Position Paper of the ESC Working Group Cellular Biology of the Heart: Cell-Based Therapies for Myocardial Repair and Regeneration in Ischemic Heart Disease and Heart Failure

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Abstract

Despite improvements in modern cardiovascular therapy, the morbidity and mortality of ischemic heart disease (IHD) and heart failure (HF) remain significant in Europe and worldwide. Patients with IHD may benefit from therapies that would accelerate natural processes of postnatal collateral vessel formation and/or muscle regeneration. Here, we discuss the use of cells in the context of heart repair, and the most relevant results and current limitations from clinical trials using cell-based therapies to treat IHD and HF. We identify and discuss promising potential new therapeutic strategies that include *ex vivo* cell-mediated gene therapy, the use of biomaterials, and cell-free therapies, aimed at increasing the success rates of therapy for IHD and HF. The overall aim of this ESC Working Group Cellular Biology of the Heart Position Paper is to provide recommendations on how to improve the therapeutic application of cell-based therapies for cardiac regeneration and repair.

# 1.	Stem cells in the context of heart repair

Stem cells are defined as cells with the ability (i) to self renew by dividing to make copies of themselves and (ii) to differentiate to at least one other cell type (1). In the context of cell transplantation and heart repair, the term "stem cells" has been widely used but in retrospect, some of the cells used do not match the definition of a stem cell. Cells with various molecular and functional properties have been isolated from the heart and termed "cardiac stem cells" (CSCs), "cardiac progenitor cells" (CPCs) or "cardiomyocyte progenitor cells" (CMPCs) (2, 3). These cells can self renew in culture, and differentiate into different lineages (endothelial cells and mesenchymal cells) but for example have limited cardiogenic differentiation capacities except under exceptional circumstances. By the addition of compounds that induce demethylation, human CMPCs do form cardiomyocytes (3a). Otherwise the only "stem cells" that form cardiomyocytes using mixtures of growth factors, that have been collectively referred to as "cardiogenic cocktails", are pluripotent stem cells (PSCs). PSCs can be of embryonic origin (embryonic stem cells, ESCs) or created by re-introducing cell cycle genes into terminally differentiated cells, to make what are called induced pluripotent stem cells (iPSCs) (1). Another term now generally? regarded as incorrectly used is "endothelial progenitor cells" (EPCs). These cells were originally isolated as populations that grew in culture from peripheral blood samples (reviewed in (4)). They could form networks that resembled vasculature, but they turned out not to be true endothelial cells. Finally, cells that adhere onto tissue culture plastic in serum-containing growth medium and have adipogenic, osteogenic and chondrogenic differentiation potential in culture were termed mesenchymal stem cells (MSCs) (5). However, these cells have not been isolated clonally as single cells and could therefore be heterogeneous cell populations. Moreover, with the exception of those derived from bone marrow, these effects are not observed *in vivo*. Thus despite

them all expressing a similar set of surface markers, these cells are now called "bone marrow derived mesenchymal stromal cells" (BM-MSCs) or adipose tissue-derived mesenchymal stromal cells (AT-MSCs) (6). These MSCs have not been shown to spontaneously differentiate into cardiomyocytes. For the purposes of this position paper, we use the terminology as in the (historic) literature for the sake of clarity but are aware of the caveats in the terminology itself.

2. Translation of cell therapy: successful preclinical stories with uncertain clinical efficacy

Ischemic heart disease (IHD) and heart failure (HF) remain major causes of morbidity and mortality worldwide (7, 8). Potentially valid clinical strategies aimed at repairing damaged heart muscle and ischemic tissue, and increasing the heart's regenerative potential, are currently being developed in clinical trials (2, 9). Despite originally high expectations fueled by exciting scientific progress, and although long-term, randomized clinical trials have shown reassuring safety profiles for intracoronary delivery of cells (2, 11-16), regenerative therapy for cardiovascular disease has had inconsistent and modest efficacy thus far (9, 17-23). Several limitations of most previous clinical trials of cell-based therapies were raised and should be addressed before we can fully understand the potential of these approaches (see Table 1).

As a consequence, several strategies have been developed to further improve cardiac function in response to cell delivery. The different strategies and protocols, collectively referred to as 'cell enhancement', are discussed in the section "Critical issues on protocols for cell-based therapy".

In this ESC WG Cellular Biology of the Heart Position Paper, we critically review the current approaches using stem cell or cell-based therapies to treat IHD and HF, and discuss promising new strategies for stem cell therapy enhancement, with the aim of

increasing the efficacy and outcome of stem cell therapies in the future. The overall objective of this ESC Working Group Cellular Biology of the Heart Position Paper is to provide recommendations on how to improve cell-based therapies for cardiac regeneration and repair in IHD and related HF.

