

# Autologous peripheral blood mononuclear cells for the treatment of lower extremity lymphedema: A preliminary report

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## Abstract

Lymphedema is a chronic devastating disease characterized by the accumulation of fluid in the extremities, tissue progressive changes such as adipose tissue deposition and fibrosis. To restore the functionality and structural integrity of the damaged lymphatic vessels, autologous peripheral blood mononuclear cells (PBMNC) was implanted in 3 sessions, 4 weeks apart, in the affected limb. Each patient was followed for 6 months, monitoring changes in the limb volume. Lymphangiogenesis was evaluated by lymphoscintigraphy, and the monitoring of quality of life. A rapid reduction in the volume of the limbs was observed: Twenty-four point 5 percent of volume reduction after the first implant, 18.5% after the second, and 15.3% at 6 months after the third ( $p < 0.05$  vs baseline). Lymphoscintigraphy showed a hyper fixation of the tracer along the ipsilateral iliac axis not appreciable at baseline. Implants of autologous PBMNC in patients with primary lower limb lymphedema seems to be a feasible, effective therapy option.

## Introduction

Lymphedema is the clinical manifestation of damaged lymphatic transport due to impaired lymphatic drainage which cause proteins and lipids build up in the interstitial space, tissue progressive architectural changes such as adipose tissue deposition and fibrosis. These changes are also strongly associated with inflammation which cause resistance to current therapies making lymphedema both under-recognized and under-documented clinical condition that still lacks a cure.<sup>1-3</sup> Thus, innovative treat-

ments are needed to restore the functionality and integrity of the lymphatic vessels damaged in this pathology. Recently, cell therapy has emerged as a novel therapy in lymphedema treatment with the aim to restore the lymphatic vasculature from the capillary to the collector.<sup>4</sup> Autologous peripheral blood mononuclear cell (PBMNC), consisting of autologous monocytes/macrophages, lymphocyte and hematopoietic stem cell, showed to be able to induce therapeutic angiogenesis through paracrine release of growth factors, cytokines, messenger molecule and exosomes, in the treatment of no-option critical limb ischemia and more generally in the healing of chronic wounds.<sup>5-7</sup> Moreover both monocyte/macrophages and lymphocytes T regulatory (Treg) populations, play a key role in tissue regeneration in not-healing trophic lesions,<sup>8</sup> also through polarization from macrophages inflammatory M1 phenotype to the anti-inflammatory regenerative phenotype M2.<sup>9-11</sup> Recently it has been observed that PBMNCs significantly reduce limb amputation in critical limb ischemia patient non feasible for revascularization.<sup>12</sup> Recent trials show the beneficial effects of stem cell therapy in lymphedema treatment, providing reversal of pathological reorganization associated with lymphedema progression.<sup>4,13,14</sup>

Both blood vascular microcirculation and lymphatic circulation are the conduits for the entry and the exit for monocyte-derived macrophages in almost every tissue. The role of macrophages as key players in both angiogenesis and lymphangiogenesis has been extensively described.<sup>15</sup> Macrophages support lymphangiogenesis either by transdifferentiating and directly incorporating into the endothelial layer or by stimulating division of pre-existent local lymphatic endothelial cells through paracrine effect.<sup>15,16</sup> These findings confirm the plasticity of macrophages, which are already known to transform from naive monocytes into vascular endothelia growth factor-C (VEGF-C) producing cells.<sup>17</sup> Macrophages play a key role in lymphedema by controlling lymphangiogenesis through the release VEGF-C, while macrophage depletion in recognized lymphedema, decreases lymphatic transport activity and VEGF-C expression.<sup>18</sup> It has been also observed that Treg lymphocytes limit the pathological changes in lymphedema tissue, while depletion of Treg cells exacerbates edema and fibrosis.<sup>19</sup> Based on this rationale we decided to treat patients affected by lower extremity primary lymphedema not responder to standard therapy with implantation of autologous PBMNC in the lymphedema tissue.

