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The adipose tissue–derived stromal vascular fraction cells from lipedema patients: Are they different?

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Abstract

Background aims. Lipedema is a hormone-related disease of women characterized by enlargement of the extremities caused by subcutaneous deposition of adipose tissue. In healthy patients application of autologous adipose tissue–derived cells has shown great potential in several clinical studies for engrafting of soft tissue reconstruction in recent decades. The majority of these studies have used the stromal vascular fraction (SVF), a heterogeneous cell population containing adipose-derived stromal/stem cells (ASC), among others. Because cell identity and regenerative properties might be affected by the health condition of patients, we characterized the SVF cells of 30 lipedema patients in comparison to 22 healthy patients. *Methods.* SVF cells were analyzed regarding cell yield, viability, adenosine triphosphate content, colony forming units and proliferative capacity, as well as surface marker profile and differentiation potential in vitro. *Results.* Our results demonstrated a significantly enhanced SVF cell yield isolated from lipedema compared with healthy patients. In contrast, the adipogenic differentiation potential of SVF cells isolated from lipedema patients was significantly reduced compared with healthy patients. Interestingly, expression of the mesenchymal marker CD90 and the endothelial/pericytic marker CD146 was significantly enhanced when isolated from lipedema patients. *Discussion.* The enhanced number of $CD90^+$ and $CD146^+$ cells could explain the increased cell yield because the other tested surface marker were not reduced in lipedema patients. Because the cellular mechanism and composition in lipedema is largely unknown, our findings might contribute to a better understanding of its etiology.

Key Words: *adipogenesis, adipose tissue, adult stem cells, CD146, lipedema, stromal vascular fraction*

Introduction

Lipedema is a progressive disease characterized by subcutaneous bilateral deposition of adipose tissue in the extremities and buttocks [1,2]. In 60% of lipedema cases, genetic background with familial predisposition has been described [3]. In a recent study, we observed that 89% of lipedema patients had maternal and paternal predisposition over three generations [4]. The genetic background of lipedema was demonstrated in a clinical report with 330 family members, although the involved genes have not been completely identified [5]. Patients suffer for a long time because their symptoms, such as reduced joint mobility, hematoma, and edema, are frequently misdiagnosed with adipositas or lipohypertrophy [6–8]. In fact, the increase in fatty tissue is a consequence of adipocyte hypertrophy and hyperplasia [9] accentuated by alterations of the connective tissue [10]. Furthermore, the enhanced adipocyte growth leads to capillary permeability and insufficient lymphatic backflow associated with hematoma [11]. Lipedema symptoms have primarily been treated with compression [12] or lymph drainage [13–15]. Because lipedema does not respond

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to diets, drugs or physical activity, the only efficient treatment option with demonstrated long-term benefit to date [16] is micro-cannular liposuction in tumescent anesthesia [17–20]. Additionally, radial shockwave therapy is able to enhance lymphatic decongestion in lipedema after micro-cannular liposuction [19], and water-jet-assisted liposuction provides long-term improvement by preserving lymph vessels in lipedema [21].

Lipedema is a hormone-related disease mainly affecting women starting in puberty, after pregnancy or during menopause [5,22]. Estrogen contributes to decreased lipolysis in the gluteal, compared with the abdominal, region due to a distinct pattern of estrogen receptors [23]. Moreover, estrogen regulates bone morphogenetic protein (BMP)-2, which can stimulate adipogenesis but inhibits it by knockdown PPARgammma2; thus, PPARgamma2 may play a role in BMP-induced adipogenesis [24]. However, the exact etiopathogenesis of this disorder is still largely unknown. Examination of adipose tissue of lipedema patients exhibit differences in adipocyte morphology. Suga *et al.* analyzed adipose tissue of lipedema patients and observed infiltration of macrophages and the presence of necrotizing adipocytes. In parallel, enhanced proliferation capacity of adipose-derived stem/progenitor/stromal cells ($Ki67^{+}CD34^{+}$ cells) was described, which seems to promote adipogenesis [25]. Additionally, hypertrophy-induced hypoxia and subsequently enhanced angiogenesis of pathologic vessels might contribute to capillary permeability [26,27]. This would explain the fluid increase in the interstitium and consequently the emerging orthostatic edema.

All in all, little is known about the cell composition including the properties of adult stem and precursor cells in lipedema. The aim of this study was to investigate adipose tissue–derived cells obtained from lipedema patients by liposuction compared with healthy patients. Because adipose tissue is a highly vascularized tissue containing a broad variety of regenerative cells, we concentrated on the adipose-derived stromal vascular fraction (SVF) including adipose-derived stromal/stem cells (ASC). Cells isolated from liposuction material from healthy and lipedema patients were analyzed regarding phenotypic and functional criteria for the identification of adipose-derived cells as defined in the statement of the International Federation for Adipose Therapeutics and Science (IFATS) together with the International Society for Cellular Therapy (ISCT) [28].

