Non-reconstructable peripheral vascular disease of the lower extremity in ten patients treated with adipose-derived stromal vascular fraction cells

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Abstract
We present a series of ten patients with non-reconstructable peripheral vascular disease (PVD), secondary to arteriosclerosis (AS) and/or diabetes mellitus (DM), treated with local injection of non-expanded autologous, adipose-derived stromal vascular fraction (SVF) cells for the purposes of enhancing neovascularization and chronic wound healing. Adipose tissue was surgically harvested and processed to yield the heterogeneous SVF cells for immediate point-of-care injection. The gastrocnemius muscles and ulcers or wounds where present were locally injected with the resulting SVF. Response to treatment was evaluated both clinically based on pain-free ambulation, wound healing capacity over time and ankle/brachial index (ABI) measurements, and by imaging using MRI-based angiography. All patients exhibited clinical improvement (reduction in rest pain and claudication and improvements in ABI), with imaging signs of neovascularization in the majority (5 of 6) of patients in whom the evaluation was feasible. Similarly, 5 of 6 chronic wounds healed without further surgical intervention. This series highlights the utility of non-expanded adipose-derived heterogeneous SVF cell population processed at the point-of-care, to treat patients with end-stage PVD as an alternative to palliation or amputation.

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1. Introduction
Peripheral vascular disease (PVD) due to arterial insufficiency is a common problem, affecting up to 6.5% of individuals >45 years of age (Stoffers et al., 1996). The most common causes in the U.S. population are macrovascular lesions due to arteriosclerosis (AS) (70%–80%), and microvascular lesions due to diabetes mellitus (DM) (20%–30%) (Marso and Hiatt, 2006), with a large proportion of patients presenting concomitant infrapopliteal arteriosclerotic disease (Chen et al., 2013).

Local and regional ischemia follow a clinical progression, starting with claudication, referred as muscle pain distal to the occlusion site brought on by predictable levels of exertion. Later, critical limb ischemia (CLI) presents with rest pain typically at the level of the metatarsal foot, unrelenting, and relieved by dangling the foot, and when blood flow falls below a critical perfusion pressure, ulceration or frank ischemic necrosis ensues. This symptomatic progression can be classified using the criteria of Fontaine and Rutherford (Nehler et al., 2003; Rutherford et al., 1997). The diagnosis is confirmed by vascular examination including pulse doppler and the ankle/brachial index (ABI) calculation (the ratio between the systolic pressures at the ankle and the mid-arm). Normal ABI or stage I is ≥1.0. Stage II occurs at an ABI of 0.9, while rest pain at stage III is characterized by an ABI of 0.5. Additional imaging-based modalities, such as computerized tomography (CT) angiography and/or magnetic resonance (MRI) angiography, localize sites of occlusion and help to determine if surgical bypass is feasible.

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Thirty percent (30%) of patients with CLI present with non-reconstructable disease, a situation particularly critical for diabetic patients, due to calcific and fibrocalcific disease in the distal vasculature (Bishop et al., 2008; Dormandy and Rutherford, 2000; Norgren et al., 2007). Furthermore, 40% of diabetic patients diagnosed with CLI will progress to tissue necrosis and/or gangrene, versus 5% of patients with AS disease (Lévigne et al., 2013; Tobalem et al., 2015). Given these numbers, the need exists for novel cost-effective treatments for CLI. Current treatment alternatives such as percutaneous transluminal angioplasty (PTA) have a variable therapeutic efficacy, with patients resulting in partial revascularization and higher rates of restenosis, especially in infrapopliteal arteries (Dormandy and Rutherford, 2000). In addition, endovascular revascularization procedures are precluded in about 30% of patients with CLI due to high operative risks and/or unpropitious vascular anatomy (Mamidi et al., 2012). In an effort to reduce the already high incidence of amputations and other complications, alternative approaches have been proposed. Gene therapy was introduced as a way to provide angiogenic growth factors (e.g.: VEGF, HIF1a, HGF, FGF1), based on their known inductive capacity to form vascular structures (Sedighiani and Nikol, 2011), a fact initially appreciated in cardiac ischemia (Isner and Ashara, 1999). Cell-based therapies have also been proposed as promising alternatives, on the basis of providing cell progenitors capable of fabricating new blood vessels in ischemic areas. For instance, bone marrow-derived and peripheral blood circulating (with or without previous mobilization) mononuclear cells (MCNs) containing a population of endothelial progenitor cells (EPCs) have been used in various clinical trials for vascular insufficiency (evaluating safety and efficacy) with variable degrees of success (reviewed in Raval and Losordo, 2013). In those studies, intra-muscular (IM) injections of unselected or selected (CD34+) cells ameliorate the symptoms associated with poor distal blood supply.