2.1 Cell sources used in clinical trials

Several types of cells have been used in clinical trials, most of them derived from bone marrow (12, 14, 15, 17-22, 24-29), or peripheral blood (30, 31), although some studies have used mesenchymal stromal cells (MSCs), cultured from a variety of tissue sources (Table 2). These heterogeneous cell populations used in the early years of regenerative cardiac medicine, have been called "first-generation" stem cells, in contrast with contemporary "second-generation" counterparts. The latter consist of more purified cell populations with a presumed greater potential for cardiac repair and are often derived from non-bone marrow sources, or subjected to genetic and pharmacological "priming" *in vitro* to enhance their engraftment, survival, plasticity and paracrine activity. MSCs exhibit low immunogenicity, making allogeneic application feasible. Since the quality and number of cells may diminish in patients who are older or have comorbidities or genetic defects (reviewed in (32)), allogeneic MSCs can be used from young healthy individuals. Five systematic reviews and meta-analyses have reported a significant improvement in left ventricle ejection fraction (LVEF) of 2-4% and a reduction in infarct scar size and left ventricular end-systolic volume after intramyocardial transplantation of bone marrow cells, but all are regarded as surrogate endpoints and not clinically relevant endpoints (or: surrogate endpoints with uncertain clinical relevance) (23, 33-36). This is in contrast with the outcomes of studies based on small cohorts of patients. Among various possibilities (discussed in Table 1), these modest results and the variability between trials have been attributed to the different isolation protocols used, which may profoundly impact the

function and number of bone marrow cells or blood-derived EPCs actually delivered to the patient (37, 38). Therefore, the general consensus is that assessing cell number and viability along with careful cell characterization and functionality is necessary before delivering cells into patients in any clinical trials. Moreover, the effect of bone marrow mononuclear cells on incidence of death, recurrent myocardial infarction or stroke and hospitalization for heart failure remains to be determined in adequately powered prospective clinical trials.

Cardiac-derived progenitor or stem cells (CPCs / CSCs) have very recently entered the clinical trial arena. Although isolation of these cells from the heart is more invasive than bone marrow, long culture periods are required to obtain sufficient numbers for transplantation, and their number and functional activity may decline with age, their intrinsic paracrine activity (39-41) is expected to make them potentially good candidates for enhancing myocardial function in HF patients. Except for the small scale transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy (TAC-HF) trial, comparative clinical data between bone marrow-derived cells (BMCs), MSCs and CPCs/CSCs is not available in HF. A few comparisons have been done in animal models of myocardial infarction (reviewed in (42)), and MSCs seemed to transfer more benefit on systolic function than BMCs in a chronic large animal model of myocardial infarction (43). Preclinical research thus far suggests the greatest potential functional benefit for CPCs/CSCs from the heart, followed by MSCs, with BMCs having more modest effects on LVEF (44). Conclusions about the effect on mortality of BMC therapy after acute myocardial infarction (AMI) are expected to derive from the ongoing phase III BAMI trial, despite the lack of an appropriate? placebo control injected group (https://clinicaltrials.gov/ct2/show/NCT01569178). Likewise, conclusions on the use of MSCs alone or in combination with c-Kit positive CSCs will be drawn from the NHLBI CCTRN "Concert Trial", which will probably be initiated before the end of 2015.

Of note, there is still no consensus on whether transplanted cell numbers or survival *in vivo* are crucial for effect size. While trial-based meta-analysis suggested a relationship between cell numbers and effect in clinical trials, individual patient-based meta-analysis have not confirmed this relationship (45).

2.2 Pluripotent stem cells in clinical trials

Another class among the second-generation cells are pluripotent stem cells, both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Table 2). A clinical trial with ESC-derived cardiomyocytes in severe HF (ESCORT) has been initiated in France and is being monitored with both interest and caution (46, 47). Since the same differentiation protocols for ESCs are effective in iPSCs, it may be expected that this will also move forward for the treatment of the heart. iPSC-derived cardiomyocytes have not yet been tested in humans, despite the possibility of them being autologous, largely because of the extra risk of genetic mutation inherent to the reprogramming method as such. Congruently, the first results of ESC-mediated eye repair are encouraging (48) and iPSCs for this aim are in clinical trial since September 2014, but the latter study is on hold since July 2015 for the identification of a mutation in an oncogene in one of the human iPSC lines (http://www.ipscell.com/2015/07/firstipscstop/). This next-generation iPSC-derived approach is therefore still fraught with uncertainty in the absence of a regulatory framework or guidance about "allowable" levels of mutations and methods of their detection in iPSC products.