Methods

Liposuction

The collection of human adipose tissue was approved by the local ethical board with patients' written consent. Dermal and subcutaneous white adipose tissue was obtained during routine outpatient liposuction procedures from the hips and outer thighs ("saddlebags") under local tumescence anaesthesia. Table I provides patient characteristics. Tumescence solution contained one vial Volon-A 10 mg (Dermapharm), three vials Suprarenin 1 mg/mL (Sanofi), 45 mL bicarbonate 8.4% (Fresenius Kabi) and 60 mL Xylocaine 2% including Lidocaine 0.04% (Astra Zeneca). The harvesting cannulas were triport and 4 mm in diameter (MicroAire System power-assisted liposuction).

Table I. Characteristics of healthy and lipedema patients.

Characteristics	Healthy	Lipedema
N	22	30
Sex	Female	Female
Age (years)	42 ± 10	41 ± 13
Weight (kg)	75 ± 17	91 ± 18
BMI (kg/m^2)	27 ± 6	33 ± 7
Type II	—	3%
Type III	—	5%
Type I–III	—	10%
Type I–IV	—	82%
Stage 2	—	55%
Stage 2–3	—	24%
Stage 3	—	21%

SVF/ASC isolation

SVF isolation was performed as modified from Wolbank *et al.* [29]. Briefly, 100 mL of liposuction material was transferred to a blood bag (Macopharma) and washed with an equal volume of phosphate-buffered saline (PBS) to remove blood and tumescence solution. Afterward, for tissue digestion PBS was replaced with 0.2 U/mL collagenase NB4 (Serva) dissolved in 100 mL PBS containing Ca^{2+}/Mg^{2+} and 25 mmol/L N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES; Sigma), and the blood bag was incubated at 37°C under moderate shaking (180 rpm) for 1 h. The digested tissue was transferred into 50-mL tubes. After centrifugation at 1200 *g* for 7 min, the cell pellet was incubated with 100 mL erythrocyte lysis buffer for 5 min at 37°C to eliminate red blood cells. The supernatant was aspirated after centrifugation for 5 min at 500 *g*. The pellet was washed with PBS and filtered through a 100-µm cell strainer (Greiner). After another centrifugation step at 500 *g* for 5 min, the supernatant was removed, and the isolated SVF cells were cultured in endothelial growth medium (EGM-2) at 37°C, 5% CO_2 and 95% air humidity or resuspended in EGM-2 for further analyses. After seeding the SVF on plastic surface in expansion medium (EGM-2), the adherent cell fraction including ASC could outgrow as an adherent monolayer and was cultured to a subconfluent state. Medium was changed

every 3–4 days. Cells were detached with 1× trypsin/EDTA at 37°C and collected in a tube. After centrifugation, the pellet was resuspended in EGM-2 medium, and cells were quantified with trypan blue exclusion in a cell counter (TC-20, Bio-Rad). Adherent SVF from passage 0 were used for analysis of the adenosine triphosphate (ATP) concentration and immunophenotype of cells after 1 week in culture as well as the differentiation potential, whereas freshly isolated SVF was used for all other experiments.

Cell yield and viability

Cell number was determined using trypan blue exclusion and quantification in a cell counter (TC-20, Bio-Rad). For measuring cell viability, the percentage of living cells compared with total cell count was analyzed with the cell counter.

Cellular ATP

To determine the cellular ATP concentration CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used and performed according to the manufacturer's instructions. Freshly isolated SVF and cells after 1 week in culture were seeded at a density of 1×10^4 cells per well in a black 96-well plate (Greiner) in 100 µL EGM-2 medium. After 2 h, 100 µL CellTiter-Glo Reagent were added to each well, and the plate was gently agitated on a shaker for 2 min. Afterward, the plate was incubated for 10 min in the dark. The luminescent signal was detected with an Infinite M200 Multimode Microplate Reader (Tecan) at an exposure time of 2000 ms and correlated to an ATP standard curve.

Colony-forming unit fibroblast assay

A defined number of SVF cells (4, 20, 100, 500, 2500, 12 500) was seeded in each well of a six-well plate (Sarstedt) and cells were cultured in EGM-2 for 14 days. Medium was changed on day 7 after cell seeding. After 14 days of culture, cells were fixed with 4% formaldehyde and stained with hematoxylin. The cells were then washed with tap water and stained with eosin solution (Roth/Lactan). The percentage of cells that formed visible colonies was calculated and compared with the total number of seeded cells.

