

Cellular Myogenic and Angiogenic Therapy for Patients with Cardiac or Limb Ischemia

Juan C Chachques, Fabricio Duarte, Jesus Herreros⁽¹⁾, Felipe Prosper⁽¹⁾, Rubens Giambroni, Pierre Julia, Gerard Fournial⁽²⁾, Alain Carpentier and Jean-Noël Fabiani

European Hospital Georges Pompidou, University of Paris, France, (1) University of Navarra, Spain and (2) University of Toulouse, France

Abstract

While various donor cells have been studied to induce myogenesis after myocardial infarction, recent interest has arisen in promoting cardiac angiogenesis. Angiogenic growth factors (as basic fibroblast growth factor and vascular endothelial growth factor) and genes encoding angiogenic factors are being delivered to the infarcted area to induce growth of new blood vessels. Increased perfusion has also been observed after endothelial cell transplantation (removed from arteries) and following implantation of progenitor cells (directly removed or mobilized from bone marrow).

These techniques retain an important potential as an adjunct to myogenic cellular transplantation in inducing angiogenesis in injured myocardium, because muscular cells mortality after implantation in high fibrotic infarcted myocardium seems to be high since the oxygen and nutrients supply are limited within the scar. Another problem limiting hemodynamic benefits of myogenic cellular cardiomyoplasty is that even if the myoblasts survived after implantation, functionally this denervated cells cannot contract spontaneously, hence, they do not contribute to improve regional myocardial contractility. Mechanical and electrical coupling of the transplanted myoblasts with the cardiomyocytes is still not demonstrated.

The goal of angiogenic cell therapy is to recover or restore myocardial viability and function of peri-infarct and scar areas. We initiated a clinical trial based on the transplantation of angiogenic cells into ischemic myocardium and ischemic limbs, using a cell isolation kit including a magnetic separation column for pluripotent CD133+ stem cells, which have a recognized role in postnatal blood vessel formation. This approach avoids in-vitro cell culture procedures. Mononuclear autologous cells having endothelial potential, can be previously mobilized from bone marrow by administration of G-CSF (granulocyte-colony stimulating factor).

The clinical trial includes randomly patients presenting ventricular postischemic scars (akinetic and metabolically nonviable), and surgical indication for CABG in remote, viable and ischemic areas. The stem cells are isolated with specific approved technology using antibody against the surface marker AC133. Cell implantation in myocardial scars are performed during cardiac surgery, catheter-based percutaneous implantation procedures are in evaluation. In another cell-based angiogenic randomized clinical trial patients suffering from chronic limb ischemia will be included.

The ultimate goal would be to propose cellular angiogenic therapy for myocardial infarction before myogenic cell transplantation, in order to improve local conditions for muscle cells survival (preconditioning). Thus, therapeutic angiogenesis and myogenesis should be successively associated.

Key words: cellular cardiomyoplasty, heart failure, limb ischemia, myocardial infarction, myogenesis, therapeutic angiogenesis.

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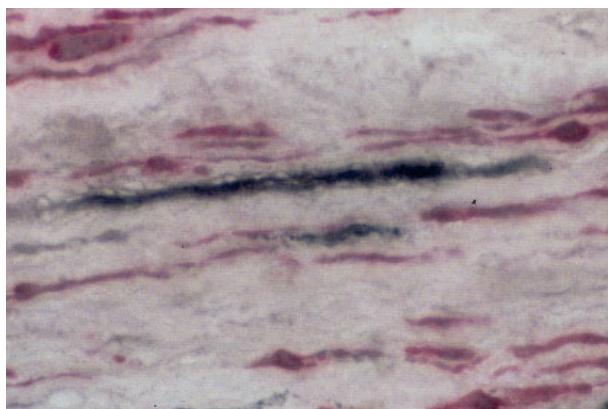


Figure 1. Angiogenesis and myogenesis developed after combined skeletal myoblast transplantation and VEGF injection in a myocardial infarct model. Myogenesis was identified using fast myosin heavychain (MHC) antibodies (blue stain). Angiogenesis was evaluated by the identification of endothelial cells with anti-von Willebrand factor antibody (DAKO): red stain.