2.3 Cell-free approaches

A general consensus is that first generation cells may exert any effects on tissue repair by secretion of paracrine factors. These largely unknown factors may stimulate the myocardium via myocyte salvage, induction of angiogenesis or stimulation of myocyte

division. Although the second generation cells, e.g. CPCs/CSCs and iPSC-derived cardiomyocytes, have been suggested (CSCs) or proven (pluripotent cells) to have greater regenerative capacity because of their ability to form new myocardium, the contribution of remuscularization vs. "paracrine effects" to overall efficacy has not been demonstrated clinically nor preclinically. Any effects they have are thought to be also mediated via paracrine mechanisms. Since functional improvement is not necessarily related to cell survival (49, 50), approaches have been developed to mimic the benefit of cell therapy without transplanting the cells. Such strategies include stimulating endogenous repair, e.g. by promoting neovascularization or activating resident progenitor cells (51, 52). Mediators of paracrine effects are thought to include growth factors (e.g. erythropoietin, G-CSF) (53, 54), episomes (55) and non-coding RNAs (56), mimicking the secretome of donor cells. These factors can also be combined by assembling them in different controlled release formulations, such as microbeads (57), large scaffolds, or injectable biomaterials (58). Recent developments for cell-free approaches that emanate from cells such as these, presently focus on secreted nanosized vesicles, called extracellular vesicles, and include microvesicles and exosomes (59, 60). These small lipid containing vesicles are capable of transferring proteins, mRNA, and miRNAs between cells, and therefore represent a way for intercellular communication and inducing cardiac repair (61). However, organ selectivity after systemic delivery or inadvertent systemic spread after intracoronary or intramyocardial delivery of these nanoparticles remains unknown and a topic for further scrutiny.

In summary, although the superiority in cardiac repair of one type of cell compared to another has not yet been proven, since very few preclinical studies compared them head-to-head, BMCs continue to be the source of cells most often used in

human clinical trials. In cardiac patients, direct comparative data between different cell types is notoriously lacking since adequately powered, randomized clinical trials with head-to-head comparisons of different cell types have not yet been performed. However, at least one study is planned to begin before the end of 2015. It is a NHLBI CCTRN trial with cardiac c-Kit cells alone and combined with other cell types versus placebo in patients with ischemic cardiomyopathy. Apart from the risk of immune rejection, which can potentially be solved using MSCs, allogeneic somatic/adult cells appear to be safe. To match reported levels of functional cardiac improvement, cell therapy without the cells via paracrine factors may be an interesting alternative. For functional improvement beyond current levels achieved via paracrine actions, new developments will be necessary for proper regeneration of lost tissue.

3. Critical issues on protocols for cell-based therapy

One major problem for cell therapy is the relatively poor levels of cell retention in the transplanted area, and this may not be limited to first generation cells but apply to all cell sources. In fact only ≤ 10 percent of injected cells remained at the targeted location. No cells survive when injected into the infarct scar, short-term engraftment is ~ 8% regardless of injected cell dose in remote normal myocardium, and in the infarct border zone the percent survival at 24 h decreases progressively from ~ 8% to < 1% (62, 63).

3.1 Improving cell coupling, differentiation, survival and retention by cell modification, conjugation with biomaterials or tissue engineering and cytoprotection pathways

To improve cell retention, several biomaterial-based approaches have been explored (e.g. hydrogels, cell sheets, prefabricated matrices, microspheres and injectable nanomatrix) (58, 64, 65). An alternative approach, explored in animal models, is the implantation of engineered heart tissue made *in vitro* from cardiomyocytes and hydrogel (66). Another method is the use of bispecific antibodies that bind to the cells and recognize a cardiac-specific antigen that is only present in injured myocardium (67). Localized hypoxia, inflammation, excessive oxidative stress, lack of supporting cells, poor supply of nutrients, and fibrosis promote apoptosis or necrosis of the grafted cells. Thus, the efficiency of cell therapies might be improved by using genetic engineering tools including overexpression of pro-survival genes (e.g. Akt, Pim-1 kinase, ERK1/2, HIF-1α, heme-oxygenase 1, GATA4, heat shock protein 27, miRNA-1, myocardin, and protein kinase G1α) or angiogenesis-initiating genes (e.g. VEGF, MYDGF, FGF-2, SDF-1, PDGF) in the cells to be transplanted or by transplanting the cells together with pro-survival or pro-angiogenic factors (42, 63, 68-74). Interestingly, exposure of cells to sub-lethal hypoxia increased the tolerance of these cells to the harsh environment after transplantation (75). These preconditioned cells showed also increased differentiation, enhanced paracrine effects leading to increased trophic support, and improved homing to the lesion site (75). Transplantation of preconditioned cells helped to suppress inflammatory factors and immune responses, and promoted heart function (75). In addition, transient modulation of cell specification towards myogenic differentiation e.g. via microRNAs, could also be beneficial in increasing the amount of myocardium. For this, miR-1 and 499 are excellent candidates as they can enhance both differentiation *in vitro* (76) and *in vivo* (77). Another approach to promote transplanted cell survival is to modulate the inflammatory environment (using TSG-6, IL-1 inhibitor) (70, 78). Finally, a significant barrier to the therapeutic use of most cell populations with the exception of ESCs and iPSCs, is their limited cardiac differentiation potential despite the use of

