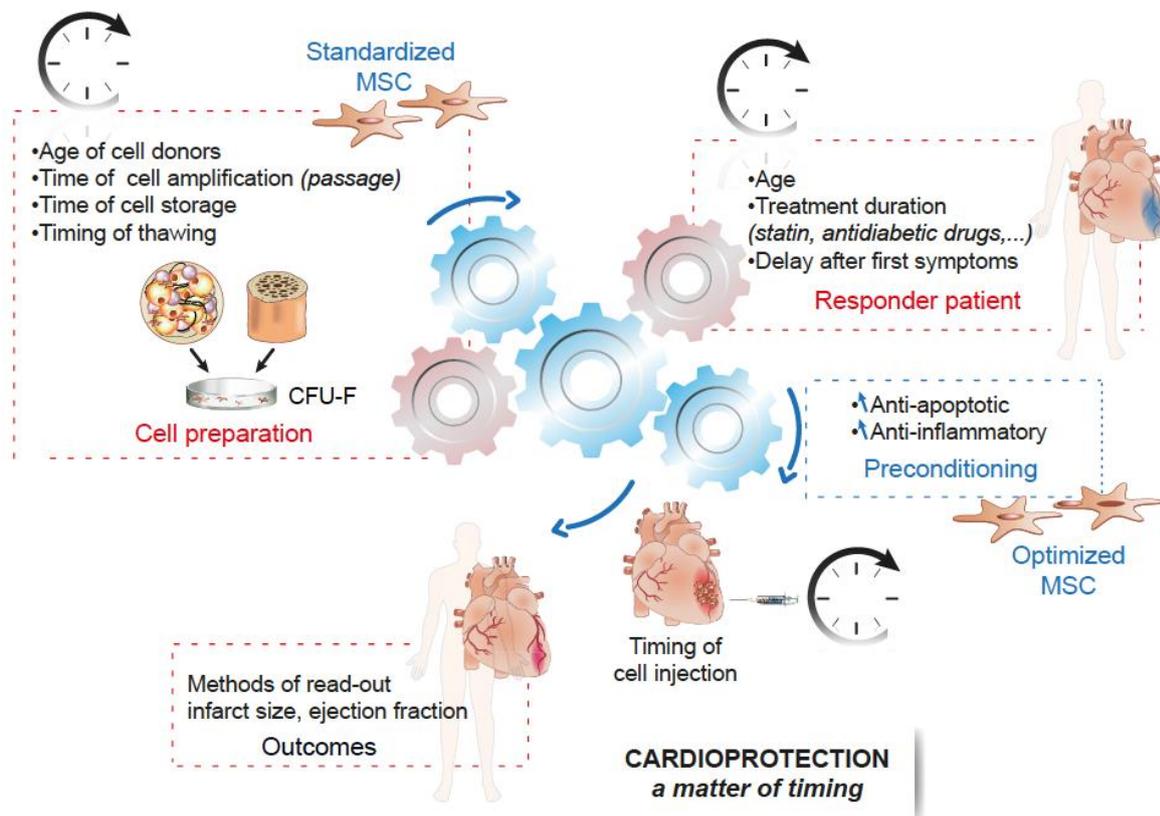
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20

21



22 **Clinical highlights**

23 The use of mesenchymal stromal/stem cells (MSC) in clinical trials has provided inconsistent outcomes
24 in patients with acute myocardial infarction (AMI), which contrasts with their promising results shown
25 in preclinical studies. The challenge for achieving success in the clinical setting is to generate an
26 optimized cell therapeutic product able to prevent cardiac injury because fully activated to release
27 cardioprotective molecules in the infarcted myocardium. In this context, data from the literature
28 highlighted the "matter of timing" as a critical parameter for the success of MSC therapy. Indeed, the
29 notion of time is involved in different ways such as between MSC culture and administration to patients,
30 the age of MSC donors and the age of AMI patients, and identified as a major parameter for the success
31 of MSC-based therapy. Current efforts are focused on MSC optimization in particular to circumvent the
32 effects related to an inappropriate timing.

33

34 **Abstract**

35 Acute myocardial infarction (AMI) is the leading cause of cardiovascular death and remains the most
36 common cause of heart failure (HF). Re-opening of the occluded artery i.e., reperfusion, is the only way
37 to save the myocardium. However, the expected benefits on infarct size are disappointing due to the
38 reperfusion paradox, which also induces specific cell death. These ischemia-reperfusion (IR) lesions can
39 account for up to 50% of final infarct size, a major determinant for both mortality and the risk of heart
40 failure (morbidity). In this review, we first provide a detailed description of the cell death and
41 inflammation mechanisms as features of IR injury, cardioprotective strategies such as ischemic
42 postconditioning (PostC) as well as their underlying mechanisms. Due to their biological properties, the
43 use of Mesenchymal Stromal/Stem Cells (MSC) has been considered as a potential therapeutic approach
44 in AMI. Despite promising results and evidence of safety in preclinical studies using MSC, the effects
45 reported in clinical trials are not conclusive and even inconsistent. These discrepancies were attributed
46 to many parameters such as donor age, *in vitro* culture and storage time as well as injection time window
47 after AMI, which alter MSC therapeutic properties. In the context of AMI, future directions will be to
48 generate MSC with enhanced properties in order to limit cell death in myocardial tissue, thereby reduce
49 infarct size, and improve the healing phase to increase post-infarct myocardial performance.

50

51 **Running head:** MSC to decrease cardiac injury

52

53 **Keywords:** mesenchymal stromal/stem cells; cardioprotection; apoptosis; myocardial infarction;
54 conditioning; inflammation.

55

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142 I- INTRODUCTION

143 Acute myocardial infarction (AMI) ranks first in the leading cause of cardiovascular death and
144 is a strong provider of heart failure. According to the *World Health Organization*, cardiovascular
145 diseases are predicted to stay at the first rank until 2030 and this burden will be worsened due to an
146 aging population, an exponential growing number of diabetic patients and the COVID-19 pandemic
147 leading to cardiovascular complications. In 2023, prompt revascularization of the culprit artery to
148 reperfuse the myocardium is the only treatment for the care of AMI patients recommended by the
149 societies of cardiology in Europe and in USA. While timely reperfusion is crucial for AMI patients, still
150 15 to 30% of patients are admitted too late at the hospital for reperfusion therapy to be effective. In
151 addition, there is also a significant number of patients for whom reperfusion is not effective at all.
152 Optimized techniques of angioplasty and care management of patients have allowed to decrease infarct
153 size, a major determinant of mortality. However, for the patients surviving after AMI, morbidity rate for
154 heart failure is still rising. There is an urgent need to develop cardioprotective strategies to reduce the
155 high morbidity and mortality rates and the total burden in terms of public health and societal costs.
156 This review gathers authors with complementary expertise with the goal of identifying in the large panel
157 of studies in the field, the best conditions for providing a successful cell therapy tool able to prevent the
158 complex myocardial injury following AMI and for which no effective treatment is available. Section II
159 describes the main mechanisms involved in ischemia and reperfusion injury that are targeted by
160 mesenchymal stem/stromal cell-based therapy. While the goal of reperfusion is to save the myocardium
161 and decrease infarct size, it has deleterious effects, paradoxically exacerbating cardiac cell death at the
162 time of patient care and leading to specific so-called ischemia-reperfusion (IR) injury. In this review,
163 we describe the historical context of the discovery of both ischemic injury and reperfusion injury as well
164 as the mechanisms of regulated cell death (RCD) and inflammation as features of myocardial infarction
165 and reperfusion. We also describe the endogenous mechanisms of cardioprotection involved in ischemic
166 conditioning, in particular ischemic postconditioning (PostC), which is clinically relevant and also
167 represents a major concept attesting the existence of IR injury. Remote conditioning is also described
168 since it allows a non-invasive care of the patient. However, it is striking that in over 30 years of research,
169 all monotherapies tested in AMI patients have failed and that nowadays there is still no clinical treatment
170 for reperfusion injury. This explains why multi-target therapies such as inhibition of both apoptosis and
171 inflammation, identified as main contributors of PostC and mesenchymal stromal/stem cell (MSC)
172 therapeutic effects, have been explored and have provided promising results. This section highlights the
173 complexity of the mechanisms underlying the lesions and their unfolding in time highlighting the fact
174 that the treatments, to be effective, must be applied in a compatible and optimal temporal window.
175 Section III describes the various embryonic origins and the heterogeneity of MSC that exists at different
176 levels. The properties of MSC, in particular their anti-apoptotic, immunoregulatory effects and anti-
177 inflammatory potential, are discussed by highlighting the underlying mechanisms, including their ability

178 to release bioactive molecules and transfer organelles such as mitochondria and extracellular vesicles
 179 (EV). The description of the phenotypic and functional heterogeneity of MSC is addressed in order to
 180 identify strategies to generate standardized and optimized cells to achieve their maximal therapeutic
 181 effect in AMI patients. Among these strategies, the enhancement of MSC survival rate and engraftment
 182 in the damaged tissue as well as their anti-apoptotic properties on cardiac cells appears as a promising
 183 approach.

184 Section IV is focused on the results achieved with MSC during acute myocardial infarction. We report
 185 the promising issues obtained in preclinical studies by contrast to the clinical trials that reveal
 186 heterogeneous and inconsistent outcomes, although the safety and efficacy of MSC in phase I and II
 187 have been demonstrated. The second part of section IV analyzes the factors that could explain such an
 188 heterogeneity of results in patients compared to preclinical data i.e., the source of MSC and the *in vitro*
 189 amplification process of MSC obtained from patients. Finally, this section describes the methods used
 190 to optimize MSC therapeutic potential and bring them into the routine clinical practice. In addition,
 191 analysis and identification of the critical parameters including MSC doses, their routes and timing of
 192 administration, should allow to further enhance the success of MSC-based therapies.

193

194

195 **II- Myocardial injury and aspects of cardioprotection**

196 In this section will be described the elements helping to understand the mechanisms of action of the
 197 MSC involved in their cardioprotective effects. In particular, since MSC have been reported to protect
 198 the heart in the context of AMI through an inhibition of both regulated cell death cascades and
 199 inflammation. All the mechanisms underlying cardiac injury at the acute and subacute phases of
 200 myocardial infarction as well as those mediating cardioprotection will be described.

201

202 **A- Mechanisms of cardiac injury**

203 **1- Historical consideration and definitions**

204 Myocardial infarction (MI) originates with an ischemic injury (**FIGURE 1A**) induced by the complete
 205 occlusion or a severe reduction of the blood flow in a coronary artery. Ischemia, whose Greek etymology,
 206 *ισχημια*, means “withholding blood”, generates alterations in the deprived cardiac region, which,
 207 depending on the severity of the reduction of the coronary blood flow, will lead to the death of cells and
 208 hence the heart muscle. The area perfused by the coronary artery downstream the site of occlusion
 209 corresponds to the area at risk of infarction. Within a given area at risk, both the severity and duration
 210 of the reduction in coronary blood flow will determine the amount of injury.

211

212 **Ischemic injury (FIGURE 1A)** In fact, infarct size depends on several parameters (1) including
 213 the site of occlusion (proximal *versus* distal), which will determine the extent of area at risk (2), the

214 density of the collateral capillary network involved in the coronary circulation (1, 3, 4), the ischemia
215 duration (3, 5) as well as the haemodynamic parameters during ischemia such as heart rate (6, 7).

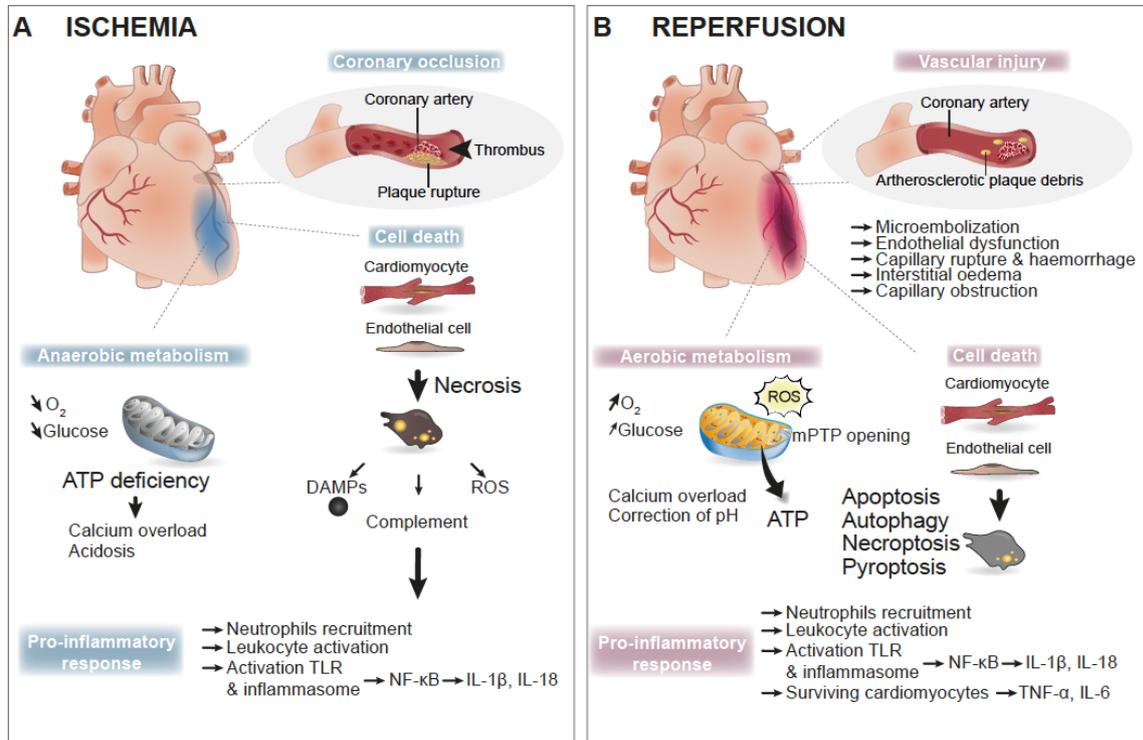
216 As a result of the interruption or reduction of blood supply, the lack in oxygen in the ischemic tissue
217 leads to the cessation of the respiratory chain in the mitochondria shifting their metabolism from aerobic
218 to anaerobic (8) *via* mechanisms well detailed in a previous review published in Physiological Reviews
219 in 2008 (9). The metabolic changes result in ATP deficiency leading to an increased production of
220 reactive oxygen species (ROS), acidosis and to impaired ion homeostasis (10). In particular, increased
221 cell resting membrane potential, reduced excitability associated to an increased potassium (K^+) efflux,
222 shorten action potential duration and decrease conduction velocity. These electrophysiological
223 modifications generate a potential substrate for the generation of arrhythmias leading to ventricular
224 fibrillation and sudden cardiac death (11, 12). Severe ischemic conditions during MI induce cardiac cell
225 death mainly by necrosis leading to the disruption of the cell membrane and the passive release of
226 DAMPS (*Danger associated molecular patterns*) (13).

227 In fact, both reversible and irreversible injuries are induced during myocardial ischemia. R.B. Jennings
228 was the first to clearly show that all myocytes of the sub-endocardium of the canine heart survived when
229 occlusion of the circumflex artery was maintained less than 15 minutes, due to reversible injury (13).
230 However, when the ischemic period was prolonged until 60 minutes, most of the subendocardial tissue
231 was dead, resulting in an irreversible injury. In those experiments, dead cells were evidenced due to their
232 degraded architecture with either membrane disruption, prominent “contraction bands”, or calcified
233 mitochondria. This princeps study clearly evidenced two major results: first, the notion of irreversibility
234 because some myocytes after reperfusion did not survive and second, restoration of arterial blood flow
235 after revascularization allowing the salvage of ischemic myocytes still alive, constituting the basis of
236 the actual reperfusion therapy (13, 14). K. Reimer and R. Jennings reported both the time line and the
237 wavefront of cell death within the transmural myocardium, starting from the sub-endocardium and
238 ending in the sub-epicardium (3). Following MI, around 1 billion cardiac cells die in response to
239 ischemia (15). The resulting “infarct size”, corresponding to the dead tissue, is mainly correlated with
240 the duration of the coronary occlusion (3, 5, 13, 16) and constitutes a major determinant of myocardial
241 functional recovery and mortality after MI (17).

242 Ischemic injury is also associated with functional changes in the infarcted tissue due to the lack of high-
243 energy phosphates. These metabolic changes take place following the switch from aerobic to anaerobic
244 glycolysis providing new high energy phosphates from glucose issued from glycogen stores. As a result
245 of lactate accumulation, the cytosol becomes acidic, the pH dumping from 6.6 after 10 minutes to 5.8
246 after 50 minutes of ischemia (18). Disturbance in calcium homeostasis is also a main characteristic of
247 ischemic injury due to the lack of ATP and the shutdown of ion pumps or channels leading to a defect
248 in contractile performance of the myocardium (19). At the acute phase, necrotic cardiac cells release
249 their cytosolic contents into the extracellular space, activating innate immune pathways, which have
250 been previously described in details by Xu et al. in Physiological Review (20). This resulting transient

251 but intense inflammatory response (see section II-A-3 below: *Contribution of inflammation*) allows
 252 repair of injured tissue through wound resorption and scar formation, but was also reported to prevent
 253 cardiac regeneration in adult mammals (21-23).

254



255

256 **FIGURE 1: Mechanisms of ischemia and reperfusion injury.**

257 **A:** Ischemia results from coronary occlusion, leading to a shift from aerobic to anaerobic metabolism.
 258 The lack of ATP induces the shutdown of ion channels and pumps leading to calcium overload. Acidosis
 259 results from the accumulation of lactate in the cytosol. Necrotic cells release DAMPs, produce ROS,
 260 and activate the complement cascade, inducing a burst of sterile inflammation: neutrophils and
 261 leukocytes are recruited, the inflammasome activates in circulating cells leading to the release of IL-1β
 262 and IL-18.

263 **B:** Upon reperfusion, aerobic metabolism is restored and mitochondria again produce ATP.
 264 Reperfusion injury is complex and multifactorial involving ROS, calcium (Ca^{2+}) overload, mPTP
 265 opening, vascular complications and cardiac cell death mediated by apoptosis, necroptosis, autophagy
 266 and pyroptosis. The pro-inflammatory response is exacerbated and mediated by TLR (2, 4, and 9) and
 267 NLRP3 signaling cascades. Activation of the inflammasome in circulating cells leads to release of IL-
 268 1β and IL-18 and to caspase-1 mediated pyroptosis in cardiomyocytes. Surviving cardiomyocyte in the
 269 border zone produce TNF-α and IL-6 pro-inflammatory cytokines.

270

271

272

273 **Reperfusion injury (FIGURE 1B)** J. Ross' laboratory was the first in 1972 to describe in a
 274 preclinical dog model of AMI that reperfusion, after 180 min coronary occlusion, reduced infarct size
 275 and restored contractile function, a cardioprotective effect maintained during 1 week after reperfusion
 276 (24, 25). With the aim to limit infarct size and improve cardiac function in patients after AMI,
 277 reperfusion therapy was translated into the clinics in the early 80's, first using intracoronary (IC)
 streptokinase infusion that allowed to restore coronary flow in 70 % to 90% of the cases and improved

278 left ventricular function compared to the non-reperused myocardium groups (26-29). However, despite
279 treatment with thrombolytics, the coronary stenoses that contributed to the infarction could only be
280 treated surgically by coronary bypass. Hartzler et al. reported the first experience in AMI patients using
281 percutaneous transluminal coronary angioplasty as an alternative to bypass surgery, alone or in addition
282 to thrombolysis (30). Percutaneous coronary intervention (PCI) later provided benefits in terms of both
283 myocardial salvage and clinical outcome, i.e. post-myocardial infarction (post-MI) recovery and patient
284 survival (31). Reperfusion therapy, preferably by PCI but also by thrombolysis, stays nowadays the only
285 treatment recommended for all types of MI (32-35).

286 The first evidence of coronary no-reflow phenomenon as a vascular complication by R. Kloner
287 highlighted the dark side of myocardial reperfusion (36, 37). In addition, the death of myocardial cells
288 in this dog model was reported to occur before the onset of capillary damage due to the lower energy
289 requirements of endothelial cells for their structural integrity conservation (36). In areas of no-reflow,
290 oxygenated blood and pharmacological agents cannot enter the ischemic region, thus preventing any
291 therapy to save the muscle in this area. A few years later, obvious signs of irreversible myocardial injury
292 such as rupture of cell sarcolemma were described in the same dog model (3, 5).

293 Despite clear evidence of direct cardiac cell injury upon restoration of the blood flow, the
294 existence of reperfusion injury has been a matter of debate since more than 30 years until the proof-of-
295 concept of PostC was provided, a main issue, which will be described in section I-B (38, 39). Ischemia-
296 reperfusion (IR) injury (see **FIGURE 1B**) includes both reversible mechanisms, such as myocardial
297 stunning, endothelial dysfunction and most of arrhythmias (with the exception of ventricular
298 fibrillations), but also irreversible damage characterized by cardiac cell death occurring in contractile
299 cardiomyocytes and vascular cells.

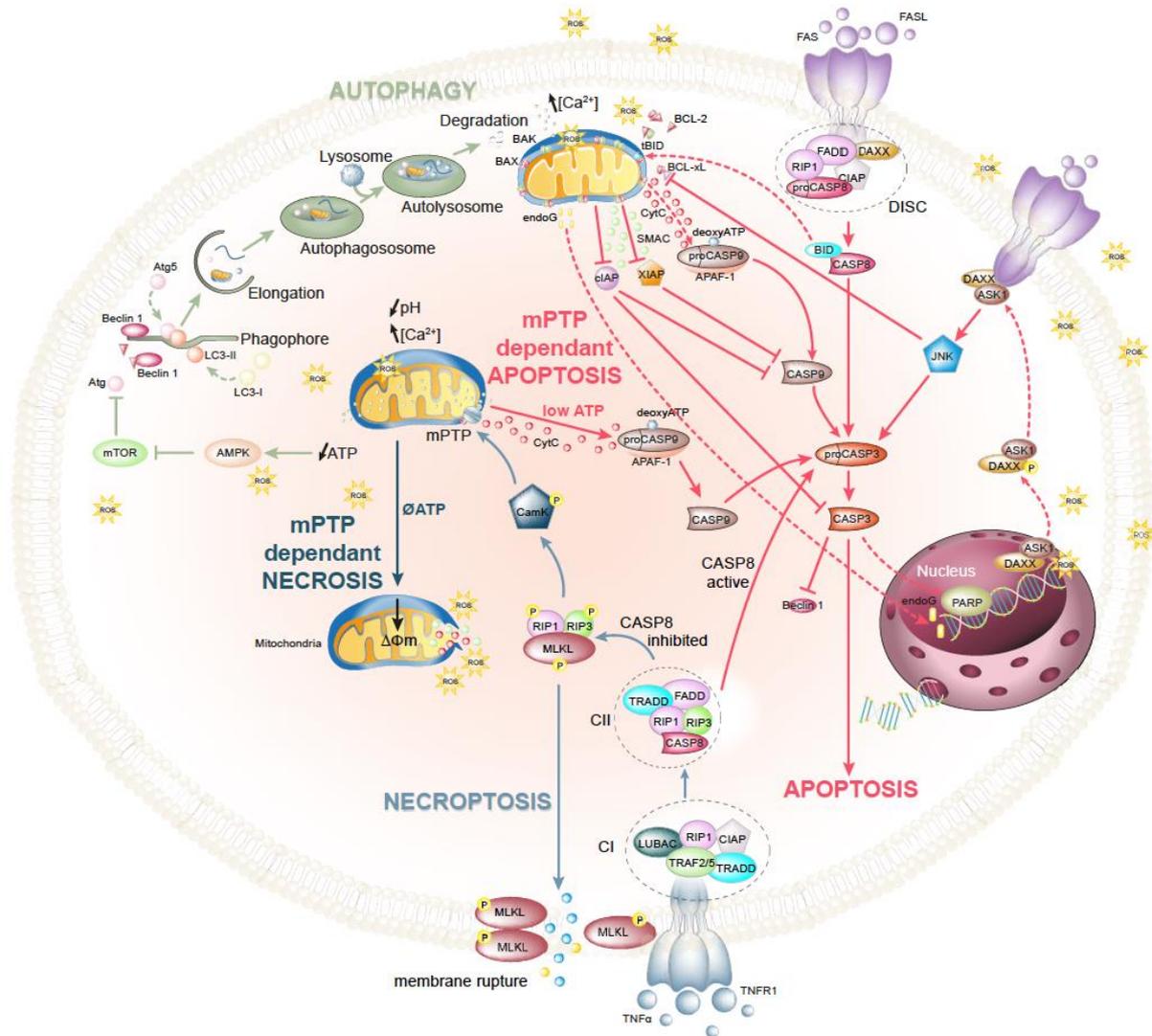
300 Upon reperfusion, the abrupt return of oxygen allows mitochondria to provide ATP after switching from
301 an anaerobic to an aerobic metabolism (**FIGURE 1B**). Due to the accumulation of the substrate
302 succinate, an overproduction of ROS occurs, leading to oxidative stress (around 6-fold more than in
303 ischemic conditions) (40, 41). As a result, IR injury includes metabolic alterations, ROS overproduction,
304 autophagy deregulation, mitochondrial dysfunction, cytosolic and mitochondrial calcium overload,
305 cardiomyocyte hyper contracture as well as apoptosis (**FIGURE 2**). At the tissue level, IR is associated
306 with a dysfunction of the microcirculation, an influx of inflammatory cells and edema (42). We will
307 focus our review on irreversible damage mediated by RCD mechanisms including necrosis, apoptosis,
308 necroptosis and autophagy.

309

310 **2- Cell death mechanisms**

311 MI is defined as cardiac cell death caused by both ischemia and reperfusion resulting from
312 necrosis, apoptosis, necroptosis, autophagy and pyroptosis cell death mechanisms as illustrated in
313 **FIGURE 2**.

314

315
316

317 **FIGURE 2: RCD mechanisms activated upon IR injury.**

318 Apoptotic or necrotic pathways activate during severe IR injury by contrast to autophagic cell death
 319 activating during moderate injury to ensure survival. Mitochondria are stimulated by various stresses
 320 including ischemia-reperfusion, oxidative/proteotoxic stress, DNA damage or increased cytosolic
 321 concentrations of Ca^{2+} . The **intrinsic pathway of apoptosis** is characterized by MOMP under the control
 322 of proteins from the BCL-2 family. Oligomerization (hetero- or homo-) of BAX and BAK to form pores
 323 in the outer membrane is dependent on BH3-only protein regulators and leads to the release of Cyt C,
 324 that binds to APAF-1 with dATP to constitute the apoptosome, in which procaspase-9 is activated and
 325 SMAC binding XIAP or cIAP, preventing them to inhibit caspase-3 and -9. Caspase-9 subsequently
 326 activates downstream procaspase-3. Active Caspase-3 cleaves PARP in the nucleus leading to DNA
 327 fragmentation. EndoG, released by mitochondria, contributes also to DNA fragmentation. In parallel,
 328 mPTP opening is triggered at the onset of reperfusion by the burst of ROS, a further increase in
 329 mitochondrial Ca^{2+} and the restoration of a physiological pH. In these conditions, the H^+ gradient
 330 across the inner mitochondrial membrane is lost, leading to the depolarization of the mitochondrial
 331 membrane potential, $\Delta\psi_m$. The permeabilization of the inner membrane leads to a swelling of the matrix
 332 due to osmotic pressure, inducing the rupture of the OMM and the release of pro-apoptotic Cyt C in the
 333 cytosol. BAX and BAK regulate or participate in the formation of the mPTP as proteins from the outer
 334 membrane.

