# Publishable summary

* 1. **Project Objectives**

The **overall objective** of ENDOSTEM is to leverage European scientific excellence through a collaborative project in order to develop new strategies to activate and mobilize muscle tissue-associated endogenous stem cells as a tool for efficient tissue repair and as an alternative to stem cell transplantation. We are focusing our research efforts on candidate compounds that target muscle and muscle vasculature progenitor cells as well as prevent a damage response. This effort will be complemented by bio-delivery strategies for effective targeting of muscle tissues. This synergistic approach will ensure that ongoing and future clinical trials have a greater chance of success. To this end, key actions have been identified:

1. Phase I and Phase IIa clinical trials for patients with muscular disorders.
2. Characterization of actual target cells in the muscle repair processes in order to design future therapeutic approaches, which can also be applied to other tissues.
3. Analysis of neo-vascularisation, a key success factor in all efficient tissue regeneration.
4. Immunological studies both *in vitro* and *in vivo* in order to determine *(i)* any adverse or beneficial effect and *(ii)* factors that can modulate the immune response.
5. Stem cells fate and nuclear reprogramming analysis using epigenetic modifiers.
6. Refinement of bio-delivery of selected compounds.
7. Characterisation of *(i*) regulatory, *(ii)* transcriptional and *(iii)* signalling pathways that control stem cell activity in large animal models

To achieve these stated goals, the programme of work focuses on six interrelated projects in which the partners bring in specific expertise and resources that are collectively leveraged.

The primary focus is on clinical trials (CT) with selected pharmaceutical agents. CT represents the core of the ENDOSTEM project: they will benefit from discoveries during preclinical trials in small and large animals and their results will lead to the identification of new therapeutic approaches.

Four clinical trials are initiated *(i)* Phase IIa Clinical trial using Nitric Oxide (NO) in combination with anti-inflammatory agents *(ii)* Phase II Clinical trial using antioxidants *(iii)* Phase Ib/IIa Clinical trial using an HDAC inhibitor *(iiii*) Phase I clinical trial using Omigapil, a small anti-apoptotic molecule. These agents serve as critical tools to dissect muscle and muscle vasculature stem cell biology and promise to generate therapeutic agents for chronic degenerative vascular and muscle diseases.

The second project focuses on discovering and understanding new signalling pathways that control muscle stem cells fate and regeneration. It relies on results produced in small animals and constitutes a key translational interface between stem cell biology and the implementation of clinical protocols.

The third focus on *(i)* the improvement of circulating vascular associated progenitors homing in the damaged tissue, to both contribute to muscle regeneration and neo-angiogenesis *(ii)* the induction of vascular stability to optimize muscle perfusion.

In this regard, strategies aimed to increase circulating vascular progenitors homing and mobilization can be increased both by chemokines and by increasing cell adhesion and mobility through the endothelial cell monolayer.

The fourth focus is on the immunological aspects that result from tissue damage and invasion of muscle by inflammatory cells. Here, we are focusing our attention on macrophages, which are rich sources of growth factors and cytokines as well as of tissue damaging species such as free radicals.

The fifth project focus on new pharmacological approaches and delivery mechanisms. In this regard, we are focusing our work on studying the relation between stem cell fate and nuclear reprogramming by analysis and intervention on the epigenetic state of some key genes that could promote stem cell based repair. In addition, strategies for efficient targeting of muscle tissue using biomaterials approaches are developed.

Finally, the sixth goal is the preclinical testing of therapies for skeletal muscles diseases in clinically relevant large animal models.

In this aim, we are focusing our work on *(i)* isolating and characterizing porcine muscle stem cells *(ii)* analysing their response to growth factors and cytokines *in vitro* *(iii)* testing therapeutics approaches outlined in the previous projects on skeletal muscles regeneration models already setup.

**1.2** **Work performed since beginning of the project**

The work performed since the beginning of the project covers all the areas mentioned above. We have initiated the development of several therapeutics that act on stem cells activation. These therapeutics are nucleic acids, peptides, anti-oxidant drugs or epigenetic modifiers and have highest impact on opportunity to meet the challenges in regenerative medicine market.



We have carried out preclinical studies on selected compounds with the aim of providing ground for their testing in clinical trials to assess their suitability as therapeutics. Of the various approaches, two have now reached clinical testing.

