




Treatment of Breast Cancer-Related Lymphedema with Adipose-Derived Regenerative Cells and Fat Grafts: A Feasibility and Safety Study

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ABSTRACT

Breast cancer-related lymphedema (BCRL) is a debilitating late complication with a lack of treatment opportunities. Recent studies have suggested that mesenchymal stromal cells can alleviate lymphedema. Herein, we report the results from the first human pilot study with freshly isolated adipose-derived regenerative cells (ADRC) for treating lymphedema with 6 months follow-up. Ten BCRL patients were included. ADRC was injected directly into the axillary region, which was combined with a scar-releasing fat graft procedure. Primary endpoints were change in arm volume. Secondary endpoints were change in patient reported outcome and safety. The study is registered with ClinicalTrials.gov (NCT02592213). During follow-up, a small volume reduction was noted but was not significant. Five patients reduced their use of conservative management. Patient-reported outcomes improved significantly over time. ADRCs were well tolerated and only minor transient adverse events related to liposuction were noted. In this pilot study, a single injection of ADRC improved lymphedema based on patient-reported outcome measures, and there were no serious adverse events in the 6 months follow-up period. In addition, half of the patients reduced their use of conservative management. ADRC therapy is a promising interventional therapy for alleviating lymphedema, but results need to be confirmed in randomized clinical trials. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:1666–1672

SIGNIFICANCE STATEMENT

Lymphedema is one of the most serious and debilitating late complications following breast cancer surgery with lack of treatment opportunities. Cell therapy has shown promising results in preclinical models of lymphedema. Autologous adipose tissue is the most convenient source of cells for clinical therapy. In the present First in Human Pilot Study, it is shown that treatment with autologous adipose-derived regenerative cells can alleviate symptoms and reduce the need for other treatment options during the 6 months follow-up. The treatment proved safe without any noteworthy adverse events. Randomized controlled trials will be needed to verify the positive results.

INTRODUCTION

Lymphedema is one of the most common and severe complications after breast cancer treatment. Breast cancer-related lymphedema (BCRL) occurs in 20%–30% of patients with lymph node involvement [1] and manifests itself with excess lymphatic fluid and swelling of subcutaneous tissues in the nearby arm due to obstruction and/or destruction of lymphatic vessels following surgical treatment and/or radiation therapy. Lymphedema impacts the patients' lives both physically and psychologically. Physically, the swelling can result in fatigue, feeling of arm heaviness and tension, reduced range of arm motion, as well as difficulties in performing daily activities, including

returning to work [2]. Psychologically, the swelling can result in negative feelings with regard to body image. In addition, lymphedema is associated with increased risk of severe skin infections [3].

Standard of care for lymphedema today is conservative nonsurgical management, which includes a mix of compression garments on the affected arm(s), manual lymphatic drainage, exercises, as well as meticulous skin care [4]. The nonsurgical management is only effective to a certain extent with excess volume reductions in the range of 20%–30% [5]. Each treatment is time consuming, and treatment is lifelong because the underlying problem has not been addressed [6]. Several microsurgical approaches are being used

experimentally with promising initial results; however, the evidence for the efficacy is poor [7, 8].

The potential ability to treat diseases through regeneration has sparked the interest in cell therapy in almost all organ systems [9], including BCRL [10, 11]. Two studies have so far examined the efficacy and safety of using bone marrow-derived mesenchymal stromal cells to treat BCRL [10, 11]. These results were promising with symptom relief and are supported by several preclinical studies using cell therapy to treat lymphedema [12]. In recent years, several cell therapeutic protocols that utilize adipose-derived stromal cells (also called stromal vascular fraction or adipose-derived regenerative cells [ADRC]) have emerged. ADRCs are easily accessible through liposuction and are an abundant cell source [13]. This pool of cells is, however, heterogeneous in nature and includes stem cells, endothelial cells, pericytes, progenitor cells, as well as hematopoietic cells. It has been proposed that these cells may work in synergistic fashion, especially through paracrine secretion of growth factors and cytokines [14, 15], and several studies, including our unpublished data, indicate that ADRC likely improves vascularity [16, 17].