On the other hand, transplantation of adult mesenchymal stem cells (MSCs) has great promise based on their regenerative capacity. These cells secrete a wide range of growth factors and cytokines acting in a paracrine fashion. Many of these factors are proangiogenic (e.g.: VEGF), inducing the formation of new blood vessels, while others assist in the repair process of injured tissues (Caplan and Correa, 2011; Caplan and Dennis, 2006). MSCs can be obtained from a variety of tissues including bone marrow and adipose tissue, the latter being particularly rich in MSCs (Zuk et al., 2001). MSCs are readily obtained, as a component of the adipose tissue-derived multicellular Stromal Vascular Fraction (SVF), after enzymatic digestion and centrifugation of liposapirate (Bourin et al., 2013). SVF is a heterogeneous population of MNCs that includes adipose-derived stem cells (ADSCs) of mesenchymal phenotype (analogous to MSCs), endothelial progenitor cells (EPCs), hematopoietic progenitors, monocytes, leukocytes and pericytes (Amos et al., 2008; Nguyen et al., 2016; Guo et al., 2016). Pericytes represent the perivascular phenotype of native MSCs (Crisan et al., 2008; da Silva Meirelles et al., 2008, 2006; Sacchetti et al., 2007) and constitute a key cell component of SVF during angiogenesis, as they stabilize nascent blood vessels (Armulik et al., 2005, 2011; von Tell et al., 2006).

A number of animal models of CLI, including the hindlimb ischemia model after femoral artery ligation in rats (Rochester et al., 1994) and rabbits (Hao et al., 2014), have been proven useful to assess the effects of various cell types and to study potential mechanisms of action. For instance, IM injections of culture-expanded ADSCs increased flow and induced a higher systemic presence of EPCs (Kondo et al., 2009). Iwase et al. (2005) demonstrated the superior angiogenic potential of bone marrow-derived MSCs over MNCs; the former were able to differentiate into both endothelial cells and vascular smooth muscle cells. Finally, Hao et al. (2014) reported the neovascularization effect of both ADSCs and bone marrow-derived MNCs. In these and other studies, ADSCs came to be recognized as a source for angiogenic factors acting through a paracrine mechanism, and in concert with other cellular players (e.g.: EPCs and macrophages) (Nakagami et al., 2005; Rehman et al., 2004; Sumi et al., 2007).

Pre-clinical data have prompted multiple groups to explore the feasibility, safety and efficacy of bone marrow-derived cell-based therapy for PVD, through the design and execution of small clinical trials (summarized in Lawall et al., 2011, 2010; Liew and O’brien, 2012; Raval and Losordo, 2013). Powell et al. (2011) reported the interim results of the RESTORE-CLI trial, where IM injections of tissue repair cells (analogous to a MNC mixture) proved no serious adverse effects, with increased amputation-free survival of patients and improved wound healing. In addition to the IM route, intra-arterial (IA) administration (through a femoral artery catheter) has been also safe and efficaciously used to inject allogeneic, expanded, bone marrow-derived MSCs (Das et al., 2013).

