# CARDIO REPAIR EUROPEAN MULTIDISCIPLINARY INITIATIVE

## Rapports

Informations projet

**CARE-MI**

N° de convention de subvention: 242038

Site Web du projet

Projet clôturé

**Date de début**
1 Avril 2010

**Date de fin**
30 Septembre 2015

**Financé au titre de**
Specific Programme "Cooperation": Health

**Coût total**
€ 14 939 184,49

**Contribution de l'UE**
€ 11 305 102,80

**Coordonné par**
CENTRO NACIONAL DE INVESTIGACIONES CARDIOVASCULARES CARLOS III (F.S.P.)
Spain

# Final Report Summary - CARE-MI (CARDIO REPAIR EUROPEAN MULTIDISCIPLINARY INITIATIVE)

Executive Summary:
The main objective of the CAREMI project is to develop broadly available and clinically applicable treatments for ischemic heart disease (IHD) by exploiting the biology of endogenous resident cardiac stem cells (eCSCs) and the molecular mechanisms responsible for their activation and differentiation in situ. The CAREMI project is divided in two main therapeutic streams A) Allogeneic Cell Therapy Development and B) Factor Therapy Development; both for Acute Myocardial Infarct, as the primary target, and Chronic

Heart failure.
A.Allogeneic Cell Therapy Development
CAREMI reached its main clinical objective, focused on the evaluation of an allogeneic cardiac stem cells based product candidate (AlloCSC-01) in patients with acute myocardial infarction (AMI). The clinical trial I/IIa (EudraCT 2013-001358-81) has recently completed the recruitment phased and has now entered in the final 12 month of follow up.
In the ongoing phase I/II study, a 6-month interim efficacy analysis is planned. The aim of this analysis is to obtain preliminary efficacy results that can help in the design of the subsequent confirmatory efficacy clinical trials in AMI (Phase III). In addition, promising results arising from this first study will help support the opening of new indications in Chronic Heart Failure. In parallel several technological implementations related with improved CSC isolation and GMP expansion are also under evaluation.
B.Factor Therapy Development
Concerning Factor Therapy Development, the main goal was to evaluate the safety and efficacy of intracoronary and intramyocardial administration of microspheres loaded with growth factors The aim was to treat ischemic heart disease by local delivery of these growth factors via intracoronary injection Due to technical limitation the workplan suffered a significant delay but, finally, appropriate preclinical formulations of IGF1-monospheres and HGF-monospheres were obtained and IGF1-monospheres tested in animals showing preliminary promising results.. The key partners involved in this development are currently exploring how to advance the preclinical programme with the expectation to obtain regulatory approval for future clinical development.
The main achievement of the CAREMI project has been the initiation of a first in man clinical trial with the allogeneic CSC product candidate. The proposed therapy aims to induce the activation of endogenous cardiac stem cells (eCSC) as a potent source of secreted growth factors during the early stage of the treatment. This first experience will serve us to define the conditions required for the development of our second proposed strategy based on the development of a non-cellular product, microspheres loaded with the combination of growth factors that could mimic the effect provided by CSCs injection, promoting activation, expansion and even differentiation of the eCSC. Success in any of the two novel therapeutic approaches explored could open a new avenue for the treatment of this highly prevalent pathological condition. Eventually, both alternatives are not mutually exclusive and it is conceivable that they could be used in combination seeking a synergistic effect.

Project Context and Objectives:
Treating Ischemic Heart Disease (IHD) is a crucial challenge for the health systems of the developed world and increasingly for developing countries due to its vast prevalence in Europe and in the developed world, and the special characteristics of the disease, particularly its long-term sequels. Percutaneous Transluminal Coronary Angioplasty (PTCA), combined with stent implantation, is successful in re-establishing the perfusion of the ischemic myocardium and has helped to significantly reduce early mortality in acute myocardial infarctions (AMI). Although this success has reduced the immediate mortality produced by the coronary artery obstruction, it does not recover the injured tissue and fails to prevent the subsequent degenerative process of cardiac remodelling that ultimately leads to the onset of Chronic Heart Failure (CHF).
CHF post-MI is a terminal disease with an annual mortality rate of 18% after the first episode, for which no curative treatment exists with the exception of heart transplantation. Unfortunately this treatment is available only to a minute fraction of the candidate patients due to scarcity of donors, its cost and the need