We therefore recently performed a case study of ADRC transplantation and fat grafting to alleviate lymphedema in one patient. Interestingly, both a patient-reported and volumetric improvement was noted [18]. To substantiate this promising result, we herein tested efficacy, safety, and feasibility of this procedure in the first pilot study performed. We here report the results from 10 patients with a 6-month follow-up period after ADRC transplantation and fat grafting for BCRL.

MATERIALS AND METHODS

ADRC Transplantation and Fat Grafting

The procedures were performed under general anesthesia at the Department of Plastic Surgery, Odense University Hospital. Approximately 300 mL lipoaspirate from either thighs or abdomen with water-jet-assisted liposuction (body-jet, Human med AG, Schwerin, Germany, <http://www.humanmed.com/en>) was obtained, and 30 mL was saved for lipotransfer to the axilla. After decantation, the lipoaspirate for lipotransfer was injected in a fan-shaped pattern for loosening the scar tissue. The patient was transferred back to the ward awaiting cell injection to avoid occupying the operating theatre for 2 hours as the ADRC were being isolated. The remaining lipoaspirate was used for immediate ADRC isolation using the Celution 800/IV system (Cytori Therapeutics, San Diego, California, <http://www.cytori.com/>) according to the manufacturer's instructions. The ADRC was resuspended in 5 mL Lactated Ringer's solution, and 1 mL was saved for *in vitro* characterization. The remaining cells were injected in the axilla at eight standardized points around the scar into the subcutaneous plane.

Cell Characterization

Cells were counted with a NucleocounterNC-100 (ChemoMetec, Denmark, <https://chemometec.com/>). ADRC surface marker analysis was performed as recommended internationally [15] and previously described [19]. The following antibodies were used according to manufacturer's (BD Biosciences, Albertslund, Denmark, <http://www.bdbiosciences.com/us/home>) recommendations: anti-CD235a (BV421, clone GA-R2/HIR2), anti-CD34 (PECF594, clone 581), anti-CD45 (FITC, clone HI30), CD31 (Alexa

Fluor 647, clone WM59), CD73 (APC, clone AD2), CD90 (APC, clone 5E10), and appropriate isotype (for single stains) or FMO control (for CD34 on multistain). Sample acquisition was performed on a BD LSRII flow cytometer and analyzed using the FACS-Diva software v8.0.1 (BD Biosciences, Albertslund, Denmark, <http://www.bdbiosciences.com/us/home>) and FlowJo 10.0.8r1 (Flowjo, LLC, Ashland, Oregon, US <https://www.flowjo.com/>). Cell doublets were excluded from all analyses by sequential gating through forward and side scatter height/width plots.

The percentage of fibroblastoid colony-forming units (CFU-F) in each ADRC sample was determined by seeding cells at low density (4 densities in triplicate ranging from 28–280 live nucleated cells per cm²) in dulbecco's modified eagle medium (DMEM) with 1 g/L glucose, 10% fetal bovine serum (FBS), 1%PS, culturing for 14 days, and counting hematoxylin and eosin (HE)-stained colonies comprising >50 cells. The differentiation potential of the adipose-derived stem cells within the ADRC was tested using adipogenesis, osteogenesis, and chondrogenesis differentiation kits from StemPro (Thermo Fisher Scientific, Denmark, <https://www.thermofisher.com/us/en/home.html>) as recommended by the manufacturer.

Volumetric Outcome Assessment

Arm volume was calculated as previously described [20]. Briefly, circumference measurements were made at five points on each arm: wrist, largest point on lower arm, elbow, middle of upper arm, and proximal on the upper arm. The length between each point was measured, and at each time point the same sites were measured. Based on these five measurements, the arm was divided in four segments and the volume of each segment was calculated based on the truncated cone formula.

$$V = \frac{h(C_1^2 + C_1C_2 + C_2^2)}{12\pi}$$

Where V is the segment volume, h is the length of the segment, and C₁ and C₂ are the two circumference measurements at the two ends of the segment. Arm volume was calculated as the sum of the four segment volumes. Excess arm volume was calculated as the volume difference between the two arms.