In sum, adult stem cell transplantation constitutes a paradigm shift in the treatment of chronic limb ischemia (CLI), especially for diabetic patients (O’Neill et al., 2012; Powell, 2012; Weck et al., 2011). The safety and efficacy of culture-expanded ADSCs derived from SVF for the treatment of CLI has been documented (Bura et al., 2014; Lee et al., 2012) and reviewed (Zhi et al., 2014), although further studies with more rigorous designs, including randomization, standard-of-care or placebo controls, are still needed. However, to the best of our knowledge, no report has been made so far with fresh, non-fractionated, un-cultured, point-of-care administered SVF in CLI. Therefore, an open label, non-randomized study to assess the safety and efficacy of non-culture-expanded adipose-derived SVF cells administered IM to ten patients with nonreconstructable CLI was designed, approved, and executed at the National Autonomous University of Nicaragua in Leon.

2. Materials and methods

2.1. Ethics

This study (not registered in clinicaltrials.gov) was approved by the Medical Ethics Committee of UNAN-Leon and by the Ministry of Health of Nicaragua (MINSA). The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national, Universidad Nacional Autónoma de Nicaragua — León) and the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all participants in accordance with standards of MINSA and the World Health Organization, and included consent to publish this study in all formats.

2.2. Patients’ enrollment

The criteria for inclusion were: rest pain or claudication at least of one half block or less, non-healing ulcer or surgical amputation site for greater than three months, and inoperable PVD due to medical reasons. Given the limited resources available in Nicaragua for endovascular intervention or bypass, all patients at this advanced stage of disease were considered candidates for amputation. Criteria for exclusion were: age <40 years, unstable cardiovascular disease at the moment of enrollment, smoking and/or the presence of chronic pulmonary disease, ongoing infection and/or sepsis, and uncontrolled diabetes.

2.3. Surgical procedure and SVF preparation

The SVF cells were obtained after enzymatic digestion of surgically harvested adipose tissue. Liposuction was performed from the flanks and abdomen with the yield of dry fat per case ranging from 250 to 350 cm³. The liposapirate was collected directly into a sterile tissue-processing canister (GID SVF-1, Louisville, CO, USA) for tissue dissociation and processing under closed conditions at all times and following the manufacturer’s instructions. It was first washed three times with sterile Lactated Ringer’s Solution inside the canister to remove red cells and residual oils, and then dissociated with GMP-grade collagenase (GIDzyme, GID, Louisville, CO, USA) in 125 ml of Lactate Ringer’s Solution, at a concentration of 200 CDU/ml of total volume. The mixture was dissociated
for 40 min by placing the canister inside an incubated benchtop orbital shaker (MaxQ 4450, Fisher Scientific) at 37 °C and 150 rpm. After dissociation, the collagenase was neutralized by addition of 2.5% solution v/v human serum albumin (Baxter Healthcare, Deerfield, IL) and then centrifuged (Sorvall ST40, Fisher Scientific) for 10 min at 800g. The resulting SVF cell pellet at the bottom of the device was removed using a 6-inch #14 gauge spinal needle connected to a 20 ml syringe with 15 ml Hartmann solution. Ten microliters of SVF were taken from the final suspension and submitted for differential staining. Two samples were then passed through an image cytometer (ADAM MC, Portsmouth, NH, USA) for counting of MNCs and to assess cell viability. The cell suspension was then administered using a 26 gauge needle into the plane between the gastrocnemius and soleus muscles in a pattern of injections (22 per muscle, 11 in the external and 11 in the internal gastrocnemius, each one 1.5 cm to 2 cm apart) of equal volume each (0.5 ml), on either side of the midline (Fig. 1B). The resulting final cell doses are shown in Table 2. The thickness of the gastrocnemius muscle was approximated so that the depth at which the cells would be deposited would be at or near the plane between the gastrocnemius and soleus. If an ulcer was present, SVF (3–4 ml) was infiltrated immediately beneath the wound and around its edges.