"cardiogenic cocktails" (containing TGF-β1, BMP-4, activin A, retinoic acid, IGF-1, FGF-2, α-thrombin, and IL-6) and overexpression of cardiac transcription factors (79-81). In addition, these stem cells fail to electromechanically integrate (82). This limitation has been partially solved by overexpressing the two key proteins, N-cadherin and connexin 43, but clinical translation remains to be fully investigated. By contrast, human iPSCs can now be routinely differentiated with high efficiency (>80%) into cardiomyocytes (83). However, the cardiomyocyte populations may contain varying proportions of atrial, ventricular and nodal cardiomyocytes (84, 85). This is a critical issue as they have unique mechanical and electrical properties and thus the implantation of a mixture of these cells harbors the risk of arrhythmias (86). In addition, all of these cardiomyocyte types are immature and beat spontaneously, another source or arrhythmogenic risk. Consequently, even though many protocols primarily give rise to ventricular like cardiomyocytes, it is important to refine the differentiation protocols to produce pure populations of defined cardiomyocyte phenotype (87, 88). In addition, a robust cardiac lineage differentiation state of all transplanted cells is critically important to avoid the formation of teratomas.

3.2   Stem cell rejuvenation

Aging or comorbidities may cause a reduction in the number and function of tissue-resident and circulating cells (32, 89, 90). Several proteins and signalling pathways have been identified that are capable of reverting the process of cell senescence, including Pim-1 kinase (91-94), NOTCH1 (95-97), telomerase and myocardin (98). Pim-1 kinase has anti-senescence and anti-apoptotic effects in CSCs as well as in MSCs (92). Activation of the NOTCH1 signaling pathway results in remarkable rejuvenation of satellite muscle cells associated with enhanced proliferation, increased telomere lengths, and decreased susceptibility to replicative senescence (95). The overexpression of telomerase and

myocardin genes increases cell survival, proliferation, cardiomyogenic (99, 100), and smooth muscle differentiation *in vitro* (101). After overexpression of genes encoding for "rejuvenating factors' and *in vitro* expansion, genetically modified cells may secrete high amounts of the regenerating factor, either transiently or permanently, at the site where they have been transplanted (68, 102). Taken together, the design of new protocols for aged cell rejuvenation would allow improved cell preparation and clinical application of cells in aged patient populations.

3.3 Enhancing endogenous cardiac regeneration

Recent studies have demonstrated that cardiomyocyte turnover occurs throughout life in mammals, including humans (103-107). While the estimated rate of human cardiomyocyte renewal is controversial, most labs find an annual turnover rate of 1%, which increases after injury. However, the intrinsic capability in humans to regenerate injured myocardium after massive ischemic cell death is too low to be of functional relevance. It has been suggested that transplanted cells may exert their beneficial effects by secreting cytokines and growth factors promoting cardiomyocyte proliferation, recruitment and activation of CPCs, induction of vessel formation, reduction of fibrotic scars, and inhibition of apoptosis (108). In addition, modulation of macrophage and regulatory T-cell function can improve healing, repair and regeneration (109; 109a). Another approach to enhance endogenous cardiac repair is the induction of cardiomyocyte proliferation, a mechanism described in neonatal mice, zebrafish and newts in response to injury (110) although never in adult mammals. However, blocking the Hippo pathway or upregulating the downstream Hippo effector Yes-associated protein (Yap), may promote cardiomyocyte regeneration after myocardial infarction (10). Alternatively, application of the human Fstl1 protein (FSTL1) via an epicardial patch stimulates cell cycle entry and division of pre-existing cardiomyocytes (110a).

3.4 Cell tracking and injection systems

In vivo cell tracking involves either 'direct' physical labelling of cells by incubating them with a contrast agent, or 'indirect' genetic labelling by transfecting cells with a reporter gene construct. The position of, and signal from these labels can then be tracked using various imaging modalities including clinical scanners, such as positron emission tomography (PET), single photon emission computed tomography (SPECT) and magnetic resonance imaging (MRI) (reviewed in (111, 112). Given its high anatomical resolution and safety profile allowing serial longitudinal evaluations, MRI has been commonly used to track cells in clinical trials (113). However, MRI might detect macrophages that ingest the marker after the cell (derivative) dies. Safety concerns regarding the effects of genetic manipulation of cells currently limit the use of genetically modified cells in clinical trials, and thus long-term cell tracking. However, combination approaches relying on the simultaneous co-registration of different imaging modalities (nuclear medicine combined with CT or MRI) might overcome the limitations of individual imaging techniques, and represent powerful tools to gain insight into the delivery, engraftment, survival, off-target and possible adverse effects of transplanted stem and progenitor cells. Given the indispensable role of cell tracking in clinical trials, the feasibility of imaging should be included in preliminary proof of concept studies, and considered among inclusion or exclusion criteria, but will limit cell transfer studies to only a few centers that have access to multimodal imaging expertise.

3.5 Controls, data reproducibility, standardization issue and data quality

Over the past few years, concerns have been increasingly voiced about experimental reproducibility across the whole biomedical research fields (114, 115), especially cell therapy. For example, a recent paper searching for errors in published cardiac clinical

trials using autologous BMCs reported that the greatest enhancement of LVEF was described in those studies with the most discrepancies or errors in factual reporting (116). The pervasive risk of neglecting basic rules of clinical trial design in stem cell trials has been demonstrated in a recent review (117). On the other hand, phase II studies, where the aim is to prove efficacy should be designed to assess several primary end-points, which might include structural evaluations, cardiovascular physiological measurements, biomarkers, functional capacity, and quality of life (118).