Part I: Cardiac Angiogenesis and Myogenesis

Objectives

An increasing number of patients are being referred for coronary artery bypass surgery despite extensive atherosclerosis that precludes complete revascularization. A variety of novel angiogenic therapies are being evaluated as adjuncts to bypass grafting or as sole therapy for patients who are not candidates for coronary artery bypass. The goal of these interventions is to induce angiogenesis and increase perfusion to ischemic regions of the heart that cannot be adequately revascularized by conventional means. Transmyocardial laser revascularization improves anginal symptoms, probably by inducing fibrosis hence reducing myocardial viable cells and pain. Vascular endothelial growth factors (VEGF) and basic fibroblast growth factors (bFGF) have been given as protein therapy or as gene therapy by transfection by naked DNA or with adenoviral vectors. Growth factors have been given as a direct injection, in biodegradable capsules or by transcatheter delivery. Angiogenesis can also be induced with endothelial cell transplantation (removed from arteries) and with implantation of pluripotent progenitor cells, e.g. CD133+ (isolated from bone marrow).

We underwent a multicenter clinical study based on the transplantation of angiogenic cells into ischemic myocardium and ischemic limbs, using a cell isolation kit including a magnetic separation column for CD133+ cells, which have a recognized role in postnatal blood vessel formation. This approach avoids in-vitro cell culture procedures. These mononuclear autologous cells having endothelial potential, can be previously mobilized from bone marrow by administration of stimulating growth factors. This cellular angiogenic therapy is being performed in myocardial infarction before myogenic

infarction before myogenic cell transplantation in order to improve local conditions for cell survival (preconditioning). The ultimate goal is the successive association of therapeutic angiogenesis and myogenesis.

Background

Myocardial circulatory insufficiency, cardiomyocyte necrosis, and apoptosis play important roles in many pathologic conditions of the heart. Therapeutic approaches aimed at promoting angiogenesis and growing new heart muscle fibers (myogenesis), currently undergoing intensive investigation and early clinical trials, therefore hold considerable promise for the future. Cardiac myogenesis and angiogenesis constitute regenerative medical and surgical therapeutic procedures, conceived for substitution or regeneration of irreversibly damaged cardiac tissues and structures. These new modalities of therapy will not be constrained by the scarcity of donor organs, immunologic rejection, or the need for mechanical devices requiring maintenance and replacement.

Genes encoding angiogenic factors and angiogenic growth factor proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, are being delivered to the target tissue to induce growth of new blood vessels. Growth factor gene delivery to myocardium demonstrated deleterious effects of unregulated expression. Angiogenesis can also be induced with aortic endothelial cell transplantation into a myocardial scar and with implantation of progenitor cells from G-CSF (granulocyte-colony stimulating factor), mobilized peripheral blood (CD133+ cells).

For myogenesis, various progenitor and stem cells are being assessed as donor cells for implantation into the ventricular wall of injured hearts. Clinical studies into myogenesis have been initiated with implantation of autologous skeletal myoblasts and bone marrow stromal cells into myocardial scar tissue. However, a number of important questions remain. The myoblasts implanted into myocardial scar have been reported to improve systolic fiber shortening, even though no syncytial integration of these cells with native myocardial fibers could be recognized because they were surrounded by the fibrous scar tissue. This is curious inasmuch as an anatomic supracellular integration seems a necessary prerequisite for synchronized contractions of these new muscle cells during ventricular systole. Clarifying such dilemmas should help the understanding of findings reported from functional studies.

Cellular cardiomyoplasty

Cellular CMP is a combination of cellular biology with cardiac surgery or with interventional cardiology. Its aim is to regenerate the myocardium in order to prolong and improve the quality of life of patients who suffer from severe chronic cardiac deficiency and who are unresponsive to medical treatment. Cellular CMP consists of in situ cell implantation intended to induce the growth of

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new muscle fibers and the development of angiogenesis and vasculogenesis in the damaged myocardium.

Cells for myocardial regeneration

Current possibilities in cell therapy for heart failure are the transplantation into the damaged myocardium of different cell types.

- * For angiogenesis and vasculogenesis the following cells can be proposed: endothelial cells, bone marrow-derived stem cells, circulating blood-derived progenitor cells.
- * For myogenesis: skeletal myoblasts, smooth muscle cells, fetal and neonatal cardiomyocytes can be used.