335 The **extrinsic pathway** is mediated by the FAS receptor (TNF family) activated by external stimuli
 336 (FASL). Homotrimers of FAS undergo a conformational change favoring homotypic interactions with

337 *intracellular death domain-containing proteins such as FADD. This pathway recruits pro-Caspase-8 in*
 338 *the DISC protein complex formed by FAS, FADD and RIP1, leading to its auto-proteolytic cleavage.*
 339 *Activated caspase 8 will in turn active caspase 3 directly or indirectly via the cleavage of cytosolic BID.*
 340 *tBID translocates and inserts into the mitochondrial outer membrane where it contributes to the release*
 341 *of Cyt C through mechanisms either involving BAX or independently of BAX.*
 342 *The nuclear protein DAXX also plays a crucial role in the intracellular signaling cascade of the FAS*
 343 *membrane receptor. DAXX nucleocytoplasmic export is triggered by oxidative stress during reperfusion*
 344 *and mediated by the MAPK kinase ASK1. DAXX possesses a critical role in FAS-mediated cell*
 345 *death to optimally form the DISC. The activation of ASK1 results in the phosphorylation and activation*
 346 *of JNK to induce cell apoptosis through the JNK-BAX-dependent mitochondrial pathway.*
 347 ***Necroptosis** is defined as a regulated death mechanism triggered by TNF, FasL and TRAIL ligands.*
 348 *Following activation of complex I (TNFR, RIP1, TRADD, cIAP1/2, LUBAC), complex II (TRADD,*
 349 *FADD, RIP1 and pro-caspase 8) activates and triggers initiation of apoptosis or necroptosis (no*
 350 *cleavage of RIP1 in case of caspase-8 inhibition). The association of RIP3/RIP1 with MLKL within the*
 351 *necrosome complex leads to MLKL phosphorylation and membrane permeabilization. CAM kinase is a*
 352 *substrate of RIP3 leading to activation of the mPTP-dependent mitochondrial pathway.*
 353 ***Autophagy** is activated in the heart during ischemia with ATP depletion and subsequent activation of*
 354 *AMPK to eliminate intracellular waste from proteins, lipids and organelles damaged during stress.*
 355 *Autophagy is inhibited by the survival proteins AKT or mTOR. Beclin-1 controls the initiation of*
 356 *autophagosomes, ATG their elongation and recruit the cytosolic protein LC3-I which, once transformed*
 357 *into LC3-II, will be integrated into the autophagosome membrane. Functional autophagy invariably*
 358 *culminates in lysosomal degradation. Beclin-1 is cleaved by caspases, showing the existence of a*
 359 *crosstalk between autophagy and apoptosis.*

360
 361
 362 **During ischemic injury**, passive mechanisms of cell death are triggered due to the lack of ATP.

363 Necrosis is the first mechanism described initially to occur during this phase in cardiomyocytes. It is
 364 characterized by plasma membrane permeability and the release of DAMPS in the extracellular space
 365 (13). By contrast to RCD mechanisms, this form of apoptosis cannot be prevented.

366 Lethal reperfusion injury is defined as the death of cardiac cells, viable at the end of the ischemic period,
 367 that occurs within the first minutes of reperfusion in cells (43, 44). This concept was supported by studies
 368 showing that the mitochondrial permeability transition pore (mPTP) opening, considered as the key
 369 event responsible for cardiomyocyte death, opens within the first 5 minutes of reperfusion (45). In fact,
 370 either apoptosis, necrosis, necroptosis or autophagy cell death mechanisms can all be activated upon IR
 371 injury, depending on the intensity of the stress (46): if the lesions are moderate, autophagy will be
 372 activated to ensure cell survival; if ischemia is more intense, cell death will be induced mainly by
 373 apoptotic or necrotic route, with a continuum between the two mechanisms. Intensive research in the
 374 last 30 years has been conducted to inhibit IR-induced cell death, however no treatment has been yet
 375 validated for its use in the clinical setting (47).

376 The existence of apoptotic mechanisms in the IR myocardium, described first by Gottlieb et al. in an *in*
 377 *vivo* rabbit model, was evidenced in different animal models and human ischemic heart disease, and
 378 considered of potential interest for therapeutic intervention in cardioprotection (48-51). Moreover,
 379 numerous studies have shown the beneficial effect *in vivo* of specific anti-apoptotic interventions against
 380 myocardial IR injury (see below in section I-C-1) (52-59). By contrast, another study using *in vivo*
 381 models of cardiac-specific caspase-3 deficient/full, caspase-7-deficient mice (Casp3/7DKO) reported

382 that caspases-3 and -7, known as executioner caspases since they can cleave target proteins leading to
383 characteristic apoptotic breakdown of a cell, do not significantly contribute to cardiomyocyte death
384 induced by transient coronary occlusion (60). These discrepancies on caspase contribution were
385 explained by a global reduction of the whole caspase-dependent pathway expression in terminally
386 differentiated cardiac cells (61). However, an immunohistochemical study performed on post-mortem
387 human hearts slices clearly showed that caspase 3-dependent apoptosis is an important mode of
388 cardiomyocyte cell death in MI in the early period (less than 24h) in particular in infarcted heart tissues
389 who received reperfusion therapy. When analysis was performed on samples taken later (more than 48h),
390 caspase-3 activity was detected in neutrophil granulocytes and inflammatory cells (62).

391
392 **Apoptosis** is a highly regulated, energy-dependent form of RCD defined as “*type I cell death*
393 *or apoptosis, exhibiting cytoplasmic shrinkage, chromatin condensation (pyknosis), nuclear*
394 *fragmentation (karyorrhexis), and plasma membrane blebbing, culminating with the formation of*
395 *apparently intact small vesicles (commonly known as apoptotic bodies) that are efficiently taken up by*
396 *neighboring cells with phagocytic activity and degraded within lysosomes” (46). The dependence on*
397 ATP production makes apoptosis incompatible with ischemic conditions and makes it specific to the
398 reperfusion phase: apoptotic cascades pre-activate during ischemia and are fully executed during
399 reperfusion, in the presence of ATP, leading to specific DNA fragmentation (63). Apoptosis retains the
400 integrity of the plasma membrane and the metabolic activity until the final stage where the plasma
401 membrane breaks down and the cells acquires a necrotic morphotype (secondary necrosis) (46). Two
402 main apoptotic pathways, the intrinsic (or mitochondrial) and the extrinsic (depending on death
403 receptors), are activated during IR injury.

404 **The intrinsic, or mitochondrial pathway** has been described in detail in 2019 by Del Re et al. (64) in
405 Physiological Reviews. It is defined as a type of RCD initiated by perturbations of both the extracellular
406 or intracellular microenvironment. It is characterized by the permeabilization of the mitochondrial outer
407 membrane (MOMP) and precipitated by executioner caspases, mainly Caspase-3 (**FIGURE 2**) (46, 65,
408 66). MOMP is mediated by homo/hetero oligomers of BCL-2 associated to X apoptosis regulator (BAX),
409 and/or BCL2 antagonist/killer 1 (BAK1 or BAK) transmembrane proteins with BH domains
410 (Homologous to *B-cell lymphoma 2*, BCL-2) (67), that form pores across the outer mitochondrial
411 membrane (OMM) and lead to a large increase in outer mitochondrial permeability (68, 69).
412 Permeabilization results in the release into the cytoplasm of the pro-apoptotic factors: (i) SMAC (Second
413 Mitochondrial Activator of Caspases)/DIABLO that binds and inhibits XIAP (X-linked Inhibitor of
414 Apoptosis); (ii) endonuclease G that cleaves DNA (caspase-independent mechanism), and (iii)
415 cytochrome c (Cyt C), the loss of which in the mitochondrial respiratory chain leads to the dissipation
416 of the mitochondrial transmembrane potential ($\Delta\psi_m$) and cessation of mitochondrial functions such as
417 ATP synthesis (70). Cyt C binds to and stimulates the oligomerization of APAF-1 (*Apoptotic peptidase*
418 *activating factor 1*) and the subsequent recruitment and activation of caspase-9. In turn, executioner

419 caspases 3/6/7 activate, leading to specific DNA fragmentation and cell apoptosis (71). The
420 characteristic apoptotic morphology results from the caspase-mediated cleavage of key proteins such as
421 poly (ADP-ribose) polymerase (PARP), actin, or lamins (72).

422 An increase in inner mitochondrial membrane permeability occurs when the mega channel mPTP (73),
423 maintained closed in acidic ischemic conditions, opens upon reperfusion. The molecular identity of the
424 mPTP is still not fully unraveled despite significant progress in the recent years (74). The adenine
425 nucleotide translocator (ANT) was proposed first as candidate for mPTP channel formation. Several
426 proteins from the different compartments have been described also as contributors: VDAC (voltage-
427 dependent anion channel), BCL-2 family proteins, Translocator protein (TSPO) called benzodiazepine
428 receptor from the OMM, and the IMM protein F1Fo ATPase as well as CyPD (cyclophilin D) from the
429 matrix (for review (75)). mPTP opening is triggered by the cellular stress generated by the burst of ROS,
430 an increase in mitochondrial Ca^{2+} and the restoration of a physiological pH (76-83). This
431 permeabilization of the inner membrane leads to a swelling of the matrix due to osmotic pressure caused
432 by the high concentration of proteins in the matrix relative to the outer component of mitochondria.

433 It is important to note that the mPTP and BAX/BAK proteins together contribute to both the
434 mitochondrial pathway and the mPTP-driven necrosis RCD mechanisms, suggesting a crosstalk
435 between apoptosis and regulated necrosis (46, 64, 84). In fact, the time frame of mPTP opening
436 determines the mechanism of RCD: short-term opening allowing restoration of ATP levels leads to
437 apoptosis while long term opening induces irreversible damage leading to necrosis (85). The
438 pathological role for PTP opening during IR injury was highlighted by pharmacological or genetic
439 approaches inducing cardioprotection (45, 86-88). Many animal studies have shown that the
440 administration of drugs interacting with cyclophilin D (CyPD), such as cyclosporin A (CsA), at the time
441 of reperfusion to target PTP opening, reduces infarct size, although studies in pig AMI models have
442 been puzzling (89). In summary, only the full elucidation of the mPTP structure would probably to solve
443 the uncertainties on mPTP involvement in the pathophysiology of IR injury.

444 **The extrinsic pathway** is activated by external stimuli (specific ligands) binding to death receptors
445 belonging to the TNF receptor superfamily such as FAS (*First Apoptosis Signal*; TNFRSF6/CD95/APO-
446 1) (90). FAS receptors are pre-associated as homotrimers in the cell membrane and undergo, after
447 stimulation by FAS ligand (FASL), a conformational change favoring homotypic interactions with
448 intracellular death domain-containing proteins (91). Among them, two adaptor proteins FADD (*FAS-*
449 *Associated protein with Death Domain* or MORT 1) and DAXX (*Death-domain associated protein-6*)
450 initiate the intracellular cascades of FAS that will converge to a common caspase cascade leading to
451 specific DNA fragmentation and cell destruction (92-95). The extrinsic pathway has been less explored
452 in the myocardium as compared to the intrinsic mitochondrial pathway. Indeed, previous experiments
453 in various knockout mouse models with inactivated FAS receptor or non-functioning FASL, have shown
454 inconsistent results on cardioprotection (96-99). In addition, FASL alone is not sufficient to induce
455 apoptosis *in vitro* in cardiac cells unless a pre-exposition to oxidative stress, suggesting that hypoxia

456 sensitizes cardiac cells to the effects of FASL (100), probably through the recruitment of key
457 contributors (such as DAXX) as additional locks for a maximal safety to trigger apoptotic cell death.
458 Furthermore, the release of FASL in the plasma of patient during the early phases of AMI and in
459 postischemic hearts *ex vivo* after the onset of reperfusion provides strong evidence for the contribution
460 of the FAS-dependent pathway in IR injury (96, 101).

461 Activation of the FAS receptor initiates the caspase cascade *via* the formation of the DISC (*Death*
462 *inducing signaling complex*) proteinase complex formed by FAS, FADD, RIP1 (*Receptor interacting*
463 *protein kinase 1*) and pro-caspase 8 (102). This FAS-FADD signaling cascade has been widely described
464 by many authors and reviewed in detail by Del Re et al. 2019 (64). Efficient DISC assembly induces an
465 auto-proteolytic cleavage of caspase-8, which in turns activates caspase 3 directly or indirectly *via* the
466 cleavage of cytosolic BID (*BH3 interacting-domain death agonist*). Truncated BID (tBID, p15)
467 translocates and inserts into the mitochondrial outer membrane where it contributes to the release of Cyt
468 C through mechanisms either involving BAX or independently of BAX (103-107). As a result, FAS-
469 mediated apoptosis is amplified by the activation of the mitochondrial pathway *via* a subsequent release
470 of Cyt C leading to the complete processing of caspases-3. The amount of caspase-8 recruited to the
471 DISC allows to define cells as Type I cells when they execute FAS-induced apoptosis by a largely
472 mitochondrial-independent pathway and type II cells when FAS-apoptosis is also dependent on the
473 mitochondrial pathway. Importantly, cardiomyocyte restricted deletion of FADD significantly reduced
474 the infarct size *in vivo* in a mouse model of IR injury (108).

475 Independently of the cascade involving FADD, the nuclear protein DAXX also plays a crucial role in
476 the intracellular signaling cascade of the FAS membrane receptor (92), allowing to optimally form the
477 DISC even in myocardial tissue (59, 92). The dual role of DAXX as an antiapoptotic transcriptional
478 repressor in the nucleus (109, 110) and as a pro-apoptotic signal mediator in the cytoplasm depends on
479 the subcellular localization of the protein (92). DAXX nucleocytoplasmic export is triggered by
480 oxidative stress or UV irradiation in cardiac cells (100, 111) or by glucose deprivation (112). The MAPK
481 kinase kinase ASK1 (*Apoptosis signal-regulating kinase 1*) insures DAXX-shuttling into the cytoplasm
482 during nucleo-cytoplasmic transport (112, 113). The activation of ASK1 results in the phosphorylation
483 and activation of JNK to induce cell apoptosis through the JNK-BAX-dependent mitochondrial pathway
484 (106, 114). Finally, JNK activates a variety of transcription factors, such as c-JUN and p53, and regulates
485 mitochondrial proteins, such as BCL-2 and BCL-XL, through phosphorylation, resulting in DNA
486 damage and apoptosis (see for review (115)). DAXX *via* its FAS-binding domain binds the death domain
487 of FAS after oligomerization (92). Interestingly, our group has shown that blocking this specific
488 interaction by either genetical and pharmacological approaches appears as a very promising
489 cardioprotective strategy against IR injury because both extrinsic and intrinsic (mitochondrial) apoptotic
490 cascades are inhibited (57, 59, 116).

491 Importantly, both intrinsic and extrinsic apoptosis generate a lot of apoptotic debris, which has
492 to be rapidly eliminated to avoid the release of cytotoxic particles after the loss of their membrane

493 integrity that are not always immunologically silent. This process serves as a waste disposal mechanism
 494 and promotes resolution of inflammation (117). In addition, apoptotic cells have to be cleared by
 495 phagocytes, predominantly macrophages that represent 7% of the noncardiomyocytes cells, a process
 496 known as programmed cell removal or “efferocytosis” (118-121).

497

498 **Necroptosis** is another form of RCD involved in IR injury triggered by oxidative stress or death
 499 receptor activation by TNF, FasL and TRAIL (*Tumor necrosis factor-related apoptosis-inducing ligand*)
 500 ligands (**FIGURE 2**) (122). Despite its similarity with necrosis, necroptosis depends on caspase 8
 501 activity, a feature found also in apoptosis. RIP1 and RIP3 protein kinases as well as death receptors play
 502 a crucial role at the crossroads of necroptosis and apoptosis. In fact, following activation of complex I
 503 including TNFR, RIP1, TRADD (*TNFR-Associated Death Domain*) and cIAP1/2 (*cellular inhibitor of*
 504 *apoptosis proteins 1 and 2*), complex II (TRADD, FADD, RIP1 and pro-caspase 8) activates and triggers
 505 initiation of apoptosis or necroptosis. The latter will only be triggered if caspase 8, responsible for the
 506 cleavage of RIP1, is inhibited. The association RIP3/RIP1 with MLKL (*mixed lineage kinase domain*
 507 *like pseudokinase*) within the necrosome complex leads to permeabilization and rupture of the plasma
 508 membrane under the action of MLKL.

509

510 **Autophagy and autophagic cell death** Autophagy is activated in the heart to eliminate
 511 intracellular waste from proteins, lipids and organelles damaged during stress (123). During ischemia,
 512 autophagy is a cardioprotective mechanism activated by ATP depletion and subsequent activation of
 513 AMPK (**FIGURE 2**). However, during reperfusion, autophagy and apoptosis can occur in the same cell
 514 and usually autophagy occurs before apoptosis because stress often stimulates an autophagic response,
 515 especially if the stimulus is not lethal (124). As a negative feedback loop, increased caspase activity in
 516 apoptosis can cleave Beclin-1 to inactivate Beclin1-mediated autophagy (125). Reduced activity is also
 517 controlled by BCL-2 or BCL-XL, by the survival proteins AKT (protein kinase B) (126, 127) or mTOR
 518 (mammalian Target Of Rapamycin) (128). Autophagic cell death in cardiac cells after reperfusion has
 519 been reported and can even spiral out of control in the final phase of reperfusion, leading to autosis (129,
 520 130). The BH3-only protein Beclin-1, a core component of the class III PI3K (phosphatidylinositol-3
 521 kinase) complex, controls autophagosome formation, which is the main step in autophagy. Beclin-1 is
 522 reported to be activated during reperfusion. However, conflicting results have been obtained concerning
 523 the role of Beclin-1 in cardiac cell death, since cardioprotection was observed both in Beclin-1^{+/-} mutant
 524 mice or after administration of a Tat-Beclin1 peptide during myocardial IR injury (129, 131).

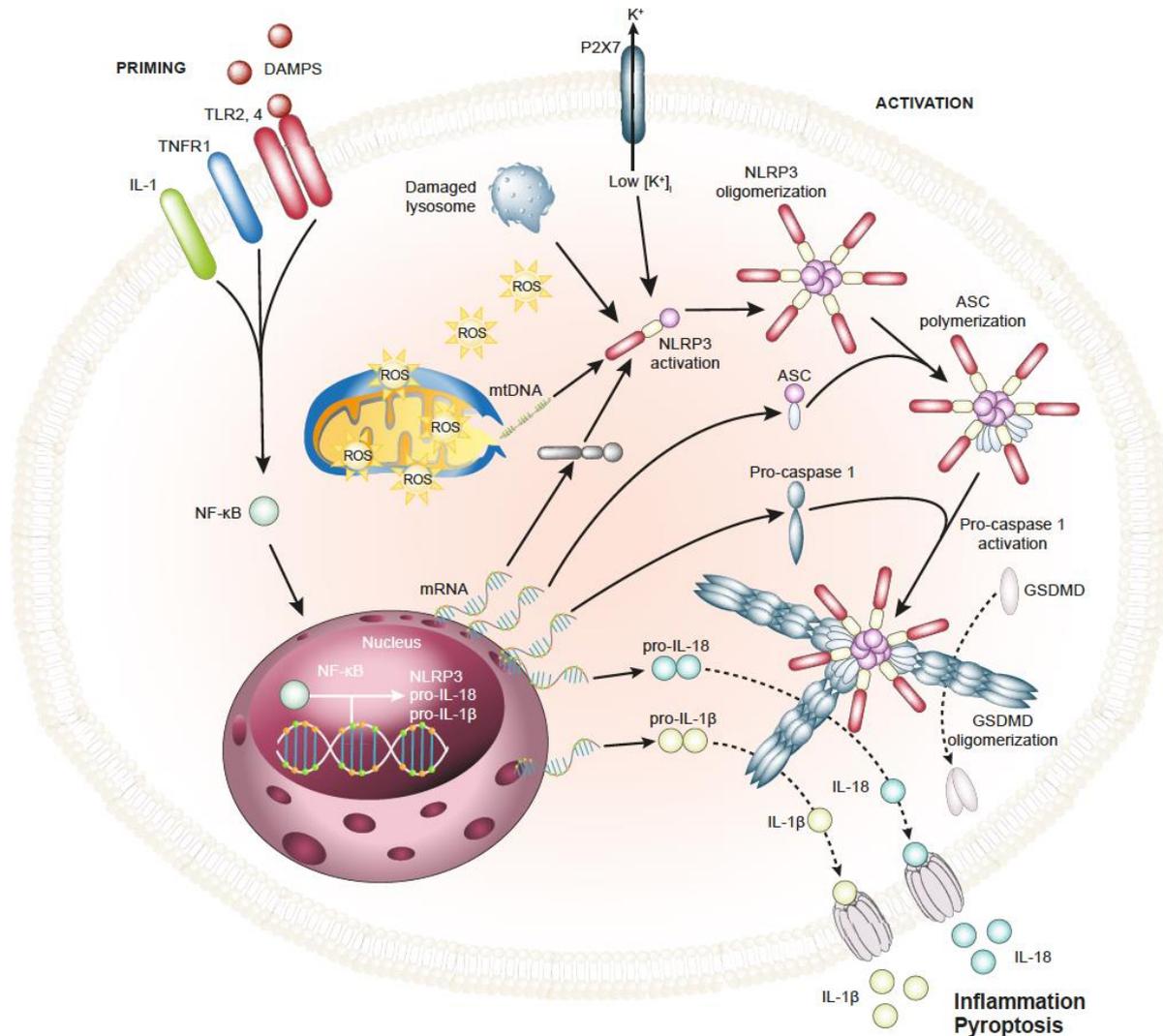
525

526 **Pyroptosis** corresponds to a pro-inflammatory programmed cell death (Greek roots: πυρο for
 527 fire and πτοσις meaning falling). Pyroptosis is characterized by the generation of pores in the plasma
 528 membrane by gasdermin D (GSDMD) activated through a cleavage by inflammatory caspases such as
 529 caspase-1 (**FIGURE 3**). To form pores, GSDMD-NT, the NH₂-terminal cleavage product, oligomerizes

530 in the plasma membrane inner leaflet and causes the release of intracellular material inducing cell death
 531 (64).

532

533



534 **FIGURE 3: NLRP3 Inflammasome and pyroptosis.**

535 The first priming signal is triggered by DAMPS binding to TLR (Toll-like Receptors) and by cytokines
 536 activating their receptors. Subsequent activation of the NF-κB cascade leads to the transcription of
 537 target genes encoding for pro-IL-1β, pro-IL-18 and NLRP3. Activation of NLRP3 is triggered by K⁺
 538 efflux, DAMPS, lysosomal rupture and mitochondrial ROS, and subsequent release of oxidized
 539 mitochondrial DNA into the cytosol. Active NLRP3 oligomerizes into NLRP3-ASC-Caspase1
 540 inflammasome, forming a circular structure functioning as a platform for the polymerization of ASC
 541 into filaments, which in turn work as a central core from which caspase 1 filaments branch, forming a
 542 star-like structure. Active caspase 1 cleaves the inactive pro-IL-1β and pro-IL-18 into the active IL-1β
 543 and IL-18. GSDMD is an additional substrate of caspase 1 that oligomerizes upon cleavage and forms
 544 pores in the cell membrane with the N-terminal fragments, which allow the extracellular release of
 545 active IL-1β and IL-18. The mature NLRP3 inflammasome works as an activation platform for caspase-
 546 1 that, in circulating cells, processes and releases IL-1β and IL-18 cytosolic inflammatory cytokines
 547 and, in cardiomyocytes, leads to caspase-1 mediated pyroptosis.
 548

549 During IR injury, the expression of ASC (*Apoptosis-associated speck-like protein containing a CARD*)
550 and NLRP3 (*Nucleotide-binding oligomerization domain-Like Receptor family Pyrin domain*
551 *containing 3*) inflammasome components are upregulated as well as the activation of caspase-1 and the
552 release of pro-inflammatory cytokines IL-1 β and IL-18 mainly in fibroblasts and to a less extent in
553 cardiomyocytes (132, 133). Activation of the inflammasome in circulating cells leads to IL-1 β and IL-
554 18 release but its activation in cardiomyocytes leads to caspase-1 mediated pyroptosis (134). GSDMD-
555 mediated cardiomyocyte pyroptosis is a key event during IR injury (135) Recently, Zhang et al.
556 demonstrated that GSDMD inhibition allows to reduce IR injury by blocking pyroptosis (136). However,
557 cardiac function was worsened by activation of poly(ADP-ribosyl)ation through the interaction of
558 GSDMD with PARP-1, leading to apoptosis of cardiomyocytes. This suggests a cross-talk between
559 pyroptosis and apoptosis.

560

561

562 Understanding cell death mechanisms underlying the acute phase of MI as well during
563 reperfusion is crucial for the development of therapeutic strategies inhibiting specifically these cascades
564 and save cardiomyocytes. It is worth noting that RCD mechanisms are interconnected as illustrated by
565 FIGURE 2, thanks to regulator proteins playing key roles in the intricate complex interplay between
566 apoptosis, necrosis, necroptosis, pyroptosis and autophagy. RCD mechanisms activate during
567 reperfusion, which coincides with the therapeutic time window of MSC administration. At the onset of
568 reperfusion, autophagy is triggered to save cellular components and steer the cell towards a survival fate.
569 Then, apoptosis is triggered when the injury is more severe during reperfusion. In parallel, the
570 inflammatory RCD mechanism pyroptosis activates. In this context of cardiac injury, targeting of
571 multiple mechanisms should offer greater efficacy in improving the outcome of MI. MSC are relevant
572 to provide a multiple target strategy because targeting either apoptosis, autophagy, necroptosis and
573 pyroptosis (see below sections III and IV).

574

575 **3- Contribution of inflammation to cardiac injury**

576 During AMI, inflammation is necessary for myocardial healing and the formation of a scar thus
577 preventing wall rupture. The inflammatory response includes both a local and a systemic component.