2.4. Outcomes assessments

The purpose of this study was to evaluate the safety and feasibility of an SVF cell-based therapy for advanced PVD in ten patients. Safety was evaluated by the absence of adverse effects after the procedure, related to inflammation, infection or local necrosis of the injection site. Feasibility was evaluated using clinical and imaging criteria. Clinical data were both subjective (pain assessment and ulcer healing when present) and objective (ankle-brachial index – ABI). The ulcers, when present, were photographed and dimensions measured recording their two main axes (in cm). Patients were evaluated for pulses and the ankle-brachial index (ABI) calculated by averaging the ratios of three consecutive measurements of systolic blood pressures of the ankle to mid-arm. Imaging data involved a pre- and post-operative MRI-based angiography. These parameters were collected before the procedure and after at 2, 4, 6–8 and 10 and 18 months (pain and ulcers), and 4 months (ABI and angiography).

2.5. Statistical considerations

To establish a potential correlation between age, lipoaspirate volume, viable cell injected (i.e.: cell dose) and the changes in ABI (delta ABI), the Pearson correlation coefficient (r), along with a 95% confidence interval and a P value were calculated and graphed using Prism 6 for Mac (GraphPad Software, Inc.).

3. Results

3.1. Patient characteristics

Ten patients (nine females and one male, ranging in age from 57 years to 85 years (average of 73 years) were enrolled in the study, with their demographics and characteristics (ambulatory status, Rutherford stage) shown in Table 1. In addition to their diagnosis, no additional risk factors were recorded. All diabetic patients were under insulin therapy and dietary control, and one patient (#8) was receiving concomitantly anti-hypertensive therapy. The oldest patient (case # 10, 85 years) was lost to follow up at four months post-op, due to cardiac sudden death experienced at home. Of the nine remaining patients, three were diabetic, four had a diagnosis of AS, and two had a mixed

![Fig. 1. Correlation analysis: Pearson correlation coefficient (r), along with a 95% confidence interval and a P value calculated for: (A) lipoaspirate volume and age; (B) viable cells injected and lipoaspirate volume; and (C) Delta ABI and viable cells injected, showing no statistical correlation between the analyzed variables.](image-url)
diagnosis of AS and DM. Six patients (cases #1, #3, #5, #6, #8 and #10) had non-healing ulcers that were characterized by a complete lack of skin cover and a chronic, fibrotic tissue bed. Three of them (cases #1, #5, #8) had previously amputated toes and the ulcers (anterior foot) had necrotic edges that required debridement prior to the treatment. One patient (case #8) had a large wound post-debridement, which was treated locally and became a candidate for skin grafting.

### 3.2. Injections and follow-up

All liposuctions and SVF injections were well tolerated and uneventful. No complications attributed to the procedures were observed either short- or long-term, including excessive pain/discomfort, allergic/immune reactions, bleeding or infection (local or systemic). The procedural data (cell yields and viability) as well as clinical outcomes (pain, ulcers, ABI ratios) are summarized in Table 2 and presented in Figs. 2 and 3. Despite the high variability in the cell yields, we could not find any statistical correlation between parameters such as age, liposaprate volume, cell yield/viable cells injected (cell dose) and quantifiable clinical outcomes (delta ABI) (Fig. 1).

— Pain: Five patients (cases #1, #4, #5, #8 and #10) started with rest pain (#5 and #8 on a wheelchair), while the remaining five had claudication triggered at walking for half a block. After the treatment, two out of the five patients with rest pain (#1 and #4) were able to ambulate pain-free for at least 200 m. The two patients in wheelchair reversed their rest pain but could not walk and case #10 was not followed (deceased patient). The five patients with claudication at half a block were able to walk from 300 m to several blocks without claudication symptoms. At an eighteen-month follow-up these results remained unchanged.

— Ulcers: Four out of six patients with ulcers achieved closure between 8 and 10 months post-op (Figs. 2 and 3). A fifth patient underwent successful skin grafting at 5 months post-procedure. The remaining patient (case #10) was showing early signs of wound healing when passed away.