Endostem regroups clinical trials using therapeutics of 4 different pharmacological approaches; several clinical trials are scheduled or have already started in the last years:

* 2010: Phase IIa Clinical trial Using Nitric Oxide (NO) + NSAID as therapeutic agent
* 2013: Phase I studies using NO-NSAID combination
* March 2013: Phase Ib/2a Study of HDACi in Duchenne Muscular Dystrophy (started march 2013)
* May 2014: Phase 1 pharmacokinetic, tolerability and feasibility study with *omigapil* in patients with CMD

A fifth compound, a soluble for Cripto is developed and it is in an advanced preclinical development and emerged directly from the ENDOSTEM consortium.

We are investigating the molecular mechanisms that will allow us to understand the control of stem cell activation and differentiation. First results have already been produced and will lead to promising new developments that will, after regulatory review, help the activation and mobilization of endogenous stem cell. For example we have identified several compounds (peptides or miRNA) that improve stem cell migration and recruitment during regeneration.

Below, we describe the work achieved since the beginning of the project focusing on clinical trials and therapeutics development.

**1.3** **Main results achieved so far**

**Clinical trials**

The participants in this consortium have carried out preclinical studies on selected compounds with the aim of providing ground for their testing in clinical trials to assess their suitability as therapeutics. Of the various approaches, three have now reached clinical testing. These are the combination of nitric oxide (NO) donating molecules isosorbide dinitrate with the non-steroidal anti-inflammatory drug (NSAIDs) ibuprofen, the histone deacetylase inhibitor Givinostat and anti-apoptotic compound Omigapil.

In 2010, we finalised a phase IIa study on 71 patients (36 of which with placebo) suffering from Duchenne, Becker and Limb Girdle Dystrophy. The aim of this open pilot study was to establish the profile of safety, tolerability and clinical response of NO and ibuprofen drug combination in a cohort of adult patients affected by muscular dystrophies. The study included 71 patients, of whom 35 treated with the drug combination for 12 months (10 with Duchenne Muscular Dystrophy, DMD, 7 with Becker Muscular Dystrophy, BMD, and 18 with Limb-girdle Muscular Dystrophy, LGMD) and 36 untreated (11 with DMD, 9 with BMD and 16 with LGMD). Based on the results of the pilot trial, done in 2010, we undertook the necessary steps needed to develop the drug combination further into a clinical setting. In 2012 we set up a protocol of development of the combination. The protocol included two phase I studies, the first on the possible pharmacokinetics interaction of isosorbide dinitrate and ibuprofen, the second to define the maximum tolerated dose. In 2013, the protocols were approved and the studies carried out, at the Phase I Centre of the L. Sacco University Hospital. The results of the studies showed that drug combination is safe and well tolerated. We started designing a protocol to test the efficacy of the combination of isosorbide dinitrate and ibuprofen in non-ambulant patients affected by Duchenne muscular Dystrophy.

A 2-part, phase 2 study has been started in May 2013 to assess the effects of Givinostat on muscle histologic parameters and on clinical parameters in ambulant children with DMD. The safety, tolerability, and pharmacokinetics of Givinostat will also be assessed.

A phase 1 study using omigapil has been registered on march 2013 and is titled officially: “Congenital Muscular Dystrophy Ascending Multiple Dose Cohort Study anaLyzing Pharmacokinetics at Three Dose Levels In Children and Adolescents With Assessment of Safety and Tolerability of Omigapil ». The study is scheduled to start on May 2014.**Pha**

**Targeting muscle stem cells**

This understanding allows the identification of molecules that can be used to activate muscle regeneration through endogenous stem cell cells mobilization. During the last three years significant progress has been achieved and candidate molecules have been identified:

1. We have shown that growth arrest and differentiation are coordinated via the Notch signaling pathway during skeletal muscle regeneration.
2. We have pursued during the last five years the fundamental characterization of the actual target cells in the muscle repair process in order to intelligently design future therapeutic approaches. A significant progress has been achieved in the comprehension of the molecular mechanisms and new targets have been identified thanks to a large scale profiling of progenitors and postnatal stem cells from embryonic day 8.5 to old mice of 24 months
3. We have identified SoxF (Sox7/17/18) as novel regulators of muscle stem cells function. Indeed, muscle-specific loss of Sox17 leads to
* Reduced muscle fiber size with increased muscle fibers number
* Impaired muscle regeneration
* Reduced satellite cell numbers
* Loss of quiescence

These results suggest that the SoxF cascade is relevant targets for modulating skeletal muscle regeneration and is a relevant therapeutic target.