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## Materials and methods

We enrolled 4 patients: 1 female aged 54 and 3 male aged 67, 74, 75 with III clinical stage lymphedema of the lower limbs, in particular with primary lymphedema in 2 cases, one post-surgical case and 1 post-lymphangitic case. Patients with acute relapsing lymphangitis were included, while secondary lymphedema to cancer intervention less than 10 years were excluded. All patients signed a statement to consent the publication of their data. All patients were treated with various press therapy and lymph drainage sessions with

poor results. The 54 years-old woman suffered from primary lymphedema for 40 years, elephantiasis for lower limbs for 20 years and repeated episodes of lymphangitis. The 75 years-old man, underwent radical prostatectomy, increase in limb volume after about 30 days from surgery. The 67 years-old man, repeated episodes of inguinal lymphadenitis and lymphangitis 10 years ago. Within a year, a progressive increase in the volume of the lower right limb, 7 years ago lymphatic surgery failure (venous lymphatic anastomosis). The 74 years-old man, primary lymphedema for 40 years, elephantiasis for lower limbs for 30 years, underwent lymphatic surgery failure (venous lymphatic anastomosis) 10 years ago, reported repeated episodes of lymphangitis.

All patients underwent metric evaluation of the circumferences back foot, calf and thighs and an instrumental evaluation with arterial and venous doppler ultrasound of the lower limbs, longitudinal ultrasound measurement of the medial portion of the knee, the calf, the internal and back foot malleolus, before and after the three PBMNC implantation (Table 1).

Diagnostic examinations were performed including high-resolution ultrasound of soft tissues in all patients (both at the beginning and at the end of treatment). After each treatment session, each patient was subjected to a zinc-oxide and coumarin multilayer bandage.

Oral therapy with 100 mg of melilotus (containing 20% coumarin equal to 20 mg), 300 mg of rutin and 100 mg of bromelin, one tablet /day was also prescribed for all patients.<sup>20</sup>

All 4 patients were treated with autologous PBMNC produced by Hematrate Blood Filtration System (Cook Regentec, Indianapolis, USA), previously called Pall Celeris/MonoCells by Athena Biomedical Innovations (Florence, Italy), a selective filtration point-of-care device with the intended use for intra-operatively rapid preparation of total nuclear cell concentrate from 120 mL of anticoagulated blood, for use in human cell therapy applications. The PBMNCs cell product obtained by this device has been extensively characterized

in terms of number of cells obtained, composition, recovery and flow cytometry cell population analysis.<sup>21</sup> All the procedures were performed in operator room with anaesthesiologic support (propofol and/or peripheral block). The PBMNCs implant procedure was repeated three times, at intervals of 30-45 days from each other. It was not possible to characterize each cell concentrate in this preliminary study. Patients' blood counts were in the standard range. Hematrate blood filtration system used concentrate MNC 4.2 folds of the baseline, with an average of  $1.06 \pm 0.28 \times 10^8$  total MNCs injected including  $0.16 \pm 0.04 \times 10^8$  Monocytes and  $0.9 \pm 0.2 \times 10^8$  lymphocytes starting from 120 mL of whole blood. Any regimen of mobilization was performed to increase CD34+ concentrations. PBMNC implanted contains a mean count of  $1.37 \times 10^6$  CD34+ cells.<sup>21</sup> PBMNCs were harvested by 10 mL of sterile saline backflush, collected and immediately implanted in lymphedemas tissue in 0.2–0.3cc in boluses, at intervals of 1–2 cm and to a mean depth of 1.5–2 cm, using a 21G needle. The implants were performed in the back of the foot, ankles, popliteal cavity, inguinal region (locations of the main lymph node stations) and along the course of the main lymphatic collectors on the leg and thigh.

Patients were assessed at baseline (T0), and one month after each cell implants (T1,T2,T3) for the following parameters: pitting, measurement of circumferences of the limbs as published by Tessari *et al.*,<sup>22</sup> measurement of ultrasound thicknesses of the superficial tissues in the affected limbs, VAS and assessment of the patient's well-being through the SF12 QOL questionnaire.

The volume measurements were made according to the disk model of Kuhnke and the volume was calculated using the following formula:  $V = (C1 + C2 + \dots + Cn) / p$ . The percentage reduction in volume at each point of measurement was calculated via the formula for each patient:  $DV\% = [(pre-treatment\ limb\ volume - post-treatment\ limb\ volume) / pre-treatment\ limb\ volume] \times 100$ .