for long-term immunosuppression.
Because CHF post-MI is the consequence of a deficit in functional myocardial contractile cells (myocytes), regenerative approaches for myocardial tissue, based on different versions of autologous cell therapy have been proposed and several of them have been experimentally and clinically tested. Unfortunately, all these treatments have proven marginally effective at best and, although interesting from the point of view of advancing a new paradigm for the treatment of IHD and CHF, none is posed to solve the severe public health problem or even to have a positive impact in the everyday clinical setting and the natural course of the disease.
Unfortunately, in addition to their disappointing results, most of the cell transplantation-based therapeutic approaches used so far are predicated on the outmoded concept that the adult human myocardium does not have intrinsic regenerative capacity. The findings by some members of this consortium, show that the adult myocardium harbours a population of resident pluripotent cells with the characteristics of true cardiac stem cells (eCSCs, for endogenous cardiac stem cells), which are able to regenerate the contractile myocytes and the microvasculature.
This proposal is addressing the clinical implementation of this recent paradigmatic change in myocardial biology.
If this or any other translational approach to regenerative cardiovascular medicine is to be clinically effective as well as applicable to a significant proportion of the target population, it should be:
• Affordable, in terms of the production costs of the medicinal product.
• Readily and widely available, implying that the product may be easily stored and readied for application at the earliest convenience of the clinicians.
• Easy to apply and compatible with current clinical standard of care for AMI, including the widespread use of PTCA interventions.
The preliminary preclinical data produced before the beginning of the project by the partners of the consortium has focussed mainly on understanding the cellular and molecular mechanisms responsible for myocardial cell homeostasis and its response to different stress and/or injury processes. The characterization of the mammalian endogenous eCSCs by CAREMI consortium, from rodents, pig and human shows that these cells are responsible for the maintenance of the cellular homeostasis of the adult mammalian myocardium. Our results in rodents and a large animal (pig) model of acute myocardial infarction show that the activation of eCSCs can be made several fold more robust and of longer duration through the paracrine effect produced by the transplantation of a specific population of either autologous or allogeneic cardiac stem cells. Surprisingly, these allogeneic CSCs that survive only transiently in the recipient set off a potent endogenous regenerative process by activating the eCSCs that could be capable of restoring the anatomy and function of damaged myocardium. This approach results in reduction of the scar area and the formation of new vascularised and functional autologous myocardium.
The evidence summarized above opened the possibility of perfecting a medicinal treatment for cardiac regeneration based on delivering a combination of regenerative agents effective in activating the eCSCs to the post-ischemic myocardium. As indicated, this can be accomplished by the administration of a dose of “stored” allogeneic CSCs, by the local delivery of a cocktail of growth factors (recombinant proteins), or by a combination of the two approaches. Based on the large animal data, each of these three therapeutic modalities would fulfil the cost, feasibility, ready availability and robustness criteria established above for a treatment of broad clinical use. The clinical testing, readying and optimization of these medicinal regenerative treatments are the primary goals of this project. The further pre-clinical work proposed here is designed to address and eliminate some outstanding bottlenecks (see below) facing the clinical

implementation and further improvement of these protocols. In addition CAREMI aims to gain further information on the biology of the eCSCs, which should be the foundation for future development of the therapies. This approach will result in optimized clinical protocols with improved efficacy and safety that will be tested within the duration of this project.

According to previously described, the main objective of the CAREMI project is to develop broadly available and clinically applicable treatments for IHD by exploiting the biology of eCSCs and the molecular mechanisms responsible for their activation and differentiation in situ. The proposed therapies act directly on the resident eCSCs resulting in their activation and differentiation into cardiac myocytes, endothelial and smooth muscle vascular cells to regenerate the contractile tissue and the microvasculature lost as a result of the ischemic event. These therapies are based on and have been validated through solid preclinical results in animal models, which are relevant to human cardiac anatomy, physiology and pathology. To accomplish this goal it will be necessary to compare the relative merits of the two therapies proposed, their relative potential as therapies for broad clinical application to activate the endogenous regenerative capacity of the myocardium before we will be able to address whether their combined use offers any additional benefits.

During the first year of the project, the consortium’s priority was to complete and expand the preclinical safety and efficacy data needed to obtain the required institutional ethical committees and regulatory authorization to perform in-patient trials employing the allogeneic transplant of CSCs. The clinical testing of this therapy continued throughout the duration of the project. The logistic aspects with respect to cell availability for these trials are already in place. We have shown that the cells can be produced in large amounts from a population of cells from different donors, to obtain batches of Finished Product, which are bioequivalence between them. Also, once harvested the cells can be maintained frozen for long periods of time (at least 30 months) without loss of regenerative capacity.

Based on the already available preclinical information, while the cell therapy arm moves to clinical application, we will complete the preclinical optimization of the factor(s) based treatment to fulfil the requirements of the ethical committees and regulatory agencies. Together, the allogeneic CSCs product and the local growth factor delivery system, will constitute the initial platform for a novel and universal myocardial regenerative therapy with characteristics not presently available: easy to apply, effective, available at all times and of moderate cost. These parameters are in contrast with those of the treatments based on autologous cell therapy which, by necessity has to be personalized and is very costly and time consuming because it has to be developed specifically for each patient. Nevertheless, it should be noticed that the generic treatments proposed in this application must result in a regenerated autologous tissue.

In addition to these main goals, the project will include the comprehensive development of basic and clinical enabling technologies needed to assure the success and the further development of the approaches proposed. The following specific aims will also be accomplished:

• Define some molecular pathways regulating endogenous cellular regeneration in the myocardium
• Elucidate the optimal time of administration and dosage of the regenerative agents
• Generate the molecular biology tools required for advancing the knowledge of the regenerative process
• Test different formulations with biodegradable polymers for the safe intravascular local delivery of the regenerative agents.
• Establish solid animal experimental models for rigorous preclinical testing, including randomized assays
• Optimise imaging techniques for the non-invasive monitoring of the delivery and fate of the regenerative agents as well as the evolution of the regenerative response with particular emphasis on the immunological response, myocardial regional contractility, degree of perfusion and metabolism of the

regenerating zone
• Perfect the clinical grade production processes of CSCs and encapsulated growth factors required for preclinical and human trials of allogeneic cells and recombinant factors
Special emphasis would be dedicated to determine the optimal regulatory path to the clinic of different versions of the two main agents described. We will design and execute early stage human trials for the allogeneic and growth factors/cytokines based treatments in close collaboration with clinicians and expert advisors included in the project and in constant dialogue with regulatory agencies.
Furthermore, to obtain regulatory approval to start clinical trials with these therapeutic approaches, we first had to overcome two short-term bottlenecks to clinical testing and a longer term development. We needed to:
1. Document further and to the satisfaction of the regulatory agencies that administration of allogeneic cells is well tolerated, immunologically innocuous and poses no risk of triggering neoplastic development originating from either the transplanted or the host's cells.
2. Obtain additional validation to the satisfaction of the regulatory agencies that our proposed drug delivery system administers the regenerative factors localized to the target region of the myocardium in a safe and time regulated manner. To eliminate or reduce any potential undesired systemic effects we need further documentation that there is either none or negligible spill over into the systemic circulation.
3. Obtain a better understanding of the identity and mechanism of action of an additional list of factors that can improve and optimize the strength and duration of the regenerative process until the restoration of the damaged tissue has been completed. In particular, we need additional information about the additive, synergistic or antagonistic effects of different factors when used in combination.
For regulatory approval we need additional tumorogenesis, toxicology and biodistribution data that, by regulation, has to be generated by a certified CRO.