Proliferation

Proliferation potential was analyzed by determining the population doubling level (PDL). Freshly isolated SVF cells were seeded at a density of 5×10^5 cells per T-25 culture flask and cultured in EGM-2 medium. Medium was changed every 3–4 days. When cells had reached a subconfluent state, they were passaged, and cell number was determined as described earlier. For further analysis of PDL, ASC were seeded at a density of 5×10^4 in T-25 culture flasks and cultured until passage 3. Cell number was determined after each passage.

Flow cytometry analysis

Freshly isolated SVF cells and after 1 week in culture were characterized using the following antibodies: CD14-FITC (BD), CD31-FITC (eBiosciences), CD34-APC (BD), CD45-PerCP (BD), CD73-FITC (eBiosciences), CD90-PE (eBiosciences), CD105-PE (eBiosciences) and CD146-PerCP (R&D). For staining, 5×10^5 cells in 50 µL PBS with 1% fetal calf serum (FCS; PAA) were incubated with 5 µL primary labeled antibodies at room temperature for 15 min in the dark. Cells were washed with 1.5 ml Cell Wash (BD) and centrifuged for 5 min at 400 *g*. The supernatant was discarded and the cell pellet resuspended in 300 µL 1× Cell Fix (BD; diluted 1:10 with aqua dest). Samples were analyzed on a FACSCalibur (BD).

Osteogenic differentiation and detection

For osteogenic differentiation, cells were seeded at a density of 2×10^3 cells per well in a 24-well plate in EGM-2 medium and incubated over night. The next day, medium was changed to osteogenic differentiation medium Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Lonza) containing 10% FCS, 2 mmol/L L-glutamine (PAA), 100 U/mL penicillin/streptomycin (Lonza), 10 nmol/L dexamethasone (Sigma), 150 µmol/L ascorbat-2-phosphate (Sigma), 10 mmol/L β-glycerophosphate (StemCell Technologies) and 10 nmol/L dihydroxy vitamin D3 (Sigma) or control medium consisting of DMEM/F12/L-glutamine (Lonza) with 10% FCS and 100 U/mL penicillin/streptomycin. Medium was changed every 3–4 days. After 21 days, osteogenic differentiation was analyzed with alizarin red staining and quantification, as well as determination of intracellular alkaline phosphatase (ALP) activity. For alizarin red staining of calcified structures, cells were fixed for 1 h with 70% ethanol at −20°C and stained with alizarin red solution (Merck) for 15 min. For quantitative analysis of alizarin red staining, the supernatant was discarded, and the cells were incubated with 500 µL 20% methanol and 10% acidic acid (diluted in aqua dest) for 15 min. After resuspension, the mixture of cells and methanol/acidic acid was transferred to a transparent 96-well plate (100 µL per well). The absorbance was measured at 450 nm with an Infinite M200 Multimode Microplate Reader. The second method to analyze osteogenic differentiation is the detection of the activity of intracellular ALP. Cells were incubated with 100 µL PBS for 1 h at −20°C. The cells were then lysed for 1 h with the addition of 100 µL

PBS containing 0.5% TritonX-100 (Sigma). For quantitative detection of ALP activity, 100 µL of substrate solution (4-nitrophenylphosphate) were added in each well and incubated for 1 h in the dark. Finally, the solution was transferred to a transparent 96-well plate (100 µL per well), and absorbance was measured at 405 nm together with a reference wavelength of 620 nm in an Infinite M200 Multimode Microplate Reader. By creating a standard curve with known p-nitrophenol concentrations diluted in a solution containing 0.5% TritonX-100 diluted in PBS 1:2 and measuring the corresponding absorption, the ALP activity of the samples was calculated.

Adipogenic differentiation and detection

For adipogenic differentiation, cells were seeded at a density of 1.4×10^4 cells per well in a 24-well plate in EGM-2 medium and incubated over night. The next day, medium was changed to adipogenic differentiation medium DMEM-high glucose (Lonza) containing 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin/streptomycin, 1 µmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX; Sigma), 10 µg/mL insulin (Sigma) and 100 µmol/L indomethacin (Sigma) or control medium consisting of DMEM/F12/L-glutamine with 10% FCS and 100 U/mL penicillin/streptomycin. Medium was changed every 3–4 days. After 21 days, adipogenic differentiation was analyzed with oil red O staining and quantification. Cells were fixed with 4% formaldehyde for 1 h. After washing with aqua dest, the cells were rinsed with 70% ethanol for 2 min and stained for 5–15 min with oil red O solution (Sigma). Then the cells were washed with aqua dest, counterstained for 1–3 min with hematoxylin solution and blued with tap water. For quantitative