To determine the efficacy of this treatment, final endpoint to evaluate improvements were decrease in the limb circumference, volume reduction in the lymphoscinti-

graphic transport index (TI), increase in the lymphatic vessel count. Lymphoscintigraphy was performed before and after the treatment. Given that the vast majority of patients had deep lymphatic vessels abnormalities, we applied a 2-compartment lymphoscintigraphy able to accurately detect lymphatic flow abnormalities in patients with limb swelling.<sup>23,24</sup>

The patients were followed for 4 months total and for the whole period subjected to a zinc oxide and coumarin multilayer bandage<sup>25</sup> with weekly replacement after decongestive treatment with combined decongestive physio lymphatic treatment (Co.De.Phy.L).<sup>26</sup> The bilateral limbs' circumference and patients' weight were recorded monthly from the beginning. At the end of the 4-month follow-up period, lymphoscintigraphy was performed and the QOL questionnaire was given to each patient. Statistical analyses were performed with the Student t-tests. Differences were accepted as significant when  $p < 0.05$ . All patients gave informed consent before inclusion.

## Results

PBMNC implantation, has given decongestive results that exceeded expectations, since the first cycle, with a rapid reduction in the volume of the limbs of 60%, after subsequent cycles, in all four patients, with rapid healing of trophic lesions and a significant improvement in the patient's quality of life, as evidenced by the VAS scale which was maintained in the subsequent sessions. One month after the first implant we observed a 24.5% volume reduction (T1), 18.5% reduction after the second implant (T2) and another 15.3 % after the third implant, which correspond to 4 months after the start of therapy (T3) with an overall volume reduction of 58,3% (Figure 1). All these volume reductions were significant compared to the baseline mean volume ( $p < 0.05$ ).

In all 4 treated patients there was a significant clinical improvement with disappearance or significant reduction in pain

**Table 1. Metric evaluations before and after peripheral blood mononuclear cell (PBMNC) implants.**

Patient	Before			After PBMNC		
	Back foot	Calf	Thigh	Back foot	Calf	Thigh
1	40 cm	64 cm	80 cm	27 cm	37 cm	67 cm
2	32 cm	57 cm	72 cm	26 cm	47 cm	60 cm
3	29,5 cm	56 cm	58,5 cm	27 cm	37 cm	55 cm
4	28 cm	70 cm	89,5 cm	23 cm	51 cm	80 cm

and/or heaviness already in the days immediately following the first PBMNC implant adequate to require any analgesic treatment: VAS scale score went from 10 to 3 after T1 to 1 at T3 (Figure 2). These data were confirmed by the completion of the SF12 questionnaire, which showed a progressive improvement in the quality of life (Figure 2). None of the patients evaluated had side effects or adverse reactions such as lymphangitis or septic lesions at the inoculation site.

In all patients, an arterial flow was recorded, present and valid up to the extreme periphery. There were no signs of deep and/or superficial venous thrombosis, no signs of venous hypertension or pathological reflux. No differences in arterial and venous doppler ultrasound were recorded before and after PBMNC implant.

Lymphoscintigraphy was performed before and after the treatment. The first case (Figure 3A) treated was a female patient suffering from elephantiasis of the lower limbs from primary bilateral lymphedema, underwent re-evaluation of the superficial lymphatic drainage, through the execution of lymphoscintigraphy, after having performed three cycles of PBMNC implantation in the most affected sites of the left limb, which was the one clinically more severe. From the comparison with the “baseline” scintigraphy study, some aspects emerged which suggest effectiveness of the treatment in patients with primary lymphedema (Figure 4).

From the very first images relating to the “dynamic” phase of the post-treatment scintigraphy study, it was possible to document a rapid ascent of the radiocolloid from the administration site of the left limb, unlike what was observed in the previous treatment which no progression of the radiopharmaceutical was appreciated from the injection site. The subsequent detection, carried out about twenty minutes after administration, keeping the patient in a supine position, also allowed us to highlight important changes in the distribution of the tracer to the left limb, which also appeared clinically more affected by the pathological process. The presence of non-homogeneous accumulation of the colloid not only in the lateral side of the foot, as previously noted, but also in the distal third of the leg and in particular along the lateral side, which was the site of implantation of PBMNC cells, was highlighted early. Further substantial variations emerged in the late phase of the study which involves the acquisition of images relating to lymphatic drainage 120 minutes after the administration of the radiopharmaceutical substance and after having invited the patient to walk. It was

possible to document the appearance of more intense accumulation of the radio compound at the level of the left inguinal lymph node station, initially only blurred, as well as further tracer hyper fixations, pertaining to the lymph node, in the popliteal cavity and along the ipsilateral iliac axis not appreciable in the previous measurement.