Arm volume was also evaluated by DXA, which was performed preoperatively and 3 months and 6 months postoperatively. The DXA was performed as two whole body scans with the patient lying in a modified position enabling the arm to be free from the trunk. For analysis, a blinded assessor drew the region around the arm with the proximal end of the arm defined as just below the deltoid muscle, which is easily visualized on the DXA. The bone mineral content, fat mass, and lean mass were used to calculate a total arm volume based on known densities as has been previously described [21].

Patient-Reported Outcome Assessment

Patients were asked to rate the feeling of heaviness in the lymphedema arm on a numerical rating scale ranging from 0 to 10, with 0 meaning no heaviness at all and 10 signifying the worst heaviness imaginable. Similarly, the patients were asked to rate the feeling of tension in the lymphedema arm. In addition, two questionnaires were used: the disabilities of the arm, shoulder and hand (DASH) outcome questionnaire [25] as well as the Lymphedema Quality-of-Life (LYMQOL) questionnaire [26] were filled out preoperatively and at each time point after treatment.

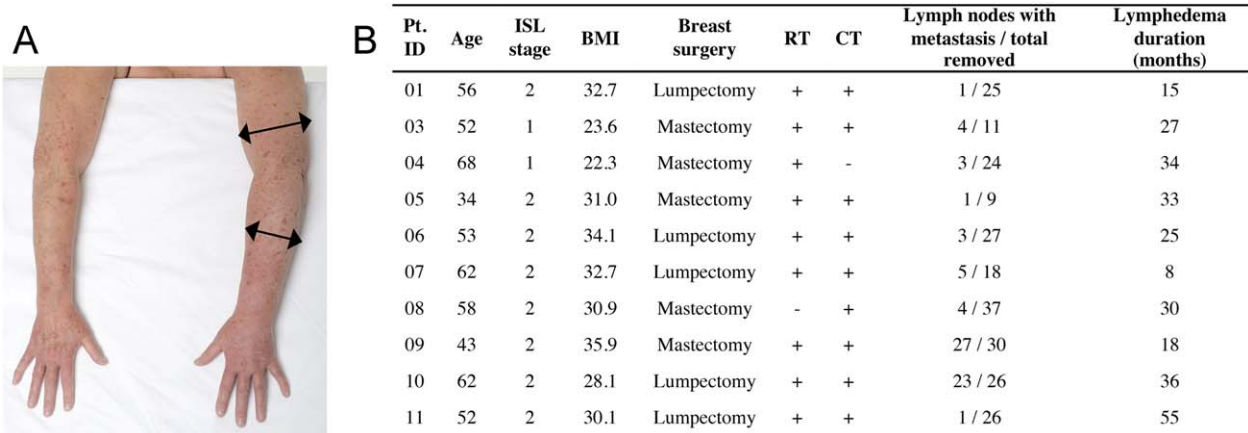


Figure 1. Patient overview. **(A):** Representative photo of breast cancer-related lymphedema in the left arm compared with the healthy contralateral side. **(B):** Table showing baseline characteristics of included patients. Patient ID 02 was excluded due to non-protocolled treatment as described in the manuscript. Abbreviations: +, yes; -, no; BMI, body mass index; CT, chemotherapy; ISL stage, International Society of Lymphology stage; Pt. ID, patient identification; RT, radiation therapy.

Safety Assessment

Safety was evaluated by a specific questionnaire given to the patients postoperatively, which was to be filled out prospectively during the first month following treatment. The questionnaire included questions regarding redness, swelling, itching, pain (which was not handled sufficiently with over the counter medicine), infection, other discomfort, and having seen their family doctor for any reason. Additionally, at the postoperative visits, adverse events were recorded by inspection of the injection and donor site and posing an open question, "Did you experience any other discomfort related to the operation since the last visit?"

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 for Windows (GraphPad Software, La Jolla, California, www.graphpad.com). Subjective scores on visual analogue scale and questionnaire outcomes were analyzed by Friedman's test for multiple non-parametric comparisons with Dunn's post-hoc test for multiple comparisons. Volumetric changes were analyzed using One-way analysis of variance (ANOVA) with Dunnett post-hoc test for multiple comparisons. For subgroup analysis based on International Society of Lymphology (ISL) stage, a two-way ANOVA was performed with Tukey's test for multiple comparisons. A two-tailed *p* value of less than .05 was considered significant.