The choice of appropriate controls and methodological rigor may be more demanding in the field of cell therapy if, for example, the need for a myocardial biopsy to harvest autologous stem cells complicates double-blinding. A pragmatic alternative is to use a crossover study design, in which each patient is randomly assigned to a sequence of treatments. However, where reagents such as cytokines are administered in conjunction with cells, a control group with cytokines alone should also be included. Another issue is the choice of the right placebo control, which, in some cell therapy trials, simply consisted of transparent saline solution which easy to distinguish visually from serum.

Standardization of cell isolation and processing procedures is highly desirable in order to facilitate comparisons between trials and to enable meta-analyses. Standardization of patient populations and stratifications should also be attempted. It has been proposed that reference MSCs be developed to facilitate comparison between studies (119).

In summary, cell-based therapies would benefit significantly from different protocols collectively referred to as cell enhancement, including possible priming of host tissue with cytokines to increase homing; preconditioning of transplanted cells, drugs and pro-survival factors; genetic engineering of cells; and the use of biomaterials. All of these strategies could contribute to improving cell retention and promote cell survival, proliferation, differentiation and induction of neo-

angiogenesis. Nevertheless, irrespective of cell enhancement, pilot studies to understand where the cells go by choosing the best tracking system in vivo, and adherence to well-established rules for the design of robust clinical trials, are minimum requirements for any cell protocol to assess actual effectiveness of cell-based clinical interventions.

4. Clinical trial design

4.1 Safety and ethical issues

The design of randomized controlled clinical trials that are able to ascertain the long-term safety of cell therapy, can be challenging from an ethical perspective, and encompass issues related to (120): 1) Public perception of cell therapy – heightened expectations may influence the patient's decision to participate in clinical studies with cell therapy and may also affect the randomization procedure, with a preference to be in the treatment arm of the study rather than in the control group; 2) conflicts of interest – commercial interests may place pressure on researchers to investigate cell therapies which are not yet ready for clinical testing; 3) risks vs. potential benefits – given the invasive nature and uncertainties surrounding cell therapy, the potential risks may be difficult to define, thereby making the consent procedure all the more challenging; 4) choice of study outcome measure – there is a fine balance between choosing a surrogate endpoint which provides mechanistic insight, and a clinically relevant endpoint.

4.2 Patient selection (co-morbidities and co-medications)

When considering efficacy of cell therapy, a better understanding of cell biology and the interaction between treatment and patient-specific cardiovascular risk factors, co-morbidities (such as age, gender, diabetes, hypertension, dyslipidemia, smoking, depression and psychological stress), and routine medications is required. All major co-morbidities and co-medications in patients with IHD are potential confounders of the efficacy of cell therapy, via affecting the quality of source cells as well as the response of host tissue to the transplanted cells (121-123). Autologous or allogeneic hematopoietic cell transplantation for hematologic diseases was the first type of cell therapy, the outcome of which was correlated with comorbidity indices (124). However, no data are available on comorbidity index or score systems to be used in clinical cell therapy trials in order to objectively and reproducibly assess the possible interference of pre-existing co-morbidities and co-medications with the outcome (121). In this regard, key points that should be considered are the following: 1) roughly equal stratification of patients into risk groups; 2) the inclusion of possible confounders in the analyses of outcomes; 3) evaluation of aging as a three-dimensional variable incorporating chronologic age (which is a poor predictor of cell therapy outcomes, probably due to a lack of data on organ dysfunctions (125)), co-morbidities, physical function, nutritional and cognitive status; 4) developing useful prognostic biomarkers and co-morbidity index that could help understanding correlations between co-morbidities with either cell biology and host response before any cell therapy.

In summary, careful attention must be given to a variety of factors (including age, gender, co-morbidities, concomitant medications, and any other cardiovascular risk factors) that may interfere with the regenerative potential of cell therapy in the setting of IHD and HF. The development of useful prognostic biomarkers and co-morbidity indexes could help to objectively assess the weight of these factors in both preclinical and clinical trials.

4.3 Clinically relevant delivery routes, cell dose, and timing of delivery

Catheter-based intracoronary cell infusion using a perfusion balloon catheter during stop flow conditions is the mostly used delivery route in clinical trials, with the following drawbacks: 1) the potential non-selective distribution pattern of the transferred cells, with exclusion of infarcted and border area in the case of an occluded coronary artery; 2) the need for the cells to transmigrate from the vessel lumen into the myocardium; 3) the possible occurrence of microembolisms with subsequent myocardial dysfunction. Intravenous administration is limited by entrapment of the donor cells in the capillaries of the lungs. Direct myocardial injection is the most precise and accurate type of delivery, however it requires anesthesia and prolonged recovery. Transcatheter transendocardial cell injection through the femoral artery and the aortic valve, is less invasive but requires expensive and time-consuming mapping systems that have a certain risk.