The second case (Figure 3B) treated was a patient with a history of operated prostate cancer post-treatment lymphoscintigraphy confirmed in the left limb, in the dynamic phase, the failure of the radiocolloid to rise from the injection site.

Also, in the subsequent acquisitions made at 20, 60 and 120 minutes (after stimulus) from the administration of the radiopharmaceutical, no significant changes were observed compared to the “baseline” study; the presence of nuanced colloidal stagnation in both feet and in the distal third of the leg bilaterally was confirmed in the later images of the study and after stimulation, unchanged respect to what previously emerged. Furthermore, in all the surveys carried out, the lymphatic passages or clear areas of accumulation of the radiopharmaceutical referable to lymph nodes were not

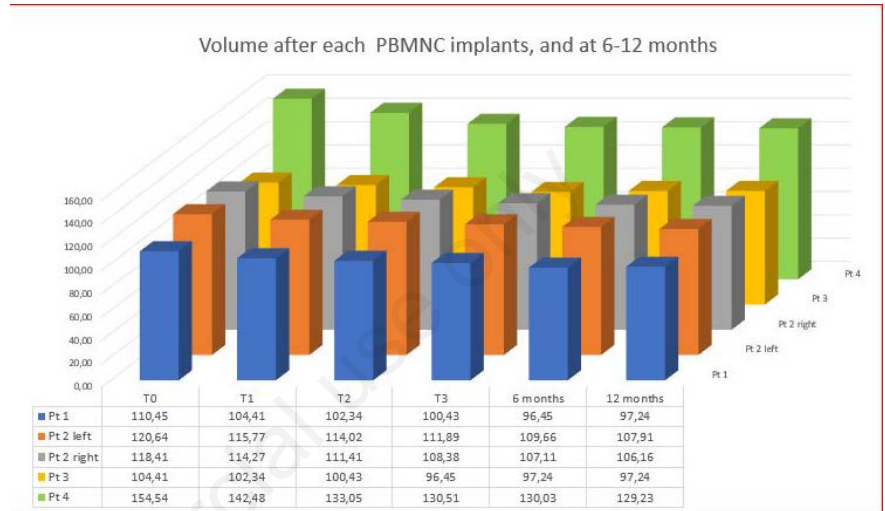
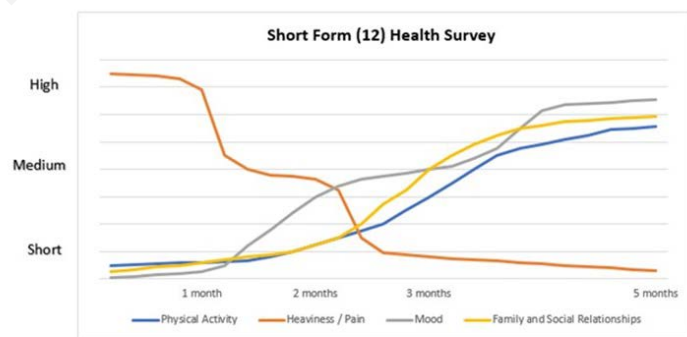


Figure 1. Lymphedema volume reduction after T1, T2, T3 peripheral blood mononuclear cell (PBMNC) implants. p value <0.005



VAS SCORE			
Patients	T0	T1	T3
1	7	3	0
2	7	4	1
3	6	5	1
4	7	2	0

Figure 2. Improvements obtained on the basis of the SF12 questionnaire submitted to patients after each treatment session and during the follow up and VAS score data.

detected bilaterally. The third case treated (Figure 3C) was a patient with a history of primary scrotal lymphedema and repeated episodes of bilateral inguocrural lymphangio-adenitis which 7 years ago underwent to lymphatic-venous anastomosis surgery with persistence of the lymphedema.