Project Results:
PROJECT PERIOD 1
Production of human CSC under GMP conditions for clinical trials established
A formulation with identified components suitable for human use (hCSC) is established. The expansion process in cell factory format for GMP-scale up using closed-system that improves productivity and maintain aseptic conditions is also established. Finally, a CMO partner has been identified and a work plan for technology transfer and scale up in 2011 has been established. Defined allogeneic banking methodology has been incorporated (CTPX).
Immunological studies. Genotyping and phenotyping for hCSC
The immunological studies have been initiated. The genotyping (HLA-class I and II, as well as HLA class I-like) and the phenotyping for immunologically relevant molecules has been accomplished for hCSC from three different donors; essential step for characterization of cellular and humoral immune response triggered by hCSC (HLA-MED).
Preclinical efficacy and safety allogenic CSC therapy
A toxicity experiment was performed to determine the highest doses of hCSC that can be intravenously injected without compromising mouse viability. The results showed that doses up to 3x106 did not induce mortality, with a 100% of survival (2 weeks). After 8 months of follow-up of 15 scid mice, injected with 3x106 did not present any significant finding (CTPX, VRS, FIS).
The preclinical work in large animal model has optimized MRI protocols for measuring the functional impact of myocardial regeneration, as well as the viability of the injected after passing through catheters;

initial experiments for optimization of dosing, timing and delivery methods have been carried out (PHILIPS, KUL, CCMIJU).

IGF-1 microsphere formulation development

Concerning the non-cellular product, the optimization of monodisperse microsphere manufacturing has been worked out, with special dedication with IGF-1. For the moment only polydisperse IGF-1 formulations with acceptable encapsulation and release characteristics are available; the developed formulations will be transferred to the Microsieve process to prepare monodisperse microspheres of the required particle size distribution. However, preliminary data indicate that the bioactivity of released IGF-1 is preserved (NANO, INNO, CTPX). In addition, HiPS-MBCP polymer-only monospheres has been produced to determine the maximum dose/number of monospheres tolerated for administration to pigs.

PROJECT PERIOD 2

Preparation of Clinical Trial Applications

Regarding the regulatory strategy for the preparation of non-clinical, clinical and quality dossiers for CSC clinical trial and preparation of the Clinical Trial Applications, as soon as the process is totally validated and ready for the Spanish regulatory agency Spanish Regulatory Authority (AEMPS – Agencia Española de Medicamentos y Productos Sanitarios) inspection, we will be able to complete the quality (Q) section of the IMPD with all the data about the manufacturing and control process of the advance therapy medicine, working at the same time with the advance therapy consultant Gradocell to develop the strategy and the different stages of the manufacturing process to ensure regulatory and quality considerations; as soon as all the final reports of the preclinical studies are available non-clinical (NC) section will be prepared.

Optimization of CSC isolation and storage. Validation of the manufacturing process

The process for human CSC (hCSC) isolation and expansion according to GMP-compliance quality has been set up at CTPX. Currently, a formulation with identified components suitable for human use (hCSC) is already defined. The expansion process, in cell factory format, for GMP-scale up using closed-system that improves productivity and maintaining aseptic conditions, has also been established and evaluated for reproducibility with independent cardiac samples. The studies for cell expansion demonstrated an improved yield when cells were expanded at 3% O2 and this is the reason it has been incorporated to the expansion protocol.

A CMO partner has been identified and a defined work plan for technology transfer and scale up is being developed during 2011/2012. The project has been presented to ethical committees of two hospitals, close to the CMO, for having a stable source of human samples. We have obtained the agreements and the Consent Inform of the donors to provide the CMO with human biopsies for hCSC isolation and expansion for clinical trials use. The production process has been agreed with the AEMPS and all the process is being implemented at the CMO with the aim of obtaining the GMP accreditation.

Defined allogeneic banking methodology has been incorporated (CTPX). The protocol for cell freezing at the WCB level has been established and we already have promising preliminary results for a freezing protocol at the FP level free of animal proteins. Cell viability after thawing is higher than 80% in all the cases. The identity and purity specifications of the cell products at the Working Cell Bank and Final Product levels have been fixed. Different controls of sterility, identity and genetic stability have been fixed along the expansion culture to confirm the safety of the cellular product.

Immunoregulatory properties of hCSC. hCSC-induced allogeneic response

An extensive evaluation of expression and regulation of key immune relevant molecules in hCSCs has been carried out. All the results suggest that hCSCs could be potentially tolerogenic rather than

immunogenic. hCSCs preferentially initiate the activation and proliferation of regulatory T cells and express high level of PD-L1, a negative co-stimulatory molecule implicated in immune modulation in various systems; demonstrated to play a significant role in the hCSC model (HLAMed). The effect of hCSCs in other cell subsets can be variable; they are potent recruiters of monocytes (CTPX) and can induce expression of cytotoxicity markers in NK cells (HLAMed). Chemokines essential for monocyte recruitment have been identified. Moreover, in vitro assays have shown that hCSC secrete paracrine factors able to protect cardiomyocytes from apoptosis. All these findings are important for mechanism definition.