578

579 **Local inflammatory response** The burst of inflammation induced by necrotic cells through
580 production of ROS, complement cascade activation and the release of DAMPS by necrotic cells, has
581 been referred as sterile inflammation since it occurs in the absence of pathogenic infection (137).
582 DAMPS (or alarmins; previously detailed in a review by Sarhan et al., (138))- such as HMGB1 (*High*
583 *Mobility Group Box 1*), eATP (extracellular ATP), pro-IL1 α , heat-shock proteins, S100-proteins and
584 histones- bind to PRRs (*Pattern Recognition Receptors*) in cardiomyocytes, fibroblasts, endothelial cells
585 and leukocytes, to induce inflammatory pathways and stimulate cytokine secretion (**FIGURE 3**) (139,

586 140). In particular, among the PRRs, the TLRs (*Toll Like Receptors*) lead to the production of cytokines
587 as well as the activation and recruitment of neutrophils to the site of injury (141, 142). In addition, the
588 cytoplasmic PRR NLRP3 is activated during the first step of priming by endogenous or exogenous
589 signals leading to the activation of NF- κ B and the transcription of inflammasome components in
590 circulating inflammatory cells and in cardiomyocytes, fibroblasts, and endothelial cells (22, 143).
591 Following the production of inflammasome components during the priming step, full activation of the
592 NLRP3 is triggered by K⁺ efflux but also DAMPS, lysosomal rupture, mitochondrial ROS, and
593 subsequent release of oxidized mitochondrial DNA into the cytosol. Thus, NLRP3 inflammasome
594 assembles into the multimeric complex of inflammasome NLRP3-ASC-Caspase 1 (143-145). NLRP3,
595 ASC, and procaspase 1 are associated to form a mature NLRP3 inflammasome functioning as an
596 activation platform for caspase-1 that controls the processing and release of cytosolic inflammatory
597 cytokines, including IL-1 β (**FIGURE 3**). Furthermore, NLRP3 inflammasome activates also caspase-8
598 in addition to caspase-1, leading to both apoptotic and pyroptotic cell death (146). Mast cells and soluble
599 complement proteins are also important initiators of inflammation. The activation of the complement
600 cascade and the release of cytokines and chemokines (TNF-alpha, IL-1 β , IL-18) induce the recruitment
601 of neutrophils and then monocytes to the injured area (142).
602 Upon reperfusion, the pro-inflammatory response is exacerbated and will contribute to cardiomyocyte
603 death and IR injury within 6 and 24 h post-reperfusion. TLR2, TLR4, and TLR9 stimulated by HMGB1
604 and NLRP3 in the NLRP3-ASC-caspase 1 inflammasome contribute to this strong inflammatory burst
605 following reperfusion through mediators such as IL-1, IL-6 and active caspase-1 acting *via* the NF- κ B
606 pathway (23).

607
608 ***Systemic inflammation*** In addition to a local inflammatory response, myocardial injury triggers
609 also systemic inflammation, stimulating the release of bone marrow-derived leukocytes. The
610 complement activates within 2 hours after AMI leading to the recruitment of neutrophils and monocytes
611 at the site of the lesion in animal models and in patients (147). Neutrophils activated in the peripheral
612 circulation infiltrate into the ischemic myocardium across the vessel wall within 6 to 8 hours after AMI,
613 produce extensive ROS and pro-inflammatory factors to attract monocytes (148). In the injured
614 myocardium, neutrophils will polarize upon activation of TLR4. Hours after AMI, the massive
615 recruitment of neutrophils peaking at 1-3 days is followed by the infiltration of monocytes and
616 macrophages with phagocytic and proteolytic functions promoting the digestion of the infarcted tissue
617 and the removal of necrotic debris (149, 150). Monocyte recruitment to the infarcted myocardium is
618 biphasic with classical proinflammatory monocytes being the predominant monocyte subset recruited
619 within the first 48h (peaking at day 3 to 4) and the anti-inflammatory populations with Ly-6C^{low} and
620 non-classical anti-inflammatory monocytes starting 4-7 days post-MI (peaking about day 7 post-MI).
621 Within the infarcted tissue, inflammatory monocytes differentiate into inflammatory macrophages that
622 will remove the cell debris after injury. They play a crucial role in the early inflammatory phase,

623 producing a variety of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases. It has
624 been shown that following AMI, B lymphocytes infiltrate the infarcted area (peaking at day 5 post-AMI),
625 and augment the pro-inflammatory response by secreting the chemokine CCL7, which in turn induces
626 mobilization from the bone marrow of pro-inflammatory Ly6C^{hi} monocytes (151). In addition, the
627 polarization of inflammatory towards anti-inflammatory macrophages is finely tuned by cytokines (152).
628 Then, anti-inflammatory macrophages produce a variety of anti-inflammatory, pro-angiogenic, and pro-
629 repair factors contributing to the resolution of inflammation by also removing cell debris (152).
630 Beside their role in immune response, macrophages participate in the crosstalk with other cells
631 (including cardiomyocytes, fibroblasts, immune cells, and vascular endothelial cells) to coordinate post-
632 MI processes within cardiac tissue. Activated monocytes and macrophages are the major source of
633 cytokines but all nucleated cell types within the myocardium including endothelial cells, smooth muscle
634 cells and cardiac myocytes also secrete themselves cytokines locally in the heart or systemically (153,
635 154). Finally, a limited short-term up-regulation of stress-activated cytokines such as TNF α , IL-6 and
636 IL-1 β leads to an inflammatory process necessary for wound repair, scar formation and compensatory
637 hypertrophy after MI. This inflammatory cytokine response mediates cardioprotection by activating the
638 survivor activating factor enhancement (SAFE) pathway (155).

639 However, an excessive, persistent and expanded pro-inflammatory response after AMI due to a
640 delay in resolution of inflammation will contribute to expand the infarcted area (23). Prolonged
641 expression of cytokines leads also to myocardial deleterious effects including myocyte hypertrophy,
642 alterations in fetal gene expression, myocyte apoptosis and subsequent adverse LV remodeling leading
643 to heart failure (154). Splenic B lymphocytes are reported to play a key role in cardiac remodeling after
644 AMI and have been identified as putative cardiac therapeutic strategy (156). Vascular complication
645 occurs during AMI when neutrophils exert detrimental effects on endothelial function and together with
646 platelets cause plugging of the coronary microcirculation. Distal microthrombus embolization
647 (microvascular obstruction) and microvascular damages during PCI lead to the no-reflow phenomenon
648 in up to 40% of patients (157), which is associated with larger infarct size and worse outcomes including
649 a higher incidence of death (158). Both no-reflow and hemorrhage carry an adverse prognosis for
650 patients after reperfusion (159).

651 In summary, a complex inflammatory response during AMI is organized by different
652 contributors leading to both pro-inflammatory and anti-inflammatory reparative phases, allowing wound
653 repair. A number of anti-inflammatory strategies have failed to reduce MI size in patients. Combining
654 multiple targets approaches such as MSC-based therapy for this complex phenomenon and using an
655 appropriate timing of administration should provide beneficial results.

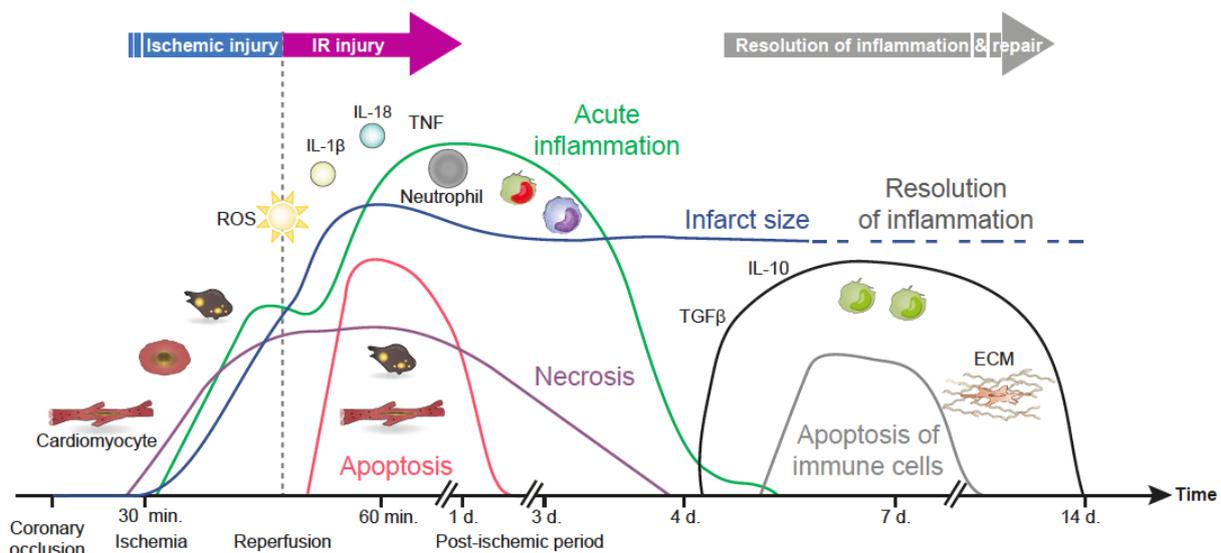
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657 **4- Temporal wave-front of cardiac injury**

658 Beside a spatial progression of cardiac injury, a dynamic wave-front of cell death occurs and
659 develops over time after reperfusion (50, 160, 161). Our group described for the first time the

660 progression over time of apoptosis after the onset of reflow in the mouse heart. A slight delay (around
 661 10 minutes) was evidenced between infarct size and apoptosis kinetics (**FIGURE 4**). Such a delay
 662 suggests that a degree of necrosis takes place in the very first minutes of reperfusion before apoptosis
 663 as previously hypothesized by other groups (38, 50). In the mouse model, the apoptotic wave-front
 664 progresses during the first 60 minutes after the onset of reperfusion to reach a plateau phase
 665 corresponding to the final infarct size. Regarding necrosis, it is predominant during ischemia and at the
 666 onset of reperfusion (38, 50) but progresses also in human hearts after infarction more than expected
 667 regarding the guidelines for timely reperfusion. Indeed, a study by Greulich et al. revealed that in their
 668 cohort of ST segment-elevation MI patients, time to reperfusion to salvage myocardium was less than
 669 suggested by current guidelines since a symptom-to-balloon time of >121 minutes causes a high grade
 670 of transmural necrosis. This study strongly suggests that immediate reperfusion is vital (162).

671 Knowing the time window of reperfusion injury i.e., exploring the timing of activation of the
 672 potential targets of interest, is essential to develop an appropriate and efficient therapeutic strategy
 673 adapted to each specific phase.
 674



675

676 **FIGURE 4: Temporal wavefront of IR injury.**

677 *Simplified diagram of the development of injury in the myocardium as a function of time, after the*
 678 *coronary occlusion leading to ischemia. Necrosis develops first during ischemia. The wavefront of*
 679 *apoptosis is very rapid, triggered by the exacerbated production of ROS due to the abrupt return of*
 680 *oxygenated blood in the artery at the onset of reperfusion. After reperfusion, the pro-inflammatory*
 681 *response is exacerbated and will contribute to cardiomyocyte death (necrosis) and IR injury within 6*
 682 *and 24 h post-reperfusion.*

683 *After the ischemic period (AMI), acute inflammation is triggered by DAMPS and is characterized by the*
 684 *massive recruitment of neutrophils peaking at 1-3 days and followed by the infiltration of monocytes*
 685 *and macrophages with phagocytic and proteolytic functions promoting the digestion of the infarcted*
 686 *tissue. The removal of necrotic debris is dominated by Ly-6Chigh monocytes activity, peaking day 3-4*
 687 *post-MI, and the anti-inflammatory phase with Ly-6Clow monocytes (peak at day 7, post-MI). The*
 688 *immune cells are eliminated in the injured tissue by apoptosis, thus contributing to the resolution of the*
 689 *inflammation. The temporal window of cardioprotection to prevent reperfusion injury is, for anti-*

690 apoptotic strategies, limited to the first 30 minutes of reperfusion. Regarding anti-inflammatory
691 strategies, this window could be extended to the first days (< at 5 days) of the post-ischemic period to
692 avoid any interference with the healing process.

693

694

695 **B- Endogenous mechanisms of cardioprotection**

696

697 **1- Ischemic conditioning stimuli**

698 Making the myocardium briefly ischemic before a more severe ischemia increases the tolerance
699 of the myocardium to ischemic injury by activating endogenous protective mechanisms (163).
700 Myocardial ischemic conditioning is an intervention protecting the heart from damages caused by
701 prolonged exposure to ischemic conditions (infarction). Conditioning protocols can be applied at
702 different times: before, during and after the ischemic period. They act as triggers of endogenous
703 mechanisms of cardioprotection.

704

705 **1.1. Preconditioning**

706 Murry et al. were the first to describe in a dog model that the application of repeated brief
707 episodes of ischemia followed by reperfusion can protect myocardial tissue from the deleterious effects
708 of a subsequent prolonged episode of ischemia by reducing the amount of necrosis (164). This
709 phenomenon was called ischemic preconditioning (PreC) and its effectiveness has been demonstrated
710 both in reducing infarct size and in limiting ventricular rhythm disorders in many animal models *in vivo*
711 (165). Piot et al. provided evidence that ischemic PreC reduces ischemic injury and infarct size after
712 prolonged ischemia and reperfusion in the rat heart *in vivo* (166). There is substantial evidence that this
713 phenomenon also occurs in humans, including the observation that the AMI patients suffering from
714 angina pectoris have decreased infarct size due to the repetitive episodes of short ischemia within one
715 vascular bed during prodromal angina compared to those who do not have pre-infarction angina before
716 the acute phase of MI (167, 168). Angina pectoris before infarction, comparable to PreC, was
717 associated with a reduction in death, cardiogenic shock and heart failure (168). Unfortunately,
718 outside of specific situations such as cardiac surgery, the application of ischemic PreC in the human
719 clinic is not feasible because, by definition, the occurrence of a MI being unpredictable.

720

721 **1.2. Postconditioning**

722 While PreC suffered from its lack of clinical application, the description by Vinten-Johansen's
723 group of PostC revolutionized the world of cardioprotection by demonstrating that application of brief
724 repeated episodes of ischemia-reperfusion immediately after coronary reopening, i.e., at the very
725 beginning of reperfusion allowed to inhibit specifically IR injury (39). Zhao et al. reported a significant
726 reduction in infarct size in the postconditioned group (3 periods of 30 seconds of ischemia and 30

727 seconds of reperfusion immediately after 60 minutes of sustained ischemia) compared with the
728 unconditioned control group. The cardioprotection conferred by PostC was identical to that induced by
729 PreC, corresponding to a reduction in infarct size (15% and 14% of the area at risk, respectively)
730 compared with 25% for the control group. Furthermore, this decrease in infarct size was corroborated
731 by a decrease in creatine kinase (CK) activity, and reduced tissue edema in the area at risk identical to
732 that observed with PreC (39). A lower amount of malondialdehyde (MDA) level in the plasma was
733 described for the PostC group compared to the control group indicating less lipid peroxidation (39).
734 Thus, the work of Zhao et al. highlighted that modifying the hydrodynamic conditions during the early
735 period of reperfusion provides powerful cardioprotection by reducing reperfusion injury, comparable to
736 PreC. Since its original description, many experimental studies have been carried out, and benefits of
737 PostC were then confirmed both *in vitro* and *in vivo* in rats, mice, and rabbits, and *in vivo* in pigs and
738 dogs (165). PostC, by contrast to PreC, is clinically applicable in AMI patients. In a "proof-of-concept"
739 clinical study in AMI patients, Staat et al. highlighted that PostC consisting in 4 episodes of 1-minute
740 inflation/1-minute deflation of the angioplasty balloon performed just after a direct stenting of the
741 occluded coronary artery lowers the release of CK, a classic marker of infarct size (169). Moreover,
742 PostC applied during PCI leads to a persistent reduction in infarct size and to an improvement in long-
743 term functional recovery (170).

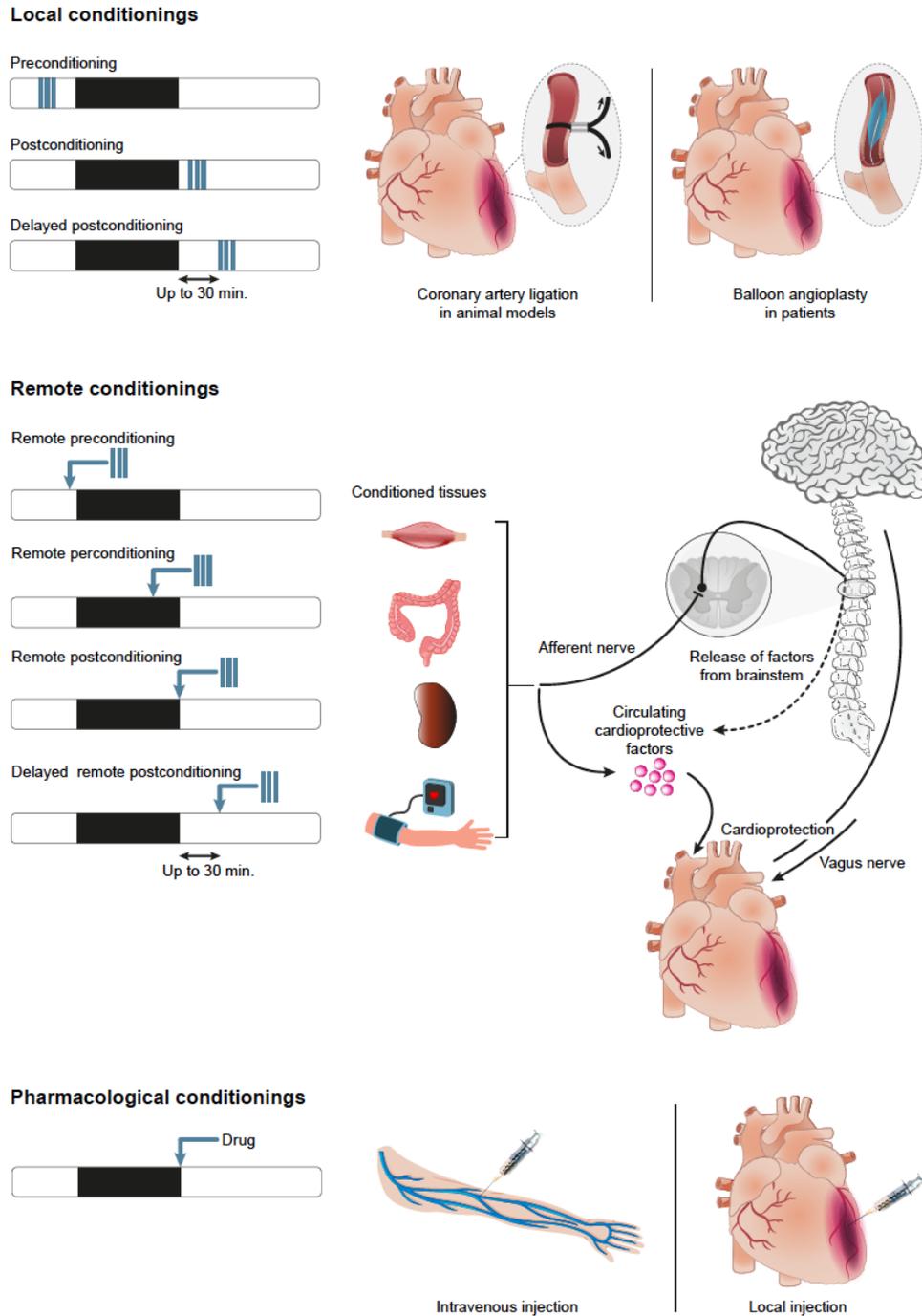
744 The potent inhibition of myocardial injury by PostC suggests a clinically applicable strategy suitable
745 with certain surgical revascularization procedures. PostC was then considered by scientists and
746 physicians as an attractive approach to protect the myocardium against IR injury and was therefore the
747 subject of many studies aimed at exploring its efficacy in different animal models and understanding its
748 underlying mechanisms of action (for review (171-173)).

749

750 ***1.3. Remote conditioning***

751 The major disadvantage of PostC treatments is the need to perform an invasive procedure
752 directly into the heart (**FIGURE 5**). Przyklenk et al. demonstrated, in a canine model, that remote
753 ischemic preconditioning (RIPC) in the circumflex artery bed, prior to a 60 min left anterior descending
754 (LAD) occlusion protected the LAD vascular bed against severe ischemia, leading to a markedly smaller
755 (38%-reduction) infarct size compared to control hearts (174). This work highlighted that ischemic
756 conditioning also applied at a distance from the heart was able to provide cardioprotection (hence the
757 term "remote" ischemic conditioning, RIC) through the production of diffusible protective factors
758 released in the circulation reaching the myocardium (174). Gho et al. demonstrated that brief anterior
759 mesenteric artery occlusion/reperfusion to induce a brief period of intestinal IR stimulus immediately
760 reduces myocardial infarct size by 28% in a rat model (175). RIPC could be applied to a number of
761 different tissues or organs far from the heart (i.e. skeletal muscle, kidney, liver and brain). It could
762 protect any other organ or tissue against a prolonged lethal episode of acute IR injury and was termed
763 inter-organ preconditioning (176). RIC has been demonstrated to be effective if applied before ischemia

764 (remote ischemic preconditioning) (175), during ischemia (remote ischemic perconditioning) (177) or
 765 as a PostC stimulus (178-184). A large number of experimental studies have established that brief
 766 ischemic events in a limb can protect the heart and reduce infarct size (see for review (185)).
 767



769 **FIGURE 5: Illustrative scheme of cardioprotective strategies in acute myocardial infarction.**
 770 Local preconditioning (PreC), postconditioning (PostC), delayed postconditioning (Delayed PostC),
 771 are applied at various time points through a coronary artery within the myocardium. PreC consists of
 772 applying occlusion-reperfusion cycles administered before the ischemic event. For PostC the stimulus
 773 is applied at the onset of reperfusion and can be achieved in animal models by reversible coronary
 774 artery ligation. PostC in patients with AMI can be applied by repeated balloon inflation during the

775 coronary angioplasty procedure. PostC induces cardioprotection even if delayed until 30 minutes after
 776 the onset of culprit coronary artery reperfusion (Delayed PostC).
 777 Remote conditionings (RIC) stimuli could be applied to a number of different tissues or organs remote
 778 from the heart (i.e., skeletal muscle, mesentery, kidney) to protect against a prolonged lethal episode of
 779 acute IR injury. In patient, RIC could be initiated by simply inflating and deflating a blood pressure cuff
 780 placed on the arm or leg for a few minutes then deflating the cuff and performing the procedure several
 781 more times to induce brief cycles of IR. Regarding the timing, protection with RIC can be accomplished
 782 when the stimulus is applied before sustained coronary occlusion (remote preconditioning), during the
 783 sustained ischemic insult (remote perconditioning), or at the time of reperfusion (remote
 784 postconditioning). The conditioning stimulus induces the release of both circulating factors and factors
 785 activating local sensory neurons leading to activation of the parasympathetic innervation.
 786 Pharmacological conditionings are induced by drugs administered intravenously or intracoronary just
 787 before or at early reperfusion.
 788 The black rectangle indicates severe ischemia and the three blue lines the conditioning stimulus.
 789

790

791 In the clinical setting, the fact that RIC can be applied by simply inflating and deflating a blood
 792 pressure cuff placed on the arm or leg of human patients makes it attractive to patients in the acute phase
 793 of their infarction, as part of perconditioning or postconditioning (186, 187). Various small clinical
 794 studies reported inconsistent data with limb RIC applied prior to, or after the PCI, showing improved
 795 myocardial recovery (188-191) and/or reduced infarct size (192-194) or no protection (190). Long-term
 796 clinical outcomes in patients with ST-elevation myocardial infarction were also improved including hard
 797 clinical endpoint of cardiac mortality and hospitalization for heart failure (195, 196). As shown by
 798 several recent meta-analyses, most of the clinical studies indicated a positive effect of RIC on decreasing
 799 IR injury in particular in patients treated by PCI (197, 198). Despite these promising studies, a recent
 800 international multicenter, single-blind, randomized controlled trial (CONDI-2/ERIC-PPCI) including
 801 5401 STEMI patients who underwent PCI indicated that RIC did not report improved clinical outcomes
 802 (cardiac death or hospitalization for heart failure) at one year in STEMI patients (199).

803

804 **2- Mechanisms underlying endogenous cardioprotection**

805 Reduction of infarct size compared with reperfusion alone is a common feature of the three
 806 types of ischemic conditioning (i.e., PreC, PostC and RIC), despite temporal and spatial differences (200,
 807 201). Experiments in animal models allowed to demonstrate that decreased infarct size was related to
 808 an inhibition of IR injury. Indeed, Piot et al. provided the first evidence that ischemic PreC decreases
 809 specific DNA fragmentation as an hallmark of apoptosis in the rat heart (166). PostC has also been
 810 shown to decrease apoptosis *in vivo* (202, 203) in addition to necrosis, both of them contributing
 811 ostensibly to overall infarct size (202, 203). Indeed, PostC diminishes known triggers of apoptosis such
 812 as oxidants (i.e. ROS, peroxynitrite), calcium overload (203, 204), and also inhibits pro-apoptotic
 813 pathways and regulatory factors *in vivo* (205). Our group evidenced that more than 80% of the cell
 814 death-related genes regulated by PreC or PostC are involved in apoptosis (206). An anti-inflammatory

815 effect is also described but the studies have been focused only on evaluation of TNF α production and
816 neutrophil infiltration (202, 207, 208).

817 Although PreC and PostC have been reported to protect the heart against cardiac injury after
818 AMI, a greater cardioprotection was reported for PreC than for PostC in the rat heart *in vivo* with several
819 differences been highlighted (209, 210). Combining PreC and PostC was reported to offer a greater
820 cardioprotection in an *in vivo* rabbit model but this additivity was not retrieved in both *ex vivo* rat heart
821 or *in vivo* canine models (211-213). The non-additive cardioprotection during combined application of
822 PreC and PostC could indicate either that the effects of the “conditionings” are species dependent, or
823 might be related to the number and duration of the sequences of the algorithms (often different between
824 studies) or that these two stimuli could activate common pathways. In this context, a large-scale
825 differential transcriptomic study comparing gene expression levels revealed that only 5 of the 16 463
826 mouse genes explored were jointly regulated upon both PreC and PostC after 1 hour of reperfusion (206).
827

828 The underlying mechanisms of protection by ischemic conditioning are multifactorial and include all
829 mediators of cardioprotection, from the physical stimulus (conditioning maneuver) to the cascades
830 blocking IR-induced damages (see **FIGURE 6**).

831

832 **2.1. Molecular mediators**

833 In summary, the currently accepted paradigm suggests that, in response to the ischemic
834 conditioning stimulus, cells release autacoid substances. Endogenously released chemical stimuli
835 triggering cardioprotection include conventional ligands that, after binding to their respective receptor
836 (*G protein-coupled receptors*, GPCR), will induce the activation of downstream pro-survival signaling
837 pathways (214). Liu et al. demonstrated that 8-sulfophenyltheophylline (8-SPT), a nonspecific blocker
838 of the adenosine receptor, blocks ischemic conditioning, and that IC administration of adenosine or an
839 adenosine A1 receptor agonist can decrease myocardial infarct size (215). Subsequently, more GPCR
840 ligands have been reported as triggering ischemic conditioning, such as acetylcholine (216),
841 catecholamines (217), opioids (218), bradykinin (219), angiotensin II (220), endothelin-1 (221) and
842 glutamate (222). Other inducers of ischemic conditioning include small molecules, as calcium ions, ROS,
843 nitric oxide (NO), hydrogen sulfide and the mitochondrial K_{ATP} channel (see for review (173)).