The relative contribution of various sources of precursor cells in postnatal muscles and the factors that may enhance stem cell participation in the formation of new skeletal and cardiac muscle in vivo have been investigated by several groups. In postnatal muscle, skeletal muscle precursors (myoblasts) can be derived from satellite cells (reserve cells located on the surface of mature myofibers) or from cells lying beyond the myofiber, e.g. interstitial connective tissue or bone marrow. Both of these category of cells may have stem cell properties. In adult hearts (which previously were not considered capable of repair), the role of replicating endogenous cardiomyocytes and the recruitment of other stem cells into cardiomyocytes for new cardiac muscle formation has recently been reviewed. The main conclusions are that although many endogenous cell types can be converted to contractile cells, the contribution of non-myogenic cells to the formation of new postnatal muscle in vivo appears to be negligible. The recruitment of such cells to the myogenic lineage can be significantly enhanced by specific inducers and appropriate microenvironment. For myocardial repair, the participation of bone marrow-derived stem cells in the repair of damaged cardiac muscle motivate our group to start cell-based angiogenic and myogenic clinical trials.

Rationale for cell-based angiogenic therapy

Myogenic cell transplantation into an infarcted region was intended to restore elasticity to the injured region and prevent cardiac thinning and dilatation. Several types of cultured cells have been transplanted into infarcted myocardium. However, cells mortality after implantation in high fibrotic infarcted myocardium seems to be high since the oxygen and nutrients supply are limited within the scar. Furthermore, in current clinical trials the survival of the transplanted myogenic cells were probably facilitated by the angiogenesis induced by simultaneous CABGs or angioplasty procedures performed simultaneously with cell implantation. For these reasons, angiogenic therapy before myogenesis seems to be justified.

Cell-based angiogenic therapy is an interesting and safe approach in comparison with the administration of growth factors in the form of proteins, which presents risks of systemic effects inducing problematic angio-

genesis in the retina or the potentiation of growth and metastasis of occult tumors. Growth factor gene therapy presents also risks related with stability, unregulated expression and adverse response to transfection vectors.

Experimental studies performed at the University of Paris (Broussais Hospital)

1) Angiogenic growth factor versus myogenic cellular therapy for myocardial infarction

The goal of this experimental study was to compare the effects of myoblast implantation versus the injection of VEGF in a model of myocardial infarct. Our results showed that cell therapy improves regional myocardial contractility and reverses LV chamber remodeling. Angiogenesis was only demonstrated in the group of animals treated with VEGF (not with cells), however any functional benefit was observed in this group. Simultaneous delivery of VEGF and cells did not demonstrate beneficial effects. It can be concluded that angiogenic growth factors should be deliver in myocardial infarction several days before cell transplantation in order to improve local conditions for cell survival (preconditioning) (fig. 1).

2) Myocardial electrostimulation after cellular CMP

The principles of electrophysiological conditioning of muscle fibres was applied by our group in cellular cardiomyoplasty since skeletal myofibers express predominantly fatigue sensitive fast myosin, which is not suitable for cardiac work. Atrial synchronized biventricular pacing is indicated in heart failure patients to correct conduction disorders associated with chronic systolic and diastolic dysfunction. We explored the possibility to electrostimulate the ventricles following autologous skeletal myoblast implantation to create an active cellular cardiac support, since skeletal myoblasts are known not to contract spontaneously. This approach induced the contrac-



Figure 2. Bioheart Myocath approach for cell implantation procedures. The catheter is introduced via the femoral artery and the left ventricle is reached by the retro-aortic approach. The injection needle is then protruded and cells injected directly into the myocardium.



Figure 3. The CliniMACS instrument (Miltenyi Biotec) provides an open platform for cell selections in a closed and sterile system.

tion of the transplanted cells (dynamic cellular support) and a higher expression of slow myosin, better adapted for chronic ventricular assistance.