In regard to cell dose (reviewed in (42)), it should be noted that in the vast majority of pre-clinical and clinical studies, dosing has been non-systematic and empirically assessed, guided more by feasibility and accessibility rather than by intentional dosage optimization. This has contributed to the still open question of how many cells should be delivered in order to achieve clinical benefit. Mean numbers of cells infused into the coronary circulation of patients with IHD and HF range from 1.2×10^7 to $2.05\pm110 \times 10^8$ bone marrow cells and from 1×10^6 to 25×10^6 CSCs (reviewed in (42)). The optimal timing of donor cell delivery also remains debated. Although no consensus has been reached, between 4 (126) to 8 days (127) after AMI onset seemed to be the optimal time point for BMCs or circulating blood-derived progenitor cells delivery into an infarct-related coronary artery, based on the results and the inflammatory response in myocardial infarction.

4.4 How to assess the clinical benefit of cell therapy (including follow-up)

In the vast majority of trials, the primary endpoint has been the evaluation of left ventricular size and global systolic function before and after treatment (reviewed (128)). Small (if any) improvements of LVEF have been observed in cell-treated patients by 2-dimensional echocardiography, MRI, left ventricular angiography, or radionuclide ventriculography performed at different time points and with different acquisition and analysis protocols (reviewed in (128)). Given the controversial outcomes of previous clinical trials, future studies should avoid imaging methodologies with poor reproducibility, should standardize timing of image acquisition and analysis protocols and more comprehensively evaluate the potential benefits deriving from cell therapy. Indeed, implementation and standardization of other techniques, such as 3D echocardiography (129), strain/strain rates (130, 131), tissue Doppler echocardiography (132, 133), and MRI might be extremely helpful to identify more sensitive markers of cardiac improvement. It is important to emphasize that, at the present time, MRI currently provides the most accurate, comprehensive, and reproducible measurements of cardiac chamber dimensions, volumes, function and infarct size compared to other techniques (134, 135), and therefore should be performed in cell-treated patients enrolled in clinical trials whenever possible at baseline, after treatment and during follow-up. In addition to MRI, myocardial viability should be determined by ^{18}F-FDG PET assessing glucose metabolism, alone or in association with dobutamine stress echocardiography, since all studies using ^{18}F-FDG have shown an improvement in myocardial viability (136, 137), but this beneficial effect has not always been paralleled by an increase in contractile reserve (138). Finally, to precisely determine the effects of cell therapies on vasculogenesis, serial quantitative PET evaluations of global and regional myocardial perfusion might be extremely valuable (20, 137, 139). Independent of the specific technology, centralized evaluation by independent and blinded core labs should be standard.

In addition to the above endpoints, real, clinically relevant endpoints should also be used in future clinical trials, as e.g. indicated in the BAMI-trial that is focused on the effect of intracoronary reinfusion of BMCs on all cause mortality in AMI (NCT01569178). Although such trials need enough power and are costly, they are essential to demonstrate the net clinical benefit for patients. Additional standard tests that should be considered, include quality of life assessment, number of hospitalizations, 6 min walk tests, and death over several years' follow-up.

In summary, what clinical endpoint should be analyzed and by which method, how patient selection takes place and what the best clinically relevant delivery routes are for cell administration and which cell dose and timing of delivery should be used, are the most crucial aspects in clinical trials investigating the effects of cell therapy. Adequately powered large-scale clinical trials, taking into account all the possible safety and ethical issues and focusing on hard clinically-meaningful endpoints, are mandatory to determine whether the observed functional improvement reported in some studies can be extended to others and indeed translates into increased survival and reduced morbidity.

5.   Recommendations

In Figure 1, we provide a flow-chart of experimental design starting from nonclinical studies and ending with the human clinical trials. To this translational pathway, we would like to make the following recommendations when assessing the clinical potential of conventional cell-based therapy, as well as novel strategies of cell enhancement for cardiac regeneration and repair in IHD and HF patients:

- Conventional cell-based therapy has been demonstrated to offer efficacy and safety in most experimental myocardial infarction models tested, including those in large animals, but in human clinical trials in IHD and HF patients only safety of cell therapies has been shown. Therefore, future pre-clinical studies using cell-based therapies should be designed to **address specific hypotheses** on modes of delivery and mechanisms of efficacy, rather than safety and efficacy endpoints only;

- Based on the expected clinical trial outcome, a careful **selection of cell source** is essential: whereas first generation cells might be useful for stimulation of endogenous repair mechanisms or angiogenic effects, second generation cells truly aim at replacing damaged myocardium. A comparison of different cell types, or a combination of cell types in randomized clinical trials has not yet been performed but are being planned in future trials of chronic ischemic heart failure;

- Assessing cell number and viability along with **full cell characterization** should be done in every clinical trial;

- Poor cell retention remains a major issue. To further boost both cellular and paracrine effects, effective **carrier materials** or engineering approaches should be further developed;

- T**o maximize** successful translation **of novel cell enhancement strategies**, it is of primary importance to ensure that the efficacy of preclinical studies is validated in the presence of confounding factors, such as age and gender and common cardiovascular co-morbidities as well as their routine medications;

- Use of **hard clinically -meaningful endpoints** is mandatory to determine whether functional improvement indeed translates into increased survival and reduced morbidity.

