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Direct Minimally Invasive Intramyocardial Injection of Bone Marrow-Derived AC133+ Stem Cells in Patients with Refractory Ischemia: Preliminary Results

Authors G. Pompilio[1], G. Steinhoff[2], A. Liebold[2], M. Pesce[3], F. Alamanni[1], M. C. Capogrossi[4], P. Biglioli[1]

Affiliations
[1] Cardiovascular Surgery, Centro Cardiologico Monzino IRCCS, Milan, Italy
[2] Cardiac Surgery, University of Rostock, Rostock, Germany
[3] Laboratory of Vascular Biology and Gene Therapy, Centro Cardiologico Monzino IRCCS, Milan, Italy
[4] Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy

Key words
- coronary artery disease
- refractory ischemia
- stem cells

Abstract

Background: Bone marrow-derived stem cells (BMSC) may represent a viable option for patients with myocardial ischemia refractory to conventional treatments.

Material and Methods: In 5 patients (4 males and 1 female, mean age 64 ± 8 years) with untreatable angina pectoris (Canadian Cardiovascular Society Class III/IV), myocardial segments with stress-induced ischemia as assessed by gated single-photon emission computed tomography were injected with 4 to 12 million CD133+ BMSC. Cells were injected into the myocardium (2 anterior, 2 lateral, 1 inferior wall) through minimally invasive approaches (left minithoracotomy [n = 4] and subdiaphragmatic approach [n = 1]). At baseline, at 6 months and at 1 year of follow-up, an exercise test, gated single-photon emission computed tomography (SPECT), 2-D echocardiography and coronary angiography were performed to assess exercise capacity, myocardial perfusion, LV function and coronary anatomy.

Results: Intramyocardial injection of autologous CD133+ BMSC cells was safe. No early or long-term complications were observed. After an average of 3.8 weeks from cell inoculation, all patients experienced a significant improvement of CCS class (from 3.8 to 1.8 at 6 months) and serial SPECT documented improvements of rest and stress perfusion in the injected territories at 6 months from operation. In 3 cases, coronary angiography showed an increase in the collateral score of the target areas. Clinical improvements still persist unchanged in 4 out of 5 cases at a mean of 36.5 months postoperatively.

Conclusions: After stand-alone BMSC transplantation for refractory myocardial ischemia, we observed long-term clinical and perfusion improvements in the absence of adverse events.

Introduction

Recent reports have suggested that the injection of bone marrow-derived stem cells (BMSCs) into ischemic myocardial territories may improve perfusion and symptoms in patients with chronic ischemia refractory to conventional treatments [1,4]. These initial clinical studies have followed a consistent basic and preclinical body of evidence [5–8] showing that BMSCs can improve myocardial vascularization in the ischemic heart. The angiogenic response was attributed to the ability of BMSC both to differentiate into fully mature endothelial cells [9,10] and to exert a supportive paracrine effect on tissue-resident endothelial cells by secreting a number of angiogenic factors [11].

The aim of the present pilot study was to evaluate the hypothesis that direct minimally invasive inoculation of autologous BMSCs into the ischemic myocardium of patients with refractory angina pectoris may safely reduce anginal symptoms and improve myocardial perfusion.

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Correspondence
Dr. Giulio Pompilio, MD, PhD
Cardiovascular Surgery
Centro Cardiologico Monzino IRCCS
Via Parea 4
20138 Milan
Italy
Phone: + 39 2 58 00 25 62■ok?
Fax: + 39 2 58 01 11 94■ok?
giulio.pompilio@ccfm.it

Material and Methods

Patients

Five patients were enrolled from December 2001 to June 2006. The patients suffered from severe chronic angina (Canadian Class III or IV) with evidence of anterior, anterolateral, or inferior ischemia on nuclear imaging (gated technetium-99m single-photon emission computed tomography [SPECT]) (**Table 1**). Before enrolment they were required to have attempted the "best" medical therapy, including long-acting nitrates, maximal use of β-blockers and calcium channel agents, without control of symptoms, and to be taking at least 2 antianginal medications. Baseline char-

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Table 1 Selected characteristics of treated patients