In the dynamic phase of the post-treatment scintigraphy study, the absence of the colloid migration from the inoculum site is confirmed in the right limb 20 minutes after administration, a faint stagnation of the radiopharmaceutical appears on the back of the right foot, not noticeable in the previous study; the finding in the later phases of the study tends to extend to a slightly greater extent than documented in the “basal” study with progressive and further involvement of the middle and distal third of the leg, especially on the medial side, site of PBMNC implant.

The fourth case (Figure 3D) was a patient with a history of primary lymphedema for 40 years; elephantiasis of the lower limbs for 30 years; 10 years ago underwent to lymphatic surgery (lymphatic-venous anastomosis); various sessions of press therapy and lymphatic drainage; he also underwent repeated episodes of lymphangitis.

Pre-treatment basal lymphoscintigraphy confirmed in the left limb, in the dynamic phase, the failure of the radiocolloid to rise from the injection site. Even in the subsequent acquisitions made at 20, 60 and 120 minutes (after stimulation) from the administration of the radiopharmaceutical, no sign of progression of the tracer was recorded. Only in the later images of the study and after stimulation, was detected the presence of nuanced colloidal stagnation in the left foot and bilaterally in the distal third of the leg. Immediately after the first implantation

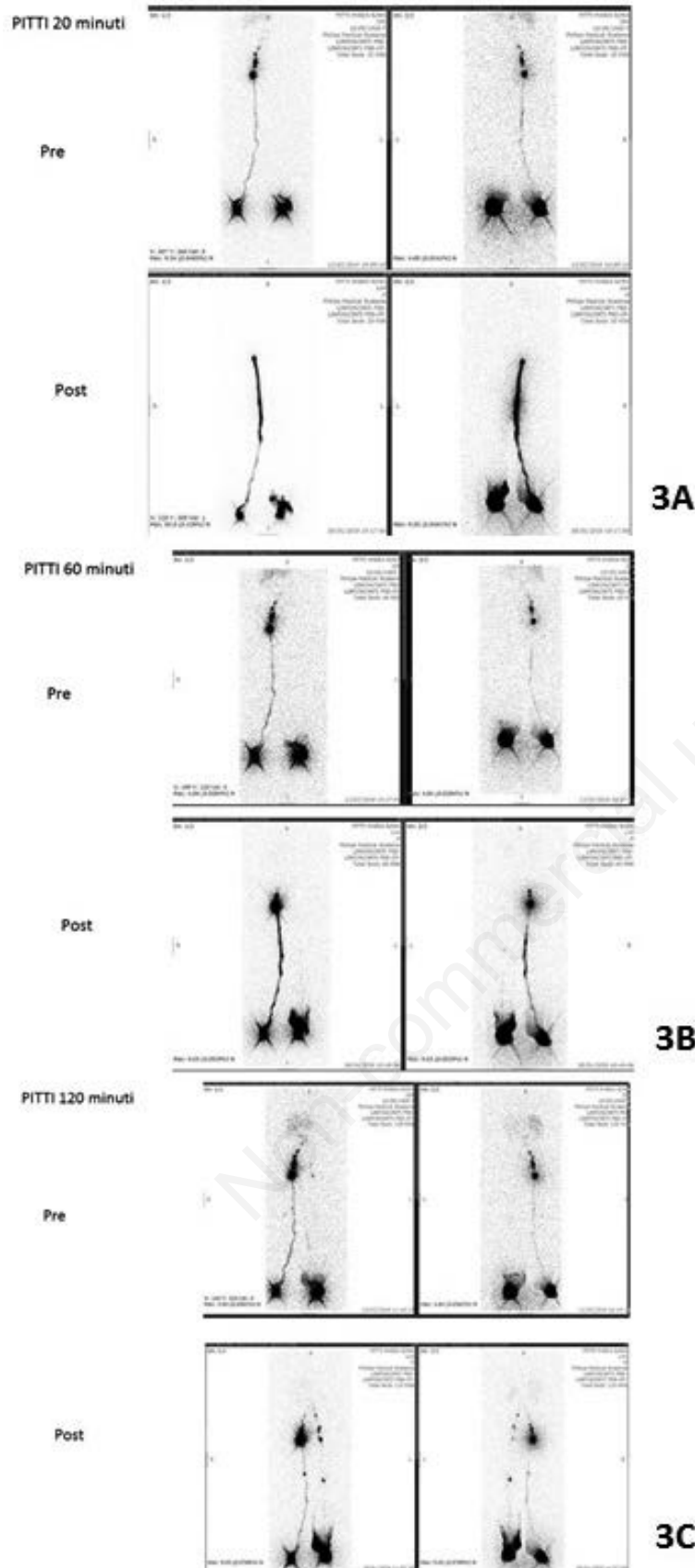
of PBMNC cells, we highlighted a significant reduction in the circumferences of the limb with a clear clinical improvement perceived by the patient as a “sense of lightness with restoration of sensitivity on the whole limb”.