1. Several partners have shown the existence of different stem cells lineages that co-exist in skeletal muscle. Most studies show the importance of the signaling and the crosstalk between these lineages. These data indicate that PICs and FAPs provide a novel source of endogenous, pharmacologically inducible, population of intramuscular cells that can be exploited to regenerate dystrophic muscles and prevent deleterious events, such as fibro-adipose infiltration of muscles that complicate DMD progression.
2. On the matter of lineage interactions and looking for new therapeutical targets, we have shown that autocrine IGF-1 is an M2 polarizing stimulus and that administration of IGF-1Ea or blocking of IGF-1 can used to modulate the macrophage population with a significant resulting impact on muscle repair.
3. We analyzed the mechanism of action of nitric oxide in the activation of myogenesis and we have found that NO actions appear coordinated to increase SC number per fibre.
4. In the aim of identifying new therapeutic targets, we characterized 2 miRNAs that have been previously poorly described with functional relevance in skeletal muscle. Both miRNAs are highly upregulated in different muscle disease models thereby representing attractive targets for further investigations.

All these data provide new avenues of investigation and novel relevant candidates regulating skeletal muscle regeneration and stem cell regulation.

**Targeting vascular associated stem cells**

In a therapeutic perspective several results should be considered with the identification of new therapeutic targets:

1. We showed that JAM-A is involved in the formation of the new skeletal myofibers on muscle vascularization and showed that the inhibition of JAM-A promotes the formation of new vessels. Therefore, JAM-A became a new target for improving vascular cell therapy for muscular dystrophy. **.**
2. We generated a HMGB1 mutant form (3S) resistant to oxidation. Interestingly, this mutant behaves as a superagonist for cell recruitment and it fails to induce the expression of proinflammatory cytokine and chemokine by immune cells. Data demonstrated that HMGB1 has beneficial effects in skeletal muscle regeneration after acute injury by increasing the number of healthy fibres and of satellite cells. Moreover, HMGB1 acts directly on primary myoblasts by inducing their migration and their fusion to form large myotubes. Remarkably, 3S-HMGB1 behaves as a superagonist of HMGB1 *in vivo*, suggesting that it is a very promising candidate for muscle repair therapies.
3. We have tested several miRNAs to improve homing of bone marrow-derived cells. We showed that miR-34a inhibition improved homing functions in vitro and in vivo. However, several other miRNAs, such as miR-92a (which is expected to target SDF-1) did not affect bone marrow-derived cell functions. We are currently testing miR-210 and let-7 as putative targets.
4. Genetic or pharmacological elimination of one regulator of tissue repair and inflammation, fibrinogen, revealed a powerful modifier of degenerative muscle disease and protected mdx mice from functional muscle deterioration.
5. Manipulating chemokine system with chemokines antagonists or agonists can improve muscle regeneration.
6. Understanding of macrophages polarization allowed us to identify new therapeutic targets such as AMPKα1 or nitric oxide signalling.

**Developing large animals for preclinical**

It is essential for pre-clinical testing of therapies for dysfunctional muscle to be carried out in a model, which is more similar than the available rodent models in tissue biology, size and physiology to the human. The porcine model represent a good compromise to optimize analysis, due to its size, rapid growth rate, well known physiology and availability, has proven a very useful and frequently used pre-clinical large animal model for many pathologies.

One challenge was to identify and to characterize the porcine muscle stem cells. The second challenge was to set up the optimized conditions to maintain muscle stem cell growth in genetic and phenotypic stabilityand to generate large quantities of pig skeletal muscle-derived stem-progenitor cells. We have succeeded to meet these both challenges.

Considering the key role of soluble factors in mediating intercellular interactions through autocrine and paracrine signalling, the secretome can be considered as a clinically relevant source for regenerative therapies. As such, the identification of growth factors and cytokines, which mediate the proliferation and commitment of skeletal muscle stem cells, is a crucial step toward the discovery of new molecular targets for clinical therapy. This last year we have identified a panel of growth factors, which are involved in cell cycle and myogenic differentiation.

* 1. **Expected final results & their potential impact and use**

The proposed projects capitalize on combined expertise in different areas of regenerative medicine. The proposal involves collaborative interactions that allow us to merge our unique and complementary expertise in the field.

**Impact on science**

Presently, there is a focus on the therapeutic use of engrafted stem cells to treat degenerative diseases or aging; however advancing our understanding of the basic biology of stem cell activation best complements such efforts. Regardless of the potential success of engrafted stem cells, such therapies will be very costly and will require tailoring for each patient given the current state-of-the-art. Approaches aimed at mobilizing endogenous stem cells become more plausible in light of a major shift in the field of adult stem cell biology that has provided increasing evidence that pluripotentstem cells with regenerative potential are present in adult tissues contrary to the generally accepted view just 5 to 10 years ago. While many tissues possess limited regenerative potential, the capacity for regeneration declines with age and chronic disease. In addition, stem cell recruitment in response to injury or disease often produces inappropriate re-patterning of the tissue culminating in scar tissue formation (fibrosis), inadequate revascularisation, or chronic inflammatory disorders.