Patient	Site of ischemia	Approach	Number of injected cells (CD133+)	EF	Prior procedures
1	lateral	MiTh	9×10^6	45%	None
2	inferior	SubDia (redo)	12×10^6	50%	CABG; SCS
3	anterior	MiTh	8×10^6	35%	CABG; PCI; TMR
4	anterior	MiTh (redo)	5×10^6	55%	CABG; PCI; SCS
5	anterolateral	MiTh (redo)	4×10^6	60%	CABG; PCI; TMR

MiTh = left mini-thoracotomy; SubDia = subdiaphragmatic approach; EF = ejection fraction; CAB = coronary artery bypass grafting; SCS = spinal cord stimulator; PCI = percutaneous coronary intervention; TMR = transmyocardial laser revascularization

acteristics of the study group included a history of hypertension in 3 patients, smoking history in 2, peripheral vasculopathy, diabetes mellitus and rheumatoid arthritis in 1 case. None of the patients underwent prior automated implantable cardioverter-defibrillator implantation. Two patients had had prior percutaneous transmyocardial laser revascularizations or prior spinal cord stimulator. All patients but one underwent prior percutaneous and/or surgical revascularization procedures (**Table 1**).

The patients were judged ineligible for percutaneous or surgical revascularization as assessed by coronary angiography. A committee comprising 3 cardiovascular surgeons and 2 cardiologists determined the ineligibility for percutaneous or surgical revascularization. The exclusion criteria were: age greater than 75 years, an acute myocardial infarction within 6 months of enrolment in the study, LV ejection fraction lower than 40%, a history of malignant disease, renal dysfunction (serum creatinine > 2 mg/dl), unexplained hematologic or biochemical abnormalities, malignant ventricular arrhythmias, or serious concomitant medical conditions (other than ischemic heart disease) not adequately controlled. The local ethics committees approved the protocol, and all patients gave their informed consent.

Study design

The severity of angina was graded according to the Canadian Cardiovascular Society Score. The Angina Seattle Questionnaire was used to assess patients' quality of life. The following laboratory exams were obtained within 1 week before cell injection: 24-hour Holter monitoring to assess ventricular arrhythmias, 2-dimensional echocardiography to assess LV function, volume, and scar tissue, and gated SPECT to assess perfusion and ischemia. The follow-up evaluations performed at 1, 3, 6, 12 and, when applicable, 24 months after the injection procedure included a clinical evaluation, a laboratory evaluation, echocardiography and 24-hour Holter monitoring to assess ventricular arrhythmias. At 6 and 12 months of follow-up, a gated exercise SPECT and at 6 months a coronary angiography were performed.

Bone marrow aspiration and isolation of CD133+ cells

Two different procedures were used for cell harvesting, according to site preferences. We selected CD133+ stem cells, because they represent a population of early precursors highly enriched for multipotent stem cells with potent angiogenic capacity [12]. In the first 3 patients (2001 – 2003), stem cells were mobilized [13,14] as follows: 10 mg/kg die of lenograstim (Granocyte 34; Aventis Pharma, Milan, Italy) were administered s.c. for 4 consecutive days in order to mobilize stem cells from the bone marrow to the peripheral blood. At the end of the mobilization therapy, the WBC count peaked to 40800/mm^3 with neutrophil prevalence, and then peripheral blood stem cells (PBSC) were collected by means of apheresis, using the COBE Spectra Apheresis System (Gambro BCT Inc., Stockholm, Sweden). This automated procedure includes blood collection, blood processing by centrifugation to collect white cells in the size range of stem cells, and blood return to the patient. The duration of the procedure ranges from 3 to 4 h. The product of apheresis collection was processed by a CliniMacs device (Miltenyi Biotech, Bergish-Gladbach, Germany) in order to obtain CD133+ cell fractions.

In the last two patients (2005 – 2006), CD133+ cells were obtained from the iliac crest, as follows [15]: 1 day before surgery, a total of 150 to 200 ml of bone marrow was aspirated from the iliac crest. The procedure was performed within a qualified operating room. Mononuclear cells were isolated by Ficoll density centrifugation. These cells were then incubated with a ferrite-conjugated monoclonal antibody against the human stem-cell marker AC133 for 30 min. AC133+ cells include a non-hemopoietic (CD34–) subpopulation of bone-marrow stem cells that have a high potential to induce angiogenesis. We used the CliniMacs magnetic cell separation device to separate AC133+ cells from the mass of mononuclear bone marrow cells. To avoid competitive binding of the labelling antibody with the AC133 antibody used for cell separation, we labelled cells for counting using CD34 monoclonal antibody (clone 8G12, Becton-Dickinson, Heidelberg, Germany).