RESULTS

Study Design and Patient Characteristics

This study was conducted as an open-label, single-arm, single-center feasibility and safety study in patients with breast cancer-related lymphedema of the upper extremity (ClinicalTrials.gov NCT02592213). The study was approved by The Regional Committees on Health Research Ethics for Southern Denmark (3-3013-1572/1), who had oversight of the study. The study was registered with The Danish Data Protection Agency (2008-58-0035).

The aim was to include 10 patients for treatment, and 34 patients were screened for eligibility, of whom 11 were found to be eligible. The reason for the extra included patient was due to non-protocolled treatment of the very first patient, who was

therefore excluded from analysis (supplemental online Fig. 1). Inclusion period ranged from December 2015 to May 2016, and all patients were treated between January and May 2016.

Eligible patients had to fulfill the following criteria: diagnosed with upper extremity lymphedema due to previous breast cancer treatment with lymph node involvement, recurrence-free for minimum 1 year, unilateral disease, lymphedema ISL stage I or II, circumference of arm a minimum of 2 cm larger than the healthy side, age between 18 and 70 years old, The American Society of Anesthesiologists Physical Status classification system (ASA) score 1 or 2, able to give written informed consent, and able to understand the Danish language. Additionally, they were excluded if they had a history of other cancer types, had diabetes mellitus, had psychiatric conditions that could interfere with participation, or used tobacco, which was not ceased in relation to the procedure (supplemental online Fig. 1). The ages of the 10 included patients ranged from 34–68 years (54.5 [12.3], median [interquartile range {IQR}]). Only two participants had ISL stage U lymphedema and the remaining participants had stage II (Fig. 1A and 1B). The majority of patients had received both radiation and chemotherapy for the primary breast cancer treatment, and the duration of lymphedema prior to inclusion in the trial was 28.5 (17.3; median [IQR]) months (Fig. 1B).

ADRC Isolation and Transplantation

For the ADRC treatment, all patients underwent liposuction under general anesthesia, during which approximately 300 mL of lipospirate (Fig. 2A) was harvested from either the abdomen or thighs depending on availability and preference of the patient. The ADRC isolation using the automated Celution system and cellular/biochemical characterization (Fig. 2B) has previously been described [19]. In addition, a lipospirate was harvested for immediate grafting in the axilla, where fat was injected in a standard fan shaped pattern to release scar tissue (28.1, 7.8 mL [mean, SD]; Fig. 2C). ADRCs (5.37×10^7 , 1.08×10^7 cells [mean, SD]) were injected in the axilla 2 hours later (Fig. 2D).

The characteristics of the isolated ADRC were comparable to those previously reported by us [19], including yield (ADRC cells/g fat tissue, 2.1×10^5 , 4.2×10^4 [mean, SD]), cell size (10.8, 0.2 [mean, SD]), viability (83.4, 3.0 [mean, SD]), and percentage of