844 Ischemic conditioning cardioprotective effects are mediated by the activation of signaling cascades, in
845 particular, RISK (*reperfusion injury salvage kinase*), SAFE (223) and cGMP/PKG (*cyclic Guanosine*
846 *Mono Phosphate /Proteine Kinase G*) pathways (224). Finally, it is proposed that this set of receptor-
847 mediated signals converges on mitochondria (end effector) and confers myocardial protection by
848 stabilizing mitochondrial membranes, inhibiting the opening the mPTP, and thereby attenuating
849 mitochondrial swelling and rupture (9, 42, 200).

850 **RISK pathway** The RISK pathway designates a group of pro-survival protein kinases, conferring
851 cardioprotection when they are specifically activated at the time of reperfusion (225, 226). The RISK

852 pathway is carried by the PI3K-AKT and p42/44 extracellular signal-regulated kinase (ERK1/2)
853 cascades. The cardioprotective benefit of both PreC and PostC is fully reversed by inhibition of the
854 RISK pathway suggesting that the RISK pathway commonly mediates ischemic PreC and PostC at the
855 time of reperfusion (212, 223, 225).

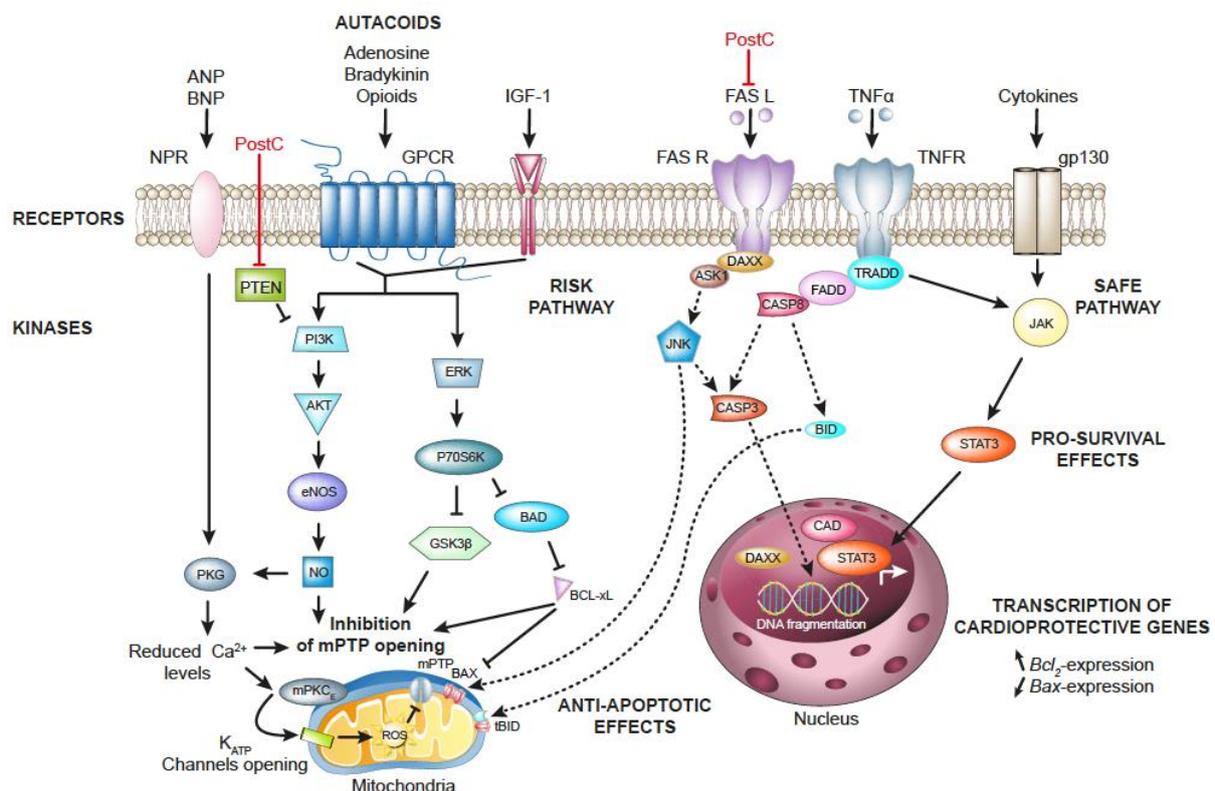
856 - The PI3K/AKT pathway is activated by G-coupled receptors reported to be involved in
857 cardioprotection, such as adenosine, opioid and glutamate receptors (222, 227). The mechanism by
858 which cell survival is mediated is essentially the inhibition of apoptosis after phosphorylation of BAD
859 (*BCL-2 associated agonist of cell death*) by PI3K (228). At the cardiac level, its protective role against
860 IR injury is mediated by the activation of the serine-threonine kinase AKT (or protein kinase B) (229,
861 230). In fact, although AKT activation follows PI3K activation, both enzymes are capable independently
862 to acutely inhibit apoptosis in cardiomyocytes (229). Furthermore, AKT, in addition to its anti-apoptotic
863 effects, preserves cardiac function *in vivo* following an episode of ischemia and decreases infarct size *in*
864 *vivo* (231). Another study showed that overexpression of AKT *in vivo* also improves calcium
865 mobilization, in particular by increasing the expression and phosphorylation of SERCA2a
866 (*sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase 2a*), a mechanism that could explain the beneficial
867 effects on cardiac function (232). While transient activation of PI3K-AKT is cardioprotective, chronic
868 activation of AKT can be deleterious as described in human cardiac tissue samples at advanced stages
869 of heart failure characterized by high levels of phosphorylated AKT. This suggests that either high levels
870 of AKT are deleterious or that, despite significant activation of AKT, there was no beneficial effect
871 against the consequences of myopathic deterioration (233). In mouse models of *Akt* overexpression
872 specifically in the myocardium, concentric ventricular hypertrophy and, in the most severe cases,
873 significant cardiac dilatation have been observed (234, 235). However, an interesting study revealed that
874 the deleterious effects of chronic AKT activation can be antagonized *via* PI3K activation (236). Indeed,
875 this study conducted on a transgenic mouse model of an activated form of AKT (Myr-Akt-Tg), reports
876 that in this case, a negative feedback mechanism inhibits upstream PI3K activity. Restoration of PI3K
877 function by gene transfer in these transgenic mouse hearts was reported to reduce the deleterious effects
878 generated by a myocardial IR episode (236).

879 PTEN *Phosphatase and tensin homologue deleted chromosome 10*) is a powerful negative regulator of
880 PI3K activity in various cell types and plays an important role in myocardial IR injury, as well as in
881 myocardial remodeling, hypertrophy, and fibrosis (237-240). PTEN inhibition is therefore an effective
882 potential target for reducing infarct size and improving cardiac function in animal models (241). By
883 dephosphorylating the product of PI3K i.e., PIP3, PTEN inhibits the activity of several downstream
884 molecules in the signaling pathway, the most important of these molecules being AKT. PTEN activity
885 is reduced upon PreC and restored as the protective effect of PreC is lost (242). PostC treatment
886 decreases also PTEN level, activates PI3K/AKT prosurvival kinase pathway and decreases significantly
887 myocardial apoptosis in an *in vivo* rat model (243).

888 The PI3K/AKT pro-survival kinase pathway has a crucial influence on the opening of mPTP by
 889 inhibiting its opening in the first minutes of reperfusion, thus preventing cell death by apoptosis or
 890 necrosis (244-246). Numerous studies have evidenced that mPTP opening is prevented at the onset of
 891 reperfusion in hearts subjected to PreC (86, 164, 247, 248) or PostC (39, 249) but not in the case of RIC
 892 (see for review (250, 251)).

893 - ERK1/2 or p42/44, members of the MAP kinase (*mitogen activated protein kinase*) family, are serine-
 894 threonine kinases involved in the regulation of cell proliferation, differentiation and survival. Among
 895 the five ERK isoforms identified, ERK1 and ERK2 are the most abundantly expressed forms in the heart.
 896 Numerous studies have shown that the MEK (*MAPK-ERK kinase*)-ERK pathway can have anti-
 897 apoptotic effects following stimulation by IGF-1, cardiotrophin-1 or catecholamines (252-254).
 898 Activation of ERK1/2 plays a protective role against reperfusion injury *in vitro* and *in vivo* (255, 256).
 899 The work of Lips et al. in the *erk2^{+/-}* and *erk1^{-/-}* mouse models, suggests that ERK1 and ERK2 would
 900 have specific roles and that only the MEK1-ERK2 pathway would protect the myocardium from IR-
 901 induced apoptosis. However, there are some differences reported between PreC and PostC, such as the
 902 fact that ERK1/2 is involved in PostC but not in PreC (257).

903



904

905

906 **FIGURE 6: Endogenous mechanisms of cardioprotection.**

907 Upon postconditioning stimulus autacoids (adenosine, bradykinin, endogenous opioids) are released
 908 and bind to their specific receptors belonging to the superfamily of GPCR. Other receptors are able to
 909 mediate the response to growth factors such as IGF-1 or cytokines (IL-6). RISK signaling pathways
 910 comprising survival kinases such as PI3K/AKT and ERK1/2 (p42/44) are downstream cascades
 911 activated upon PostC stimulus. PTEN is a powerful negative regulator of PI3K activity. The final

912 *endpoint of this intracellular cascade is the inhibition of MPTP opening via inhibition of GSK3 β ,*
 913 *increased NO (via AKT) and PKG activity (via natriuretic peptides receptors) and reduced intracellular*
 914 *Ca²⁺ levels. The opening of mitochondrial K_{ATP} channels is part of the endpoints of the postconditioning*
 915 *mechanisms.*

916 *The SAFE pathway mediated by JAK-STAT3 is also responsible for postconditioning induced-*
 917 *cardioprotective effects. STAT3 relocates to the nucleus in order to control the transcription of*
 918 *cardioprotective genes such as Bcl-xl.*

919 *The cardioprotective effects of PostC results also from the inhibition of apoptotic pathways (dotted*
 920 *lines) mediated by the extrinsic pathway involving death receptors such as FAS and TNF receptors.*
 921

922 **SAFE pathway.** The conditioning stimulus also activates the SAFE pathway involving the cytokine
 923 TNF- α and STAT3 (*Signal Transducer and Activator of Transcription-3*) (258). Indeed, Lecour et al.
 924 demonstrated the cardioprotective effect of the administration of TNF- α before the ischemic insult
 925 without involvement of the RISK signaling cascade (258). Later, they showed that TNF- α administration
 926 during reperfusion activated an alternative pathway, termed the SAFE pathway (259-261), and they also
 927 established a link between preconditioning and the activation of this pathway (262). In humans, it seems
 928 that STAT5 (*Signal Transducer and Activator of Transcription-5*), instead of STAT3, may play a
 929 relevant role in cardioprotection (263). Activation of STAT3 has a role in the long-term transcriptional
 930 regulation of cardioprotective proteins (264), but also has acute effects by enhancing mitochondrial
 931 respiration (265) particularly during PostC (266) and by attenuating apoptosis. Indirect evidence proves
 932 the involvement of STAT3 in RIC. Indeed, Apolipoprotein A has been proposed as a humoral transfer
 933 signal RIPC (267), and the decrease in infarct size by RIPC and exogenous apolipoprotein A was
 934 mitigated by blocking PI3K, ERK, and STAT 3 (268).

935 **cGMP/PKG or PKG/NO pathway** This signaling cascade involving protein kinase G (PKG) and NO
 936 has also been identified as a contributor in cardioprotection (269, 270). NO produced downstream of
 937 PI3K/AKT emerges as a key signaling element in the reperfusion salvage pathway activated by many
 938 classes of agents capable of safeguarding the organism's integrity. Activation of soluble guanylyl cyclase
 939 (sGC) and PKG depends on activation of the PI3K/AKT pathway and appears as crucial for many
 940 different forms of cardioprotection (see for review (224)). Several studies indicate that this pathway is
 941 negatively impacted in cardiomyocytes by IR injury and its activation is essential for the
 942 cardioprotective effects of PostC (211, 271, 272). However, another study shows that blockade of NOS
 943 (*Nitric oxide synthase*), sGC, and PKG, but not PI3K, abolishes the cardioprotective effects of PostC,
 944 further suggesting that activation of the cGMP/PKG pathway is NOS-dependent but independent of
 945 PI3K/AKT signaling (273).

946 In fact, PostC mitigates ROS generation at reperfusion probably by decreasing the delivery of oxygen
 947 during the controlled reperfusion (209, 213, 274) that limits tetrahydrobiopterin (BH₄) oxidation and
 948 increases endothelial NOS (eNOS) activity. The subsequent activation of the cGMP/PKG pathway leads
 949 to PKG-dependent inhibition of Na⁺/H⁺ exchanger (NHE), slowing intracellular pH normalization
 950 during reperfusion. In addition, the cGMP/PKG has a direct effect on calcium oscillations and
 951 mitochondrial permeability transition (224).

952 Despite available evidence supporting that the cGMP/PKG pathway is involved in PostC protection,
953 only a limited number of studies have identified PKG-phosphorylated target proteins that are implicated
954 in the molecular mechanisms of cardioprotection. It has been proposed that PKG activation inhibits
955 mPTP through GSK3 β phosphorylation (275, 276). Nevertheless, double GSK-3 α/β knockin mice
956 lacking GSK3 phosphorylation sites that lead to kinase inactivation could still be protected by both PreC
957 and PostC (277), proving that GSK3 β inhibition is unlikely to serve as a downstream mediator of the
958 cardioprotection induced by cGMP/PKG activation. Results from an *in situ* pig model and in *ex vivo* rat
959 hearts confirm that GSK3 β phosphorylation does not play a causal role in PostC protection (273, 278).

960

961 **2.2. Remote mechanisms**

962 The identified common mechanistic threads identified for PreC and PostC appear to extend to
963 remote conditioning (176, 214, 279-281), with a significant contribution from PKC, the RISK pathway
964 and mitochondrial function (173).

965 Unlike PreC and PostC, RIC stimulus differs considerably in that protection begins in the remote organ
966 or tissue and the cardioprotective signal must be transferred from the remote organ to the heart via
967 different pathways. Several mechanisms have been proposed in many animal studies: communication
968 via (i) circulating blood and humoral mediators (176, 282, 283), (ii) neuronal mechanisms (175, 284)
969 and (iii) modification of circulating immune cells (279, 281, 285, 286).

970 A humoral mechanism for RIC has been reported with the demonstration of perfusate transfer-
971 mediated cardioprotection between isolated perfused hearts (179, 282, 287-289). This cardioprotection
972 can be transferred by perfusing plasma into an isolated heart from a different animal species suggesting
973 cross-species protection (289, 290). In addition, it is mediated by RISK and SAFE pathways since for
974 example, blocking these cascades abolished the protection provided after infusion of plasma from pigs
975 that had been subjected to remote PostC in isolated rat hearts (290). The identification of humoral
976 mediators has been the subject of numerous studies using proteomic approaches in both animals and
977 humans, but also led to some contradictory results (267, 291, 292). Examples of proteins that may be
978 involved include apolipoprotein A-I, kallistatin, and stromal-derived factor 1 α (see for review (293)).

979 The neural hypothesis of RIC postulates that IR cycles in remote tissues or organs can promote
980 a neural reflex leading to myocardial protection against subsequent myocardial insult (294). Additional
981 mechanisms have been suggested as contributing to the remote signal, such as activation of peripheral
982 nociceptive fibers releasing unidentified molecules into the systemic circulation and/or through the
983 spinal cord activating the autonomic nervous system to release cardioprotective factors (see for review
984 (251)). Donato et al. pointed out that RIPC activates a neural afferent pathway involving the vagus nerve,
985 which releases ACh and triggers downstream cascades via a muscarinic receptor (295), resulting in
986 eNOS stimulation and elevated cGMP production (296). Humoral factors can stimulate also the
987 cGMP/PKG pathway through activation of PI3K/AKT cascade (179, 297). Intact neural connection is
988 thus necessary between the remote organ and the heart, as demonstrated in different animal models. In

989 rabbits, renal RIPC was associated with an increased sympathetic nerve activity and abrogated when the
990 renal nerve was sectioned before IR *in vivo* (298, 299). Likewise, in a mouse model, combined resection
991 of the femoral and sciatic nerves abolished the cardioprotective effect of RIPC (284). Cardioprotection
992 was abolished also after pharmacological autonomic ganglia blockade in the case of RIPC through the
993 mesenteric artery but not in the case of PreC (175). Mastitskaya et al. demonstrated that vagal pre-
994 ganglionic neurons residing in the dorsal motor nucleus of the vagus nerve (DMVN) have a crucial role
995 in RIC-induced cardioprotection.(300). The involvement of sensory nerves in the propagation of
996 protection also has been directly evidenced. Activation of sensitive fibers by nociceptive stimuli or
997 topical capsaicin results in the release of cardioprotective substances into the bloodstream, mimicking
998 the cardioprotection induced by RIPC in a rabbit model (301). Intramesenteric bradykinin, by
999 stimulating local sensory nerves, leads to RIPC-like protection, which is abolished by hexamethonium
1000 in a rat model suggesting the involvement of the autonomic nervous system in addition to sensory nerves
1001 (302). Communication between distant organs and the heart may be multifactorial, and the overlap
1002 between neural pathways and humoral mediators has also been suggested in rodents (172, 284, 299).
1003 In healthy humans, inhibition of RIPC by the autonomic ganglia blocker trimethaphan was reported but
1004 not observed after IV administration of a bradykinin-2 receptor antagonist suggesting that the
1005 mechanisms may be different in humans (303, 304).

1006 . The systemic response theory is supported by the existence of several blood-derived factors (e.g.,
1007 cytokines, nitrites, autacoids,..) constituting the "remote signal" to be delivered to the heart *via* the
1008 systemic circulation (see for review (181, 305)). Opioids are thought to be responsible for remote
1009 myocardial salvage by modulating CCR1 (C-C chemokine receptor type 1)-triggered leukocyte
1010 infiltration in the myocardium or by stimulating CCR5-mediated regulatory T cell (Treg) recruitment
1011 (286). RIC through transient forearm ischemia may alter gene expression in circulating leukocytes and
1012 suppresses their activation in human patients (306).

1013

1014 **3- Therapeutic time window of cardioprotection**

1015 AMI is an absolute emergency and recommendations in clinical practice are to reperfuse the
1016 myocardium as soon as possible in agreement with the concept
1017 that "time is muscle" (6). Whereas a 2-hour delay was recommended to obtain a beneficial effect from
1018 thrombolysis in early studies of coronary artery reopening, reperfusion therapy, mainly PCI, must now
1019 be performed within 3 hours for maximum efficacy (32, 307). Early proof-of-concept studies established
1020 the dogma that PostC, to be efficient, must be applied within the first minute after the onset of
1021 reperfusion (39, 209, 211). Since then, most PostC maneuvers have been initiated within 1 minute of
1022 the onset of reperfusion in the majority of studies, including clinical trials (169, 308, 309). A significant
1023 decrease in infarct size was demonstrated when the PostC stimulus was applied immediately after the
1024 onset of reperfusion following a prolonged ischemic insult *in vivo* in a dog (310) and rabbit models
1025 (211). Meanwhile, another study in a rat model of myocardial IR reported that PostC was effective in

1026 reducing infarct size only if applied no later than 1 min after the onset of reperfusion (209). It was then
1027 considered that PostC should be applied in the time immediately after the onset of reperfusion to be able
1028 to decrease myocardial reperfusion injury.

1029 However, several years later, our group provided evidence that PostC, delayed up to 30 minutes after
1030 the onset of culprit coronary artery reperfusion in the mouse heart *in vivo*, was still able to reduce the
1031 wave-front of reperfusion injury and prevent apoptotic cell death occurring during the first minutes of
1032 reperfusion (50). In addition, delayed PostC also exhibits a time-dependent degree of protection (50).

1033 The wave-front of apoptosis occurs as a “burst”, and prolonging the application time of anti-apoptotic
1034 strategies from 1 to 6 days was reported to be deleterious, leading to cardiac rupture, since apoptosis
1035 during the healing process is an absolute necessary mechanism (50, 311). When applied later, PostC is
1036 not able to prevent necrotic processes that occur in the late stage of apoptosis (46, 50). A very narrow
1037 therapeutic window is suggested for both preclinical and clinical studies. Many studies confirmed this
1038 idea and reported also that delaying the time point of application of the conditioning stimulus or
1039 pharmacological cardioprotective strategies is still able to preserve some cardioprotective effect (59,
1040 160, 312, 313) even in the case of anti-inflammatory strategies (314, 315). Other studies describe that,
1041 to be effective, pharmacological strategies must be applied before reperfusion, as reported for
1042 cyclosporine A administration (316).

1043 Altogether, these data clearly show that anti-apoptotic strategies need to be administered as soon as
1044 possible at the onset of reperfusion or even prior to reperfusion reinforcing the concept that
1045 cardioprotection is a matter of timing. Anti-inflammatory strategies must be administered at the same
1046 time as the pro-inflammatory burst. Indeed, colchicine has been reported to reduce infarct size when
1047 administered before ischemia (317) or 25 min. prior to reperfusion in a mouse model of AMI (318).
1048 Administered during one week post-reperfusion in mice, colchicine prevented the increased expression
1049 of inflammatory cytokines and NLRP3 activation and improved cardiac function, heart failure and
1050 survival after MI (319). In patients, colchicine administered during PCI and the 5 following days was
1051 associated either with a smaller infarct size (320) or an absence of cardioprotective effects (321). In fact,
1052 the timing to treatment initiation influences the beneficial effect of colchicine. Results of the COLCOT
1053 study have shown that early colchicine administration within 3 days after AMI allows reducing future
1054 events including major adverse cardiovascular events, cerebrovascular accidents, and need for
1055 revascularization compared to standard therapy alone (322).

1056 The therapeutic time window for treating patients with AMI appears to be crucial for targeting specific
1057 signals and should be tailored according to their targets. In conclusion, the therapeutic time window for
1058 treating patients with AMI appears to be crucial and must be adapted to each target.

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1061
1062

1063 **III- Generalities on mesenchymal stem cells (MSC)**

1064 As described above, apoptotic mechanisms are of major importance in IR injury as evidenced
1065 in various animal models and in human ischemic heart disease.

1066 A major reason for the failure to translate cardioprotection in the clinical setting may have been the use
1067 of a monotherapy approach focused on a single target involved either in IR injury or in an endogenous
1068 cardioprotective pathway. Unfortunately, this strategy might be insufficient to address the multifaceted
1069 components of myocardial reperfusion injury, comprising mitochondrial and endothelial dysfunction,
1070 oxidative stress, calcium overload and inflammation as well as microvascular obstruction (323-325).
1071 This awareness has paved the way for new multi-targeted therapies, including mesenchymal stromal cell
1072 (MSC)-based therapy, which relies on the pleiotropic properties of MSC. In this chapter, we will
1073 summarize the properties of MSC that are mainly relevant in the treatment of AMI.

1074

1075 **A- MSC definition**

1076 The acronym MSC has been used for multiple meanings that include, mesenchymal stem cell,
1077 mesenchymal stromal cell, and multipotent stromal cell. MSC have been initially discovered by
1078 Friedenstein *et al.* who identified in the bone marrow (BM) of mouse and guinea pig a fibroblastic cell
1079 type that gives rise to clonal colonies and that generates bone and reticular tissue when transplanted
1080 heterotopically (326, 327). BM-MSC represent 1/10 000 of the mononuclear cells in the BM and can be
1081 easily isolated and expanded *in vitro*. Given that a stem cell is defined for its clonal self-renewal potential
1082 and multilineage differentiation potential, the term “mesenchymal stem cells” has for a long time been
1083 criticized (328) although their self-renewal capacities have been demonstrated (329). The international
1084 society for cellular therapy (ISCT) recommends to distinguish between the self-renewing fraction within
1085 the BM that exhibits a multipotent differentiation potential *in vivo* and mesenchymal stromal cells
1086 identified in multiple tissue types and that exhibit multipotent differentiation potential *in vitro* (328).
1087 Cells that display a multipotent potential *in vitro* have been identified in the adipose tissue (AT-MSC)
1088 (330-332), dental pulp (333), cartilage (334), synovial membrane (335), tendon (336), heart (337) and
1089 lungs (338) as well as in perinatal tissues including the placenta (339), umbilical cord blood (340),
1090 umbilical cord (341) and fetal sources such as fetal tissue and amniotic fluid (342).

1091 Regardless of the niche, these multipotent cells referred all to MSC as multipotent mesenchymal
1092 stromal cells in accordance with 3 criteria established by the ISCT (343). First, MSC adhere to the plastic
1093 when cultured in standard conditions. Second, MSC are positive for a panel of surface molecules,
1094 individually not specific, that includes CD105, CD73 and CD90, and are negative for hematopoietic
1095 lineage markers such as CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR. Third, MSC
1096 are multipotent cells that can differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts. In
1097 2019, the minimal criteria defined by the ISCT were updated to classify a cell as a MSC and to
1098 rationalize the use of the MSC nomenclature in laboratories. On this occasion, the board re-emphasized

1099 the necessity of MSC nomenclature and the need for additional reportable criteria such as their tissue of
1100 origin and functional assays performed to better qualify these cells (344). Similarly, Renesme et al. have
1101 pointed to the need for increased efforts to ensure the agreement of a consensus definition of MSC and
1102 reporting guidelines to improve the robustness, repeatability, and clarity of the MSC literature and,
1103 eventually, the safe application of effective MSC-based therapies (345).

1104

1105

1106 **B- MSC developmental origins**

1107 The high number of MSC sources has highlighted the possibility that MSC might derive from
1108 different embryonic origins. Indeed, although bone marrow and adipose tissue were thought, for a long
1109 time, to host MSC originating from the mesoderm (346), elegant and convincing studies have evidenced
1110 that these two tissues also host neural crest-derived MSC (**FIGURE 7**). The first study was carried out
1111 in 2007 by Takashima and colleagues (347) by means of lineage tracing approaches. They show that the
1112 trunk of E9.5 mouse embryos possesses cells positive for Sox1, a marker for neural stem cells, that gave
1113 rise to MSC positives for PDGFR α (PDGF receptor alpha), a marker of paraxial mesoderm. They
1114 evidenced also that in postnatal BM, neural crest-derived MSC are still present, even at a low extent,
1115 suggesting that the first wave of MSC in the embryo derives from the neural crest and that following
1116 birth, a second wave of MSC from another source takes place (347). The origin of adult MSC is still an
1117 open question and the function of MSC from different developmental origins has been addressed more
1118 recently. While neural crest-derived stem cells are quiescent in the BM where they exhibit a
1119 hematopoietic stem cell niche function during the *in-utero* development and after birth, mesoderm-
1120 derived MSC participate in fetal skeletogenesis and lose their activity after birth (348). Similarly, the
1121 subcutaneous fat of adult mice was shown to contain neural crest-derived MSC that are phenotypically
1122 and functionally distinct from MSC derived from non-neural crest (349).

1123 These studies that improve our knowledge on the developmental origin of MSC found in
1124 different adult tissues are critical for the optimal use of MSC in clinic. Indeed, MSC from different tissue
1125 sources, with no regard to their embryonic origin, have been or are currently used in 663 clinical trials
1126 enrolling patients with heart disorders. However, the studies described above have brought to our
1127 attention that depending on their embryonic origin, MSC might exhibit different functions once injected
1128 *in vivo*. Of note, for clinical applications, neural crest-derived MSC may be of interest, given recent
1129 studies demonstrating the contribution of the neural crest to several cardiovascular structures in
1130 vertebrates, such as cardiomyocytes and to cardiac regeneration in adult zebrafish (350, 351). Moreover,
1131 these studies raise the question about the intrinsic properties of MSC from a given embryonic origin and
1132 the subsequent phenotypic and functional heterogeneity among MSC within a same tissue source that
1133 are ontogenically distinct.