Irrespective of the procedure used, the purity of the stem-cell suspension was > 80% in all patients. CD133+ positive cells were stored at 4 °C and resuspended in a CE approved medium until intramyocardial injection the next day. The number of cells injected varied from 4×10^6 to 12×10^6 (**Table 1**).

Direct intramyocardial injection of CD133+ cells

We elected to perform minimally invasive surgical approaches to minimize the surgical trauma (**Table 1**). In 4 cases with anterior or anterolateral myocardial ischemia, a left anterior mini-thoracotomy was performed; the chest was opened via an approximately 10-cm anterior or anterolateral incision, the pericardium was opened and the myocardial regions to be injected with cells were identified. Then the solution containing the autologous CD133+ stem cells was injected into the target myocardial areas of the beating heart by means of gentle hand injection through a 22-gauge butterfly needle, as previously described [13]. The plastic cover around the needle was left in place and shortened by approximately 3 mm to control the injection of PBSC into the myocardium at a constant depth, avoiding insufficient or excessive penetration. Fifteen to 20 injections of 0.5 of the solution containing BMSC were performed in the target myocardial regions.

In 1 patient with inferior wall ischemia, the heart was visualized via a transdiaphragmatic minilaparotomy approach. The abdo-

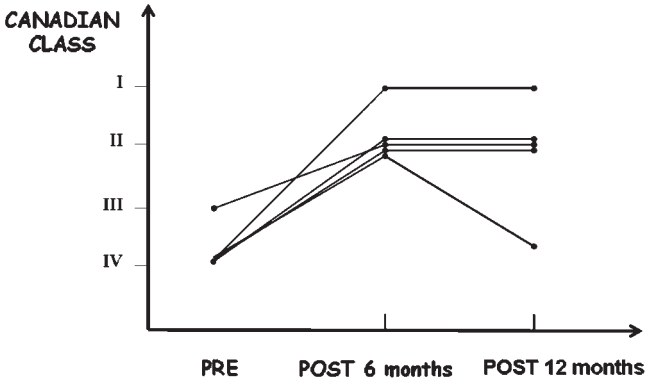

Fig. 1 Individual CCS class at baseline (pre) and at 6 and 12 months (postoperatively) after autologous BMSC inoculation.

men was opened via a 10-cm median incision under the xiphoid process, and an Olivier (Aesculap, Tuttlingen, Germany) retractor was positioned at the left and right costal arches to better expose the abdominal side of the diaphragm. Then a transverse incision of approximately 5 cm was made on the central tendon of the diaphragm to expose the inferior wall of the left ventricle. Injections were performed as described above. Each heart received 15 – 20 injections in the ischemic territory and a total of 0.5 ml of cell suspension was injected.

Periprocedural evaluation

Patients were monitored in the cardiac intensive care unit for 24 hours after the injection procedure. Immediately after the procedure, an electrocardiogram was performed and 2-day continuous heart rhythm monitoring was started. The complete blood count and serum C-reactive protein, erythrocyte sedimentation rate, creatine kinase, and troponin T levels were assessed before, immediately after the procedure and after 6, 24, and 48 hours. Before discharge, 4 to 5 days after the injection procedure, 2-dimensional echocardiography and 24-hour ECG monitoring were performed to exclude pericardial effusion and malignant arrhythmias.