3) Myogenic cellular therapy for dilated cardiomyopathy

Non ischemic cardiomyopathies are responsible of almost 50% of the indications to cardiac transplantation. Dilated cardiomyopathy mostly concern young people in their working age (20-40 years) and it is related to high mortality rate. Until now, most of the research on cellular cardiomyoplasty have focused on improving the regional impaired myocardium in ischemic hearts, but little is known about the effects of cell therapy in dilated cardiomyopathy. We created an experimental model of dilated cardiomyopathy induced by ventricular rapid pacing to study the effects of cell therapy on non-ischemic heart failure. In another protocol, dogs presenting non-ischemic cardiomyopathy were treated with cellular cardiomyoplasty using autologous cultivated skeletal myoblasts. Dilated cardiomyopathy is a well-recognized cause of spontaneous heart failure in large- and giant-breed dogs. We took advantage of this to investigate the clinical and hemodynamic impact of cellular CMP on treating this pathological condition. Autologous intramyocardial skeletal myoblasts implantation was carried out in 5 dogs weighing 30 to 70 kg;

these dogs were followed for 2 years. From a biopsy performed in the sartorius muscle, myoblasts were isolated and cultured during 10 days obtaining 50 million cells (93% myogenic). Implantation in the LV was performed through 20 epicardial injections. Myocardial function and viability were evaluated. Two dogs died due to ventricular arrhythmia and one of pulmonary embolism. The long term follow-up of the survival dogs showed clinical improvement and clear evidence of the restoration of myocardial viability and function.

4) Functional evaluation of myogenesis in a cardiotoxin model

In this study a reproducible method for the creation of a myocardial lesion was developed, using locally delivered snake toxins (Sigma Chemical Co.). The functional benefit of cell implantation was evaluated by 2-dimensional echocardiography for global contraction and color kinesis echocardiography for regional LV wall motion.

5) Catheter-based versus surgical cell implantation for myocardial regeneration

We studied the possibilities of endoventricular cell implantation in an experimental model of myocardial infarction, using a specific catheter by percutaneous transfemoral endoventricular approach (fig. 2). The goal of this study was to compare 2 methods of cell delivery into infarcted myocardial areas: 1) percutaneous endoventricular cell implantation, 2) epicardial implantation by thoracotomy. Cell implantation was performed using a specific catheter (NOGA Biosense, Cordis Inc) guided by electroanatomical mapping.

Cellular myogenesis: mechanisms of action

The mechanism by which implanted myogenic cells improve heart function remains controversial. The 2 most investigated cells for myocardial repair are skeletal myoblasts and bone marrow stem cells.

After skeletal myoblast implantation in myocardial infarcts, it is not certain whether improvement in left ventricular performance is mediated by increased systolic function caused by synchronus contraction of the graft, since skeletal myoblasts are known not to contract spontaneously and histological-electromechanical connections of transplanted myoblasts with the myocardium have not been demonstrated. Moreover, denervated skeletal myoblasts could progressively become atrophic. Future studies should be oriented on the long-term evolution of denervated cells, myotubes and myofibers into myocardial scars, evaluating survival time and capacity for re-growth (perhaps by phasic functional electrostimulation, FES). Electrostimulation was proposed by our group to transform passive cell therapy into «dynamic cellular support».

Bone marrow stem cells can undergo milieu-dependent differentiation: when transplanted in normal myocardium can become cardiocytes, but when these cells are implanted in a myocardial scar they may differentiate into

fibroblasts, becoming a «scar within a scar». Experimentally, bone marrow stromal cells can be induced to differentiate into myocytes prior to transplant using a co-culture system with cardiomyocytes or by including 5-azacytidine in the cultures. These approaches may be effective in driving myocardial remodeling, however, clinical trials can be compromised in terms of potential cell mutations by azacytidine.

Cardiac angiogenesis and vasculogenesis

Injected myogenic cells in myocardial scars could release growth and/or angiogenic factors that could partially explain engrafted cell survival. This has not been demonstrated to be significant and is confounded with the triggering of angiogenesis by epicardial-puncture alone.

The formation of new blood vessels in ischemic tissues can be due to two different processes. The first process, vasculogenesis, implies the in situ differentiation of endothelial cells (ECs) from hemangioblasts and their subsequent organization into a primary capillary plexus. The second process, termed angiogenesis, is defined as the formation of new vessels by sprouting from preexisting blood vessels. At present, primary differentiation of ECs from hemangioblasts or angioblasts seems to be a process that is restricted to early embryogenesis, whereas angiogenesis occurs both during development and postnatal life.