1134

1135

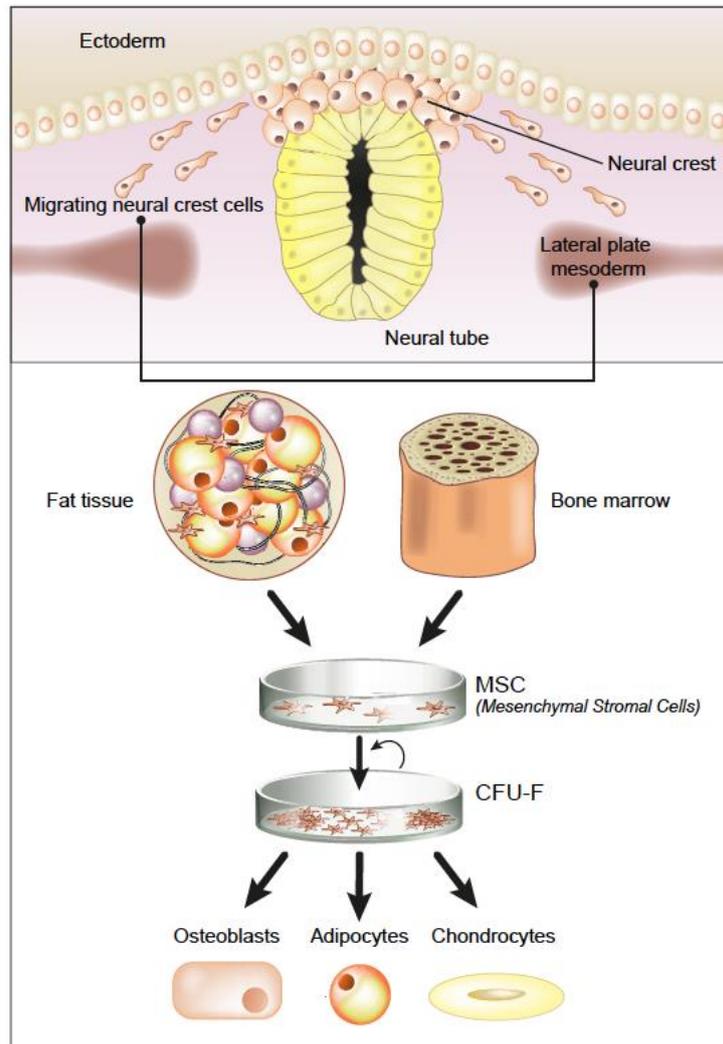


FIGURE 7: Embryonic origin, isolation, and differentiation potential of MSC derived from adipose tissue (AT-MSC) and bone marrow (BM-MSC).

AT-MSC and BM-MSC originate from both neural crest cells and the lateral plate mesoderm. Both AT-MSC and BM-MSC display the capacity to adhere to the plastic, to form CFU-F (CFU-fibroblasts), and to differentiate into osteoblasts, adipocytes, and chondrocytes.

C- MSC heterogeneity

MSC heterogeneity exists at different levels. First, MSC derived from different donors exhibit phenotypic and functional differences that are well documented (352). Second, MSC derived from different tissue sources display features that refer to their tissue of origin (353, 354). Finally, as discussed above, MSC derived from the same tissue may show differences that can be explained by the fact that MSC from the same source may have different embryonic origins (348, 349).

At the functional level, MSC from different tissue sources exhibit different properties. Regarding MSC immunoregulatory properties, while BM and umbilical cord (UC) have similar immunosuppressive capacities, placenta-derived MSC exhibit a lower capacity to inhibit CD4⁺ and

1174 CD8⁺ T cells (for review (355)). Compared to BM-MSC, AT-MSC display a stronger suppressive
1175 potential on IFN γ -producing Th1 (*T helper 1*) cells (for review (355)). Similarly, superior suppressive
1176 effects of AT-MSC were reported on macrophages compared to BM-MSC (356). With regard to MSC
1177 differentiation potential, tissue-specific abilities have been reported. For example, in a comparative
1178 study using donor-matched AT-MSC and BM-MSC, while AT-MSC showed significantly higher
1179 proliferation rate and adipogenic potential, BM-MSC showed significantly higher osteogenic and
1180 chondrogenic potential (352). Of note, a significant heterogeneity among the donors regarding the
1181 proliferation and differentiation potential of the two MSC types was observed in this study.

1182 Heterogeneity is also reported between MSC from a same tissue sample. Indeed, multiple
1183 studies from the early 2000s report variations in phenotype, proliferation and differentiation properties
1184 (330, 357-359). While 30% of BM-MSC clones display a tri-lineage differentiation potential, 70%
1185 exhibit a bi-lineage or uni-lineage potential (359). For AT-MSCs, only 1.4% of the clonal
1186 subpopulations were tripotent and the rest were either bipotent or unipotent (330).

1187 MSC heterogeneity has direct consequences on MSC phenotype, functions and therapeutic
1188 potential. MSC exert different functions including immunomodulation, anti-apoptotic, anti-fibrotic and
1189 pro-angiogenic properties (360). Thus, one can speculate that either a single subpopulation of MSC
1190 would exert these pleiotropic activities and thus effectively treat multifaceted IR lesions, or that each
1191 subpopulation would exert a specific function explaining the pleiotropy of this heterogeneous cell panel.
1192 This would explain that the use of MSC in clinical trials have led to various results going from moderate
1193 to inconsistent benefits (361-363). The frequency of MSC derived from NC or mesoderm might vary
1194 among the tissue sources, tissue donors and clonal subpopulations. Amplification protocols and culture
1195 media used for MSC could also favor the enrichment of one subpopulation of MSC rather than another.
1196 Thus, verification of the way that culture conditions can impact on the frequency and the functions of
1197 MSC subpopulation is critical step of quality control. Altogether, the multiple sources of variations
1198 would explain, in part, the phenotypic and functional heterogeneity found among MSC isolated from
1199 different tissues, donors and different laboratories. Thus, to overcome the subsequent clinical
1200 inconsistencies obtained with MSC and guarantee the therapeutic potential of these cells administrated
1201 into the patient a current strategy consists in standardizing and even enhancing the MSC therapeutic
1202 product.

1203 The development of assays to evaluate MSC functions and predict their efficacy *in vivo* before
1204 their administration in patients is essential to guarantee trustworthy and reproducible results. Thus, assay
1205 development and standardization are ongoing to facilitate the choice between different sources and
1206 improve safety and efficacy of MSC-based therapies.

1207

1208 **D- MSC homing/biodistribution in the heart**

1209 Since the first demonstrations of cardiac differentiation, data from *in vivo* studies based on cell
1210 tracking techniques have clearly shown that only very few transplanted MSC differentiate into

1211 cardiomyocytes to directly repopulate cardiac tissue. A few data evidenced that transplanted MSC can
1212 fuse with recipient cardiomyocytes (364, 365). Thus, recent investigations focus mainly on MSC
1213 paracrine effects as the main underlying mechanisms mediating cardiac repair. In this context,
1214 substantial efforts have been made to define the biodistribution and fate of MSC after their
1215 administration. IV administration of stem cells to increase the pool of endogenous stem cells homing to
1216 injured myocardium was first assessed by post-mortem histology. However, the dynamic redistribution
1217 of peripherally infused stem cells as well as cell trafficking was investigated using the high sensitivity
1218 of the combined single-photon emission computed tomography technology after IV injection of MSC
1219 after AMI. Focal and diffuse recruitment of MSC in the infarcted myocardium was visible on images
1220 within the first 24 hours and remained up to 7 days after but also in the lungs and non-target organs
1221 including the kidney, spleen and liver within 24 to 48 hours after injection (366). By contrast, MRI was
1222 unable to detect MSC after IV injection within the heart in part because of the lower sensitivity of the
1223 technique (366). MSC engraftment into the heart was significantly increased with their local
1224 administration (367, 368). Thus, currently, two main delivery strategies are used in cardiac MSC-based
1225 therapies: IC and intramyocardial (IM) injection. Because IM cell injection is a more invasive approach,
1226 IC infusion is more attractive for the clinic. However, relying on bioluminescent signals for live MSC
1227 imaging, high focal intensity signal was reported after IM delivery, while obviously a weaker signal was
1228 observed after IC delivery. These results suggest a poor retention of MSC after IC delivery compared to
1229 IM delivery (369, 370). Whether the reduced number of “homing” MSC in the myocardium contributed
1230 to higher rate of MSC biodistribution into remote organs (spleen, lung, liver...) remains to be
1231 investigated.

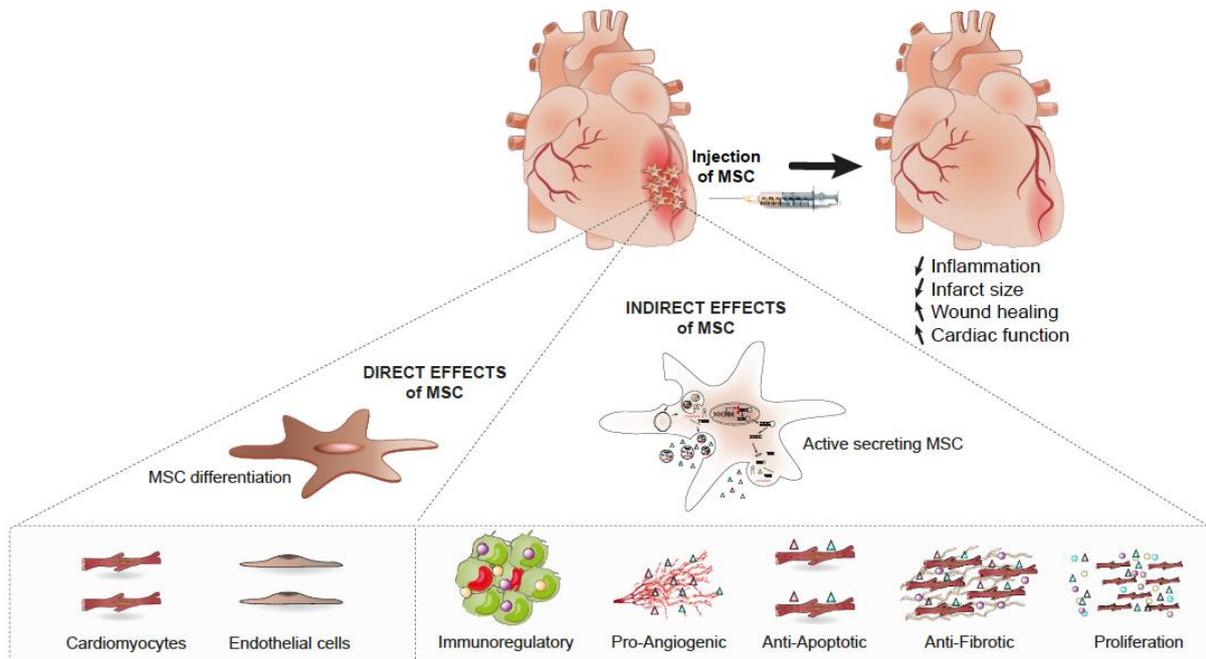
1232

1233 **E- MSC properties**

1234 The cardioprotective effects of MSC, through direct or indirect mechanisms, are multifactorial
1235 and lead to a decrease in both infarct size and post-infarction remodeling (**FIGURE 8**). However, it is
1236 well-admitted that MSC interest resides less in their differentiation abilities into cardiomyocytes and
1237 endothelial cells than in their aptitude to modulate their microenvironment by targeting injured cells
1238 through the release of a plethora of mediators.

1239 *In vitro*, MSC require induction with appropriate factors to differentiate into cells of the three
1240 main components of the cardiovascular system including cardiomyocytes, endothelial cells and vascular
1241 smooth muscle cells (371, 372). Over the last two decades, different strategies have been developed to
1242 induce MSC differentiation into cardiomyocytes, including the use of growth factors (373), chemical
1243 inducers (374), and coculture with primary cardiomyocytes (375). However, these reports also argue
1244 against the use of certain chemical inducers, such as 5-azacytidine, and highlight the necessity to identify
1245 alternative induction procedures. In this context, using sternal marrow-derived MSC, the
1246 cardiomyogenic potential of bFGF (*basic FGF*) combined to hydrocortisone was compared with that of

1247 5-azacytidine, revealing that induction with bFGF and hydrocortisone results in the differentiation of
 1248 MSC into cardiomyocytes with a slightly higher efficiency (376).
 1249
 1250



1251
 1252 **FIGURE 8: MSC-based therapy of AMI relies on the pleiotropic properties of the cells.**
 1253 *MSC cardioprotective effects can be mediated through direct effects relying on the capacity of MSC to*
 1254 *differentiate into cardiac cells or by indirect effects limiting the immune response and apoptosis while*
 1255 *increasing angiogenesis and cardiac cell proliferation. When in presence of specific differentiation*
 1256 *inducers, MSC differentiate both in vitro and in vivo in cardiomyocytes and endothelial cells which*
 1257 *contribute, at least in part, to their ability to repair injured myocardium.*
 1258

1259
 1260 *In vivo*, MSC engraftment and further differentiation into cardiomyocytes and endothelial cells
 1261 after MI have been demonstrated. The efficacy of AT-MSC and BM-MSC has been compared in a
 1262 porcine AMI model showing a similar beneficial effect of the two cell types on cardiac function and
 1263 angiogenesis but a better effect of AT-MSC on left ventricular (LV) remodeling. This therapeutic effect
 1264 was associated with the engraftment of the cells positive for CD31, a marker of endothelial cell, 4 weeks
 1265 after their transplantation (377). Also, the retrograde infusion of bFGF into the coronary vein increases
 1266 the graft uptake and differentiation potential of implanted MSC, thereby restoring cardiac function and
 1267 preventing unwanted remodeling (378).

1268 However, although the differentiation of MSC into cardiomyocytes has been documented, it is
 1269 important to note that a substantial proportion of transplanted MSC is rapidly lost mainly through
 1270 apoptotic mechanisms and that a modest fraction of MSC engraftment happened without differentiation.
 1271 Therefore, paracrine effects have been proposed as the main effect of transplanted MSC for the treatment

1272 of cardiac tissues. The multiple trophic factors produced by MSC are able to repress cardiomyocyte
1273 apoptosis, favor angiogenesis, induce the recruitment and proliferation of endogenous stem cells,
1274 decrease inflammatory oxidative stress as well as fibrosis in the border zone of the scar (379). In 2009,
1275 Shabbir et al. showed for the first time that non-invasive delivery of MSC or their trophic factors by IM
1276 injection can be successful in rescuing the failing heart (380). This study, which reveals that the MSC-
1277 conditioned milieu is as effective as MSC per se in treating experimental heart failure, offered the
1278 definitive proof of the major role of trophic factors in the field of MSC-based therapy for the
1279 myocardium. The set of factors secreted by MSC, also referred to as the MSC secretome, acts on
1280 neighboring cells through a paracrine phenomenon. MSC secretome is a complex product released *in*
1281 *vitro* in the conditioned medium and *in vivo* in the extracellular medium, consisting mainly in soluble
1282 factors and EV (extracellular vesicles). MSC EV and organelles can also be transferred from MSC to
1283 another cell *via* tunneling nanotubes.

1284

1285 **1- Anti-apoptotic properties**

1286 We will discuss the paracrine effects of MSC, which appear to be the primary mechanism by
1287 which they promote cardiomyocyte viability and protect them from apoptosis (**FIGURE 9**).

1288

1289 **1.1. Soluble factors**

1290 MSC release paracrine factors such as cytokines, chemokines and growth factors that can bind
1291 to their specific receptors on cardiac injured cells and regulate processes such as stress-induced apoptotic
1292 pathways to promote cell survival. Among the factors secreted by MSC, we will focus on the role of
1293 VEGF, IGF-1 and HGF (*Hepatocyte Growth Factor*) that display anti-apoptotic properties.

1294 **VEGF**, a vascular osmotic molecule, belongs to the family of the vascular endothelial growth
1295 factor and exhibits various physiological functions including pro-angiogenic properties and the capacity
1296 to enhance cell proliferation, differentiation, and viability. VEGF released by MSC (381) is significantly
1297 upregulated in MSC treated with pro-inflammatory cytokines such as IFN- γ and IL-1 β (382). VEGF
1298 overexpression by MSC has been shown to activate stromal cell-derived factor-1 α (SDF-1 α)/CXCR4
1299 axis and to induce cardiac stem cell mobilization and migration into infarcted areas (383) resulting in
1300 an enhanced angiogenesis. The high level of VEGF released by MSC provides multiple beneficial
1301 effects, ranging from their angiogenic effect and anti-hypertrophic properties to their anti-apoptotic
1302 properties on injured cardiomyocytes (382). The paracrine factor of VEGF is mediated through the
1303 downregulation of microRNA-23a (miRNA-23a) and miRNA-92a in injured cardiomyocytes (384).

1304 **IGF-1** displays an important role in cell growth and differentiation and was reported to prevent
1305 apoptosis of myocardial cells, ventricular dilation and wall stress in infarcted hearts (385). IGF-1,
1306 through the activation of IGF-1R, prevents oxidative stress and cardiac cell apoptosis by repressing
1307 DNA fragmentation, decreasing BAX induction, and inhibiting caspase 3 activity (386). IGF-1
1308 protective mechanisms also involve the preservation of the mitochondrial potential and the retention of

1309 Cyt C, preventing mitochondrial dysfunctions (387). IGF-1 is released by MSC and has been described
1310 to be involved in their cytoprotective effects. *In vitro*, IGF-1 increases cardiomyocyte proliferation and
1311 migration while inhibiting their apoptosis induced by hypoxia (388). In addition, IGF-1 ameliorates the
1312 healing capacity of MSC by improving angiogenesis, decreasing scar formation and accelerating muscle
1313 structure reconstitution (389). Furthermore, the exposition of MSC to a combination of IGF-1 and HGF
1314 decreased their rate of apoptosis after transplantation and promoted their protective effects by
1315 attenuating LV remodeling and improving cardiac function (390). In line with these studies, Wnt11
1316 overexpression in MSC was shown to enhance their anti-apoptotic and anti-necrotic properties on
1317 cardiomyocytes exposed to hypoxia. This increased effect of MSC was associated with the significantly
1318 greater release of VEGF and IGF-1 by MSC overexpressing Wnt11 (391). The addition of anti-VEGF
1319 and anti-IGF-1 antibodies in the co-cultures inhibited this protective effect.

1320 **Basic FGF** (bFGF) is an important mitogen factor that protects cardiomyocytes from iNOS-
1321 mediated apoptosis through the MEK-1-ERK pathway (392). Although MSC are not important
1322 producers of bFGF, they are regularly supplemented with bFGF to, in part, maintain their multipotent
1323 differentiation ability, enhance their expansion and their secretory profile (393). Moreover, bFGF
1324 represses apoptosis in human MSC and *bFGF*-transfected MSC (394, 395). *In vivo*, in a canine model
1325 of coronary ligation, the combined therapy with bFGF and MSC promoted neovascularization and
1326 reduced apoptosis in the infarct border zone. This was associated with an increased engraftment of
1327 transplanted MSC resulting in decreased myocardial fibrosis, an improved recovery of cardiac function
1328 and prevention of post-MI LV remodeling (378). Among the modifications of the secretory profile
1329 induced by bFGF on MSC, HGF secretion ability was significantly increased (393). HGF, a pleiotropic
1330 cytokine, is known to regulate several biological processes including angiogenesis, inflammation and
1331 cell survival. HGF overexpression in MSC resulted in an optimization of MSC therapeutic effect in MI
1332 (396). This enhanced therapeutic effect was due to the enhanced survival of MSC linked to HGF
1333 overexpression under hypoxic conditions and mediated by the regulation of the AKT signaling pathway.
1334 Moreover, the transplantation of MSC overexpressing HGF reduces cardiomyocyte apoptosis, increases
1335 cardiomyocyte proliferation and enhances angiogenesis presumably by augmenting their paracrine
1336 effects. Indeed, via the activation of the Akt pathway, HGF increases VEGF production as well as bFGF
1337 and EGF.

1338

1339 **1.2. Extracellular vesicles**

1340 Extracellular vesicles (EV) represent important intercellular messengers involved in the transfer
1341 to recipient cells of biological signals, especially proteins, lipids and RNA. EV derived from MSC
1342 (MSC-EV) have shown promising protective properties on injured cardiac cells, in part, through the
1343 transfer of mitochondria. The anti-apoptotic effect of EV derived from MSC was shown in coculture
1344 experiments with induced pluripotent stem cell-derived cardiomyocytes (iCM) exposed to doxorubicin
1345 (DOX) using a transwell system allowing to physically separate the two cell types (397). In these

1346 experiments, the authors showed that MSC protect stressed iCM from apoptosis and improved their
 1347 viability. This anti-apoptotic effect exerted by the secretome of MSC was mediated by their capacity to
 1348 release large EV (>200 nm) enriched in mitochondria and up-taken by iCM. Indeed, DOX-injured iCM
 1349 receiving mitochondria contained in MSC-EV exhibited improved both biogenesis and contractility as
 1350 well as increased ATP production and PGC-1 α (Peroxisome proliferator-activated receptor gamma
 1351 coactivator 1-alpha) transcription. The inhibition of the mitochondrial functions in MSC-derived EV
 1352 using 1-methyl-4-phenylpyridinium (MPP⁺) decreased their beneficial effect on injured iCM (397).

1353 The cardioprotective effects of EV are also mediated by transferring growth factors and various
 1354 micro-RNA (miR) to recipient cardiac cells. A large body of evidence suggests that miR contained in
 1355 MSC-derived EV could be a promising cardioprotective therapy. For instance, miR-144 contained in
 1356 MSC-derived EV decreases the expression of PTEN and increases levels of pAKT in cardiomyocytes
 1357 and thereby protects them against hypoxia-induced cell apoptosis (398). Of note, miR-144 was described
 1358 to promote mitochondrial biogenesis and reduce cardiomyocyte apoptosis through the activation of
 1359 AMPK phosphorylation and PGC-1 α deacetylation by targeting RAC1 (RAC family small gtpase 1)
 1360 (399). In ischemic hearts, anti-apoptotic effect of MSC-EV was reported to be mediated by miR-19a,
 1361 which down-regulates PTEN activation, increases BCL-2 protein expression and enhances AKT
 1362 phosphorylation (400, 401). PTEN was also shown to be decreased in cardiomyocytes receiving miR-
 1363 21a-5p contained in MSC-EV. Consistently, the delivery of miR-21a-5p in recipient cardiac cells targets
 1364 genes products involved in cell survival or apoptosis including PDCD4 (*Programmed Cell Death 4*),
 1365 PTEN, FASL and PELI1 (Pellino E3 Ubiquitin Protein Ligase 1) and decreased apoptosis (402).
 1366 Similarly, miR-22-enriched EV, secreted from MSC following a PreC stimulus, were shown to be up-
 1367 taken by cardiomyocytes and to reduce apoptosis in neonate cardiomyocyte subjected to an ischemic
 1368 stress (403). miR-210 and miR-4732-3p were also shown to be enriched in MSC-EV and able to protect,
 1369 in a paracrine manner, hypoxic cardiomyocytes against apoptosis by decreasing the expression levels of
 1370 BAX, BAD, and cleaved caspase 3 and increasing BCL-2 expression (404, 405). Exosomal miR-25-3p,
 1371 is another example of miRNA from MSC that confers cardioprotective effects in MI by targeting pro-
 1372 apoptotic proteins (406).

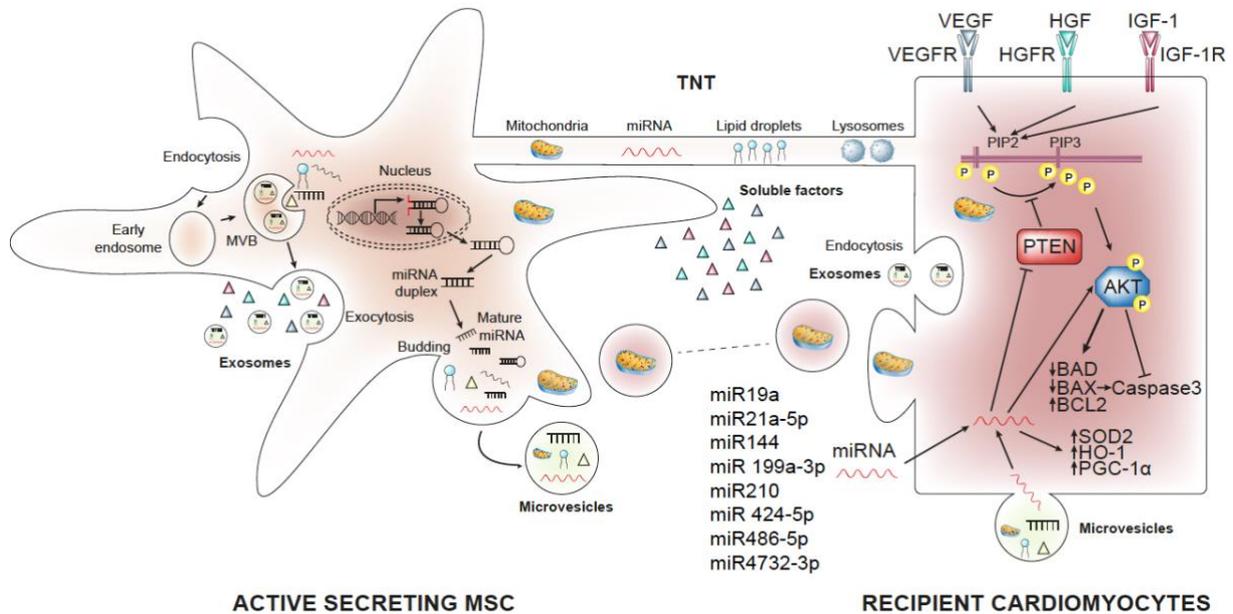
1373 Altogether, these studies strongly suggests that EV derived from MSC could be a potent
 1374 therapeutic vehicle to protect injured cardiac cells from apoptosis in ischemic conditions through the
 1375 delivery of multiple anti-apoptotic miRNAs.

1376

1377 ***1.3. Tunneling nanotubes and organelle exchange***

1378 Tunneling nanotubes (TNT) are the structures allowing the selective transfer of membrane
 1379 vesicles and organelles (407). These structures can be formed between MSC and many other cell types
 1380 including endothelial cells (408) and cardiomyocytes (409). TNT allow the transfer of organelles such
 1381 as mitochondria, lysosomes, microRNA and lipid droplets (**FIGURE 9**). The transfer of mitochondrial
 1382 DNA from stem cells to hypoxic endothelial cells leads to a restoration of their aerobic respiration and

1383 the prevention of apoptosis (410). In addition, mitochondria from BM-MSC provided anti-apoptotic and
 1384 anti-degenerative effects through the rescue of endogenous mitochondrial function restoring SOD2,
 1385 BCL-2, BAX and PGC-1 α expression and inhibiting ROS production (411).