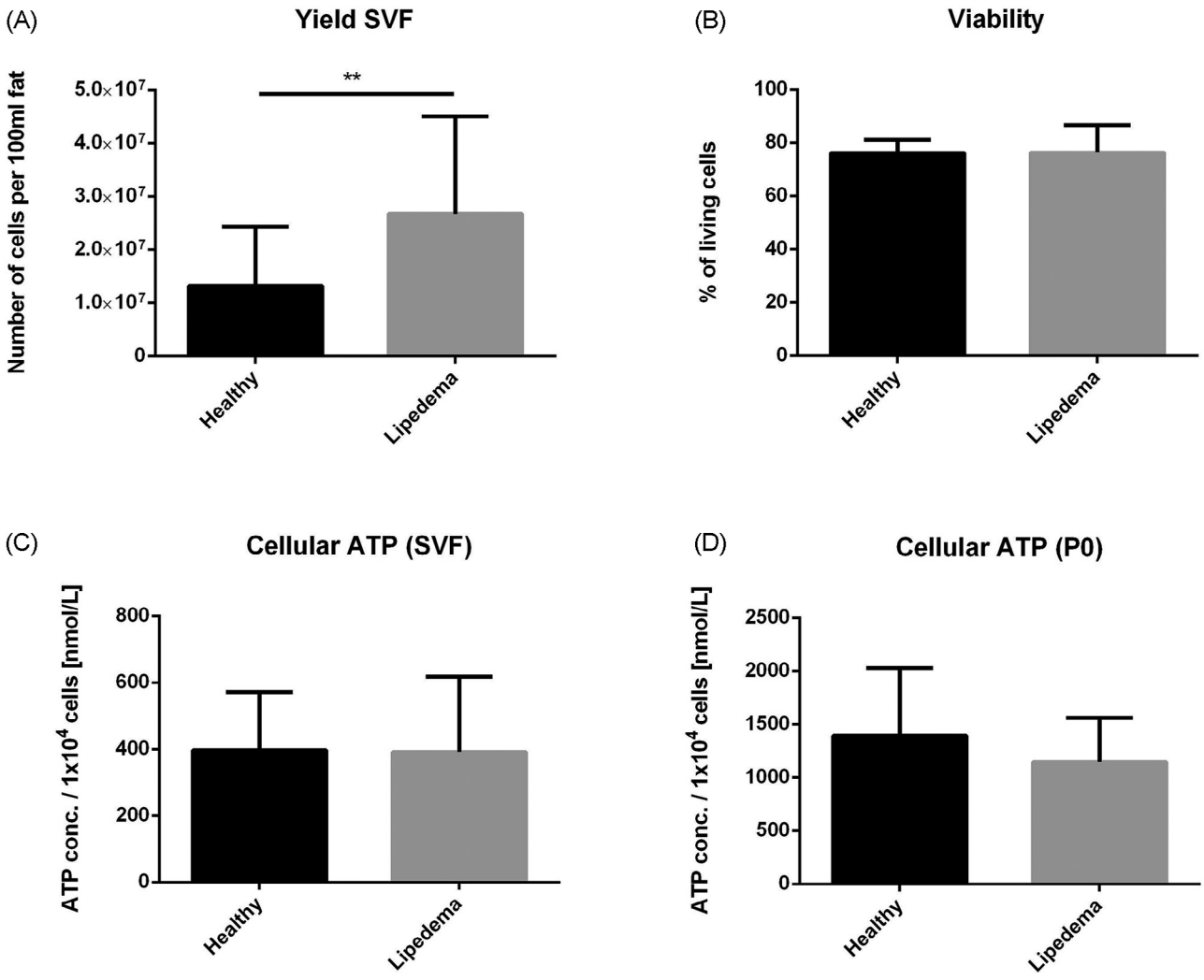

Figure 1. Cell yield, viability and ATP concentration. The SVF cell yield from liposuction material was significantly enhanced for lipedema patients compared with healthy patients (A) (n = 22 and 30), whereas there was no difference in viability (B) (n = 19 and 29) and ATP concentration of cells directly after isolation (C) (n = 13 and 19) and after 1 week in culture (D) (n = 9 and 12). $^{**}P < 0.01$.

detection of oil red O staining, the supernatant was discarded, and 500 µL isopropanol were added in each well. After resuspension, the mixture of cells and isopropanol was transferred to a transparent 96-well plate (100 µL per well). The absorbance was measured at 510 nm with an Infinite M200 Multimode Microplate Reader.