— ABI ratios: An increase in the calculated ABI ratios was documented in all patients. The increments ranged between 1.29 and 2.67 times (Post/Pre) with an average increase of 1.92 times. Of note, two out of six patients with DM had “normal” preoperative ABI ratios (1.4), which in diabetic patients with small vessel vasculopathies may be a normal finding (ABI ratios ≥ 1.0). Nevertheless, those two patients also experienced an increase in their postoperative ABI ratios (1.8 and 2.0, respectively).

— Angiography (Fig. 2 and Table 3): The MRI-based angiography was performed in six patients. Three patients had medical contraindications for the procedure (e.g.: compromised kidney function), and one patient (case #10) passed away before the time for the evaluation. From the six patients successfully studied, five demonstrated neovascularization in the leg, foot, or both. One patient presented no demonstrable changes.

### 4. Discussion

This study describes clinical outcomes obtained when fresh, non-fractionated, not culture-expanded, adipose tissue-derived SVF was used to treat patients with CLI. The rationale for using SVF instead of expanded ADSCs was twofold: first, to test the feasibility of a point-of-care administration of a cell-based therapy approach; and second, to take advantage of the presence of additional regenerative cell populations within the SVF (e.g.: EPCs, hemopoietic progenitors and pericytes), documented as having angiogenic and blood vessel-stabilizing properties. This pilot, small, open-label, non-randomized, no control group safety/feasibility study documented the clinical outcomes, evaluated both clinically and by imaging, of 10 patients with CLI (Rutherford stage 3–6)

### Table 2

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Lipo volume (cc)</th>
<th>Total cells injected (×10⁶)</th>
<th>Viability (%)</th>
<th>Injection location</th>
<th>Pre-op ABI</th>
<th>Post-op ABI</th>
<th>Post-op ambulation without claudication</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>222</td>
<td>30.78</td>
<td>81.5</td>
<td>IM, wound</td>
<td>1.4</td>
<td>1.8</td>
<td>200 m</td>
<td>No pain</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>272</td>
<td>53.2</td>
<td>75</td>
<td>IM</td>
<td>0.3</td>
<td>0.8</td>
<td>400 m</td>
<td>No pain</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>316</td>
<td>80.4</td>
<td>88.5</td>
<td>IM</td>
<td>0.7</td>
<td>1.5</td>
<td>8 blocks</td>
<td>No pain</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>362</td>
<td>34</td>
<td>86</td>
<td>IM</td>
<td>0.3</td>
<td>0.8</td>
<td>200 m</td>
<td>No pain</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>344</td>
<td>126</td>
<td>82</td>
<td>IM, wound</td>
<td>1.4</td>
<td>2.0</td>
<td>On a wheelchair</td>
<td>No pain</td>
</tr>
<tr>
<td>6</td>
<td>76</td>
<td>257</td>
<td>42.5</td>
<td>83.5</td>
<td>IM, wound</td>
<td>0.4</td>
<td>0.8</td>
<td>15 blocks</td>
<td>No pain</td>
</tr>
<tr>
<td>7</td>
<td>77</td>
<td>324</td>
<td>157.8</td>
<td>73</td>
<td>IM</td>
<td>0.4</td>
<td>0.7</td>
<td>3 blocks</td>
<td>No pain</td>
</tr>
<tr>
<td>8</td>
<td>78</td>
<td>282</td>
<td>19.1</td>
<td>83</td>
<td>IM, wound</td>
<td>0.3</td>
<td>0.8</td>
<td>On a wheelchair</td>
<td>No pain</td>
</tr>
<tr>
<td>9</td>
<td>78</td>
<td>278</td>
<td>44.52</td>
<td>85.5</td>
<td>IM</td>
<td>0.4</td>
<td>0.6</td>
<td>300 m</td>
<td>No pain</td>
</tr>
<tr>
<td>10</td>
<td>85</td>
<td>300</td>
<td>130</td>
<td>88</td>
<td>IM, wound</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Wound closed at 4 months (Patient died)</td>
</tr>
</tbody>
</table>