1386

1387 **FIGURE 9: Mechanisms involved in MSC anti-apoptotic effects of cardiomyocytes.**

1388 MSC promote cardiomyocyte viability and protect them from apoptosis through a variety of mechanisms
 1389 that include organelles (mitochondria, lipid droplets, lysosomes) transfer via the formation of TNT as
 1390 well as the secretion of various trophic factors or EV such as exosomes and microvesicles. Microvesicles
 1391 are generated by plasma membrane budding. The MSC extracellular constituents and surface proteins
 1392 form the early sorting endosome by endocytosis with internal budding of the plasma membrane. The
 1393 early sorting endosome gives rise to the late sorting endosome and multivesicular bodies (MVB). The
 1394 MVB release their vesicles into the extracellular zone in the form of exosomes. Exosomes can release
 1395 cargo to recipient cardiomyocytes by three modes: endocytosis, direct membrane fusion, and receptor-
 1396 ligand binding. MSC microvesicles and exosomes can contain a number of different molecules as cargo
 1397 such as proteins, chemokines, growth factors (VEGF, IGF-1 and HGF), cytokines, DNA, mRNA, miR
 1398 (miR19a, miR21a-5p, miR144, miR199&-3p, miR210, miR424-5p, miR486-5p and miR4732-3p) and
 1399 other nucleic acids as well as organelles (mitochondria, lipid droplets, lysosomes) which will protect
 1400 the injured/stressed cardiomyocytes from apoptosis. These molecules and organelles will target genes
 1401 involved in cell survival or apoptosis, in part, through Akt activation.

1402

1403

1404 In the injured tissue, suffering cells release DAMPS to promote the adaptative MSC reparative
 1405 response. Anti-apoptotic functions of MSC are also triggered, in part, via mitochondria transfer to
 1406 injured cardiomyocytes (412). As a response, MSC donate their healthy mitochondria to help injured
 1407 cells to overcome oxidative stress injury, in part, through the cytoprotective heme-oxygenase 1 (HO-1)
 1408 enzyme stimulating mitochondrial biogenesis (412). HO-1 is an inducible stress protein that protects
 1409 cells from oxidative stress, apoptosis, and inflammation. MSC pre-treatment with a potent inducer of
 1410 HO-1, haemin, was shown to inhibit mitochondrial fragmentation and apoptosis of MSC induced by

1411 serum deprivation and hypoxia. At 4-week after transplantation, compared with naïve MSC, haemin-
 1412 treated MSC significantly enhanced cardiac function in mice subjected to MI. This therapeutic benefit
 1413 was associated with decreased apoptosis of cardiomyocytes, improved angiogenesis and higher cell
 1414 survival (413). Therefore, pharmacological pre-treatment might improve MSC survival rate under
 1415 ischemic conditions through the repression of mitochondrial fission and then improve MSC-based
 1416 therapy for MI treatment.

1417 To conclude, MSC have strong anti-apoptotic effects mediated by different mechanisms. They
 1418 can increase pro-survival pathway by direct or indirect effects to limit oxidative stress and inflammation
 1419 and increase angiogenesis. However, the main benefits observed in MSC-based therapy including their
 1420 anti-apoptotic properties, are provided by their secretome.

1421

1422 **2- Anti-inflammatory/Immunoregulatory properties**

1423 MSC have also been of high interest for the treatment of cardiovascular diseases because of their
 1424 immunoregulatory and anti-inflammatory properties. Indeed, MSC not only exert immunoregulatory
 1425 properties through the innate and/or the adaptive immune system but also regulate the trafficking of
 1426 inflammatory immune cells. To exert such functions, MSC need to be “primed”/activated with pro-
 1427 inflammatory molecules. Indeed, in response to their environmental stimuli, MSC can adopt different
 1428 phenotypes, morphologies, a migratory potential, anti-inflammatory and immunoregulatory functions.

1429

1430 **2.1. MSC priming/activation with inflammatory cytokines**

1431 Pro-inflammatory factors released in the local injured milieu, massively impact on MSC
 1432 properties and thus modulate the outcome of MSC-based therapies. MSC express several receptors
 1433 among which the inflammation sensing receptors and the toll-like receptors enabling them to sense and
 1434 respond to tissue damage (414). Thus, MSC stimulation with a specific ligand effectively prime MSC
 1435 to induce and/or modulate one or more of their functions and their therapeutic effects (415).

1436 Several studies have shown the pivotal role of IFN- γ for MSC activation (416-418). Indeed,
 1437 IFN- γ , a proinflammatory cytokine produced by activated T cells, primes the immunomodulatory
 1438 property of MSC by upregulation of PD-L1 (*Programed Death-Ligand 1*), an inhibitory surface
 1439 molecule. Conversely, PD-L1 knockdown using siRNA abolished MSC immunosuppressive potential.
 1440 In line with these studies, it was shown that IFN- γ induces MSC immunosuppressive properties alone
 1441 or in combination with others pro-inflammatory cytokines including IL-1 β , IL-1 α and TNF- α (416).
 1442 These pro-inflammatory cytokines induce, in part, the expression of VCAM-1 and ICAM-1 in MSC
 1443 (419). Thus, when MSC are cocultured with activated T cells producing high levels of pro-inflammatory
 1444 cytokines, they significantly increase the adhesion capacity of T cells to their surface via their increased
 1445 expression of VCAM-1 and ICAM-1. Consistently, MSC immunoregulatory properties were
 1446 significantly reduced *in vitro* and *in vivo* when VCAM-1 and ICAM-1 were functionally inactivated or
 1447 genetically silenced. TNF/TNFR1 axis also enhances the production of molecules referred as mediators

1448 of MSC immunosuppressive properties such as PGE₂ by inducing the expression of COX2
1449 (*cyclooxygenase 2*) in MSC. In turn, PGE₂ released by primed MSC stimulates the production of IL-10
1450 by macrophages through the prostaglandin EP2 and EP4 receptors (420). In addition, TNF- α and
1451 hypoxia priming of MSC enhances the production of paracrine factors including b-FGF, VEGF and
1452 TGF- β (*Transforming Growth Factor- β*) that work in synergy to regulate the microenvironment and
1453 reduce inflammation in MI (421).

1454 More recently, our group and others showed that MSC activation with IFN- γ and TNF α induces
1455 their metabolic switch towards glycolysis (422-424). Going further we demonstrated that this metabolic
1456 reprogramming significantly enhanced MSC immunoregulatory properties *in vitro* and *in vivo* in an
1457 AMPK- and HIF-1 α (*Hypoxia Inducible Factor-1 alpha*)-dependent manner (422). Indeed, we showed
1458 that the significant increased level of AMPK phosphorylation in MSC after TNF α and IFN- γ stimulation
1459 induced their glycolytic switch. The inhibition of AMPK activity abolished the enhancement of
1460 glycolytic MSC suppressive activity. TNF- α and IFN- γ stimulation of MSC also significantly increased
1461 the expression and the nuclear translocation of HIF-1 α (425). HIF-1 α knockdown in MSC induced a
1462 metabolic switch from glycolysis to OXPHOS, a decrease of the immunoregulatory potential and a
1463 reduction of the expression or production of some MSC immunosuppressive mediators including ICAM,
1464 IL-6, and NO respectively (425).

1465 Among the master regulators of cell metabolism, mitochondria are key organelles that have been
1466 shown to orchestrate MSC immunoregulatory potential. MSC have the unique ability to export their
1467 own mitochondria to neighboring cells in response to inflammation. Indeed, the pro-inflammatory
1468 environment induced by TNF promotes TNT formation and mitochondrial transfer between MSC and
1469 cardiomyocytes to rescue cardiomyopathy (426). Moreover, mitochondrial transfer induced by pro-
1470 inflammatory stimuli between MSC and immune cells has been shown to regulate the immune response
1471 (427, 428). Altogether these studies indicate that the inflammatory microenvironment may be a critical
1472 factor to reprogram the metabolic status of MSC and enhance their paracrine effects and mitochondrial
1473 transfer to modulate their anti-inflammatory and immunoregulatory properties.

1474

1475 **2.2. Anti-inflammatory properties**

1476 During MI, distant inflammatory cell-generating organs, including the spleen and bone marrow,
1477 are stimulated, thereby maintaining a continuous supply of monocytes to the site of inflammation (429,
1478 430). Thus, the systemic response can transfer the inflammation to organs that are not initially involved
1479 (430). While systemic and local inflammatory responses after MI are well described, the mechanisms
1480 of infarct zone containment have been addressed recently. The authors showed that serum of patients
1481 with AMI exerts anti-inflammatory actions on healthy cardiomyocytes, thereby contributing to limit
1482 infarct size and protecting healthy tissue. However, ventricular fibrillation, a complication of MI,
1483 promotes the switch of healthy cardiomyocytes towards a proinflammatory phenotype (431). During MI,

1484 tissue injury-related signals including DAMPs released from dying or stressed cells are detected by MSC
1485 leading to increased pro-repair abilities and anti-inflammatory outcomes (432).

1486

1487 **Effect of MSC on inflammation** Experiments on cultured naive human BM-MSC spheroids
1488 exposed to TNF α , and cocultured with adult human cardiomyocytes, or with conditioned media pooled
1489 from TNF α -stimulated adult cardiomyocytes, showed that TNF α -induced inflammation altered BM-
1490 MSC survival and differentiation into mature cardiomyocytes, while promoting matrix protein synthesis
1491 and cytokine release conducive to the MI remodeling (433). While inflammation repressed naïve MSC
1492 functions, different results were reported using primed MSC. Indeed, the increased expression of
1493 inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 in the ischemic heart was shown to be inhibited
1494 upon the transplantation of IGF-1-primed MSC, which also reduced cardiac dysfunction, increased cell
1495 engraftment and reduced apoptosis of myocardium cells (434). Similarly, MSC overexpressing HGF
1496 down-regulate inflammatory cytokine secretion and up-regulate anti-inflammatory cytokine release,
1497 suggesting that HGF is a critical contributor to the regulation of cytokine secretion by MSC (435).

1498

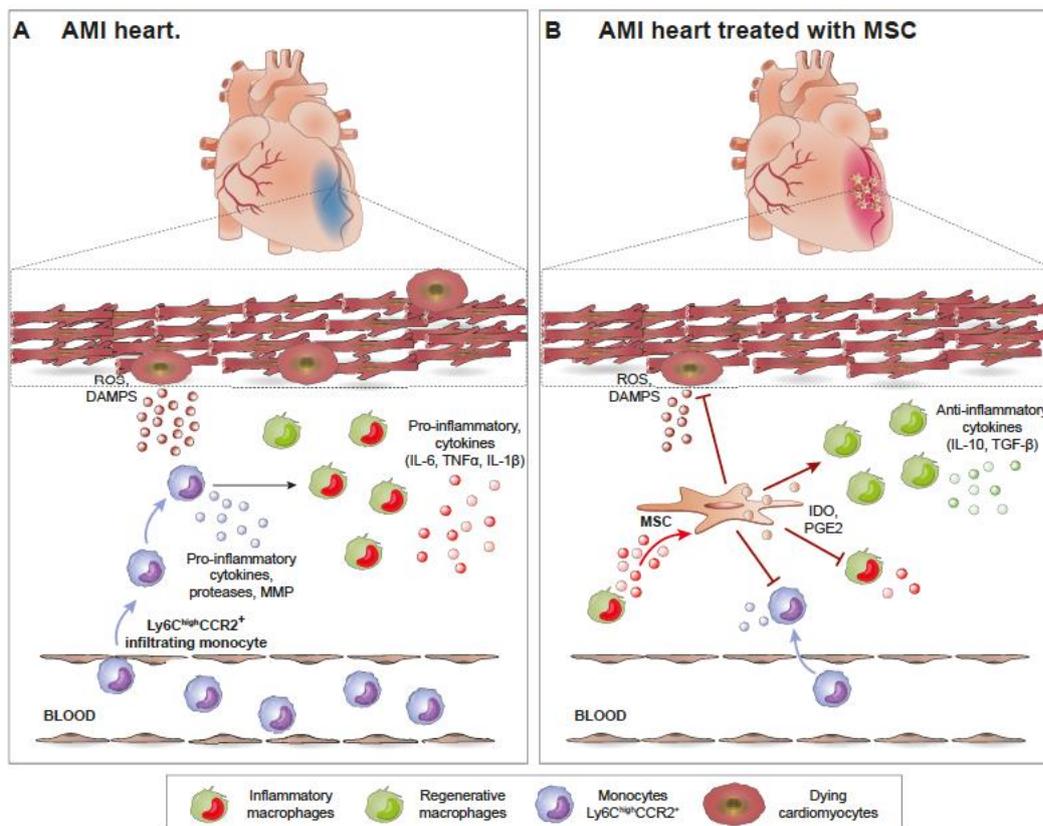
1499 **Effect of MSC administration on the monocyte response** Pro-inflammatory cytokines are
1500 mainly derived from monocyte-derived macrophages, the most abundant immune cells in the heart after
1501 MI and IR injury (436). After MI, monocytes migrate to the infarct area and differentiate into
1502 macrophages that release chemokines, growth factors and cytokines, to clear injured and apoptotic
1503 myocardial cells (**FIGURE 10**). Macrophages after MI are activated and polarized towards different
1504 subsets with different immune functions, mainly into pro-inflammatory macrophages producing IFN- γ ,
1505 TNF- α and IL-23 and promoting the destruction of the extracellular matrix (437). Therefore, the
1506 regulation of monocyte trafficking and mobilization has emerged as an attractive therapeutic approach
1507 for myocardial inflammation and fibrosis. In an experimental model of myocarditis, the capacity of MSC
1508 to decrease the severity of the disease in mice was associated with a reduced percentage of pro-
1509 inflammatory Ly6C^{high} and Ly6C^{middle} monocytes and an increased percentage of Ly6C^{low} monocytes in
1510 the heart and the blood. This study demonstrates that MSC promote the resolution of cardiac
1511 inflammation by repressing the cardiac infiltration of pro-inflammatory monocytes while enhancing
1512 cardiac influx of anti-inflammatory monocytes (438).

1513 More recently, the combined treatment with MSC and anti-CCR2 significantly reduced monocyte
1514 accumulation at the site of infarction in AMI mice by comparison with injection of MSC or anti-CCR2
1515 alone. In addition, the reduced monocyte infiltration was associated with a decreased secretion of pro-
1516 inflammatory cytokines at the injury sites, a better tissue regeneration and an enhanced improvement in
1517 cardiac function (439).

1518

1519 **Effect of MSC-EV on inflammation** Similarly, to their parental cells, MSC-EV were shown
1520 to regulate cardiac inflammation and ameliorate cardiac function in preclinical studies through the same

1521 pathway. Indeed, EV can inhibit the monocyte influx into the injured region via different mechanisms.
 1522 The first evidence of the anti-inflammatory effect of MSC-EV was provided by a study in a mouse
 1523 model of myocardial IR injury, where the EV were injected intravenously before reperfusion (440).
 1524 Similarly, MSC-EV administered in rat hearts after permanent ligation significantly reduced the
 1525 percentage of macrophages in the peri-infarct zone one week after MI (441). These anti-inflammatory
 1526 effects of EV might be mediated through the transfer of different molecules including miRNA and
 1527 therefore regulate leukocyte influx in the heart. For instance, miR-24 was shown to regulate vascular
 1528 inflammation by controlling macrophage behavior (442, 443). Moreover, miR-24-3p was suggested to
 1529 repress the recruitment of bone marrow-leukocytes by downregulating their CCR2 expression level
 1530 (444).
 1531



1532

1533 **FIGURE 10: MSC anti-inflammatory and immunoregulatory effects in hearts after ischemic injury.**
 1534 **A.** The death of cells induced by ischemic injury leads to the release of DAMPs and ROS, which induces
 1535 the inflammatory phase. Indeed, dying cardiomyocytes produce pro-inflammatory cytokines and
 1536 chemokines resulting to a substantial inflammatory monocytes influx into the cardiac ischemic area,
 1537 responsible for phagocytosis of the dying cells and the generation of an inflammatory environment
 1538 promoted by the presence of pro-inflammatory macrophages and Ly6C^{high}CCR2⁺ infiltrating monocytes
 1539 that adopt transcriptional active states and participate to adverse cardiac remodeling. **B.** MSC
 1540 administrated in the ischemic hearts will receive pro-inflammatory signals from injured cardiac cells
 1541 and the inflammatory monocytes/macrophages and an active secreting phenotype. The subsequent
 1542 primed secreting MSC will release soluble factors, microvesicles and exosomes that will mediate anti-
 1543 inflammatory and immunoregulatory properties. First, primed MSC will regulate the influx of CCR2⁺
 1544 infiltrating macrophages derived from circulating monocytes. Second, primed MSC will promote
 1545 resident or monocyte-derived macrophages polarization towards an anti-inflammatory phenotype.

1546

1547 **2.3. MSC immunoregulatory properties**

1548 In addition to their capacity to control monocyte trafficking and mobilization, MSC can also
 1549 potentially regulate the immune response. MSC immunoregulatory properties have been discovered in the
 1550 early 2000s. MSC have been initially described for their capacity to inhibit T cell proliferation and
 1551 functions both *in vitro* and *in vivo* (445). A few years after, the inhibitory potential of MSC on other
 1552 adaptative immune cells such as B cells (446) and innate immune cells, including dendritic cells (DCs)
 1553 (447), natural killer cells (NK cells) (448) and macrophages (449), was reported.

1554 MSC and macrophage interactions increase the expression of CD206 and the production of IL-10 by
 1555 macrophages while reducing IL-12 and TNF- α . Thus, it was proposed that these MSC-trained
 1556 macrophages constitute a new type of alternatively activated macrophages that would play a potential
 1557 important role in tissue repair (450).

1558

1559 **Priming of MSC immunoregulatory properties** To exert such immunoregulatory properties
 1560 and switch the inflammatory immune response into a balanced immune response, MSC sense danger
 1561 signals *via* receptors including TLR and respond to excessive pro-inflammatory signals *via* TNF- α and
 1562 IFN- γ receptors. MSC immunoregulatory properties have been described by our group and others to be
 1563 mediated through both direct cell contacts and soluble factors. Thus, MSC need to be primed by
 1564 inflammatory conditions or also hypoxia to exert their immunoregulatory functions. For example, MSC
 1565 do not exhibit at steady state any indoleamine 2,3-dioxygenase (IDO) activity, pivotal for their
 1566 immunosuppressive functions, while IFN- γ induces it. Similarly, the production of PGE₂ by MSC (451),
 1567 a soluble mediator of MSC immunoregulatory properties, is stimulated by inflammatory cytokines such
 1568 as the combination of IFN- γ and TNF- α (452). IDO and PGE₂ produced by MSC induce the switch
 1569 from pro-inflammatory to anti-inflammatory macrophages (453). However, PGE₂ can modulate both
 1570 the innate and adaptive immunity, with either beneficial or adverse effects (454). Indeed, PGE₂ produced
 1571 by MSC can, on one hand, promotes an inflammatory response by enhancing LPS (lipopolysaccharide)-
 1572 induced IL-23 production by dendritic cells, which favors Th1 and Th17 cell differentiation (for review
 1573 see (455)). On the other hand, PGE₂ produced by MSC can generate an anti-inflammatory response
 1574 through its binding to its receptors EP2 and EP4 on macrophages that enhances their polarization toward
 1575 anti-inflammatory macrophages (420).

1576

1577 **Factors involved in the immunoregulatory properties of MSC on monocytes/macrophages** In
 1578 a mouse model of AMI, the injection of MSC, 2 days after MI induction reduced the proportion of
 1579 macrophage/monocyte including pro-inflammatory macrophages, while increasing the percentage of
 1580 anti-inflammatory and reparative macrophages (F4/80⁺CD206⁺) both in the circulation and in infarcted
 1581 heart (456, 457). The increased immunoregulatory effect on macrophages observed in response to MSC
 1582 treatment in cardiomyopathies is lost when MSC are pretreated with a COX-2 inhibitor prior their

1583 injection (458). Anti-inflammatory macrophage polarization promoted by MSC *via* the COX-2-PGE2
1584 pathway will increase the concentration levels of TGF β , IL-10, HGF, PGE2 and IDO in the infarcted
1585 myocardium and thus suppress the proinflammatory state of the tissue. This paradoxical effect exerted
1586 by PGE2 depends on its concentration (for review see (455)). Other soluble factors produced by MSC
1587 such as IL-1Ra (*Interleukin-1 receptor antagonist*), IL-6 and granulocyte macrophage-colony
1588 stimulating factor (GM-CSF) mediate, in part, the shift of macrophages toward an anti-inflammatory
1589 phenotype (420, 459, 460). Recently, it has been shown that the intracardiac injection of living or dead
1590 stem cells similarly provides an accumulation of macrophage positives for CCR2 and CX3CR1 inducing
1591 a functional repair process of the injured myocardium (461). This latter study suggests that stem-cell
1592 based therapy in cardiac ischemic injury promotes wound healing response and restores the mechanical
1593 properties of infarcted heart *via*, in part, an acute immune response. However, the recent findings
1594 indicating a functional distinction between CCR2⁺ and CX3CR1⁺CCR2⁻ macrophage subsets in cardiac
1595 wound healing (462, 463) raise the question of the role of MSC-based therapy on these macrophage
1596 subsets.

1597 Modified MSC were shown to display enhanced immunoregulatory potential and subsequently
1598 improved therapeutic properties for MI. Indeed, in an experimental model of MI, MSC overexpressing
1599 IL-33 (MSC-IL-33) were shown to decrease inflammation in the hearts with MI, to increase the
1600 expression of CD206 and reduce the expression of iNOS. Moreover, genes related to inflammation
1601 including IL-6, TNF- α , and IL-1 β were found to be reduced in the border zone of hearts treated with
1602 MSC-IL-33 compared to the heart treated with control MSC (464).

1603 More recently, the role of hypoxia on MSC immunoregulatory properties was investigated. First,
1604 the authors showed that hypoxia increase the expression levels of HIF-1 α and TGF- β 1 in MSC and that
1605 the hypoxic conditioned media of MSC promotes macrophage polarization towards an anti-
1606 inflammatory-like phenotype through TGF- β 1/Smad3 signaling pathway. Finally, they report that these
1607 macrophages attenuate cardiac fibrosis and apoptosis and increase the density of the microvessels
1608 density after MI (465).

1609 Therefore, MSC based-therapy of MI is, in part, mediated through the control of the monocyte
1610 trafficking and mobilization and the regulation of macrophage polarization.

1611

1612

1613 **IV- MSC for the treatment of IR injury**

1614

1615 **A- Results obtained in preclinical studies and clinical trials**

1616

1617

1618 **1- In preclinical models**

1619 Highly encouraging results were obtained from pioneering studies in preclinical models in the
 1620 early 2000s showing that MSC in the adult heart were able to replace damaged tissue after engraftment
 1621 although contested thereafter (466). Then, MSC-based cardiomyoplasty was investigated in different
 1622 animal models, showing the differentiation of MSC into cardiomyocytes, endothelial and smooth
 1623 vascular cells (467-469). Evidence of MSC differentiation into functional cardiomyocytes (CM-MSC)
 1624 was reported *in vitro* after treatment with various drugs such as 5-azacytidine (an hypomethylating agent)
 1625 and *in vivo* with improvement of contractile function (470, 471). However, due to safety concerns, this
 1626 differentiation protocol has been replaced by insulin, dexamethasone and ascorbic acid (472) or bone
 1627 morphogenetic protein-2 associated with FGF (473). In addition, the risk of immune response more
 1628 pronounced with differentiated MSC led researchers to use undifferentiated MSC instead for *in vivo*
 1629 studies (474, 475). The safety of undifferentiated MSC was subsequently demonstrated, with the
 1630 exception of one report of arrhythmias in a porcine model of AMI (476), as well as their efficacy on
 1631 infarct size and heart function.

1632

1633 ***Effects of MSC administration on cardiac performance*** Ejection fraction parameter (EF, %),
 1634 the simplest and most widely used parameter for the global assessment of myocardial function in patients
 1635 (477), was measured in animals either using echocardiography (in most cases), MRI or SPECT (*Single*
 1636 *Photon Emission Computed Tomography*). Kanelidis' metanalysis reports a 12%-overall increase in EF
 1637 in rodents after MSC administration compared to the untreated group (478). Results in large species
 1638 such as pigs showed a 7%-improved EF obtained from only 7 out 16 studies revealing MSC positive
 1639 impact. Jansen Of Lorkeers' analysis confirmed that MSC treatment was able to increase EF by an
 1640 average of 8.7% in 832 treated animals of large species including pigs, dogs, and sheep *versus* 583
 1641 control animals (479). Accordingly, a 8%-decrease in infarct size was evidenced in 50% of the
 1642 preclinical studies performed in rodents (mouse and rat) (478) and a 6.4%-reduction in pig hearts (mean
 1643 obtained from 7 positive *versus* 5 neutral studies) (478). Cardiac remodeling was also prevented by
 1644 MSC therapy as assessed by the decreased volume of the cardiac chamber (LVEDV, *Left Ventricular*
 1645 *End-Diastolic Volume* and LVESV, *Left Ventricular End-Systolic Volume*), the absence of hypertrophy
 1646 and also a less extent in cardiac fibrosis (479-483).

1647

1648 **Immune response following MSC administration** For technical reasons, the most frequently
 1649 used MSC source are allogeneic in preclinical models but also autologous usually in large species (484),
 1650 or xenogeneic MSC (481, 485, 486) have been studied, showing similar efficacy (for review see (478,
 1651 479). Both autologous and allogeneic cell therapies have been reported to be safe in preclinical studies
 1652 on large animals (pig, sheep, dog) (479). The risk of immune rejection after transplantation has been
 1653 widely discussed since MSC exhibit an immune evasion phenotype marked by low MHC (*Major*
 1654 *Histocompatibility Complex*) class I levels and the absence of expression of MHC class II (474, 487).
 1655 MSC, due to their low immunogenicity, can survive for more than one month after transplantation in
 1656 allogeneic hearts by contrast to differentiated MSC that are not immune-privileged (474, 475). Their
 1657 short lifespan (<1month) after injection could be explained by immune rejection due to expression of
 1658 MHC class II *in vivo* (488). However, despite the existence of an immune response, an improvement in
 1659 the left ventricle EF was evidenced even with xenogeneic MSC (489).

1660

1661 **2- In AMI patients**

1662 Results obtained in clinical trials evaluating the benefits of MSC for cardiac repair in AMI
 1663 patients are variable and sometimes even contradictory (see **Table**). Safety was the first primary clinical
 1664 outcome evaluated in those studies, clearly showing the near absence of complications during either the
 1665 procedure (except for one case of periprocedural complication of coronary artery obstruction) or the
 1666 follow-up (490-492). Importantly, neither tumorigenicity nor ectopic tissue formation was reported 24
 1667 months after allogeneic MSC injection (493).