Preclinical safety allogenic CSC therapy: absence of tumorogenicity of hCSC

During 2011, hCSC produced at CTPX has been used to set up the conditions for biodistribution and tumorougenic experiments. The study protocol for biodistribution studies has been agreed with Vivotecnia and will be done using an immunodeficient rat model. Conditions for detection in the different organs of the injected human cell using quantitative PCR have been established. Biodistribution data are expected to be ready before summer. On the other hand, for tumorogenic assays we will wait for having the first GMP-like batches as suggested by the regulatory agencies. Initial toxicity experiments were performed to determine the highest doses of hCSC that can be intravenously injected without compromising mouse viability. The results showed that doses up to 3x106 did not induce mortality, with a 100% of survival (2 weeks). Preliminary results, using cells produced at CTPX in an immunodeficient mouse model, have shown the absence of tumorogenicity of highly expanded CSC. After 8 months follow-up of scid mice (15) injected with 3x106 of hCSC, we could not find any tumour (CTPX, VRS, FIS). These data are required for the IMPD preparation and will be ready before the end of 2012.

Preclinical efficacy CSC therapy: established and optimized MRI protocols

The preclinical work in large animal model has optimized MRI protocols for measuring the functional impact of myocardial regeneration, as well as the viability of the injected after passing through catheters; initial experiments for optimization of dossing, timing and delivery methods have been carried out (PHILIPS, KUL, CCMIJU). Pig CSC (pCSC) produced by CTPX, following the same protocol used for human cell expansion, have been used for the dose experiments. During 2011 modifications of the chest close infarction model in pigs have been done to reduce animal mortality during procedure. Recently experiments done with 25x106 cells intracoronary administrated, at time 0 or 7 days after myocardial infarction, revealed that the pig model is robust. Only one animal out of 10 infarcted and cell injected pigs died. The experiments to complete the dose and timing experiments are on-going and are planned to be finished in the coming months.

Acute toxicity induced by of the administration buffer was analyzed using non-infarcted animals. No elevation of cardiac enzymes was observed.

IGF-1 formulation development: polydisperse IGF-1 formulation available

Concerning the non-cellular product, the optimization of monodisperse Microsphere manufacturing has been worked out, with special dedication with IGF-1. For the moment only polydisperse IGF-1 formulations with acceptable encapsulation and release characteristics are available; the developed formulations will be transferred to the Microsieve process to prepare monodisperse microspheres of the required particle size distribution. However, preliminary data indicate that the bioactivity of released IGF-1 is preserved (NANO, INNO, CTPX). In addition, HiPS-MBCP polymer-only Monospheres has been produced to determine the maximum dose/number of Monospheres tolerated for administration to pigs (NANO, INNO, CTPX, CCMIJU). Microsphere doses that can be safely injected intracoronary in pigs without cardiac enzymes elevation have been identified. Histological analysis showed only mild inflammatory response around the microspheres at these doses.

Advanced characterizations of eCSCs. microRNAs prevent skeletal muscle differentiation in postnatal cardiac progenitors

Chronic cardiac diseases are frequent findings in several forms of muscular dystrophy, including limb-girdle muscular dystrophies, caused by mutations in the sarcoglycan proteins that are involved in the maintenance of muscle integrity during contraction. Mutations in the Sgcb gene cause LMD2E (limb-girdle muscular dystrophy type 2E), often characterized by severe cardiomyopathy and mild muscle wasting. Although not much is known on the control of cardiac differentiation in adult progenitor cells, recent studies have highlighted the role of microRNAs (miRNAs) in controlling different aspects of muscle functions. So far, all the identified muscle miRNAs indirectly promote myogenesis, rather than acting directly on key regulatory factors for muscle differentiation. To develop an ex vivo gene therapy approach for LG-MD2E, we isolated and characterized cardiac progenitors from Sgcb-null mice on the basis of different cardiac progenitor markers. We found that Sgcb-null cardiac progenitors display an aberrant activation of skeletal muscle genes that are normally silenced in healthy cardiac progenitors and differentiate into skeletal muscle fibers both in vitro and in vivo. This is because of the lack of miR669q, a novel identified miRNA encoded by the Sgcb gene, and the down-regulation of miR669a. To date, among the miRNAs known to regulate skeletal myogenesis, only miR669a and miR669q directly inhibit the MyoD 3' untranslated region (UTR) and, consequently, skeletal myogenesis. Gain and loss of function experiments show that these miRNAs act within a network to control cardiacskeletal muscle fate switch in vitro and in vivo. A delay of skeletal muscle regeneration in muscles overexpressing miR669a confirms its important role in myogenic regulation. These data indicate that ex vivo gene therapy for muscle disease might not work in all cases and show that miR669a and the novel miR669q are able to rescue, at least partially, post-infarct cardiac degeneration in Sgcb-null mice by inhibiting MyoD expression that otherwise impairs cardiac progenitors. These results have been published in the prestigious Journal of Cell Biology.

Development of a scalable strategy using a microcarrier-based stirred culture system to efficiently improve the expansion of hCSCs

An efficient protocol for hCSC expansion using microcarrier-based stirred culture systems was implemented (iBET). Different microcarrier types and medium formulations were tested in spinner flasks. Microcarrier colonization, cell concentration, viability and metabolism were further evaluated through culture time, as well as cell phenotype, and differentiation potential after expansion. Preliminary characterization assays show that hCSCs expanded in microcarrier-based stirred culture systems are phenotypically and functionally similar to the ones cultivated in traditional static monolayers, retaining their characteristics along culture time.

Characterization of the Receptome of the CSC

Selection and implementation of proteomics methodologies that could overcome the difficulties in receptors identification due to the hydrophobic nature and relative low abundance of integral membrane proteins were pursued. The extraction of plasma membrane proteins and consequent fractionation steps were optimized, previous to mass spectrometry analysis. Approximately 20% of the identified proteins were assigned as plasma membrane proteins, including several receptors and proteins with numerous predicted transmembrane domains (TMDs). Cardiac specific proteins were also found. The proteomics platforms explored have shown to be efficient in achieving a more complete qualitative description of the receptors present in hCSCs membrane (iBET, CTPX, CNIC).