6.   Conclusions

The early promise of cell therapy has not yet been fulfilled. First-generation cells and their secretomes, that aim at myocardial salvage and stimulating the endogenous repair mechanisms of the heart through pro-angiogenic or prosurvival activity, should be carefully selected depending on the desired effect. Second-generation cells such as pluripotent stem cells are indisputably capable of forming beating contractile cardiomyocytes, but large surviving grafts of injected cells are rarely observed (140). Combining these cell types with biomaterials may enhance the outcome of present cardiac cell transplantation therapy, by truly replacing the damaged myocardium with muscular grafts. Other strategies to empower the donor cells, referred to as cell enhancement, may further stimulate paracrine effects, but new developments will be necessary to achieve cardiac regeneration e.g. by stimulating endogenous cardiac regeneration. Moreover, the selection of appropriate clinical endpoints, patient population, and delivery strategies are crucial aspects to understand the clinical effects. Furthermore, focusing on hard clinical endpoints in future cell-based trials is mandatory to determine whether any observed functional improvement translates into increased survival and reduced morbidity.

Conflict of interest statement

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References

See Online supplement

Table 1: Limitations of cell-based therapies

Drawbacks	References
1. Low engraftment of BMCs and blood-derived EPCs	141-144
2. Poor survival of transplanted cells in ischemic tissue	62
3. Failure of adult stem cells to differentiate efficiently into mature and functional cardiomyocytes	145
4. Inadequate recruitment of circulating or resident CSCs	2, 146
5. Anomalous electromechanical coupling between the transplanted cells or between the transplanted and host cells with consequent arrhythmias	147
6. The use of LVEF for assessing the effects of cell therapy, as this is a load-dependent variable and loss of contractility may be compensated by increases in preload and decrease in afterload that determine changes of the Frank-Starling forces	33
7. Incorrect target population of not very sick patients with baseline LVEF ~ 50%, with generally a favorable out	33
8. Existence of well established alternative therapeutic strategies (PCI, FL, ACE-, β-blockers) that might mask potential of cell therapy effects	123

Legend: BMCs, bone marrow cells; EPCs, endothelial progenitor cells; CSCs, cardiac stem cells; LVEF, left ventricle ejection fraction; PCI, percutaneous coronary intervention ; FL, fibrinolysis; ACE-, angiotensin-converting enzyme inhibition.

Table 2: Cell source for therapeutic cardiac regeneration

Cell type	Name	Sources/Origin	Commonly used markers	Advantages/Therapeutic considerations	Disadvantages	References
First generation	Bone marrow and peripheral blood PCs	Bone marrow Peripheral blood	$CD117^+$, $CD34^+$	Limited regenerative potential Clinical trial phase 3 Minimal improved cardiac function, Limited engraftment Readily cryopreserved Readily genetically manipulated Safety profile Easy accessibility Lack of ethical or immunological problems Extensive clinical experience	Limited differentiation potential Limited yield depending on source	12, 14, 15, 17-22, 24-26, 28-31, 38, 126
	Mesenchymal SCs	Fetal origins (Wharton's jelly and cord blood) Adult tissues (i.e. bone marrow or adipose tissue) Developing tooth bud of the mandibular third molar	$CD105^+$, $CD117^+$	Limited regenerative potential Clinical trial phase 3 Minimal improved cardiac function, Limited engraftment Readily cryopreserved Readily genetically manipulated Source of paracrine factors Easy accessibility Safety profile	Limited differentiation potential Limited yield depending on source	148
	Side population cells	Cardiac biopsy	$Abcg2^+$, $Mdr1^+$	Limited regenerative potential No current clinical strategy	Limited yield depending on source	149-152
	Epicardial PCs	Developing heart	$Wt1^+$, $Tbx18^+$, $CD90^+$, $CD44^+$	Limited regenerative potential No current clinical strategy	Limited yield	153-156
	Isl^+ PCs	Developing heart	$Isl1^+$	Limited regenerative potential No current clinical strategy	Limited yield	157-159