### Table 1

Patients’ demographics, diagnosis (Dx) and characteristics at baseline. DM = diabetes mellitus, AS = arteriosclerosis, CRF = chronic renal failure.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Dx</th>
<th>Ambulatory status</th>
<th>Rutherford category</th>
<th>Wound dimensions (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>F</td>
<td>DM</td>
<td>Rest pain</td>
<td>Category 6</td>
<td>Ulcer: 5 × 4 cm</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>F</td>
<td>AS</td>
<td>Claudication at 1/2 block</td>
<td>Category 3</td>
<td>Ulcer: 3 × 3 cm</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>F</td>
<td>AS</td>
<td>Claudication at 1/2 block</td>
<td>Category 6</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>F</td>
<td>AS</td>
<td>Rest pain</td>
<td>Category 4</td>
<td>Ulcer: 4.5 × 3 cm</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>M</td>
<td>DM</td>
<td>Rest pain; on a wheelchair</td>
<td>Category 6</td>
<td>Ulcer: 7 × 5 cm</td>
</tr>
<tr>
<td>6</td>
<td>76</td>
<td>F</td>
<td>DM</td>
<td>Claudication at 1/2 block</td>
<td>Category 6</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
<tr>
<td>7</td>
<td>77</td>
<td>F</td>
<td>DM-AS</td>
<td>Claudication at 1/2 block</td>
<td>Category 3</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
<tr>
<td>8</td>
<td>78</td>
<td>F</td>
<td>DM-AS</td>
<td>Rest pain; on a wheelchair</td>
<td>Category 3</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
<tr>
<td>9</td>
<td>78</td>
<td>F</td>
<td>DM-AS</td>
<td>Claudication at 1/2 block</td>
<td>Category 6</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
<tr>
<td>10</td>
<td>85</td>
<td>F</td>
<td>AS-CRF</td>
<td>Rest pain</td>
<td>Category 3</td>
<td>Ulcer: 4 × 6 cm</td>
</tr>
</tbody>
</table>

— Cell therapy: doses and results. IM = intramuscular, STSG = split-thickness skin graft, ABI = brachial systolic blood pressure/ankle systolic blood pressure.
treated with multiple IM injections of SVF. One patient was dropped from the study due to sudden cardiac death at 4 months post-treatment, leaving nine patients for full analysis.

Despite a wide range of cell doses (viable cells injected), the patients demonstrated positive clinical responses in all assessments. In fact, of the nine patients that were followed completely, at the end of the study all had experienced significant improvement in terms of pain control and in the ability to ambulate without claudication (in the seven patients in which the evaluation was feasible). ABI ratios were increased in all patients but the significance of this finding is unclear. Four of the five patients with non-healing ulcers had a complete closure in eight to nine months with no reported ulcerations after 18 months. Angiographies showed evidence of neovascularization in five of the six patients in whom the imaging procedure was feasible. No particular pattern, either by localization (e.g. proximal, middle, or distal 1/3 of the leg), or by responding artery (e.g. peroneal, tibialis anterior and posterior, dorsalis pedis, etc.) was seen in the angiograms. Most importantly, the degree of revascularization in the leg was not as striking as the overall clinical response. We recognize the limitations of angiography, as these studies may be subject to variations by ambient temperature, heart rate, other medications (no patient was taking vasodilators), and technical difficulties with assessing collaterals (Gates and Hartnell, 2000). Our impression was that angiography in cases of severe distal disease involving small-vessels is not very sensitive as was expected and is of limited clinical use.

These results with fresh, non-fractionated, not culture-expanded, adipose tissue-derived SVF are comparable with other two previously reported clinical series, using cultured adipose-derived ADSCs with controlled cell doses. The adipose tissue contains large populations of mesenchymal cells (ADSCs) and other progenitors (e.g.: EPCs) with tested angiogenic properties (Amos et al., 2008; Bourin et al., 2013; Crisan et al., 2008). Furthermore, a multi-differentiation capacity of ADSCs into both endothelial cell and smooth muscle cell phenotypes has been proposed (Zhi et al., 2014). Consequently, initial attempts to study the therapeutic effect of culture-expanded ADSCs have shown to be very promising.