PROJECT PERIOD 3

Preparation of Clinical Trial Applications

Regarding the regulatory strategy for the preparation of non-clinical, clinical and quality dossiers for CSC clinical trial and preparation of the Clinical Trial Applications, for the Q documentation, the tech-transfer with the CMO has been finished and they have just initiated the GMP production phase of the project to manufacture the cell therapy medicinal product to be used in humans in the clinical trial. Once all these data related with the GMP manufacturing process are ready, they will be incorporated into the manufacturing section of the IMPD and the CMO will request the inspection to AEMPS to obtain the GMP accreditation of the cellular products and the authorization for using this medicine in clinical trials.
For the C documentation, at the time of the second Annual Report of CAREMI (March 2012), as the clinical trial protocol was not designed yet, CTPX took the decision of going ahead with this design and speed up its preparation. Due to it, Dr. Isabel Portero was contracted by CTPX who was responsible for preparing the protocol design with the help of an Advisory Board selected by CTPX (Dr. Fernández-Avilés from Hospital of Gregorio Marañón and Dr. Sádaba from Hospital of Navarra). This design has been shared with the clinical team of CAREMI in different teleconferences and meetings held in 2013. CTPX progress with the preclinical studies was also showed during these meetings.
CTPX is participating actively in the clinical trial protocol design with the clinical partners of the consortium. In addition, CTPX has formed a Clinical Advisory Board for the design of the clinical trial protocol. CTPX already has a working document with a preliminary version of the clinical protocol. When the draft of this protocol is available, it will be submitted to a Scientific Advice to the Spanish Regulatory Agency (and to the Belgium Agency if finally decided) and as soon as their advice is obtained the final protocol will be written.
Finally, once the final protocol is finished and the rest of documentation of the clinical trial ready (inform consent form, case report form, investigator brochure, IMPD, insurance, etc) the clinical trial application (CTA) will be submitted to the Ethic Committees and the CTA and the IMPD to the Regulatory Agencies for their evaluation.
Process for GMP cell production defined. Agreements reached with hospitals providers of samples
A GMP manufacturing process has been defined and transferred to a CMO for preparation of cellular product to be used during the clinical trial. We reached to agreements with hospitals near the CMO to obtain human cardiac samples for cell banks preparation. All samples were collected following GMP requirements and under the supervision of hospital's ethical committees. The inclusion criteria of donors as well as transport conditions of samples from hospitals to the CMO facilities have been fixed.
GMP-like cellular batches have been produced and used for pre-clinical experiments and for checking stability of the cellular product. We tested the identity of cells produced at the working cell bank and the final product, showing that manufacturing process is robust and that cells obtained from different donors are bioequivalent. Product acceptance criteria and quality tests have been defined. Identity, purity and potency markers are already defined as well as the assays to check for genomic stability of the cellular products. In addition, the freezing protocol and the vial that will be used for final product preparation were tested and implemented, and the delivery system of the cellular product from CMO to hospitals tested.
The CMO has obtained the GMP accreditation of its facilities and it is producing cell batches from three different Working Cell Banks to obtain the final product for the clinical trial. Once these products are obtained (it is expected to have the three GMP cell batches before summer) the CMO will ask for the inspection of AEMPS to obtain the GMP accreditation of the cellular product. This is a requirement for IMPD preparation.
Immunoregulatory properties of hCSCs: hypoimmunogenicity and modulation of NK activity
An extensive evaluation of expression and regulation of key immune relevant molecules in hCSCs has

been carried out. All the results suggest that CTPX cells analyzed are suitable for their administration in allogenic settings and that those cells are hypoimmunogenic. It seems that the inherent immune features of the characterized CSCs shift signalling capacities of T cells, within the allogenic setting, towards delivery of signals that promote the development, maintenance, and functioning of an anti-inflammatory immunoregulatory response. They also possess a demonstrated ability to modulate NK activity which and further supports their capacity to modulate an inflammatory microenvironment towards healing (HLA-MED).

Preclinical safety CSC therapy: absence of tumorogenicity of hCSC in immunodeficiency conditions and biodistribution in a small animal model

The preclinical work in a small animal model yielded positive results. Safety assays showed no tumours appearance when hCSCs prepared under GMP-like conditions were subcutaneously injected into immunodeficient mice. Ten million cells produced from two different donors were injected and the formation of tumors followed up for 4 months (FIS). Biodistribution assays have been performed in rat (VRS) and pig models (KUL, CCMIJU). During this third period, the biodistribution capabilities of hCSCs prepared under GMP-like quality conditions were tested in an infarcted pig. Similar to the results observed with pig cells injected, 18F-FDG labelled human cells were found in the myocardium around the infarcted area and also in the lungs. Animals were sacrificed 24h after cell injection and histological analysis is ongoing to look for the injected cells (KUL). An additional group of pigs injected with pig CSCs has been done and we are checking for the presence of administered cells 21 days after administration (CCMIJU). Moreover, biodistribution experiments using hCSCs in an immunodeficient rat model are in progress. Cells have been intramyocardial injected in infarcted animals and the presence of human cells in different rat tissues tested by qPCR checking for the amplification of Alu sequence (VRS).

Preclinical efficacy CSC therapy: NOAEL dose established and timing study

The preclinical work in a large animal model has optimized MRI protocols for measuring the functional impact of myocardial regeneration, as well as the viability of the injected after passing through catheters; initial experiments for optimization of dossing, timing and delivery methods have been carried out (PHILIPS, KUL, CCMIJU). During this year we have also implemented associated tools to analyze the data obtained with the proposed acquisition protocols.

NOAEL experiments (No Observed Adverse Effect Level) have been performed with hCSCs using a pig model. Acute toxicity was tested after intracoronary injection of different doses of human CSCs in healthy animals (without infarction) and the presence of cardiac enzymes checked in blood 6 and 24 hours after cell injection. 100x106 human cells were injected without toxicity and fixed as NOAEL dose.