Chondrogenic differentiation and detection

For chondrogenic differentiation and three-dimensional micromass pellet cultures, 3×10^5 cells were centrifuged in chondrogenic differentiation medium consisting of hMSC Chondro BulletKit (Lonza) with 10 ng/mL BMP-6 (R&D) and 10 ng/mL transforming growth factor-ß3 (Lonza) in screw cap microtubes. The tubes were placed in an incubator at 37°C, 5% CO_2, and 95% humidity with slightly open cap for gas exchange. After 2 days, the pellets were transferred to 96-well U-bottom plates (Greiner) with fresh medium. Medium was changed every 2–3 days. After 35 days of differentiation, micromass pellets were fixed in 4% phosphate-buffered formalin overnight for histological analysis. The next day, the pellets were washed in 1× PBS and dehydrated in increasing concentrations of alcohol. After rinsing the pellets in xylol and infiltration with paraffin, deparaffinized sections were stained with alcian blue for 30 min and counterstained for 2 min with hematoxylin. For collagen type II staining, sections were treated with pepsin for 10 min at 37°C (AP-9007 RTU, Thermo Scientific). Endogenous peroxidase was quenched with freshly prepared 3% H_2O_2 for 10 min at room temperature, followed by normal horse serum 2.5% (Vector RTU) to block unspecific binding. Sections were incubated 1 h with monoclonal anti-collagen type II (MS-306 P0 Thermo Scientific) at 1:100. After washing with TBS, sections were incubated with the secondary antibody (anti-mouse DAKO EnVision + System HRP-labeled Polymer, Dako) for 30 min and rinsed in TBS again. Bindings were visualized using Nova Red (SK4800 Vector Labs) for 6 min. Counterstaining was performed with hematoxylin for 2 min.

Adipose tissue biopsies

Adipose tissue biopsies were excised from healthy and lipedema patients and stored in fixation buffer. Dehydration, paraffin embedding and staining with hematoxylin and eosin (H/E) were performed by the lab Dr. Edith Beck, institute for cytological, histological and bacteriological investigations (Linz, Austria).

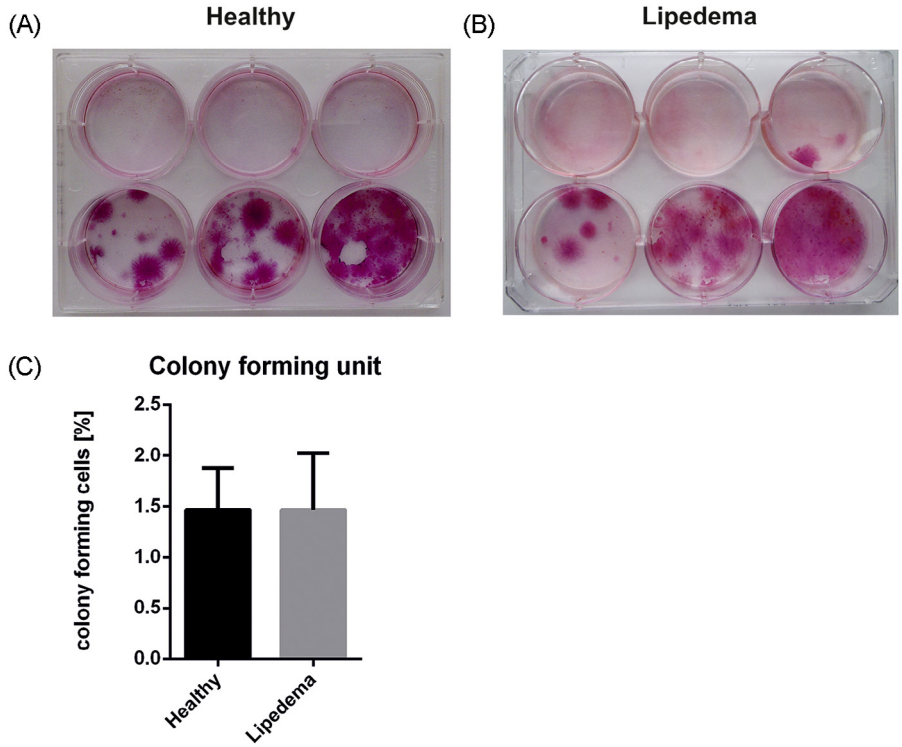

Figure 2. Colony-forming unit fibroblast assay. SVF cells derived from healthy and lipedema patients were seeded at defined numbers of cells (4, 20, 100, 500, 2500, 12 500), and the resulting colonies after 2 weeks cultivation were stained with hematoxylin/eosin (representative pictures) (A, B). Quantitative analysis revealed no difference in the colony-forming potential of cells from healthy and lipedema patients (C). n = 3 and 9.

Statistical analysis

Data are presented as mean ± SD and statistical analysis was performed using PRISM6 (GraphPad), parametric two-tailed *t*-test or one-way analysis of variance Tukey's post hoc. *P* values < 0.05 were considered to be significant.