The first reported trial with ADSCs for CLI, enrolled 15 subjects [12 with thromboangiitis obliterans (TAO) and 3 with diabetic foot] exhibiting rest pain with/without non-healing ulcers or tissue necrosis. Treatment consisted of IM injections of culture-expanded ADSCs (Lee et al., 2012). Clinical improvement, based on pain scores, claudication distances and ulcer healing, was documented in 66.7% of the patients.

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Fig. 2. Case #1: A) Right foot with amputated toe, prior to cell injections. B) Multiple injection sites at the gastrocnemius. C) Four (4) months post-injections showing a smaller wound with underlying granulation tissue and improved perfusion. D) Eight (8) months post-injections showing full wound closure. E) Pre-op angiography showing a cut-off of arteries at the level of the right ankle (yellow rectangle amplified on the right figure). F) Post-op angiography at four (4) months showing continuity between arteries of the foot with anterior and posterior tibial arteries and extensive neovascularity with anastomoses of dorsalis pedis and tibialis.
Digital subtraction angiography (DSA) at 6 months revealed a significant improvement in the collateral vessel formation score in eight out of ten TAO patients and in two out of three of the diabetic patients.

In 2014, Bura et al. reported a phase 1 clinical trial (ACellDREAM) with culture-expanded autologous ADSCs in seven patients [six with AS (3 with concomitant DM) and one with TAO] with non-reconstructable Rutherford stage III-6 CLI (Bura et al., 2014). Injections into each leg were placed into the anterior compartment and into each of the gastrocnemius muscle. Feasibility and lack of adverse effects (safety) were achieved. Efficacy was measured by pain scores, number of ulcers and hemodynamic parameters (transcutaneous tissue Oxygen pressure - tcpO₂ - and ABI index). Despite the major amputations in three out of the seven patients and no changes in the ABI index in the majority of patients, clinical improvement was seen at 6 months (pain reduction and number of ulcers).

Notwithstanding the observed clinical and imaging similarities between our study and the ones reported with cultured-expanded ADSCs, a major difference relates with the effective cell dose used. The studies reported by Lee and Bura used a fixed number of ADSCs, significantly higher than in our study. Two considerations need to be taken into account for this contrast: first, the reported percentage of SVF constitutive cell populations is variable. For instance, ADSCs can be present in a range between 10 and 30%, while EPCs and pericytes can comprise 10–20% and 3–5%, respectively (Bourin et al., 2013). Therefore, the absolute number of ADSCs administered in our study was significantly lower than the one used in the other two studies. Second, DM can exert an effect over different progenitor cell populations within the adipose tissue, which can alter even more the percentage distribution of cells within SVF, and more importantly their functional effects (Nguyen et al., 2016; Guo et al., 2016). For instance, Rennert et al. (2014) described that DM impairs the angiogenic potential of ADSCs (both in vitro and in vivo) by selectively depleting specific cellular sub-populations. Remarkably, this observation has been also made in bone marrow-derived MSCs (Januszyk et al., 2014). These findings can serve as the basis to hypothesize a potential therapeutic benefit of SVF when compared with culture-expanded ADSCs alone, again, based on the presence of additional cells with angiogenic capabilities. In fact, Zimmerlin et al. (2010) identified within the SVF mix two populations of endothelial cells (one mature and one progenitor, discriminated by the expression of CD34 and CD90), and two populations of perivascular stabilizing cells (pericytes and supra-adventitial cells discriminated by the expression of CD146, α-SMA and CD34). Collectively, these cell populations along with ADSCs may have a stronger and faster angiogenic response than ADSCs alone, and may help explain the slightly better results with non-fractionated SVF than with culture-expanded ADSCs.

Remarkably, we could not find any statistical correlation between the lipoaspirate volume obtained and parameters such as age and the resulting viable cell yield. Moreover, the cell dose administered (viable...
two of the patients that received significant neovascularization with anastomoses to tibialis anterior and posterior.