For dose and timing experiments 25x106 and 50x106 pCSCs were injected in infarcted animals 2 hours or 7 days after infarction. 30 animals have been analyzed to this end and we have confirmed that administration 7 days after infarction is safe. On the contrary, some toxicity was observed when cells were injected during the acute phase after infarction (2h after reperfusion) precluding cell administration during this phase. CMR analysis of cardiac function of infarcted pigs injected with pCSCs 7 days after infarction showed an improvement on cardiac function (positive evolution of left ventricular ejection fraction values) when compared with pigs injected with vehicle. To strengthen the robustness of the assays more animals are going to be tested in the following months. Histological evidence corroborates these data, showing a lesser extension of the fibrotic tissue in pigs injected with pCSCs (CCMIJU, CTPX).

A draft of the clinical trial protocol based on the results obtained during preclinical studies was prepared and agreed with clinicians. Consultations regarding the need of a new IMPD for a different administration route than the one described for CAREMI (acute myocardial infarction treatment after CSCs administration

by intracoronary route) has been made to the AEMPS.

IGF-1 formulation development: improvements in IGF-1 loading in monospheres

Concerning the non-cellular product, important improvements have been done in the development of the process for growth factor loading into monospheres. IGF-1 loading has been upgraded and release times assayed. The bioactivity of the IGF-1 released from the polymer was confirmed using an in vitro cellular system. Moreover, experiments in rat models have been performed to check the ability of IGF-1 to prevent cell dead and to promote proliferation. HGF supplier has been identified and formulation experiments are ongoing. A suitable buffer for this growth factor formulation has been identified.

Advanced characterizations of CSCs: importance of miRNAs in CSC activation and regeneration

Regarding hCSCs characterization, the influence of the miRNAs on the activation and differentiation of the CSCs have been also studied revealing the importance of notably miR-92b*, miR-598-3p, miR-3563-5p, miR-541, miR-3558-5p, miR-196c*, miR-103-1*, miR-667* and miR-122* in cell activation and -miR-1 or CSCs-miR-133a in the regeneration/repair capacity of the cells (LJMU, CNIC, UNI-GOE). These results have been published in high impact factor journals, such as Molecular Therapy and International Journal of Cardiology. Moreover, gene expression profile of CSCs and MSCs has been analysed and compared using 60K probes expression arrays. This platform contains all known genes as well as non-coding RNAs. More than 1800 genes differentially expressed between CSCs and MSCs (fold change >2 and p-value <0,05) were identified. These results demonstrate CSCs are different than MSCs having a specific gene expression profile. We have established a molecular signature based on the expression of 332 genes with a fold change >10 and a p-value < 0,001. Ingenuity analyses showed these genes are related with cell-cell contact, cell proliferation and cardiovascular development functions. This technology was also used to check for gene expression stability during the expansion process.

Advanced characterizations of CSCs: characterization of hCSC surface markers in order to demonstrate the immunoregulatory capacity

Proteomics technologies have been used to deeply characterize the receptome and secretome of hCSC in comparison to human mesenchymal stem cells and dermal fibroblasts; around 3,500 different proteins have been analyzed. The analysis reveals that hCSCs have a similar immunoregulatory capacity to MSCs. The analysis reveals that hCSCs have a similar immunoregulatory capacity to MSCs. We have also identified several factors CD26, myoferlin and podocalyxin-like protein 1 (PODXL), suitable to be considered as identity markers for CSCs populations. Our findings suggest that changes in PODXL expression correlate with the ability of hCSCs to attach to endothelium and migrate, and to modulate immune responses, demonstrating the immunoregulatory capacity of hCSCs. The computational approaches developed by UL will help to predict the differentiation stage can be predicted from genome-scale cell characterizations, like those of microarray or transcriptional phenotypes (CNIC).

Besides, we have identified in mice a Bmi1 expressing cells negative for c-kit and Sca-1 positive which play a major role in physiological cardiomyocytic turnover and tissue repair after injury, indicating that Bmi1 might be a defining marker of adult cardiac stem cells (CNIC).

Identification of growth factors involved in cardiac repair after AMI

We besides have identified a total of 26 growth factors up-regulated in physiologically stressed myocytes from these analysis we can remark IGF-1 and TGF-β1 and more interestingly neuregulin-1 (NRG-1), periostin (POSTN), and BMP-10. Currently we are working with the hypothesis that these new identified growth factors (i.e. NRG-1) could be effective in vivo to improve cardiac regeneration and repair after myocardial damage and dysfunction. In vivo experiments of the CSC activation through specific growth

factors have been already performed (LJMU). These data have been published in the journal of high impact factor, European Heart Journal.

Aging impact on eCSCs biology

Interesting advances have also been obtained in the study of aging of human CSCs. We can affirm that there exist differences in the stemness/multipotency and proliferation markers in % in c-kitpos CD45neg human eCSCs when isolated from young (<40 years) and old (>65 years) hearts demonstrating the effect of aging in eCSCs is also relevant in humans (LJMU).

Implemented protocol for hCSCs expansion

In addition, stirred culture systems for hCSCs expansion were tested by iBET. The results show that an efficient protocol for hCSCs expansion using microcarrier-based technology in stirred tank bioreactors was successfully implemented, providing higher cell expansion factors than traditional static monolayers systems. This is expected to be incorporated for cell manufacturing process in the future.

Conclusions from the results for this period

As conclusion, it seems that hCSCs produced by CTPXs cells present a robust phenotype suitable for their administration in allogenic settings and that those cells are hypoimmunogenic. In vivo studies in large animals have demonstrated positive results with heart function improvement in acute myocardial infarction models with no acute toxicity or tumour formation, although more experiments will be necessary in order to have statistically significant data. Finally the data obtained in the basic stream will serve in the future to improve the protocols and enhance the action of the possible cellular product.