Results

SVF isolation from lipedema patients results in a higher cell yield

Comparison of the SVF cell yield per 100 mL of liposuction material revealed a significantly increased cell number when isolated from lipedema patients. Adipose tissue from lipedema patients yielded double the amount of cells compared with adipose tissue from healthy patients (Figure 1A). Viability of the freshly isolated cells was similar for healthy and lipedema patients (Figure 1B). ATP concentration of freshly isolated cells (Figure 1C) and after 1 week in culture (Figure 1D) was also similar for healthy and lipedema patients, but higher after 1 week in culture. Analysis of the colony-forming capacity after 2 weeks in proliferation medium revealed no difference between cells derived from healthy or lipedema patients (Figure 2A–C).

Morphology of the isolated SVF cells was analyzed on day 3 (Figure 3A,B) and on day 7 (Figure 3C,D) of culture. Both cells from healthy and lipedema

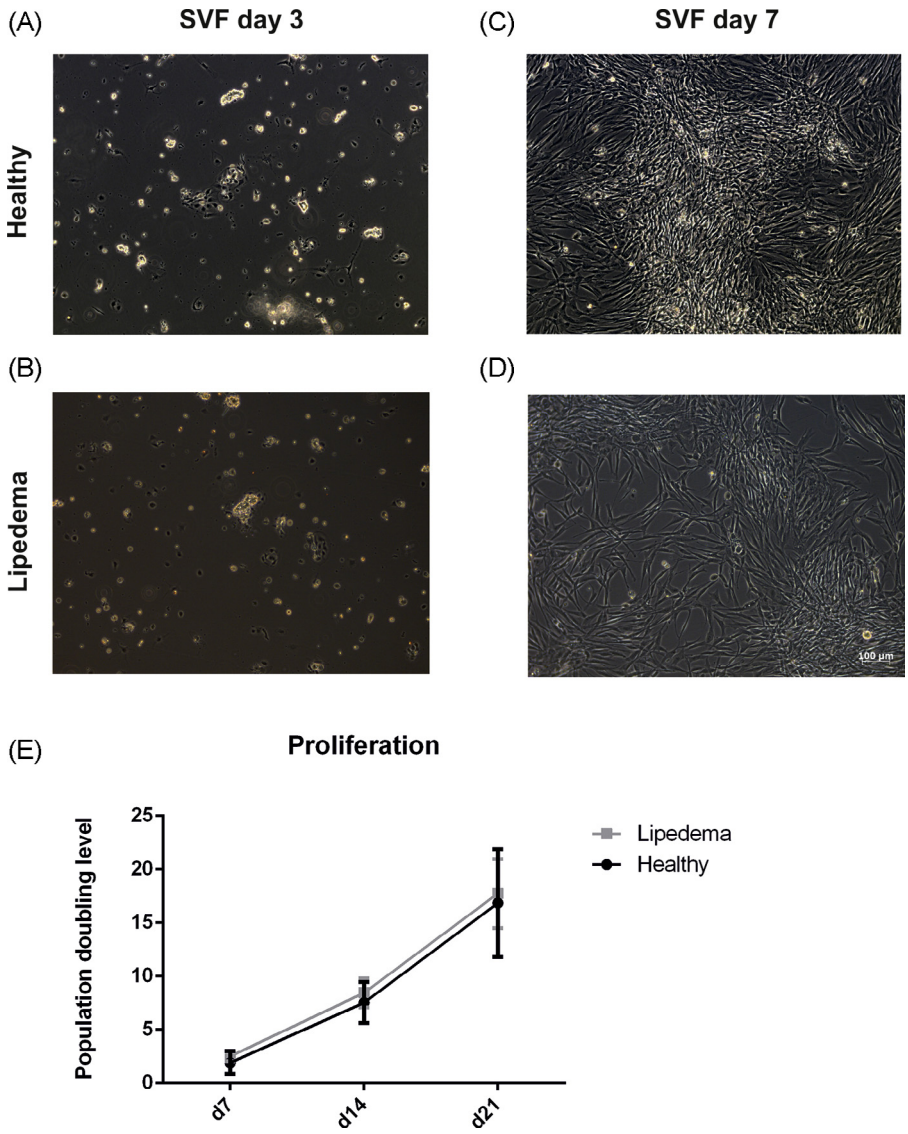

Figure 3. Morphology and proliferation potential. SVF cells derived from healthy and lipedema patients were examined by light microscopy after 3 (A, B) and 7 days (C, D) in expansion medium. Cells from both healthy and lipedema patients adhered to the plastic surface and showed spindle-shaped cell morphology. Size bar = 100 µm (A–D). Analysis of the proliferation potential after 7, 14 and 21 days in expansion medium showed similar PDL of cells derived from healthy and lipedema patients (E). n = 12 and 11.

patients were able to adhere to the plastic surface and showed characteristic ASC spindle-shaped cell morphology on day 7 (Figure 3A–D). Cells from lipedema patients showed a minor but not significant increase of the PDL after 7, 14 and 21 days (Figure 3E).