The hemangioblast has recently been identified and was shown to be a transient cell stage that develops early and is lost quickly during embryonic development. It is important to note that these findings are based on in vitro experiments with murine embryoid bodies and may not necessarily reflect developmental steps in humans. The possibility that hemangioblasts or more mature endothelial progenitors persist into adult life whereby they may circulate, differentiate, and contribute to the formation of new blood vessels remains to be determined. In this context, many studies have demonstrated the existence of mature ECs in the peripheral circulation. Recently, it was reported the presence of CD133+ endothelial progenitors in human peripheral blood. Investigations showed that the progenitors differentiated into ECs in vitro and can be incorporated in vivo into sites of neovascularization. Other studies provided strong evidence that CD133+ cells isolated from human bone marrow, umbilical cord blood, and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood contains endothelial progenitors.

Characteristics of CD133+ cells

In humans, there are pluripotent cells expressing surface antigen AC133. These cells are rich in hemangioblast progenitors and can be isolated from bone marrow or from G-CSF mobilized peripheral blood. Thereafter these cells can differentiate along the endothelial or the hematopoietic pathway forming colonies. Many scientific observations demonstrated that the CD133+ cell population includes endothelial precursors, which is of clinical relevance (eg, in the field of ischemic disorders).

vance (eg, in the field of ischemic disorders). The CD133+ cells fulfill many criteria of true hemangioblasts.

Several studies have proven the endothelial potential of the CD133+ cells in the adult and its role in postnatal blood vessel formation. The majority of the cells CD133+ co-express CD34+, only a low percentage are CD34 negative. Thus, CD133+ cells represent a subset of CD34+ stem and progenitor cells becoming an ideal starting population to generate endothelial cells.

Clinical trial

Therapeutic cell angiogenesis is evaluated by means of an European multicenter clinical trial. Indications include adult patients presenting LV wall postischemic scars (akinetic and metabolically nonviable), and surgical indication for CABG in remote, viable and ischemic areas. The protocol includes the injection of CD133 positive cells obtained from peripheral blood by mobilization with G-CSF and selection using magnetic microbeads (Myltenyi, Biotec) (fig. 3 and 4).

The study is based on the following principles:

- CD133+ cells are able to differentiate into endothelial cells and to contribute to the angiogenesis and vasculogenesis.
- Stimulation of angiogenesis and/or vasculogenesis in experimental myocardial infarct models contribute to improve cardiac function.
- Administration of hemangioblastic progenitors enriched in CD133+ cells in an animal model of myocardial infarct induces vasculogenesis and angiogenesis in the infarct region and contributes to improve cardiac function, preventing cardiomyocyte apoptosis.
- It is possible to obtain a high number of CD133 cells from peripheral blood by means of administration of G-CSF.
- CD133+ cells can be selected by a commercial system approved for clinical use.

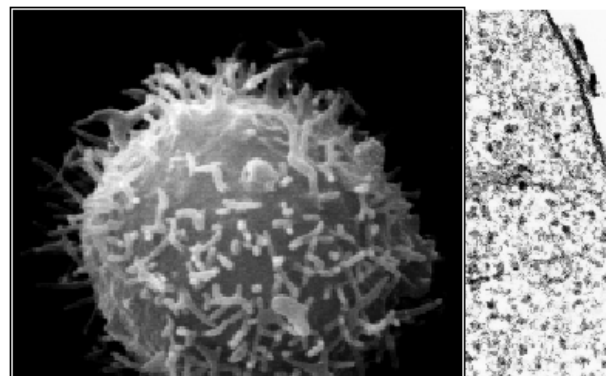


Figure 4. Cell selection technology (MACS Micro Beads, Miltenyi Biotec). The stem cells are isolated after a magnetic labeling using particles of iron oxide and dextran conjugated to monoclonal antibodies against the surface marker AC133.

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- There are populations of stem cells in the bone marrow with the ability to differentiate into cells with characteristics of cardiac muscle, improving the ventricular function.
- Intramyocardial injection of autologous skeletal myoblasts in the context of cardiac revascularization surgery, does not increase the surgical risk and it is not associated to severe adverse effects.
- There are no contraindication for giving G-CSF in patients with previous record of myocardial infarct. However, side effects during cell mobilization from bone marrow should be carefully evaluated (e.g.: leucocytosis and coagulation abnormalities).