PROJECT PERIOD 4

Process for GMP cell production completed. Final approval of CTA by Spanish Regulatory Agency

During the fourth year CAREMI has completed the GMP production of the cell therapy medicinal product to be used in the clinical trials. A complete set of Quality Controls (QCs) and Internal Process Controls (IPCs) of the manufacturing process for GMP cell production have been implemented and the manufactured process have been audited for GMP compliance with positive results. All the information related to the manufacturing process of the bank, active substance and final cell medicinal product such as the control of materials, product characterization, impurities control and its characterization, analytical procedures, batch analysis, bank and medicinal product stability, bank and medicinal product specifications, validation processes, excipients, containers (primary and secondary), labelling, etc, have been included in the Investigational Medicinal Product Dossier (IMPD) that has been submitted for evaluation to the Spanish and Belgian Regulatory Agencies (AEMPS and FAGG respectively). A complete Clinical Trial Application (CTA) has been submitted to Spanish and Belgian Regulatory Agencies (AEMPS and FAGG respectively), the Spanish Regulatory Agency has approved the Clinical Trial Application dated on 16th April 2014 allowing CAREMI to reach one of the major milestones of the projects (CTPX, KUL, ICS). The Belgian Regulatory Agency has approved the Clinical Trial Application in June 2014.

A GMP complete manufacturing process have been carried out. The analytical and production activities have been carried out in line with the approved protocols. With the methodology used 3 complete manufacturing processes were carried out obtaining 3 batches of final product hCSCs from different donors. The 3 validation processes indicated in this report have satisfied the specifications established for the in-process controls, the Working Cell Bank as well as the Finished Product Quality controls. The process has been inspected by the Spanish Regulatory Agency and the EU-GMP certification has already been obtained. Three GMP batches are already produced and ready to be used during the clinical trial.

Validation of the storage conditions for the stability of the samples

Stability studies of the cellular material once produced and stored in liquid nitrogen have been conducted

at CTPX. The stability of the FP samples has been determined for storage conditions in a vapour phase liquid nitrogen tank. CTPX is evaluating four batches of FP (one produced in CTPX at process set-up phase, one from the GMP-like phase and two produced under GMP conditions). The stability of these samples is determined in terms of cell viability, cell concentration and phenotype (FACS, qPCR). These studies will be required to fix the shelf life of the stored product. All the samples analyzed at 6 months have complied with the specifications, indicating stability of the Finished Product up to this point. Two FP samples have also been tested at the 9-month point complying with specifications. Stability studies on FP will continue up to a period of three years.

Immunoregulatory properties of hCSCs: human CSC crosstalk with major adaptative and innate immune cells

Our results on adaptive T (HLA-MED) cells and innate NK cells interactions with human cardiac-derived stem/progenitor cells (hCSC) in allogeneic settings strongly suggest that besides being safe triggering and expanding regulatory anti-inflammatory rather than inflammatory immune cells, allogeneic hCSC administration to inflamed myocardium might engender a “crosstalk” with adaptive and innate immune system but also between various actors of the immune response, whether cellular or humoral, which would ultimately emphasize an allogeneic beneficial over detrimental effect reinforcing cardiac repair. In vivo studies using humanized mice are required to confirm, nevertheless, our immunological studies allow proposing a model for allogeneic hCSC immune behavior upon their administration to injured myocardium. These results have been published in the high impact factor journal Circulation Research.

A whole solution established for measuring the functional impact of myocardial regeneration

During this period, a complete acquisition + analysis procedure to estimate absolute quantitative flow information from cardiac dynamic contrast enhanced studies have been developed (PHILIPS). The whole methodology has been established for acquisition using dual saturation approach and dual bolus approach, this approach will improve the MRI techniques for the assessment of the functional impact of the myocardial regeneration.

Preclinical efficacy CSC therapy: safe and effective dose established in large model animal

Animal testing of intracoronary administration to determine the safe dose for patient administration determining that injection of 25x106 allogeneic pCSCs is safe, both early and 7 days after experimental AMI, and alleviates myocardial dysfunction, with a greater limitation of left ventricular remodelling when performed at one week. On the other hand, the intracoronary delivery of 35x106 pCSCs in healthy swine appears to be safe. Our results suggest that human trials with these cells can be initiated. The increase in cTpnI seen after injection could be attributed to the cardiac catheterization, as suggested by the lack of myocardial injury on the MR and pathological examinations (CCMIJU). Additional studies have been taking place in order to provide a further support to the clinical trial application work.

Preclinical safety CSC therapy: CSCs show high tropism for the heart tissue

Regarding preclinical safety allogenic therapy, the biodistribution studies carried out in the rat model indicated that CSCs are preferentially retained in the heart after intracoronary or intramyocardial delivery showing a high tropism of these cells for the heart tissue. Administered cells were not found in the brain or in the gonads in animals studied. In addition, injected cells do not stay in the host and most of them are eliminated during the first three weeks after administration.

IGF-1 formulation development: readiness of microspheres for animal trials

Concerning the non-cellular product, the optimization of monodisperse microsphere manufacturing has been worked out, an improved formulation has been developed and IGF monodisperse microspheres are ready for the animal trials (NANO, INNO, CTPX). In addition, HGF monodisperse microsphere formulation

is being produced. Finally, the Basic Research stream, has produced new results regarding the role of CSCs in the regeneration of the myocardium after injury, identification and linage tracing of the cardiac progenitors and identifying the molecular signals that control CSCs senescence with the aim of determine new monitoring methods of CSCs behavior when intended to be used for clinical intervention.

Improvement of hCSCs culture using microcarriers and stirred tank bioreactors

A robust, GMP-compatible strategy has been developed using microcarrier-based technology in stirred tank bioreactors, not only to to efficiently improve the expansion factors of hCSCs, but also to generate a more controlled and advanced cell model system that will allow to simulate in vitro the insult situation that hCSCs could confront in an acute myocardial infarction (AMI). This setup will help to clarify the biological pathways underneath hCSCs activation. Our results showed that, the three hCSC isolates expanded in the stirred system presented comparable cell growth profiles and kinetics when expanded in the optimized bioreactor protocol. In all experiments, cells attached on microcarriers presented high viability and cell expansion factors higher than the ones achieved using standard static monolayer culture systems.