We are developing new strategies to activate and mobilize tissue-associated endogenous stem cells as a tool for efficient tissue repair and as an alternative/complementary approach to stem cell transplantation. The starting point for our proposal is the combined research effort by the partner groups focused upon candidate agents that target muscle and muscle vasculature progenitor cells as well as preventing tissue damage to optimize endogenous stem cell function.

The first four years were flourishing **in the identification of new target genes and new bioactive molecules that act on the muscular and vascular systems as well as the interactions between the several stem cell subpopulations present in the muscle tissue**. Moreover we have initiated **four clinical trials** using anti-oxidant drugs, epigenetic modifiers and chemical entities.

**Impact on society**

Degenerative and age related diseases create a life-altering experience for the person with injury, for their partner, parents, siblings, and children. The subsequent diminishment of body functions associated with the diseases can cause depression and loss of self-esteem. It has been considered essential, based on European policy consistent with human rights principles, that people with disabilities should be treated with dignity, encouraged to have independence, be given equality of opportunity, encouraged to have an active participation, a full citizenship and a high quality of life. Given the diversity of degenerative diseases indicated above, pathological manifestation can occur at any age: either as a child, during an individual's most productive years, or as an aged person. The trauma frequently results in morbidity, and as a result, patients typically require continuous physical and medical care depending on the disease, severity of manifestation, degree of disability, and location of injury.

**Economic impact**

**The prevalence of degenerative diseases is on the rise because aging population is increasing and this has created the need for biomaterials.** Over the past 50 years, average life expectancy at birth has increased globally by over 20 years, from 46.5 years in 1950-55 to 65.2 years in 2002. Today there are 600 million people in the world aged 60 years or over, and this will double by 2025 and reach 2 billion by 2050. While degenerative diseases are not the exclusive domain of the aged, they do impact this sector of society the highest with subsequent increased social and economic burdens on the health care systems on which they depend.

The direct healthcare costs of organ replacement are about € 240 billion globally (about 8 percent of global healthcare spending) arising from therapies that keep people alive (such as kidney dialysis), implanted replacement devices, and organ transplants. With a € 240 billion global industry already built on first generation tissue and organ therapy products and substitutes, regenerative medicine has a potential to exceed € 600 billion by 2030.

EndoStem and its partners have demonstrated this year a proof of principle through the interaction and integration of the fundamental, pre-clinical and clinical partners that have expertise in different fields such as muscular, vascular, epigenetic, immunologic fields.

By providing new and efficient therapeutics, Endostem will decrease certain social burdens while increasing the economic potential of Europe’s leading innovators in this exciting field.

* 1. **Project Contact Details and Logo**

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# Project objectives for the period

The objectives of the fourth 12 months of EndoStem involved the implementation of following clinical, scientific and organisational activities.

Scientific objectives of this period, involved the implementation of the following scheduled tasks:

1. Definition of parameters for clinical trials of selectedcandidate molecules (M5)
2. Characterize candidate genes involved in muscle stem cell quiescence (M7)
3. To expand the family of particulate formulations for the local muscle delivery of regenerative substances in large animals (M51)
4. Provide support for the use of the pig model of muscle damage to the members of the consortium (M52)

Organisatonal, communication and exploitation objectives of this period, involved the implementation of the following:

1. Continued capacity building and networking of the scientific teams
2. Reviews of progress of the biopharmaceutical development matched with market need to assess which avenues to prioritise for expenditure and impact
3. Liaison with patients associations and charities to establish stronger links and profile for the project

# Work progress and achievements during the period

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| --- | --- | --- | --- |
| **Participant no.** | **Participant name** | **Participants initials** | **Institution** |
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| 2 | **Gabriella Minchiotti** | GM | CNR |
| 3 | Emilio Clementi | EC | MEDEA |
| 4 | Jeffrey Hubbell | JH | EPFL |
| 5 | Nadia Rosenthal | NR | EMBL |
| 6 | David Glass | DG | NOV |
| 7 | Silvia Brunelli | SB | HSR |
| 8 | Elisabetta Dejana | ED | IFOM |
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| 11 | Stefanie Dimmeler | SD | GUF |
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