Single-photon emission computed tomography

For the SPECT examination, a 2-day stress/rest protocol was used. The stress protocol included a symptom-limited bicycle exercise test. Technetium-99m tetrofosmin (500 MBq) was injected intravenously at peak exercise, which was continued for 1 minute after tracer injection. On the second day, rest images were obtained: 500 MBq technetium-99m tetrofosmin was injected and images were acquired in a gated mode after the patient's daily dose of nitrates. Reconstruction yielded long- and short-axis projections perpendicular to the heart axis. The short-axis slices were displayed in a polar map format and adjusted for peak myocardial activity (100%). The myocardium was divided into 17 segments, as previously described [17]. Segmental tracer activity was expressed as a percentage of the maximum on a 4-point scale: 1 = normal tracer activity > 75%; 2 = tracer activity 50% to 75%; 3 = tracer activity 25% to 50%; and 4 = tracer activity < 25%. Perfusion defects on the stress images were considered present when the tracer activity was < 75% of maximum. When significant fill-in (> 10%) of perfusion defects was observed on the images at rest, segments were classified as ischemic [3]. Summation of the patients' segmental rest and stress scores yielded the summed score at rest and the summed stress score, respectively. Patients' segmental stress scores were summed and divided by 17 to yield the patients' segmental stress score. Patients' segmental rest scores were derived in a similar manner[18]. Segmental stress and rest scores of the injected segments were then calculated.

Coronary angiogram

The coronary investigation technique was performed in a standard fashion. Angiograms were assessed by two reviewers blinded to each other and to the patient's treatment. Myocardial blush into the target area was graded according to dye density score proposed by van't Hof et al. [17]: 0 = no myocardial blush; 1 = minimal blush; 2 = moderate blush but less than obtained during angiography of contralateral or ipsilateral artery; 3 = normal myocardial blush.

Statistical analysis

Data are reported as means ± SDs. Quantitative data were compared using a paired, 2-tailed Student's *t*-test. Categorical data were compared using the Wilcoxon signed rank test. A p value < 0.05 was considered significant.

Results

Early and mid-term outcome

No deaths or major complications were observed postoperatively. One patient presented with pleural effusion. Periprocedural laboratory evaluations demonstrated an absence of inflammation or myocardial injury as indicated by a peak C-reactive protein of 28 ± 19 mg/L and peak troponin T level of 0.33 ± 0.17 µg/L. No ventricular arrhythmias were recorded during hospitalization. Post-procedural pericardial effusion was excluded in all cases by 2-D echocardiography.

All patients experienced a significant improvement of angina class at a mean of 3.8 weeks after cell injection, and the mean Canadian Cardiovascular Society Score improved from 3.8 at baseline to 1.8 after 6 months (**Fig. 1**). The frequency of angina episodes per day decreased from 3.1 ± 4 at baseline to 0.3 ± 0.9 at 6 months ($p < 0.01$). The quality of life improved from 46 ± 8% to 74 ± 12% at 6 months ($p < 0.01$). It is worth noting that improvements were found in all 5 scales of the Seattle Angina Questionnaire at 6 months compared with baseline. The 24-hour Holter recordings at 1, 3, and 6 months after injection did not reveal ventricular arrhythmias.

At 6 months' follow-up, the number of segments with stress-inducible ischemia per patient decreased from 5.6 ± 2.8 to 2.4 ± 2 ($p < 0.01$). Rest and stress scores improved in the injected segments from 1.3 to 0.9 and from 1.8 to 1.3, respectively (**Fig. 2**). The summed stress score per patient improved from 25.2 ± 5.0 to 21.1 ± 3.2 ($p < 0.01$). Perfusion at rest also improved with an increase in the summed score at rest from 20.3 ± 3.1 to 18.2 ± 3.1 ($p < 0.05$). The mean ejection fraction did not change significantly from baseline (49% preoperatively vs. 51.3% at 6 months). Coronary reinvestigation at 6 months showed an improvement of myocardial blush into the injected area in 3 out of 5 patients (mean blush score variation: from 0.6 at baseline to 1.1 at 6 months).

Late outcome

No deaths, myocardial infarction, malignant ventricular arrhythmias, or new ventricular masses were observed after an average of 36.5 (range 8 – 62) months of follow-up. The improvements in

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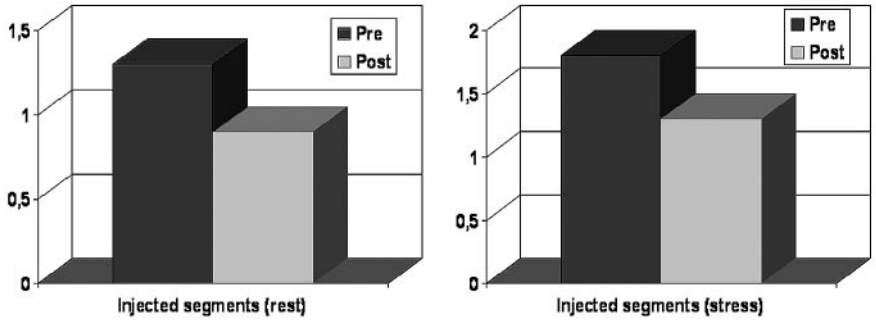