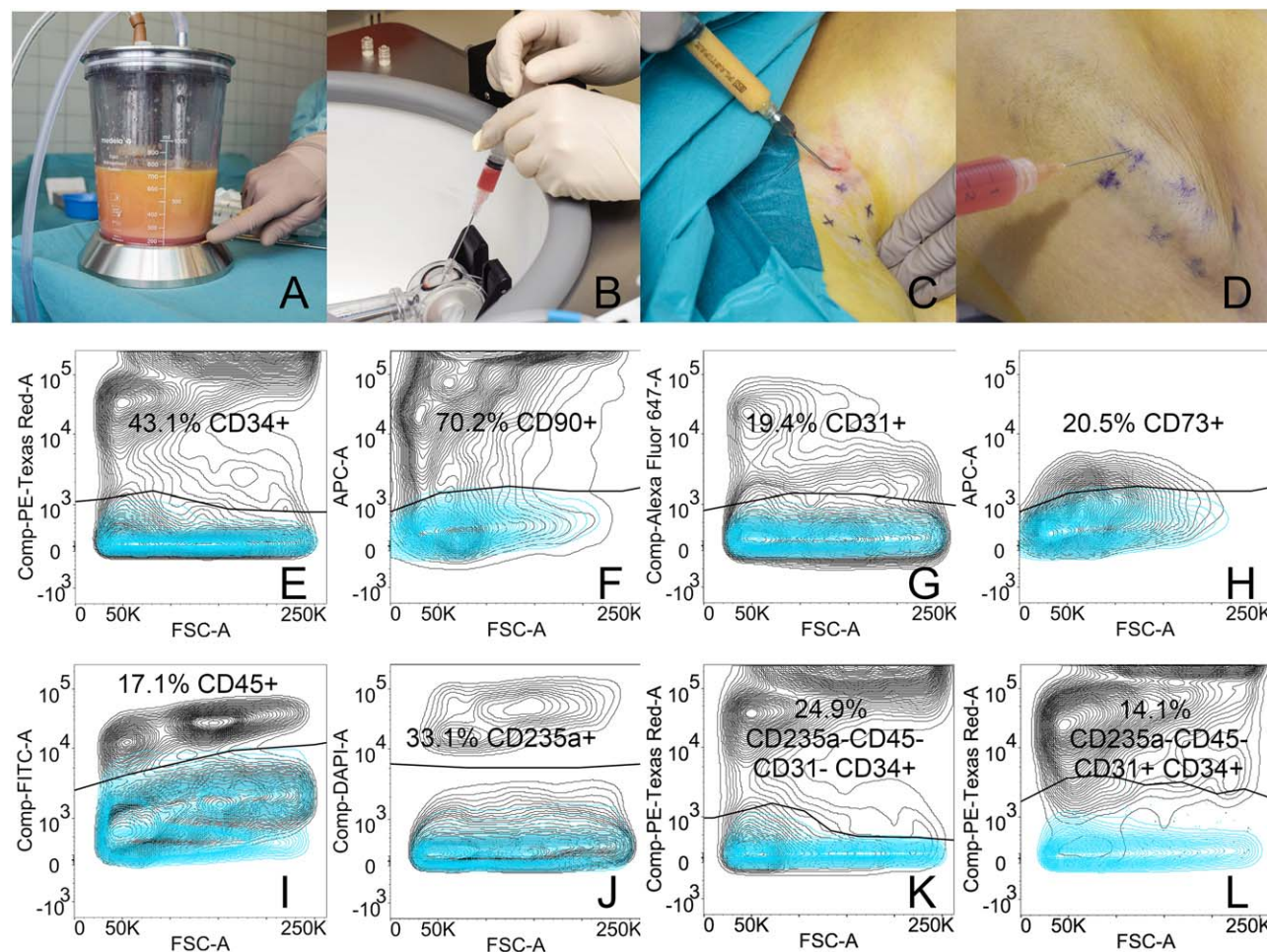


Figure 2. The procedure and cell characterization. **(A):** Approximately 300 mL of lipospirate was harvested for adipose-derived regenerative cell (ADRC) isolation. **(B):** ADRC were isolated using the automated Celution IV system yielding a 5-mL cell suspension in about 2 hours. **(C):** During surgery, 20–30 mL of lipospirate was injected in the axilla in a fan-shaped pattern to loosen scar tissue. **(D):** Immediately following ADRC isolation, the cell suspension was injected subcutaneously in the axilla. Flow cytometric evaluation of ADRC surface markers was performed on fresh cells and analyzed for CD34 **(E)**, CD90 **(F)**, CD31 **(G)**, CD73 **(H)**, CD45 **(I)**, CD235a **(J)**. The stromal stem cell subpopulation was defined as CD235a-CD45-CD31-CD34+ **(K)** and the endothelial progenitor cell subpopulation was defined as CD235a-CD45-CD31+CD34+ **(L)**.