Perspectives

It can be considered that cellular angiogenic therapy should be performed in myocardial infarction before myogenic cell transplantation in order to improve local conditions for cell survival (preconditioning). In this way, it would be suitable to recommend a clinical trial associating successively therapeutic angiogenesis and myogenesis.

Attempts are also being made to transfect myoblasts and stem cells with angiogenic genes during culture before their implantation into the myocardium, in an effort to achieve both cardiac angiogenesis and myogenesis simultaneously.

Part II: Cellular Therapy for Limb Angiogenesis and Vasculogenesis

Angiogenesis for limb ischemia can be achieved either by use of growth-factors or genes encoding these proteins. However, these approaches are related to the potentiation of pathological angiogenesis (eg, malignancy and development of angiomas) and so-called bystander effects of delivered factors (eg, effects on kidney or atheroma).

Endothelial progenitor cells in the CD133+ stem-cell fraction of adult human peripheral blood take part in postnatal neovascularisation after mobilisation from bone marrow. It was demonstrated that CD133+ cells may differentiate into endothelial cells of newly formed vessels. This differentiation can be beneficial for patients presenting severe limb ischemia, without indication of surgical or percutaneous revascularization.

Clinical trial - rationale

To assess the feasibility of angiogenic cell therapy for patients with peripheral artery diseases, we organized a randomized controlled clinical trial using CD133+ cells implanted in ischemic limbs. The goal of the study is to demonstrate that intramuscular implantation of autologous human CD133+ cells into ischemic limbs effectively induces collateral vessel formation, improving function and trophic ischemic lesions.

Endothelial progenitor cells can be sorted from the peripheral blood of patients with peripheral artery diseases and can be implanted into ischemic limbs in order to increase collateral vessel formation and to secrete vari-

ous angiogenic factors or cytokines. Although this novel angiogenic cell therapy seems to be feasible, remote angiogenic actions should be considered as possible side effects, and the clinical efficacy should be tested by specific studies.

Inclusion criteria

Randomly adult patients with ischaemia of the leg and without indication of surgical or percutaneous revascularization, are selected to be injected with CD133+ cells into the gastrocnemius of the ischaemic limb. Side effects during cell mobilization from bone marrow are carefully evaluated (e.g. coagulation abnormalities).

Exclusion criteria

Patients presenting poorly controlled diabetes mellitus and proliferative retinopathy as well as patients presenting evidence of malignant disorder during the past 5 years.

Evaluation of efficacy and safety

The following studies are performed:

- Ankle-brachial index (ABI).
- Transcutaneous oxygen pressure.
- Rest pain.
- Pain-free walking time.
- Digital subtraction angiography.
- Evaluation of cutaneous and muscular ischemic lesions.

General conclusions

There is a need of new therapeutic approaches to myocardial regeneration, whose aim is to augment the effects of the loss of cardiomyocytes, which is generally considered as irreversible, and the cause of the cardiac insufficiency. Although terminal differentiation of cardiomyocytes is disputed, it appears like a permanent turnover of contractile cells, which also happens in case of cardiac insufficiency. This might not be enough to compensate the cell death due to a pathological process. In particular, myocardial infarction leaves an akinetic fibrotic scar, which with remodeling leads to ventricular dilatation and an overall loss of the mechanical function of the heart.

The mechanisms responsible for vasculogenesis and angiogenesis are not completely understood. Several growth factors are involved in regulation of endothelial differentiation, proliferation, migration, and formation of functional vessels.

The idea of transplanting single cells has a number of attractive attributes and is dependent on an ever expanding understanding of molecular basis of angiogenesis and myogenesis. Cellular therapy primary objective is to ensure the recolonization and restoration of the muscular viability and improved functional capacities of the injured cardiac tissue.

In summary, cell transplantation already offers the promise of restoring regional ventricular function, limit remodelling and stimulate angiogenesis for patients who have had an extensive myocardial infarction and probably for patients presenting dilated cardiomyopathy.

Clinical feasibility of this new surgical technique has already become apparent.

Address correspondence to:

Juan C Chachques, Hospital Broussais 96 rue Didot-Paris, 75014 France e-mail: j.chachques@brs.ap-hop-paris.fr

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