Fig. 2 Patients' gated 99 mTc tetrofosmin■ SPECT segmental stress and rest scores (baseline to 6 months postoperatively) in injected segments. ■ok? or Tc 99m tetrofosmin?

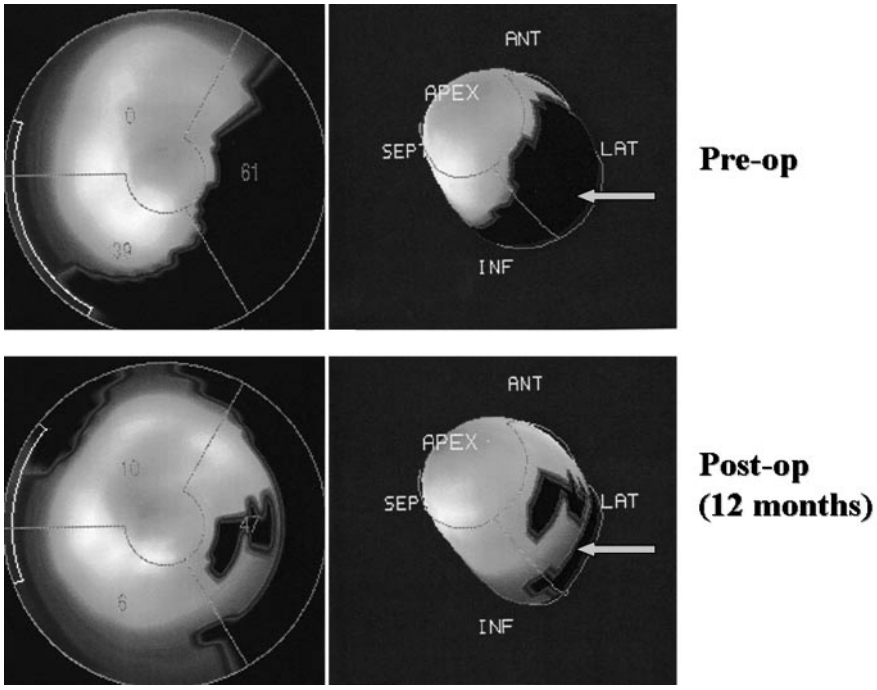

Fig. 3 Representative example of posterolateral ischemia improvement 12 months after cell injection vs. baseline.

the angina status noted at 6 months were found to be unchanged in 4 patients. One patient with rheumatoid arthritis and chronic steroid therapy required one rehospitalization for recurrence of angina 10 months from the operation, and experienced a worsening of his angina class (from Canadian Class 2 at 6 months to 4 at 1 year postoperatively) (**Fig. 1**). The mean Canadian Cardiovascular Society Score increased from 1.8 at 6 months to 2.2 one year after cell injection. No significant differences were noted in the mean left ventricular ejection fraction (EF) 12 months postoperatively when compared to the 6-month controls. The number of segments with stress-inducible ischemia per patient changed very little from 2.4 ± 2 at 6 months to 2.5 ± 1 at 1 year. An example of SPECT polar maps before and 12 months after autologous BMSC injections is shown in **Fig. 3**.

Discussion

The present study is, to our knowledge, the first evidence that myocardial therapeutic angiogenesis in a setting of refractory myocardial ischemia can be induced by direct BMSC injection using a minimally invasive surgical approach. Klein and coworkers [19] have recently proposed a less invasive surgical approach (left thoracothomy) for sole stem cell therapy in patients with severe post-infarctual cardiomyopathy (10 patients with EF < 22 %) ineligible for bypass surgery, irrespectively of the amount of regional ischemia. A mid-term significant improvement of EF in all patients was reported. Direct stand-alone inoculation of CD34+ enriched stem cells has been previously proposed by Archundia and co-workers in patients with a severely scarred and dysfunctional myocardium [20]. Our data support the hypothesis that epicardial BMSC inoculation into ischemic territories may enhance perfusion and lead to significant and long-term enhancement of angina class.