fibroblastoid colony forming units (%CFU-F, 0.4, 0.4 [mean, SD]). A large proportion of the ADRC expressed the surface markers CD34 (43.1, 14.5% [mean, SD]; Fig. 2E) and CD90 (70.2, 9.3% [mean, SD]; Fig. 2F), whereas CD31 and CD73 each defined smaller subpopulations (19.4%, 8.1% and 20.5%, 17.7%, respectively [mean, SD]; Fig. 2G and 2H). The amount of blood as determined by the hematopoietic and erythropoietic markers CD45 (17.1, 5.7% [mean, SD]; Fig. 2I) and CD235a (33.1, 16.0% [mean, SD]; Fig. 2J), respectively, was similar between samples. The fraction of stromal stem cells as defined phenotypically by the markers CD235a-CD45-CD31-CD34+ encompassed $24.9 \pm 8.4\%$ (mean \pm SD; Fig. 2K) of the parent ADRC, whereas the fraction of endothelial progenitor cells CD235a-CD45-CD31+CD34+ comprised $14.1 \pm 6.1\%$ (mean \pm SD; Fig. 2L).

Safety and Lymphedema Alleviation Using ADRC Transplantation and Fat Grafting

In total, nine minor adverse events in 4/10 patients were noted in the follow-up period. Three patients noted bruising of the donor site following liposuction, accompanied by pain in two cases. Another patient noted itching of the axilla and donor site. All pain

and itching resolved spontaneously within the first week after treatment. One patient complained of back pain 2 weeks after treatment, for which she sought a chiropractor. At 3 months follow-up, one patient complained of reduced sensation at the donor site, which resolved spontaneously at 6 months follow-up. One patient noted a slight irregularity in the skin surface at the donor site. All the above events are expected complications of liposuction and fat grafting and were therefore not attributed to the ADRC transplantation. There was no evidence of cancer recurrence during the follow-up period.

Patients were evaluated 1, 3, and 6 months following ADRC transplantation. In general, the patients reported a decrease of their lymphedema symptoms over time (Fig. 3; see Materials and Methods for detailed assessment tools). The median (IQR, $n = 10$) score for heaviness of the arm at baseline was 5.5 (4.0), after 1 month 4.0 (2.5; $p = .2498$), after 3 months 3.5 (3.5; $p = .0097$), and after 6 months 2.5 (4.3; $p = .0030$; Fig. 3A). Likewise, we found a median score for arm tension at baseline to be 5.0(1.5), after 1 month 3.0 (3.0; $p = .0281$), after 3 months 3.0 (2.3; $p = .0459$), and after 6 months 2.5 (4.3; $p = .0097$; Fig. 3B). Finally the DASH questionnaire was also used, in which the median score

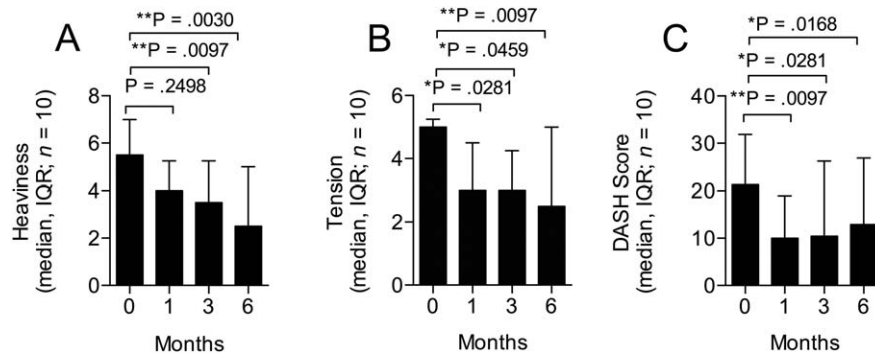


Figure 3. Patient reported outcome evaluation. Patient-reported outcome was evaluated after 1, 3, and 6 months following treatment. There was a significant improvement in patient reported outcomes based on the degree of heaviness (A): as well as tension (B): in the arm measured on numerical rating scales (0–10) as well as the DASH questionnaire (C). Abbreviations: DASH, disabilities of the arm, shoulder and hand; IQR, interquartile range.