2. Cut-off of dorsalis pedis and tibialis posterior at the ankle.

3. The femoral, popliteal, and tibio-peroneal trunks have observable flow but the vessels are beaded. The peroneal has a cut-off at the proximal 1/2. Tibialis anterior and posterior are visible. At the level of the ankle and foot, multiple tortuous vessels are seen.

4. Stenosis of the superficial femoral artery in the distal 1/3. The peroneal artery is occluded in the middle 1/3. Tibialis anterior and posterior completely absent.

5. Stenosis of superficial femoral artery at the distal 1/3. Peroneal, tibialis anterior and posterior have fibrofatty rod-like beak configuration.

6. Fibromuscular stenosis of the superficial femoral artery. The peroneal artery is patent in the distal 1/3 as is the tibial. Peroneal and proximal tibialis anterior continue to be occluded. Below the level of the peroneal fossa fibrofatty flow is now seen in tibialis anterior and posterior.

7. Stenosis of superficial femoral artery in the distal 1/3. Tibialis anterior is now patent in its distal 1/3. Tibialis anterior fills the dorsalis pedis and dorsal interosseous arteries (not seen previously); Tibialis posterior is seen in the foot as far as its plantar surface.

8. Femoral, popliteal and tibio-peroneal trunks are patent but have a rosy bead appearance. After the trifurcation tibialis anterior is identified in its proximal 2/3 but not its distal 1/3. Filiform flow is observed in the peroneal. Tibialis anterior and dorsalis pedis cannot be identified.

9. Stenosis of the superficial femoral artery. The peroneal artery is patent in the distal 1/3. Tibialis anterior fills the dorsalis pedis and dorsal interosseous arteries (not seen previously); Tibialis posterior is seen in the foot as far as its plantar surface.

cells injected) was not correlated with the degree of clinical improvement. For instance, the patient with the highest cell dose (#7) was the one with no demonstrable angiographic changes. On the other hand, two of the patients that received significantly lower doses (#1 and #6) exhibited impressive wound healing results and angiographic changes. Of note, the patient that received the lowest dose of all (#8) corresponds to the one that underwent successful skin grafting, suggesting a potential minimum cell concentration required for successful skin grafting, suggesting that given the point-of-care nature of the procedure, associated with a short duration for SVF processing and administration, an "in situ" characterization to complement our basic analysis results (cell count, viability, etc.) is difficult, leaving as an option a "confirmatory" analysis of an SVF aliquot performed after the treatment. Future studies involving more rigorous designs will incorporate such "cell product" characterization.

Future studies need to incorporate this in order to establish reproducible clinical results. In addition, due to logistical difficulties in bringing patients to the university hospital more often, the average clinical time to closure could not be ascertained in our study, a parameter that gives important clinical information regarding wound healing. This limitation will be taken into consideration in subsequent studies.

5. Conclusions

We consider the injection of adipose-derived SVF cells to represent a novel alternative for a point-of-care cell-based therapy for symptomatic patients with non-reconstructable PVD, that offers the possibility of relief from ischemic pain, improved healing of chronic wounds, and the potential for tissue preservation. Our study is preliminary and promising but its small size and lack of a control group are design factors that do not permit definitive conclusions to be drawn. Further larger controlled trials are required to confirm the potential of SVF as an easy and effective therapeutic modality for the treatment of PVD.

Author contributions

Michael Carstens: conception and design, data analysis and interpretation, manuscript writing and editing;
Arturo Gomez: conception and design and manuscript writing;
Ronald Cortes: collection and/or assembly of data;
Elizabeth Turner: collection and/or assembly of data, manuscript writing;
Cecilia Perez: collection and/or assembly of data;
Marlon Ocon: administrative support, coordinate study;
Diego Correa: conception and design, data analysis and interpretation, manuscript writing and editing and final approval.

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Disclosures of potential conflicts of interest

Michael Carstens consults for the GID Group, in which he holds stock options.
Diego Correa participates in the scientific advisory board of RegenMed, LLC.

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