1668

1669 **Heterogenous protocols of MSC administration** EF (%) parameter was also measured to
 1670 evaluate cardiac function using echocardiography in most cases (490, 492-501), MRI (493, 494) or
 1671 SPECT (490, 492, 494-497, 499, 502). A highly significant 3.78%-increase in EF for the MSC group
 1672 *versus* control was evidenced by Attar's metanalysis (13 clinical trials), confirming the results of a
 1673 previous metanalysis (3.22%-increase) (503, 504). Remarkably, a record 5%-increase in EF was
 1674 reported also with the combination of 6.10^6 MSC injected *via* the IC route, 1-week post-MI (<1 week
 1675 and $<10^7$) suggesting that the finely tuned combination of the right timing and the right dose injected is
 1676 determinant for the effectiveness of MSC therapy (495). However, considering the positive results
 1677 obtained with a treatment protocol of timing of injection <1-week combined to a dose of MSC $<10^7$
 1678 (495, 502) or the protocol with a timing <1-week and a dose $>10^7$) (493, 494) and the negative ones
 1679 obtained with a timing of injection >1 -week and a dose of MSC $>10^7$) (490, 497, 500), the "timing of
 1680 injection" seems to be the crucial parameter for success. Importantly, cardiac function improvement was
 1681 stable for up to 24 months after MSC-based therapy (505).

1682

1683

1684

Author, year	Source of MSC	Origin	Route	Timing (days after PCI)	Number /Age	Dose	Readout	Follow-up duration (months)	Results	LVEF Treated	LVEF placebo
Chen, 2004	Autologous	BM-MSC, fresh	IC	18 ± 0.5	69 / 57,5	8-10 .10 ⁹	ECHO, PET	6 m	-Increased EF (vs placebo) -Decreased perfusion defects, LVEDV and LVESV	At 3 months 67 ±11	At 3 m : 53±8
Hare, 2009	Allogeneic	BM-MSC	IV	<7	53 / 57	80 . 10 ⁶	ECHO/MRI	12 m	-safety of hMSC allogeneic -Improved EF vs baseline -reduced ventricular tachycardia	5.2 ± 1.9 changes vs placebo (p*)	1.8 ± 1.5 changes vs baseline
Yang, 2010	Autologous	BM-MSC Vs BM-MSC (no control group)	IC via the LAD (group I) or via a non-infarct coronary artery (group II)	21	16 / 60.2	12.2±17.7 (group I) or 13.2 ±17.6 10 ⁶ (group II)	ECHO, SPECT	6 m	Increased LVEF + myocardial viability vs baseline	52.0 ± 8.9	50.5 ±9.1
Penn, 2012	Allogeneic	BM-MSC	IC	3.3	25 / 53	20. 10 ⁶ , 50. 10 ⁶ 100. 10 ⁶	ECHO, Angiography	4 m	improved EF vs baseline (50 10 ⁶). Improved EF + stroke volume for doses ≥50 10 ⁶ in patients with EF<45%	8.7 % Absolute change (vs baseline) (50 10 ⁶)	
Houtgraaf, 2012	Autologous	Adipose : first study	IC	<1	13 / 58	17.4 ±4.1 x10 ⁶	SPECT/CMR	6 m	-Safety - decreased infarct size and perfusion defects vs placebo - no change in EF	56.1%	50.3%
Gao, 2013	Autologous	BM-MSC	IC	17.1	43 / 56.8	3.08. 10 ⁶	ECHO, SPECT	12 m	- no change in EF	54.9 ±1.6	55.1± 1.8
Rodrigo, 2013	Autologous	BM-MSC	IM	17 to 25	54 / 58.5	46. 10 ⁶	ECHO, SPECT	60 m	-safety /5 years - no change in EF vs placebo	57.0 ± 3	50 ± 12
Wang, 2014	Autologous	BM-MSC no control group	IC	14	58/57	10 ⁸	ECHO, angiography	6 m	-decreased Adverse events and rehospital rates -no change in EF	50.1 ± 3.4	49.1 ± 2.3
Lee, 2014	Autologous	BM-MSC	IC	28.8	69/54	72. 10 ⁶	ECHO, SPECT	6 m	-Safety -Improvement of EF vs baseline	50.0 ± 8.4 (5.9% ± 8.5% vs baseline)	50.4 ± 9.4 (1.6% ± 7.0% vs baseline)
Chullikana, 2015	Allogeneic	BM-MSC	IV	2	19/47,5	2.10 ⁹ /kg of body weight	ECHO, MRI, SPECT	24 m	Safety and tolerance - No improvement in EF	46.96 ±7.56	45 ±10.21
Musialek, 2015	Allogeneic	WJ-MSC	IC	5-7	10 / 55	30. 10 ⁶	Safety procedural	12 m	Safety		
Gao, 2015	Allogeneic	umbilical	IC	6.2	116/57	6. 10 ⁶	ECHO / PET / SPECT	18 m	- increase in EF -decreased infarct size and remodeling	7.8 ± 0.9 vs baseline (+ vs placebo)	2.8 ± 1.2 Vs baseline
Kim, 2018	Autologous	BM-MSC	IC	25	26/56.5	72 ± 9 x 10 ⁶	ECHO, SPECT	12 m	-improved EF vs baseline and vs placebo	8.8 ± 2.9% vs baseline	4.8 ± 1.9% vs baseline
Zhang, 2021	Autologous	BM-MSC	IC	14.07 ± 9.53	43/59	3.31 ± 1.7 .10 ⁶	ECHO	12 m	- no change in EF	57.2 ± 10.2	62.0 ± 6.8

1685 **Table: summary table of clinical trials conducted on AMI patients with MSC-based therapy**

1686

1687 **Effects of MSC on patient prognosis** Outcomes on infarct size were assessed by cardiac
 1688 magnetic resonance (CMR) (502), MRI (494) or ventriculography (500). In most cases, a lack of effect
 1689 was reported using either allogeneic (494) or autologous BM-MSC (500) except in one study showing a
 1690 decreased infarct size with an IC injection of freshly prepared autologous AT-MSC (502). Contrasting
 1691 results were reported also for myocardial viability after autologous BM-MSC administration showing
 1692 either improvements (506) or neutral effects (492, 501). Regarding myocardial perfusion, improvements
 1693 were observed after injection of autologous AT-MSC and BM-MSC (490, 502, 506) or Wharton's jelly-
 1694 derived MSC (495) but an absence of effects was also described despite quite similar protocols (492,
 1695 494, 501). By contrast to preclinical studies, long-term progression towards heart failure was quoted in
 1696 patients, showing a reduction LVEDV or LVESV measurements as indicators of LV remodeling (490,
 1697 493, 495) and in the number of rehospitalizations for heart failure (500). However, no change was
 1698 observed for the six-min walk test at 6-month post-MI (493) and also, more importantly, in the survival
 1699 rate (499, 500).

1700

1701 **Allogeneic versus autologous MSC** For a clinical application, bone marrow and adipose
 1702 human tissues are the two main sources of cells widely used to ensure large amounts of MSC (507) even

1703 though umbilical cord-derived stem cells have been shown recently to have a more positive effect on
1704 EF (503). Both autologous (490, 492, 496, 497, 499-502, 508) and allogeneic (493-495, 509) MSC have
1705 been tested in AMI patients. After a pioneering study showing safety and promising results using
1706 autologous MSC (490), puzzling data have been reported in following studies with either positive results
1707 (496, 497, 500, 502) or a lack of efficacy (492, 499, 501). Because autologous stem cells must be
1708 amplified *ex vivo* for several weeks (1 to 4) to obtain a sufficient number for the treatment (496, 501),
1709 they cannot be easily used in the acute phase of MI to treat reperfusion injury. Thus, the use of allogeneic
1710 MSC appear more adapted and less expensive than autologous cells that require cell expansion, product
1711 characterization and batch release testing for each patient. More importantly, allogeneic MSC in AMI
1712 patients are safe and provide promising results (362, 493, 509) highlighting the difference in quality of
1713 MSC from young and healthy donors compared to AMI patients. For these reasons, allogeneic MSC
1714 therapy is preferred over autologous MSC therapy (510). They come from healthy donors by contrast to
1715 autologous ones and, as “off-the-shelf” product, they are immediately available for urgent treatment.

1716

1717

1718 **B. Origin of the gap for the clinical translation**

1719

1720 While MSC is a promising cell therapy product, the heterogeneity of results raises the question
1721 of how to use them optimally to guarantee positive results to the patients. To this end, it is necessary to
1722 analyze the reasons underlying this heterogeneity.

1723

1724 **1- Influence of the cell therapy product and the recipient**

1725 The difference between the results obtained in the preclinical studies and those reported in the
1726 large-scale clinical trials could be explained by several issues including the heterogeneity of cell
1727 preparations, the injected doses and the therapeutic time window of administration.

1728

1729 ***1.1 Regarding the cells***

1730 ***Age of cell donors*** The first investigations performed with patient-derived cells revealed a
1731 decreased number of cells and an impaired functional activity as compared to healthy cells (511). In
1732 AMI patients, disease and/or age reduce the impact of BM-MSK on their function (492) as compared
1733 with other studies using allogeneic MSC from young and healthy donors (493-495, 512). As a result,
1734 most clinical trials used allogeneic MSC instead of autologous MSC coming from patients with age-
1735 related diseases that have decreased regenerative capacities (513, 514). In fact, compared with aged
1736 donors, MSC derived from young donors have a higher proliferation rate, a slower progression toward
1737 senescence during the *in vitro* expansion phase (515) and a lower rate of trophic factor secretion (for
1738 review see (516)). For instance, decreased secretion of brain-derived neurotrophic factor (BDNF) and
1739 PDGF in human MSC derived from aged donors was associated with less atrophy-preventing capacities,

1740 anti-inflammatory effects, vessel maturation and lower levels of MCP-1 in peri-infarct tissue compared
 1741 to MSC derived from young donors since PDGF is a potent inducer of MCP-1 and the PDGF family is
 1742 closely related to the VEGF family (516). The same negative correlation was reported for age and
 1743 immunoregulatory potential of BM- and AT-MSC (517). Old MSC display a reduced expression of TNF
 1744 receptor (TNFR), IFN- γ receptor (IFNGR), and CCR7, required for their activation by TNF- α and IFN-
 1745 γ , which stimulate/prime their immunosuppressive properties (518).

1746 Therefore, disadvantageous characteristics of MSC from aged donors could adversely affect the success
 1747 of cell therapy for MI and consequently allogenic source of MSC from young and healthy donors are
 1748 preferred in clinical trials. Further investigations are ongoing to elucidate the mechanisms associated to
 1749 MSC aging in order to find new directions for counteracting cell age loss.

1750 **Anatomical depots** In mammals, white adipose tissue, involved in energy storage, is anatomically
 1751 classified into two major fat deposits, visceral adipose tissue (e.g., epididymal adipose tissue (EAT))
 1752 and subcutaneous adipose tissue (e.g., inguinal adipose tissue (IAT)). The different characteristics of
 1753 EAT and IAT are mediated, in part, by the distinct properties of ASC. For instance, IAT-derived ASC
 1754 actively repress monocyte infiltration via secreted molecules, thereby inhibiting inflammatory responses
 1755 in IAT (519). Moreover, in a study comparing the biology of ASC from 5 different subcutaneous adipose
 1756 locations (superficial abdominal, deep abdominal, arm, thigh, and trochanteric), it was shown that the
 1757 susceptibility to apoptosis was lowest in the superficial abdominal depot and in the younger age groups
 1758 (520). This observation was in line with a study showing that the superficial abdominal depot was less
 1759 susceptible to apoptosis when compared with adipose stem cells from the omental depot (521).

1760 In addition to differences in susceptibility to apoptosis, different functions have been associated to the
 1761 ASC subsets identified in response to obesogenic stimuli. Indeed, ASC positive for syndecan 1 (SDC1)
 1762 predominant in EAT promote fibrotic remodeling, while IAT-derived ASC positive for CXCL14 inhibit
 1763 macrophage infiltration (522).

1764 The most commonly used source of adipose tissue for isolating ASCs is subcutaneous white adipose
 1765 tissue harvested from the abdomen, hips, thighs or buttocks, usually during plastic surgery procedures.
 1766 However, there are no preclinical studies comparing the therapeutic potential of these different types of
 1767 adipose tissue.

1768 **Conditions and time of cell amplification** Preclinical murine models use adapted MSC in
 1769 culture, whereas clinical trials rely on MSC stored in different conditions (bio- or cryo-preserved) and
 1770 thawed just before administration without a culture period allowing recovery. Deleterious effects of
 1771 immediate thawing on MSC properties such as their immunomodulatory properties have been described
 1772 *in vitro* (523). In addition, certain functions of MSC such as their differentiation potential decline after
 1773 sustained passages due to the average decrease in CFU (*Colony Forming Unit*) and colony size observed
 1774 *in vitro* (524). After multiple serial passages in culture (30-40 population doublings), MSC cease to
 1775 divide and enter a state of *in vitro* aging, also known as replicative senescence or the Hayflick limit
 1776 (525). Moreover, MSC start to become hypertrophic (MSC size about 4-fold larger at passage 6 than at

1777 passage 1) and their stemness gene expression profile is altered (526, 527). Regarding the expression
1778 profile of markers characteristics for MSC including CD73, CD90, CD105, and CD106, they were
1779 shown to be down-regulated during culture expansion compared to MSC in the stromal fraction (527,
1780 528). Thus, for the use of MSC in the clinic, the dilemma comes from the fact that the culture of MSC
1781 for the clinical application must have few passages while it is necessary to amplify them in a sufficient
1782 number (529). MSC amplification in a large scale will inevitably confront MSC with epigenetic, genetic,
1783 phenotypic, metabolic, functional modifications and their senescence being associated to reduced
1784 proliferative, regenerative capabilities and decreased life expectancy.

1785 A standardized manufacturing process for successful clinical trials is required. Surface marker
1786 profiling is one method to detect and isolate *in vitro* aging senescent cells. For instance, CD264, is a
1787 surface marker of *in vitro* MSC aging that is not linked to donor age (530). Compared to MSC negative
1788 for CD264, MSC expressing CD264 exhibit increased SA- β -gal (*Senescence-Associated beta-*
1789 *galactosidase*) activity as well as a reduced differentiation potential and colony-forming ability (531).
1790 In contrast, CD146, downregulated in MSC derived from aged donors (*versus* young donors), is also
1791 decreased in MSC after prolonged *in vitro* expansion (532). The decreased expression of CD146 in MSC
1792 was suggested to be associated with their reduced migratory potential in degenerative tissues (533).
1793 Although some molecules are expressed to different extents during aging and may be related with
1794 functional changes, there is no current consensus that a molecule can serve as a gold standard for young
1795 or aged MSC population selective purification. Senolysis, the process that consists in clearing senescent
1796 cells from a proliferative cell niche using specific agents called senolytic drugs, is currently tested in
1797 preclinical studies and the translation of this process to the clinical field is a promising topic to improve
1798 MSC-based therapy in MI (534).

1799
1800 ***Time of manufacturing process and storage*** Following the *European Union (EU) Regulation*
1801 *1394/2007*, MSC are an advanced therapy medicinal product that must be manufactured according to
1802 the standards of good manufacturing practices (GMP). About 21 to 28 days of manufacturing process is
1803 necessary from the BM collection to the obtention of P2-frozen/thawed MSC that will be either
1804 administrated to the patients or stored (535). Furthermore, this process must ensure an efficient long-
1805 term storage without altering MSC properties. Existing cryopreservation procedures consist in storing
1806 MSC in cryopreservation solutions at temperatures below -135°C and include cooling and thawing steps,
1807 avoiding rapid temperature changes that lead to stress and changes in material characteristics. Slow rate
1808 freezing results in cell dehydration with related stress and osmotic imbalances leading to oxidative stress
1809 and apoptotic cell death by contrast to fast MSC freezing that improves osmolality but increases the
1810 intracellular ice content. In general, a uniform rate of cooling of -1.0°C per minute has been shown to
1811 be effective for the majority of cells. The current standard practice for cell recovery in cryovials is to
1812 promote rapid thawing using a 37°C water bath (536). Of note, the effect of an inappropriate baseline
1813 cryopreservation process can be noticed within the first 36 hours after thawing (537).

1814 Several investigations have been carried out to validate cultures and study the short- and long-
1815 term stability of fresh or thawed products. In a recent study, Lechanteur et al. reported their efforts to
1816 implement GMP standards in their manufacturing process of allogeneic MSC (529) allowing to obtain,
1817 in an academic setting, a GMP license. To validate the steps of freezing and thawing steps, P2 (*Passage*
1818 2)- frozen MSC were thawed and seeded for one week prior the predefined quality criteria (QC) analysis
1819 including viability, sterility, mycoplasma, endotoxin, doubling time, population doubling time,
1820 phenotypic identity (CD73, CD90 and CD105), karyotype, viability, potency and morphology (535).
1821 MSC from three different donors were thawed and QC assessments were conducted at 0, 1, 2 and 4
1822 hours. Results show that one hour was the maximum time from the end of the MSC thawing step to
1823 administration to the patient (535).

1824

1825 *1.2. Regarding patients*

1826 Among the critical factors impacting the results of MSC-based therapy, one needs also to
1827 consider the recipient of the cell transplantation. AMI patients, as generally elderly patients, are
1828 frequently multi-morbid and are often treated with multiple therapies that can modify the intracellular
1829 cascades of endogenous mechanisms of cardioprotection.

1830

1831 *Aging of recipients* is an immutable cardiovascular risk factor that is attracting increasing
1832 attention due to its rising prevalence (538). If many studies have highlighted aspects of MSC aging, only
1833 a few studies have shown that aging significantly impedes the effectiveness of MSC migration and
1834 transplantation in both preclinical and clinical studies in other applications (539, 540). Preclinical studies
1835 described that aging impairs the angiogenic response of implanted cells after ischemic injury (541) while
1836 by contrast, a clinical trial with a small cohort of 49 patients showed that the recipient's age does not
1837 influence MSC efficiency for the treatment of patients with ischemic cardiomyopathy (511). However,
1838 it is critical to study the impact of aging since MI preferentially affects old patients, with an average age
1839 of about 65 years in men and 72 in women, and that all the preclinical studies of both cardioprotection
1840 and biotherapy are performed on young adult laboratory animals. For the treatment of AMI in old
1841 patients, allogenic transplantation of stem cells derived from young donors should be considered to
1842 potentially overcome aging-related limitations since autologous MSC will be less efficient (542). This
1843 is particularly relevant in view of the increased susceptibility to IR injury reported in old animals in
1844 preclinical studies as a result of the attenuation of the RISK/SAFE signaling cascades without any
1845 changes reported for PKG or PKA pathways (543). Indeed, Akt, ERK1/2, PKC and STAT3 undergo
1846 post-translational modifications and altered expression, leading to impaired endogenous mechanisms of
1847 cardioprotection in the elderly (544, 545). In particular, impaired Akt activation is related to a higher
1848 vulnerability to hypoxic injury and impaired paracrine efficiency of BM-MS (546).

1849

1850 **Diabetes** Diabetic patients are at high risk of MI due to increased adverse cardiovascular events.
1851 It is currently admitted that diabetic hearts are more sensitive to IR injury in human but contrasting
1852 results have been obtained in preclinical models (for review see (547)). Hyperglycemia is an important
1853 inducer of cardiac injury, and even in nondiabetic patients, hyperglycemia on hospital admission is
1854 associated with increased infarct size and should be considered a prognostic marker in all patients with
1855 AMI (548). Hyperglycemia and increased oxidative stress probably contribute to the impairment of
1856 PTEN-Akt signaling in human cardiac tissues (549). As a result, a resistance of diabetic hearts to
1857 cardioprotective strategies such as ischemic conditionings is observed both in preclinical models (for
1858 review see (547)) and in patients (550). Similar loss of cardioprotection was obtained after
1859 transplantation of human MSC in the infarcted border zone of a preclinical model of IR injury showing
1860 that diabetes impacts negatively MSC-induced cardioprotection (551).

1861
1862 **Treatments** Pharmacological treatments should be considered since they can interfere with
1863 MSC properties. Indeed, most of the patients at risk of AMI are usually treated for a coronary artery
1864 disease. In the arsenal of drugs prescribed in this case, statins (3-hydroxy-3-methylglutaryl-CoA
1865 reductase inhibitors) allowing to lower cholesterol amounts in the blood of patients, have shown
1866 contrasting results in cardioprotection depending on their duration of administration. For instance,
1867 chronic atorvastatin prevents the cardioprotective effect of PostC, while an acute treatment in
1868 combination with PostC is cardioprotective (552). In addition, atorvastatin or rosuvastatin increase
1869 proliferation rates of human MSC upon hypoxic conditions *in vitro*, and have a beneficial impact on
1870 transplanted MSC activity in preclinical studies (553-556). The impact of chronic statin treatment in
1871 AMI patients receiving MSC therapy is questioned because it has been reported that statins, evaluated
1872 as a cardioprotective drug against IR injury, are not protective when chronically administered due to an
1873 up-regulation of PTEN expression (557). Since cardioprotection by MSC therapy is mediated through
1874 downregulation of PTEN expression (398, 400, 401), it is uncertain whether MSC treatment will be
1875 strong enough to counteract the effect of statins on the PI3K pathway.

1876 Another example of treatment given to patients at risk of infarction concerns antidiabetic drugs,
1877 including metformin (biguanide) and gliflozins (*sodium-glucose cotransporter 2*, SGLT2 inhibitors)
1878 described as reversing diabetes-induced alterations in the signaling pathways, leading *per se* to
1879 cardioprotection (558-560). Indeed, metformin has been reported to induce apoptosis in MSC and
1880 impede their therapeutic when co-administered in hearts during AMI (561).

1881 All these treatments given to the population of AMI patients can compromise the therapeutic
1882 effect of MSC in clinical trials, so they need to be considered.

1883

1884 **2- Various MSC doses**

1885 **2.1 Preclinical studies**

1886 Dose-response was obtained for the improvement of cardiac function with allogeneic MSC
1887 tested at various doses 10^3 , 10^4 , 10^5 and 10^6 cells *per* kg body weight in the MI pig model. Only the
1888 highest doses provided significant benefits for cardiac function but not for a decrease in infarct size,
1889 where a U-curve was observed (562). An apparent dose-response was also observed for the values of
1890 systolic function (assessed by pressure-volume analysis) in a pig model of IR injury after IV
1891 administration of allogeneic MSC at 1, 3 and 10 millions of cells, the highest dose giving the best
1892 benefits (563). However, in another study, the highest dose of MSC, 10 million/kg, was reported to
1893 induce cardiopulmonary collapse and a subsequent loss in cardioprotection as evidenced by infarct size
1894 measurement (563). Similarly, an absence of dose-response was reported for allogeneic BM-MSC after
1895 transendocardial (TE) injection (20, 240 or 440 million) in pigs submitted to IR injury (564). These
1896 studies raise controversies regarding the dose-dependence of the therapeutic potential of MSC and an
1897 inverted U-shaped dose-response curve showing poorer results with both lower and higher doses has
1898 been described. In agreement with this observation, we reported a U-shaped curve for infarct size
1899 reduction induced by MSC treatment in a mouse model of IR injury treated with allogeneic MSC (565).

1900

1901 **2.2. Clinical trials**

1902 A recent metanalysis of clinical trials evaluating MSC therapy for AMI patients revealed that
1903 the number of cells injected, mostly intracoronary in 10 of 13 studies, ranged from 2.3 to $85 \cdot 10^6$ cells
1904 (503). This contrasts with the higher doses inducing cardioprotection in patients with ischemic
1905 cardiomyopathy, ranging from 100 to $150 \cdot 10^6$ cells, and mostly injected intramyocardially (566). The
1906 metanalysis also points out that the dose to be injected in patients with AMI should be less than 10^7
1907 MSC, within 1 week of AMI (combination timing/dose), to improve EF (503) and that administration
1908 of more than 10 million MSC has an opposite effect (504). A randomized clinical trial BOOSTER-
1909 TAHA7 is currently ongoing and recruiting patients with the aim to evaluate if increasing the frequency
1910 of cell injections will induce greater beneficial effects. This study will evaluate the paracrine theory of
1911 MSC: increasing the number of cells will increase the amount of paracrine stimuli in the recipient heart
1912 (567).

1913

1914 **3- Various timings of administration**

1915 **3.1. Preclinical studies**

1916 The time window to prevent IR injury after MI is very narrow and endogenous mechanisms or
1917 strategies of cardioprotection must be activated before the full activation of cell death mechanisms, in
1918 particular apoptosis (50, 568). The optimal timing for MSC injection to generate therapeutic benefit in
1919 AMI is unknown. In preclinical models of MI, various timing for the administration of the same dose of
1920 MSC have been tested leading to variable and contrasting results in large species. Three studies were
1921 performed using an injection protocol of autologous BM-MSC (30 million) at 30 min after reperfusion.
1922 The first one reports no beneficial effects on EF or reduction of infarct size and a poor survival rate of

1923 implanted cells in the post-infarct environment (554). The second describes only a decreased infarct size
1924 and no changes in EF (555) and the last one reports an improvement of both EF and infarct size (480).
1925 An earlier time point of injection, 15 min after the onset of reperfusion, underlined that early IV injection
1926 of allogeneic BM-MSc is effective for the treatment of IR injury (563). Later administrations, 3 days
1927 post-MI or 3 months after complete infarct healing, promoted significant reversed remodeling in pig
1928 hearts (486, 569). However, in a more recent study in rats, a detrimental effect of the adverse ventricular
1929 remodeling at 30-day post-MI on MSC survival and function was evidenced (570).
1930 The environment of the injured tissue impacts MSC function after their injection, either by positively
1931 priming them or by inducing apoptosis. We can therefore assume that, according to the timing of
1932 injection, MSC might encounter either a favorable or a detrimental tissular environment that will
1933 respectively either promote/prime their therapeutic properties or induce their apoptosis and clearance.

1934

1935

3.2. Clinical studies

1936

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1943

The “timing” parameter appears to be critical for the efficacy of stem cell therapy in AMI patients. The metanalysis from Attar et al. comparing 13 clinical trials showed that MSC improve EF when injected within 1 week after MI (503). Similar results were obtained using allogeneic Wharton’s jelly derived-MSc evidencing positive results for EF, myocardial perfusion and viability parameters, when cells were injected 5 to 7 days post-PCI (495). For early injections, within 24 hours after AMI, contrasting results were obtained since Houtgraaf et al. by contrast with Zang et al evidenced reduced infarct size using autologous freshly prepared adipose-derived regenerative cells comprising large amounts of MSC without any vascular complications (502, 571).

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1950

In conclusion, most authors recommend that MSC should not be injected after one week to achieve beneficial effects. Indeed, injecting MSC earlier, in the acute phase or up to 5 days after infarction, will impair the survival and functions of transplanted cells due to the hostile environment of the injured myocardial tissue. These results are based on naive MSC that have not been modified to optimize their resistance to the hostile conditions of the immediate post-MI period. Section IV-C will describe strategies to improve the therapeutic effects of MSC through the generation of an optimized and standardized “MSC 2.0” therapeutic product guarantying a benefit whatever the conditions.