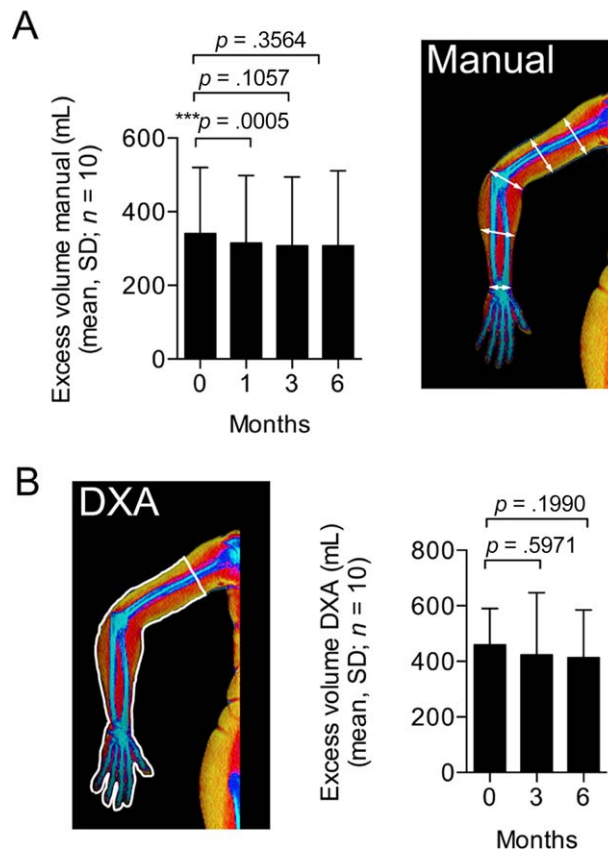


Figure 4. Volumetric outcome evaluation. (A): Volume was calculated manually by circumference measurements at five points along the arm and measurement of the distance between each point. A transient significant reduction in arm volume was seen after 1 month but results were insignificant after 3 and 6 months. (B): Volume was also assessed by DXA after 3 and 6 months after treatment. A subregion was drawn around the arm and based on known densities of bone, fat, and lean tissue types, the volume was calculated. Similar to the circumference measurements, volume change over time assessed by DXA also showed a small non-significant decrease at the 3 and 6 month time points. Abbreviation: DXA, dual-energy x-ray absorptiometry.

at baseline was 21.3 (23.5), after 1 month 10.0 (14.5; $p = .0097$), after 3 months 10.4 (24.8; $p = .0281$), and after 6 months 12.9 (26.0; $p = .0168$; Fig. 3C). Moreover, five patients reduced their use of conservative treatment. A significant mood improvement was also observed at 3 and 6 months follow-up with the LYMQOL questionnaire. However, only minor insignificant improvements

were reported in the other LYMQOL subscales (supplemental online Table 1).

The change in excess arm volume was evaluated with two separate methods. As previously described [20], the volume of the arm can be estimated by five manual circumference measurements along the arm using the truncated cone formula (Fig. 4A).

The arm volume can also be described by dual-energy x-ray absorptiometry (DXA; Fig. 4B). A subregional analysis of the arm will reveal the mass of bone, fat and lean mass, and, based on known densities of the tissue types, the volume of the arm can be calculated [21]. For each method the difference in arm volume between the lymphedematous and healthy arm was defined as excess arm volume. Although a slight difference in absolute volume is present (Fig. 4A and 4B) between the results obtained from the two methods, they correlated significantly ($r = 0.848$, $p < .0001$, Pearson). A modest decrease in excess arm volume was observed after 1 month; however, after 3 and 6 months, the change was still decreased but failed to meet the significance level (Fig. 4A and 4B).

We performed subgroup analysis based on ISL stage I ($n = 2$) and II ($n = 8$). Visual interpretation showed a trend towards greater response for ISL stage I patients (supplemental online Fig 2). Two-way ANOVA testing revealed no significant differences between the groups but for feeling of tension ($p = .0583$), DASH questionnaire ($p = .0971$), as well as volume ($p = .0947$). The p value neared significance despite the low number of patients. Interestingly for volume, there was a significant decrease in volume after 6 months for ISL stage I ($p = .0253$) but not ISL stage II patients.