Legend: SCs, stem cells, PCs, progenitor cells

Table 2: Cell source for therapeutic cardiac regeneration

Cell type	Name	Sources/Origin	Commonly used markers	Advantages/Therapeutic considerations	Disadvantages	References
Second generation	Cells from cardiospheres	Cardiac biopsy	c-kit$^+$, Sca-1$^+$ CD105$^+$, CD29$^+$, CD45$^-$	High regenerative potential Clinical trials phases 1 and 2 Improved cardiac function Limited engraftment	Limited yield	3
	c-kit	Cardiac biopsy	c-kit$^+$, CD45$^-$	High regenerative potential Clinical trials phase 2 Improved cardiac function Limited engraftment	Limited yield	160-163
	Sca-1	Cardiac biopsy	ckit$^+$, CD31$^+$, CD14$^-$, CD34$^-$, CD105$^+$, CD45$^-$	High regenerative potential Preclinical stage Limited engraftment	Limited yield	164-166
	Embryonic SCs	Inner mass of blastocyst	Oct4$^+$, Nanog$^+$, SSEA4$^+$	High regenerative potential Preclinical stage FIM phase 1	Ethically controversial source Teratoma formation Immunogenicity	167
	Induced PSCs	Derived from somatic cells using reprogramming technologies	Oct4$^+$, Nanog$^+$, SSEA4$^+$	High regenerative potential Preclinical stage Improved cardiomyocyte differentiation	Tumorigenicity	168-171

Legend: SCs, stem cells, PSCs, pluripotent stem cells.

Figure legends

Figure 1: Flow-chart of experimental design starting from preclinical studies and ending to the human clinical trials
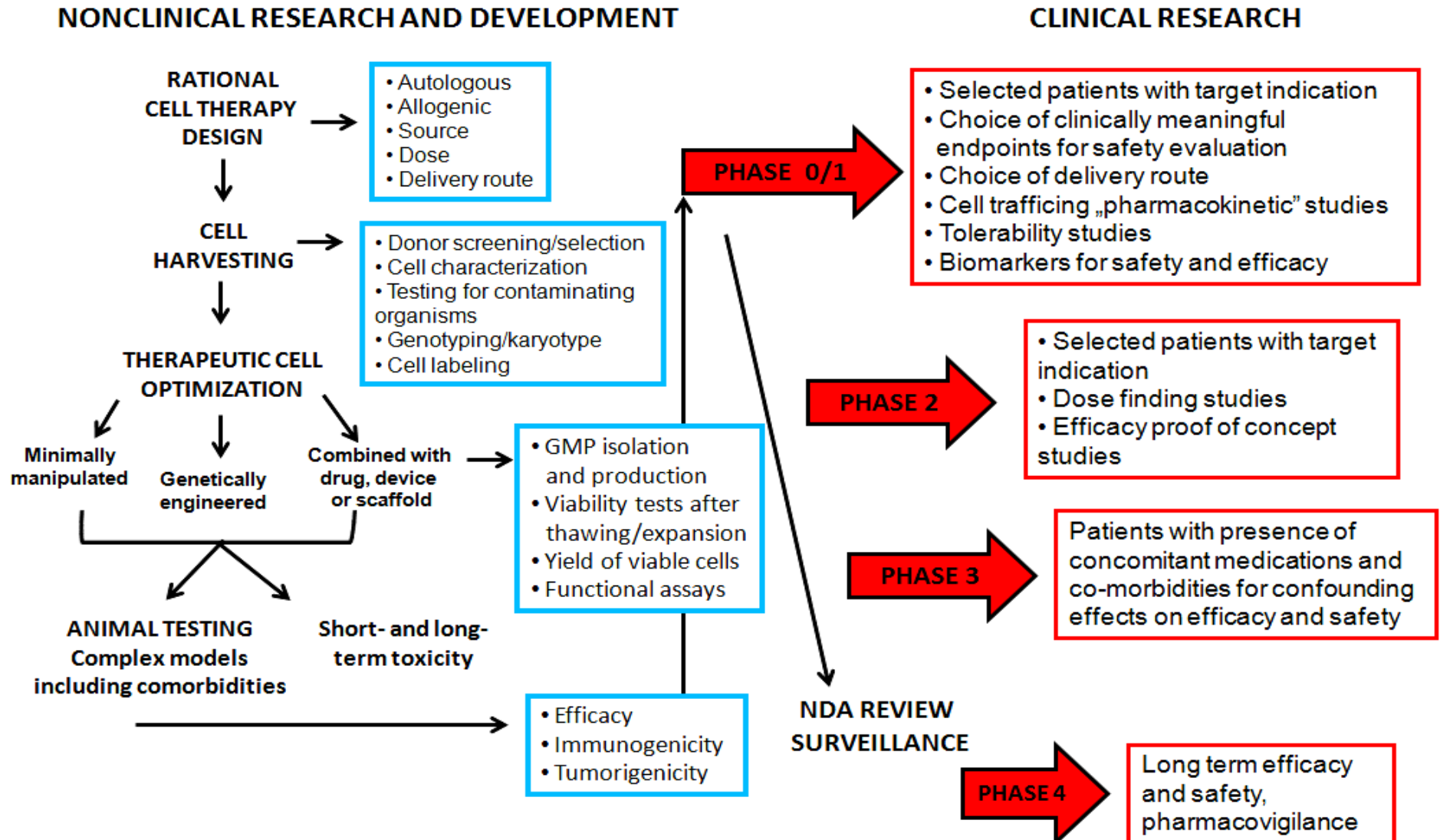

Fig. 1