Identification of the components of CSC secretome

An extensive identification of hCSC secreted proteins, using cell growth conditions essentially identical to those intended to be used in the clinical trial, through biochemical, proteomics and transcriptomic approaches has been performed this data that will enable a better definition of hCSC secretome (CNIC, IBET and CTPX). Using different approaches we have tried to obtain a detailed composition of the profile of proteins secreted or "secretome" of the hCSC (referred as CSC for simplicity) in growth conditions close similar to those used in the clinical trial CAREMI. The panel of secreted proteins was analyzed in three CSC independent cell isolates (H1, H4 and H3) and directly compared with secreted proteins in human dermal fibroblasts (HDF) and human mesenchymal stem cells (hMSC).

In summary, we have identified a total of 921 extracellular proteins as components of cardiac stem cell as CSC “secretome” combining different approaches. From this panel, 375 of protein were detected using NGS experiments, 47 proteins detected from total CSC proteome and 167 of them were exclusively identified from secretome approach. Analysis and functional validation of these proteins would be contributed to identify and define factors that would be used as complements or alternative soluble paracrine or autocrine factors from human cardiac stem cell therapies.

PROJECT PERIOD 5

In this last period, the consortium decided to set as sole priority the initiation of the clinical trial once the Regulatory Agencies had approved it, allowing CAREMI to reach one of the major milestones of the project. The consortium decided privileged studies from the immunological point of view that were judged essential and critical to reach the ultimate goal of CAREMI; the translation of hCSCs to clinic and launching of the first European trial with allogeneic hCSCs. This included beside the critical basic research for allogeneic cellular and humeral immune response to hCSCs:

1) Optimization of the conventional clinical transplantation immune assays, which is necessary for clinical immunomonitoring within clinical trial; and

2) Immunomonitoring of the escalation phase patients.

The primary objective of the clinical trial was to check for safety of intracoronary administration of allogeneic hCSC in acute AMI during the first 30 days after this cellular medicine administration:

None of the patients developed an untoward side effect during the intracoronary infusion of hCSCs in increasing doses. Thereafter, there were no signs of microvascular obstruction, myocardial ischemia or necrosis or a general inflammatory reaction to the infusion of allogeneic cells.

This clinical trial has been able to include all the proposed patients (n=49) in the double blind, randomized phase 2 of the study by 20th November 2015 and hence be able to provide reliable and robust data that will be uniquely informative on the role of hCSCs in improving cardiac post-infarction repair by reducing infarct size. The quintessential parts of the trial that protect its scientific rigor are twofold:
1) The independent MRI core lab, which guarantees proper inclusion of patients that meet all the predefined MRI entry criteria and is responsible for careful comprehensive analysis of MRI studies blinded to treatment assignment.
2) An academic independent steering committee that has the responsibility to unblind the study after all data have been validated for integrity and interpret its results after the final lock of the database.
That the randomized phase of the clinical trial is currently in progress is the best testimony for its achievement and success. Final results of CAREMI study are expected by 1st trimester 2017.

Finally, regarding Factor Therapy development, during this year, we did achieve developing IGF-1 and HGF-loaded SynBiosys Monospheres and their production processes suitable for studies in a pharmaceutically relevant large animal model: infarcted pigs.

Potential Impact:
CAREMI has provided significant advances both in the development of Allogeneic Cell Therapy and the development of Factor Therapy. R&D, innovation and trials carried out in these areas during the project are entitles to have continuation both by R&D centers and biomedical enterprises in Europe:

A.Allogeneic Cell Therapy Development
CAREMI reached its main clinical objective, focused on the evaluation of allogeneic cardiac stem cells (AlloCSC-01) in patients with acute large infarcts. The clinical trial I/IIa (EudraCT 2013-001358-81) has recently completed the recruitment phased and has now entered in the final 12 month of follow up. Depending on the results obtained, the clinical trials promoter will evaluate the continuation of the clinical evaluation. In the ongoing phase I/II study, a 6-month interim efficacy analysis is planned. The aim of this analysis is to obtain preliminary efficacy results that can help in the design of the subsequent confirmatory efficacy clinical trials (Phase III). Other indications in the AMI context are being evaluated, also. Once results on the AMI context will be available, if they would be sufficiently promising, full evaluation of allogeneic cardiac stem cells preparations (AlloCSC-01) in the Chronic Heart failure scenario is also considered. Finally several technological implementations related with improved CSC isolation and GMP expansion are also under evaluation.

B.Factor Therapy Development
Concerning Factor Therapy Development, the main goal was to evaluate safe dose and efficiency for the intracoronary (and intramyocardiac for chronic) administration of microspheres loaded with growth factors, formulated in SynBiosys Monospheres, with the aim to treat ischemic heart failure by local delivery of these growth factors via intracoronary injection. The therapy was envisaged as either a single growth factor therapy, a dual growth factor therapy, or as a combined therapy with the cell therapy as developed in other parts of the CAREMI program. Due to different technical and logistics reasons the workplan suffered a significant delay but, finally, appropriate formulations of IGF1-monospheres and HGF-monospheres were obtained at preclinical grade. Only was possible to evaluate IGF1-monospheres in vivo, but the results were quite promising. There is an agreement of the main partners involved to conclude

this preliminary study with both monospheres in the acute infarct context and, depending on results, evaluate their use in the Chronic Heart failure scenario.

List of Websites:
www.caremiproject.eu

**Dernière mise à jour:** 2 Juin 2016

**Permalink:** https://cordis.europa.eu/project/id/242038/reporting/fr