DISCUSSION

In this clinical trial, we show that a single treatment of cell-assisted lipotransfer using ADRCs can alleviate lymphedema symptoms and moreover reduce the need for conservative treatment in 50% of patients. The data from the numerical rating scales and the DASH questionnaire were significant, whereas only minimal trends were seen for the LYMQOL questionnaire, which concerns quality-of-life perception. Four patients had previous cellulitis/erysipelas infection in their lymphedema arm, and there were no episodes of infection in the arm following treatment, a finding which has also been documented for microsurgical procedures [22].

Although, our qualitative measures all point towards the conclusion that ADRC-assisted lipotransfer is a promising future intervention for lymphedema patients, we did not find any significant reduction in excess volume as measured by two quantitative techniques. The latter may be explained by the use of adjunct conservative management in our patients, because Hou et al. recently reported that patients without prior conservative treatment experience a substantial volume reduction 3 and 12 months after bone marrow-derived stromal cell treatment as compared to combined treatment that includes conservative management [10]. Likewise, Maldonado et al. have shown that bone marrow-derived stromal cell therapy alone was as effective as compression therapy with a 200-mL volume reduction in the lymphedema arm [11]. In this regard, it seems limiting for our assessment of volumes (manual and DXA) that our patients wore their compression garment up until the point of examination, leaving no window for “stress testing” of the lymphatic drainage of the arm. Currently, we are developing a new quantitative measure based on lymphoscintigraphy that enables us to determine changes in lymphatic drainage when using conservative treatment in parallel.

Importantly, we did not observe any adverse events within a 6-month time frame. While this is an important result, a long-term evaluation of the safety is necessary, especially regarding the risk of cancer recurrence, in light of ADRC injection into an area

with previous malignancy [23]. Yet hereto, the safety profiles of using ADRC transplantation and fat grafting are in agreement with other recent studies using bone marrow-derived mesenchymal stromal cells for lymphedema [10, 11]. Still, the field needs to document long-term (years) safety of cellular therapy in these patients.

The study was not without limitations. First, the trial was unblinded. It is possible that the added visits and the patients' own expectations of stem cell therapy may have caused a placebo effect. The trial was also one-armed, and the lack of a control group is a weakness. However, lymphedema is a condition that very rarely improves and, if anything, only worsens, so it could be speculated that any positive change might be attributed to the given treatment. Another potential weakness lies in the patient population, as most patients were late ISL stage II, in which chronic changes are dominant. ISL stage I patients might have a higher chance of successful treatment based on our very preliminary subgroup analysis, which is in agreement with microsurgical procedures, where it is generally accepted that the chance for successful treatment is greatest for early-stage lymphedema without chronic changes [24]. It is important to increase the chance of treatment success through the optimization of the selection criteria for patients. We aimed to harvest of 300 mL lipoaspirate for cell isolation, as we estimated that it could be harvested from almost any patient, so that the treatment was made as uniform as possible. Further studies are needed to determine an optimal dose. Additionally, it will be interesting to examine in the future if either ADRC or fat grafting can stand alone as treatment, which could possibly simplify the procedure.

CONCLUSION

The principal findings in this study are that autologous ADRC combined with a scar-releasing fat graft was safe during the 6 months follow-up period and can alleviate symptoms of breast cancer-related lymphedema, minimizing the need for conservative treatment. As the effect was primarily noted on patient-reported outcomes, it is important to confirm the benefit of this treatment modality in a properly blinded randomized controlled trial, in which sensitive methods to quantify excess volumes are included also included and patients are followed for several years to ensure long-term safety. Finally, future studies may also seek to test if the observed beneficial effect is caused by the ADRC or the fat graft alone, or a combination.

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SUPPLEMENTAL MATERIAL

Three items were included as supplementary material. First is a figure illustrating the study flow chart. The second is a table that summarizes all results during the study. Third is a figure illustrating

the subgroup analysis of the patient-reported outcomes of feeling of heaviness and tension in the arm grouped by ISL stage.

AUTHOR CONTRIBUTIONS

N.T. and C.J.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; S.P.: conception and design, revision/enhancement of manuscript for intellectual content, final approval

of manuscript; J.S.: conception and design, collection and/or assembly of data, revision/enhancement of manuscript for intellectual content, final approval of manuscript; D.A.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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