Immunophenotype is similar for healthy and lipedema patients

The composition of the cellular phenotype in the SVF was similar for healthy and lipedema patients as analyzed by surface marker expression (Figure 4A). Directly after SVF isolation, there was a minimal percentage of the monocyte/macrophage marker CD14 for healthy and lipedema patients. A higher percentage of SVF cells expressed the endothelial marker CD31 and the hematopoietic progenitor/vascular endothelial marker CD34. Less than 32% of SVF cells were positive for the hematopoietic marker CD45 for healthy and lipedema patients.

The percentage of mesenchymal marker was >30% for CD73 and CD105 and >50% for CD90 for SVF cells derived from healthy and lipedema patients. The expression levels of CD73 and CD105 were similar for healthy and lipedema patients. However, the number of CD90 cells was 12% higher for lipedema patients compared with healthy patients. Similarly, the expression level of the endothelial/pericytic marker CD146 was 20% higher for lipedema patients compared with healthy patients.

After 1 week in culture, the composition of the cellular phenotype was also similar for healthy and lipedema patients (Figure 4B). There was still a minimal percentage of cells positive for the monocyte/macrophage marker CD14 for healthy and lipedema patients. The endothelial marker CD31 and the

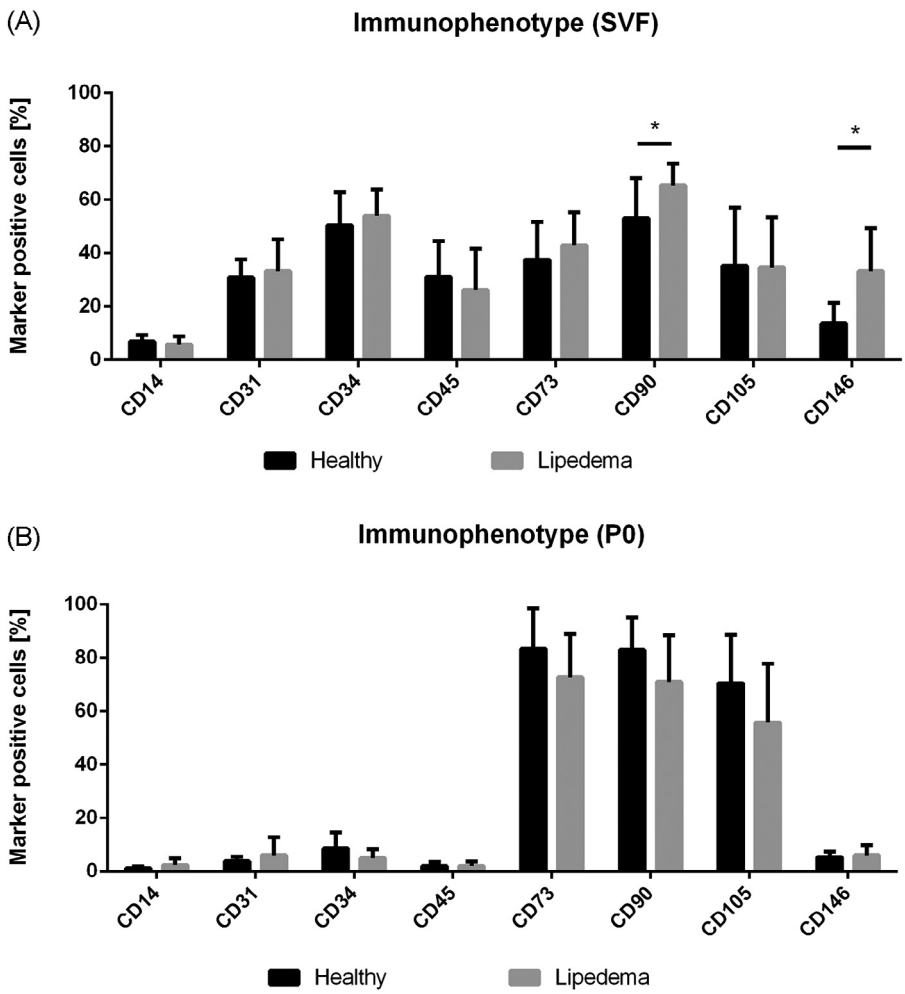

Figure 4. Surface marker profile of isolated cells. The composition of the cellular phenotype of SVF cells was similar between healthy and lipedema patients directly after isolation (A) (n = 3 and 20) and after 1 week in culture (B) (n = 3 and 5) as analyzed by flow cytometry of surface marker expression. The percentages of the mesenchymal stromal cell marker CD90 and the endothelial/pericytic marker CD146 were significantly increased in freshly isolated cells from lipedema patients compared with healthy patients (A). $^{\star}P < 0.05$.