1951

1952

4- Various routes of administration

1953

4.1 Preclinical studies

1954

1955

1956

1957

1958

1959

A meta-analysis performed on 58 studies (n=1165 mice, rats and pigs) using MSC in AMI and chronic ischemic cardiomyopathy reveals that the efficacy of MSC depends on the routes of injection (IM, IV, TE and IC) although all of them provide EF improvement (478). After analysis, TE administration appears to provide better results in terms of infarct size decrease and EF improvement, probably due to specific myocardial beneficial effects in addition to MSC systemic immunomodulatory functions (478). Moreover, TE injection was reported to be safe, well tolerated and associated with a

1960 decreased remote engraftment compared to IC and IV deliveries. TE injection resulted in a higher cell
 1961 retention in the canine heart (clusters of MSC in the core infarct) associated with a greater functional
 1962 improvement compared to the IC route (572). In addition, IC administration was associated with a higher
 1963 incidence of decreased coronary blood flow after injection, obviating the advantage of such a delivery
 1964 approach. Regarding IV injection of MSC, studies in the pig model of reperfused MI have described
 1965 beneficial effects (476, 563) despite a low engraftment rate within the infarcted area compared to other
 1966 routes (573). The decreased efficiency after systemic IV delivery of MSC results from their entrapment
 1967 in lungs as reported in rodent AMI model. Accordingly, a short half-life of about 24 hours was evidenced
 1968 for human MSC injected in mice (574, 575). The retention in the lungs is related to both, MSC adhesion
 1969 to the pulmonary vasculature endothelium through the expression of VCAM-1 (576) and a size issue
 1970 because the 20 μm diameter of cultured MSC is larger than the size of pulmonary microcapillaries (577).
 1971 Accordingly, *in vivo* experiments studying the distribution and homing of MSC after IV injection
 1972 showed that vasodilator sodium nitroprusside prior to MSC injection allowed MSC to clear the lungs
 1973 and reach the target organs in greater quantity (578). Surprisingly, instead of providing negative effects,
 1974 retention in the lung activates MSC, which in turn express TNF- α -induced protein 6 (TNAIP6 or TSG-
 1975 6) that suppresses the excessive inflammatory response induced by MI, the subsequent fibrotic scarring
 1976 and impaired cardiac function (575).
 1977 More studies comparing the routes of MSC injection are required to validate these observations and
 1978 define the most effective route for the design of clinical trials.

1979

1980 **4.2 Clinical trials**

1981 Probably in relation with PCI procedures used for reperfusion therapy, most MSC were injected
 1982 using the IC route in AMI patients at the acute phase (STEMI patients) whatever their autologous or
 1983 allogeneic nature, using the culprit artery (490, 492, 495-497, 500, 502, 503, 509) or through a remote
 1984 coronary artery (506). In addition, IC infusion in the culprit artery is minimally invasive and allows to
 1985 concentrate the cell product at the site of the lesion, facilitating the homing of MSC. Furthermore,
 1986 inflation of the angioplasty balloon at a low pressure downstream the stent allows maximizing the
 1987 contact time of MSC with micro-circulation of the infarction territory, the only precaution being to
 1988 infuse the cells through various short periods to avoid periprocedural MI. Some limitations to this
 1989 procedure are reported, such as the rapid release of MSC into the systemic circulation and the issue of
 1990 applying a PostC stimulus with angioplasty balloon inflation-deflation sequences (579).

1991 Local injection is also performed through IM administration using the *NOGA* system. This
 1992 system allows injecting cells directly into the infarct border zone (8–12 IM injections) as reported by one
 1993 study showing both feasibility and long-term (up to 5 years) safety (499).

1994 However, for the clinic, a simple IV approach would be ideal, due to its easy access and the
 1995 absence of time constraints. Thus, IV administration of allogeneic MSC cells was tested in patients
 1996 showing EF improvement only in one study (493, 494).

1997 First, the heterogeneity of results obtained with MSC therapy might be related to the design of
1998 both preclinical studies and to the protocols used in clinical trials. Second, in clinical trials, the
1999 heterogeneity of the patients arises from other parameters such as the age of the patients, the severity of
2000 their infarction, chronic treatments, comorbidities, etc... Finally, focusing on additive parameters
2001 including the cell source, amplification protocols, storage and thawing conditions, it appears obvious
2002 that the heterogeneity observed between studies occurs at multiple levels and that a standardization of
2003 the protocols becomes mandatory.

2004

2005

2006 **C. Strategies to improve MSC-based cell therapy**

2007

2008 **1- How to overcome the influence of time on MSC-based therapy?**

2009 Although the transfer of MSC from the laboratory to the bedside is theoretically feasible, many clinical
2010 trials have failed at early and late stages due in part to time-related issues that need to be clarified and
2011 resolved in the short term. These issues will be discussed in this section.

2012 ***1.1. Which patients and protocol for the best benefits?***

2013 Several factors have been described as contributing to clinical trial failure in cardioprotection,
2014 including those related to patients who are included in trials evaluating MSC therapeutic effects. Then,
2015 to design future clinical trials capable of showing the therapeutic effects of MSCs, it is necessary to take
2016 into account the factors that may compromise their cardioprotective role.

2017

2018 ***Co-morbidities*** A careful selection of the patient population is needed to avoid inclusion of
2019 patients with confounding factors including age, diabetes, cardiovascular risk and medications that could
2020 alter both MSC therapeutic properties and the response of the target tissue i.e., the myocardium to the
2021 transplanted cells as already discussed above.

2022

2023 ***Clinical protocol*** The protocol of MSC administration needs to be carefully designed and
2024 standardized among centers for clinical trials to provide data that can be compared. For inclusion of
2025 AMI patients likely to be good responders to MSC therapy, cardiac lesions must be compatible with
2026 myocardial salvage by reperfusion and additional MSC-based therapy that would be applied to further
2027 decrease the infarct size. However, ischemia duration must also not be too long for a possibility to rescue
2028 the targeted tissue. An ideal window for revealing cardioprotective effects in human would be within a
2029 range of 2 to 3 hours between the onset of symptoms and the application of the cardioprotective strategy,
2030 as previously demonstrated for PostC (580-582).

2031 The optimal timing of MSC injection within the first week post-PCI seems to be optimal, in
 2032 particular within the 4- to 7-day time window. The route of administration could be preferentially IM or
 2033 IC, both methods have their advantages and disadvantages. An appropriate method for the evaluation of
 2034 the clinical outcomes should be also chosen, probably new 3-D echocardiography and strain evaluation,
 2035 methods that will compete with CMR, for limited access and cost at the moment. Finally, the important
 2036 point is that the method used must be always the same.

2037 However, since it is rarely possible to select patients, MSC therapy must be open to as many
 2038 patients as possible and on the other side, researchers and clinicians are developing protocols to prime
 2039 MSC in order to provide a standardized cell product capable of delivering the highest benefit to any
 2040 patients.

2041

2042 ***1.2 - Which protocol(s) to obtain MSC with optimal functions?***

2043 ***Strategies for overcoming MSC age-related issues*** MSC used in the clinic are obtained from
 2044 donors of different ages and some studies have reported an effect of age on MSC functions. In this
 2045 context, efforts have to be made to elucidate the molecular mechanisms of MSC aging in order to
 2046 develop new methods to prevent this negative effect and rejuvenate aged altered MSC. Pharmacological
 2047 priming and genetic modifications have been investigated to increase the expression level of
 2048 genes/molecules decreased in aged MSC such as BDNF, PDGF-BB (PDGF with two B subunits) and
 2049 MCP-1 and that impact their therapeutic properties. The system for gene editing, CRISPR/Cas9, has
 2050 also been explored as a way to improve MSC potential in treating diseases such as MI (583). Targeted
 2051 *pdgfra* knock-in promoted MSC differentiation capacity and in turn, favored the functional insufficiency
 2052 of local cells (584). Although genetically modified MSC have been tested in clinical trials showing their
 2053 safety, further investigations are still required to produce unequivocal evidence of an enhanced
 2054 therapeutic effect of MSC.

2055 Another way to overcome the age-related problems of MSC is to study other tissue sources.
 2056 Autologous BM- and AT-derived MSC are relatively easier to collect compared to UC-MSC. However,
 2057 it is also important to consider the patient age, gender and health status when using BM- and AT-derived
 2058 MSC. Moreover, compared to these latter cells, UC-MSC show higher lifespan, proliferation and
 2059 differentiation capacities as well as an enrichment in angiogenic factors (585-588). Moreover, given the
 2060 advantages of UC-MSC, the therapeutic properties of their vesicles and trophic factors are currently
 2061 subject to intense research.

2062 As an alternative to aged tissue-derived MSC, the generation of functional MSC from induced
 2063 pluripotent stem (iPS) cells is presently a main issue (589-591). A great advantage of iPS-derived MSC
 2064 (iMSC) is that a high number of cells can be produced using *in vitro* culture based systems, an important
 2065 requirement for cell therapy. While iMSC are very similar to primary MSC isolated from diverse human
 2066 tissues in terms of morphology, phenotype, immunoregulatory and differentiation capacities, they
 2067 possess a greater regenerative potential as shown in animal disease models (For review see (592)). In

2068 addition, iPSC can be expanded almost indefinitely, so the source of iMSC can be unlimited and iMSC
2069 generated from one iPSC cell or clone are more homogeneous in theory and their biological properties
2070 are more predictable because their molecular signature is minimally different between batches. The final
2071 iMSC product quality is stable, easily controlled, and can reach the highest therapeutic efficacy for
2072 specific clinical applications and personalized patient treatment (For review see (592)). In this context,
2073 *Cynata Therapeutics* received the approval in 2016 to initiate the first trial using allogeneic iPSC (CYP-
2074 001) for the treatment of steroid-resistant acute GVHD (*Graft Versus Host Disease*). Based on positive
2075 safety and outcomes, iMSC are consider in Phase II trials for the treatment of critical limb ischemia and
2076 in a Phase III trial with 440 osteoarthritic patients (593-595). Therefore, iMSC outstanding properties
2077 enable their preferential commercial applications beyond other types of primary MSC isolated from
2078 diverse human tissues with their guaranteed quality control. However, the functions of these cells have
2079 to be further investigated in different preclinical models of MI in order to compare their therapeutic
2080 capacity when administrated in different experimental protocols when several “time” parameters are
2081 changed (including injection time, injection duration, etc.). iMSC offer the possibility to produce
2082 millions of “off-the-shelf” copies under Good Manufacturing Practice (GMP) procedures for further
2083 therapeutic applications to treat complex and multifactorial diseases. However, properties of iMSC need
2084 to be further optimized to warrant their therapeutic efficacy to treat patients with MI whatever their age,
2085 the timing of patient care and the timing of injection (after reperfusion). To that end, the production of
2086 iMSC should be perhaps combined to the MSC preconditioning protocols as discussed below.

2087

2088 ***Strategies for overcoming MSC storage-related issues*** The availability of human MSC off-
2089 the-shelf to treat patients with cardiac IR injury requires the development of cryopreservation protocols
2090 that are nontoxic, safe, and efficient to maintain MSC survival and functions. However, negative impacts
2091 of MSC cryopreservation on their therapeutic efficacy have been reported (596).

2092 To avoid physical damages of freezing and thawing, the most commonly followed steps include freezing
2093 MSC at a slow cooling rate and thawing them at a high rate as well as cryoprotecting MSC. To do this,
2094 trehalose (597), anti-freezing protein mimics (598, 599) and plant proteins (600) have been mixed with
2095 low DMSO concentrations to improve the cryopreservation of MSC (601). MSC vitrification with a low
2096 cryoprotectant concentration was accomplished *via* the introduction of microfluidic-based hydrogel
2097 microencapsulation or droplet-based cell printing technology (602, 603). Although promising, these
2098 MSC cryopreservation advances have not considered MSC properties and functions *in vivo*. More
2099 recently, protocols for cryopreservation of MSC without DMSO have been developed such as using
2100 zwitterionic compounds combined with electroporation able to retain normal *in vitro* proliferation and
2101 differentiation functions, as well as *in vivo* distribution potential (604). MSC long-term low-temperature
2102 storage has a significant impact on the recovery and function of MSC due to repeated exposure to room
2103 temperature (605). In this context, the effect of minimizing ambient temperature fluctuations on stored

2104 cells by designing an automatic cryopreservation system that handles cells in controlled temperatures
2105 was evaluated and identified as a priority to maintain MSC survival and functions (606).
2106 Of note, the detrimental effects caused by cryopreservation and reported in clinical trials using
2107 cryopreserved MSC thawed immediately prior to infusion (523, 607) were shown to be reversible.
2108 Indeed, to counteract the adverse effects of cryopreservation, the successful phase 3 clinical trial of
2109 Alofisel for perianal fistulas used “rescued” MSC in cell culture for a period of at least 24 hours after
2110 thawing, to allow “recovery” of the freshly thawed MSC before administration to the patients (608).

2111

2112 **2- Which protocol(s) to enhance MSC functions**

2113

2114 To guarantee a therapeutic effect of the MSC injected to the patients and to get over the
2115 limitations discussed above, efforts are being made to (i) protect MSC during their administration to
2116 patients in particular against apoptosis and, (ii) standardize and improve their therapeutic effects in order
2117 to decrease the number of MSC needed to obtain an optimal beneficial effect for the patients. We will
2118 then discuss below the strategies currently developed to address this clinical challenge.

2119

2120 ***2.1. MSC preconditioning to improve their therapeutic potential***

2121 MSC display a poor survival and engraftment rate *in vivo*. Moreover, the results obtained in
2122 clinical studies using MSC in various applications have often reported worse outcomes when MSC were
2123 injected at the highest dose, resulting in an inverted U-shaped dose response curve (for review see (609)).
2124 To overcome these limitations, extensive studies aimed at generating MSC with a better therapeutical
2125 potential to reduce the dose of injected cells. The enhancement of cell survival and homing to improve
2126 the paracrine activity of MSC has been also investigated as well as strategies to protect both MSC and
2127 cardiomyocytes from apoptosis. Different preconditioning protocols applied to MSC (hypoxia,
2128 pharmacological and chemical agents) and novel approaches (engineered MSC, MSC-derived EV and
2129 MSC trapped on innovative biomaterial/patches) have been developed. Among these strategies, the “*in*
2130 *vitro*” priming of MSC is a simple and attractive approach.

2131

2132 ***Hypoxic preconditioning*** MSC death and caspase-3 activation were significantly lower (40%-
2133 decrease) in an hypoxic situation when compared with MSC cultured under normoxic conditions both
2134 *in vitro* and *in vivo*, leading to a better vascularization in the infarcted myocardium and better therapeutic
2135 efficacy in rat hearts (610). This preconditioning increased pro-survival and pro-angiogenic factor
2136 expression levels such as HIF-1 α , VEGF and its receptor, BCL-2, and BCL-xL. As a preconditioning
2137 stimulus, moderate hypoxia induces MSC proliferation, while severe hypoxia promotes MSC glycolytic
2138 metabolism and quiescence, and short exposures are better than longer (611, 612). Under hypoxia

2139 exposure, MSC exhibit an increased expression level of leptin, which is pivotal for enhancing survival,
2140 chemotaxis, and therapeutic properties of MSC (613).

2141

2142 **Chemical and pharmacological preconditioning** Biological compounds and drugs, including
2143 hormones, growth factors, pharmacological and chemical substances, have been employed as an
2144 alternative to hypoxia-preconditioned MSC for the preconditioning of MSC to optimize their properties.

2145 **Hormones** The priming of MSC with adipokines has been shown to enhance their survival in an
2146 ischemic microenvironment (614, 615). The role of adipokines on MSC survival was first discovered in
2147 a study of hypoxia preconditioning of MSC, which evidenced their capacity to enhance their therapeutic
2148 properties (615). Five years later, the protective role of resistin, another adipokine, on MSC was reported
2149 (614). The injection of MSC pretreated with resistin in a murine model of IR injury significantly
2150 increased angiogenesis and reduced myocardial apoptosis and fibrosis resulting in improved cardiac
2151 performance, as compared to vehicle-treated MSC. Also, an enhanced homing of the cells into the
2152 damaged heart in a ERK1/2-dependent manner was observed. In line with these studies, it was shown
2153 that asprosin, a newly-discovered adipokine, also increases the survival of MSC in MI, improved their
2154 homing and significantly improved EF (616). *In vitro*, the authors showed that asprosin-pretreatment of
2155 MSC generates a protective effect on H₂O₂-induced damage and apoptosis of MSCs *via* the activation
2156 of the ERK1/2-SOD2 pathway. Similarly, the priming of MSC with haemin, a potent HO-1 inducer,
2157 before their transplantation into the peri-infarct region in MI mice, promotes the survival of MSC and
2158 enhances their cardioprotective properties (413).

2159 **Growth factors** IGF-1 pretreatment of MSC had a positive effect on survival, adverse infarct outcomes
2160 including infarct size and ventricular remodeling and the release of pro-inflammatory cytokines (373,
2161 434). This current trend to augment MSC viability is mediated through various pathways including PI3K,
2162 HIF-1, MAP Kinase and Hippo (617). More recently, it was described that SDF-1 α MSC pretreatment
2163 resulted in improved vasculogenesis and recovery of cardiac function in infarcted myocardium 6 weeks
2164 after treatment (618). However, the priming effect is not retained during cryopreservation/thawing
2165 because pro-survival factor expression levels are largely dependent on the environment. Of note the use
2166 of a hypothermic preservation solution allowed to keep the benefit of preconditioning.

2167 **Pharmacological substances** Statin preconditioning were shown to improve the therapeutic effects of
2168 MSC: atorvastatin was reported to enhance CXCR4 expression in MSCs and promote them homing
2169 toward the injured myocardium, leading to an improved cardiac function in an AMI rat model (619).

2170 Sevoflurane preconditioning decreases MSC apoptosis, the loss of mitochondrial membrane potential
2171 and upregulates VEGF, HIF-1 α , HIF-2 α protein levels and pAKT/AKT ratio (620).

2172 In line with this study, we have recently evidenced that optimization of the anti-apoptotic properties of
2173 MSCs has a substantial impact on their therapeutic effects in myocardial IR injury. Indeed, by using a
2174 PPAR β/δ (*Peroxisome Proliferator-Activated Receptor β/δ*) agonist to prime MSC, we found a
2175 significant increase in the survival of MSCs when exposed to an IR-simulated stress as well as an

2176 enhancement of their anti-apoptotic properties on cardiomyocytes and endothelial cells exposed to
 2177 oxidative stress compared with naive MSC. In addition, we observed that priming MSC with the
 2178 PPAR β/δ agonist permitted us to halve the dose of cell injected into the heart since the cardioprotective
 2179 effect obtained *ex vivo* using PPAR β/δ -primed MSC injected at a dose of $3.75 \cdot 10^5$ MSC was comparable
 2180 to that observed with untreated control MSC injected at the optimal dose of $7.5 \cdot 10^5$ MSC (565).
 2181 Concerning the clinical translation, the C-CURE trial reported that MSC pretreated with a cardiogenic
 2182 cocktail-based priming medium showed improved ventricular function and physical performance and
 2183 quality of life (361). While the injection of MSC is well tolerated by patients with MI, their therapeutic
 2184 benefit still deserves to be improved. Such a possibility relies on a full understanding of the molecular
 2185 mechanisms underlying the cardioprotective effects of MSC.

2186

2187 **2.2. MSC genetic modifications**

2188 Genetic modifications consist in upregulating the expression of specific genes involved in the
 2189 therapeutic properties of MSCs in order to reduce premature senescence, increase their survival and then
 2190 their therapeutic effects *in vivo*. For example, the overexpression of *EphB2* (ephrin receptor B2)
 2191 inhibited premature senescence by repressing mitochondrial ROS accumulation that triggers senescence
 2192 in MSC (621). A main target was also apoptosis and overexpression of *Bcl-2* in MSCs injected after MI
 2193 significantly increases MSC survival for up to 6 weeks compared to control MSCs and potentiates their
 2194 efficacy on vascular density, cardiac function, infarct size and myocardial fibrosis (622). *In vitro*, *Bcl-2*
 2195 overexpression decreased the apoptosis of MSC by 32% and increased the secretion of VEGF by more
 2196 than 60% under hypoxic conditions (622). Moreover, *Hgf* overexpression in MSC was shown to increase
 2197 the expression of BCL-2 while reducing BAX and Caspase-3 levels in response to hypoxia *in vitro*. *In*
 2198 *vivo*, the engraftment and survival of MSC was enhanced when the cells were both overexpressing HGF
 2199 and encapsulated in a small molecular hydrogel (20).

2200 MSC overexpressing CCR1, the receptor for CCL7 significantly upregulated in the heart post-MI, are
 2201 more protected from apoptosis and are more prone to accumulate in the infarcted myocardium as well
 2202 as to reduce the infarct size and cardiomyocytes apoptosis in injured myocardium as compared to their
 2203 control counterpart (475).

2204 The overexpression of the macrophage migration inhibitory factor (MIF), a proinflammatory cytokine,
 2205 in aged MSC rejuvenated them by activating autophagy and increased their therapeutic potential in MI
 2206 (616). In line with this study, EV from MSC overexpressing MIF significantly, were shown to increase
 2207 heart function and reduce heart remodeling, apoptosis and ROS production as compared to EV from
 2208 naïve MSC (623).

2209 Altogether these studies reveal that MSC priming to protect them from apoptosis once administrated *in*
 2210 *vivo* not only prolongs their presence and survival in the injured heart but also increases their therapeutic
 2211 efficacy in MI.

2212

2213 **2.3. Use of MSC-derived EV or microparticles**

2214 One of the particularities of MSC is their plasticity and versatility. Indeed, according the
2215 inflammatory environment, MSC are going to adopt different phenotypes and functions. *In vivo*, MSC
2216 versatility can represent an advantage since the cell can sense and adapt their properties to their
2217 environment but in some cases this versatility/plasticity can lead to an unpredictable response. To
2218 overcome this limitation, experimental animal models have addressed the therapeutic benefits of MSC-
2219 derived EV in the context of AMI.

2220 EV enriched with miR-22 secreted by MSC exposed to PreC and applied to cardiomyocytes
2221 were shown to inhibit their ischemia-induced apoptosis. EV derived from MSC exposed to PreC
2222 significantly reduced infarct size and myocardial fibrosis *in vivo* by targeting *Mecp2* *via* miR-22 (403).
2223 Similarly, miR-221 overexpression in EV derived from MSC transduced with GATA-4 (MSC-GATA4)
2224 displayed increased cardioprotective effects by reducing the expression of p53 upregulated modulator
2225 of apoptosis (PUMA) in cardiomyocytes (624). In line with this study, EV-derived from MSC-GATA4
2226 were shown to be a reservoir of anti-apoptotic miRNA responsible for activation of the cell survival
2227 signaling pathway and thus cardioprotection (625).

2228 Concerning the MSC-mediated promotion of angiogenesis and subsequent improvement of impaired
2229 infarcted cardiac function, MSC-EV were found to mediate this property through a miR-210-Efna3-
2230 dependent mechanism (626). Overall, these studies suggest that MSC-EV may exert greater
2231 cardioprotective effects (627) and a lower risk of tumorigenicity (628) compared with MSC-based
2232 therapies. This therapeutic effect mediated by EV might be more dependent on the administration
2233 window since MSC-EV will not be able to adapt to their pathological environment in contrast to their
2234 parental cells and thus will have to possess the required properties *per se* to be therapeutically useful.
2235 Indeed, one study showed that administering EV 30 min post-MI had no beneficial effect (629). Also,
2236 EV have a short lifetime and restricted efficacy due to their poor persistence in the myocardium (630-
2237 632).

2238 A new concept with synthetic MSC has been evaluated for the treatment of AMI in mice, showing
2239 enhanced therapeutic effects compared to native MSC. These synthetic MSC were obtained by
2240 packaging factors secreted by human BM-MSC into Poly (lactic-co-glycolic acid) PLGA microparticles
2241 and coating them with MSC membranes. This novel particle exhibited similar surface antigen and
2242 secretome profiles than MSC. When injected in mouse heart with MI, angiogenesis, improved cardiac
2243 function and attenuated LV remodeling were observed. Furthermore, their high stability to both
2244 cryopreservation and lyophilization and their immune privilege, makes this concept attractive for
2245 various applications in different organs (633).

2246

2247 **2.4. Combined therapies**

2248 Combinatorial delivery of MSC with their derived EV was assessed in rat hearts. In particular,
2249 the sequential administration of EV (30 min post-MI) and then MSC (3 days post-MI) effectively

2250 decreased scar size and restored cardiac function after AMI (634). The authors proposed that the
2251 beneficial effect of the combinatorial treatment was due to an improved inflammatory microenvironment
2252 induced by the EV administration resulting in a better recruitment and retention of MSC. Regarding
2253 combination of cells with drugs, a new clinical trial evaluating the association of atorvastatin and
2254 injected BM-MSC is ongoing. These patients undergoing PCI will receive autologous BM-MSC with
2255 regular or high dose of Atorvastatin treatment to improve the survival of implanted cells (635).

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2257

2258 **V- CONCLUSION**

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2260 Acute myocardial infarction is a leading cause of cardiovascular disease in the world. With the
2261 improvement of the revascularization techniques and patient care, the rates of the mortality have
2262 decreased by contrast to the rising rates of morbidity leading to heart failure. The World Health
2263 Organization predicts that this burden will be worsened since cardiovascular disease are expected to stay
2264 at the first rank until 2030 because of the aging of the population and an exponential growing number
2265 of diabetic patients. Therefore, the challenge is to discover new treatments with cardioprotective activity
2266 in particular able to reduce IR injury to further decrease infarct size which is a major determinant for
2267 the morbi-mortality rates.

2268 In this context, MSC are appropriate for this challenge because their pleiotropic properties appear
2269 relevant to overcome this multifaceted IR injury. The description of the kinetics of the events leading to
2270 IR injury in regard to the underlying mechanisms of action of MSC should allow the design of the most
2271 appropriate protocols to fully benefit from the pleiotropic properties of MSC of interest for AMI patients.
2272 While the mechanisms underlying the therapeutic properties of MSCs are not exclusively limited to their
2273 survival rate, it is necessary to identify the optimal timing conditions (duration of culture, age of MSC
2274 donors, duration of cell storage, timing of thawing) to obtain the most effective MSC possible endowed
2275 with optimal therapeutic properties. Moreover, these cells must be delivered at the right time to the
2276 damaged heart tissue to enable them to reverse the pathways that trigger cell death. Finally, AMI patients
2277 are expected be good responders to MSC, providing a status for MSC to deliver their therapeutic
2278 properties. Analyzing the various critical points to be overcome and developing protocols to enhance
2279 MSC therapeutic properties including their own survival in the cardiac tissue, will facilitate the success
2280 of clinical translation.

2281 Application of various strategies of conditioning appears as a way to provide a standardized cell product
2282 capable of delivering the highest benefit to any patients, whatever their age, status and treatments.

2283 In this context, our review suggests that cardioprotection is a matter of timing.

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2285

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2293 No conflicts of interest, financial or otherwise, are declared by the authors.

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2295

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2301 **IX- AUTHOR NOTES**

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2305 **X- AUTHOR CONTRIBUTIONS**

2306 S.B.-L., F.D. and A.V. drafted manuscript; S.B.-L., F.D., A.V. prepared figures with the support of
2307 Muriel Asari ; C.P., J.N. and C.J. corrected and revised manuscript ; S.B.-L., F.D., A.V., C.P., J.N. and
2308 C.J. approved final version of manuscript.

2309

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