## Discussion

Despite the few preliminary cases, the observed data suggest that the PBMNC implantation was followed by decongestion and a significant volume reduction and no side effects, even by patients suffering from lymphedema of the lower limbs easily susceptible to infections and lymphangitic reactions. A clear clinical and functional improvement was noted in all patients treated, regardless of the cause of the lymphedema. All patients, both those with primary and secondary lymphedema, reported in the



**Figure 3.** Before and after peripheral blood mononuclear cell (PBMNC) implant. A: Female Patient, 54 years old, primary lymphedema for 40 years, elephantiasis for lower limbs for 20 years, with repeated episodes of lymphangitis. B: Male Patient, 75 years old, radical prostatectomy, increase in limb volume after 30 days from surgery, primary scrotal lymphedema and repeated episode of lympho-angio-adenitis. C: Male Patient 67 years old, history of primary scrotal lymphedema and repeated episodes of bilateral inguocrural lymphangio-adenitis which 7 years ago underwent to lymphatic-venous anastomosis surgery with persistence of the lymphedema. D: Clinical case. Male Patient – 74 years old, Primary lymphedema for 40 years, Elephantiasis for lower limbs for 30 years, 10 years before lymphatic surgery failure (venous lymphatic anastomosis), repeated episodes of lymphangitis.



**Figure 4.** Lymphoscintigraphy patient A at 20, 60 and 120 minutes (A, B, C), pre and post treatment of peripheral blood mononuclear cell (PBMNC) implantation.

period following the first session, a sense of “lightness” and a fluency in limb movements never experienced with the current pathology. Further controlled clinical trials are needed to demonstrate the efficacy of autologous PBMNC implantation in the treatment of primary lymphoedema. Our data are comparable to a single-center, prospective, non-randomized clinical trial on 10 patients with primary lymphedema, treated with two implants of mobilized PBMNC three weeks apart in terms of volume reduction and improvement of quality of life.<sup>27</sup> Selective filtration device concentrates all populations of lymphocytes 4.25 folds, including CD45+ CD3+ T lymphocytes and CD45+ CD19+ B lymphocytes.<sup>21</sup> Despite we did not measure how many Treg are present in our cell concentrate, it could be possible that Treg population concentrated and injected can contribute to reverse all of the major hallmarks of lymphedema, such as edema, inflammation, and fibrosis, also promoting lymphatic drainage function as observed in animal model.<sup>19</sup> This study presents a strong limitation due to a global approach, with compression therapy administered using a multilayer bandage, phytotherapy with melilotus, Co.De.Phy.L plus PBMNC therapy and clearly no control group. Controlled studies are needed to prove the efficacy of PBMNC implants in these critical patients. Treatment with PBMNC is autologous, non-invasive for the patients, repeatable, easy and fast to perform in operating room. Autologous peripheral blood mononuclear cell injection is a feasible method for treatment of primary lymphedema with promising preliminary results and without serious adverse effects. From these first encouraging results, others form of lymphedema that are little known and difficult to treat, such as podoconiosis. Debilitating lymphatic disease could be treated with PBMNC with the aim to restore lymphatic circulation.<sup>28</sup> We believe that we are at the very beginning of this exciting adventure, but the good clinical data response obtained in these critical patients, together the lymphoscintigraphic measurement, suggest that we could be on the right path.

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