These findings agree with previous reports on intramyocardial endocavitary BMSC inoculation in patients with refractory angina and stress-inducible ischemia, in which cell injection was accomplished through a percutaneous approach by means of left ventricular electromechanical mapping with the NOGA system and endoventricular injection catheter [1 – 4, 21]. Taken together, these studies suggest that stem cell delivery into the ischemic myocardium can reduce angina and improve exercise capacity without the occurrence of ventricular arrhythmias. These clinical benefits were associated with enhanced perfusion in the injected segments, while the extent of myocardial scar tissue was unchanged [4, 22]. Perfusion improvements have been documented with different techniques, such as scintigraphy or magnetic resonance [2, 22]. Interestingly, an increase in left ventric-

ular function after 3 months' follow-up was also noted [23], concomitantly with a reduction of systolic volumes [3]. It has also been shown that stem cell transplantation can induce a long-term improvement in angina class and quality of life [4,14,22]. Very recently, Losordo and co-workers [24] published a phase I/II randomized placebo-controlled trial of intramyocardial endocavitary inoculation of stem cells isolated from circulating blood (CD34+) in patients with intractable angina. They provided evidence that this strategy may improve perfusion and reduce symptoms in patients with advanced coronary disease who have exhausted the currently available therapeutic armamentarium.

Our preliminary results suggest that the aforementioned clinical and perfusion improvements may be achieved with an epicardial approach and this strategy may be an alternative to endocardial injection under NOGA guidance. The more pronounced surgical trauma, even if reduced by the less invasive approach, may be compensated by a greater precision of cell delivery into the target areas [25]. Moreover, direct inoculation may be useful when a percutaneous approach is not advisable, for instance in cases with concomitant severe peripheral arteriopathy.

Cell therapy is a novel and promising potential treatment for patients suffering from angina pectoris chronically resistant to conventional treatments [26]. The clinical profile of these patients is often difficult to objectify, as is the success of potential treatments [27]. According to Kornowski and co-workers [26], the present and previous results of BMSC intramyocardial delivery seem to satisfy all the proposed major criteria for efficacy (improved perfusion, lowered ischemia and level of chest pain symptoms) in a setting of refractory ischemia. In particular, we observed an improvement of at least 4 of the 6 points to be fulfilled in order to accept a treatment effect [27]: reduced myocardial perfusion defects; improved symptomatic angina (minimal requirement ≥ 1 CCS class but ideally ≥ 2 CCS classes); improvement in standard quality of life questionnaire (e.g., Seattle Angina Survey) parameters; improved regional myocardial blush score using selective coronary angiogram. In the past, other potential treatments of refractory ischemia have failed to parallel subjective with objective improvements [28].

The limitations of the present study need to be acknowledged. This preliminary pilot report is aimed at providing a proof of the concept, without any claim for a demonstration of its efficacy, due to the very low number of patients and the absence of a control group. However, a case-controlled study is not advisable because of ethical issues related to the use of a surgical approach in the control group. Magnetic resonance was not included as non-invasive imaging modality. However, since the main goal of the study was to document perfusion improvements in viable tissue, we believe that SPECT may provide sufficient information.

Many issues still remain to be elucidated. It will be critically important to establish criteria to select patients who are likely to be the best responders to cell therapy as well as those patients who are less likely to respond because of risk factors severely impairing cell activity, such as age, diabetes, etc. [29].

We also believe that further opportunities to possibly enhance the therapeutic potential of cell therapy will be offered by basic research. For instance, engineered human EPCs transduced with vascular endothelial growth factor ($VEGF^{164}$) have been shown to have a 30-times higher angiogenic activity compared to native cells [30].

In conclusion, our study has explored the feasibility of stem cell inoculation into ischemic cardiac territories to induce therapeutic angiogenesis using a minimally invasive surgical approach. Our preliminary results agree with current data indicating a possible durable benefit in terms of ischemia relief. On this basis, we believe that this treatment option deserves to be investigated in more depth in the future with larger and controlled trials.

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