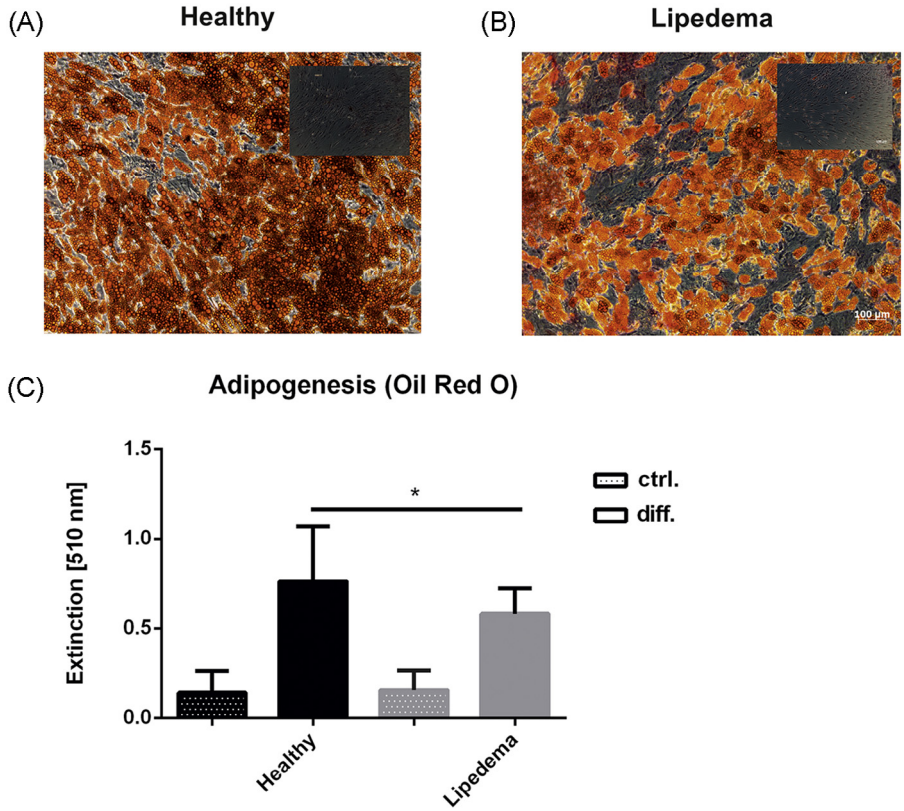

Figure 5. Adipogenic differentiation potential of isolated cells. *In vitro* adipogenic differentiation analyzed by oil red O staining 3 weeks after adipogenic induction was lower for lipedema patients compared to healthy patients (A, B), which was confirmed through quantitative analysis of the staining (C). Under differentiation conditions, the degree of lipid accumulation was significantly reduced in cells from lipedema patients compared with cultures from healthy patients. Small inserts show cells in control media (A, B). Cells in differentiation conditions showed a significant enhancement compared with undifferentiated cells in control media (C). ctrl., cells in control media without growth factors and stimuli; diff., cells in differentiation media. Size bar = 100 µm; n = 11 and 13. $*P < 0.05$.

hematopoietic progenitor/vascular endothelial marker CD34 were both reduced in regard to freshly isolated cells for healthy and lipedema patients. A minimal percentage of cells derived from healthy and lipedema patients expressed the hematopoietic marker CD45 after 1 week in culture.

In contrast to freshly isolated cells the percentage of mesenchymal stromal cell marker was clearly enhanced. For healthy patients, more than 80% of cells were positive for CD73 and CD90 and approximately 70% expressed CD105. For lipedema patients, more than 70% of cells expressed CD73 and CD90 and approximately 55% were positive for CD105. In contrast, the expression level of the endothelial/pericytic marker CD146 was reduced after 1 week in culture for healthy and lipedema patients.

Differentiation potential of cells derived from lipedema patients

Cells from healthy and lipedema patients were analyzed for their *in vitro* adipogenic, osteogenic and chondrogenic differentiation potential. Interestingly, cells from lipedema patients showed a reduced potential for adipogenic differentiation analyzed by oil red O staining (Figure 5A,B). This observation was confirmed by quantitative analysis of the staining with a significantly higher extinction for healthy patients than for lipedema patients (Figure 5C). Upon osteogenic induction, cells from healthy and lipedema patients showed equally high levels of alizarin red staining (Figure 6A,B), which was verified through quantitative analysis (Figure 6C). Similarly, analysis of ALP activity did not show a difference between healthy and lipedema patients (Figure 6D). Moreover, cells derived from both healthy and lipedema patients demonstrated a high chondrogenic differentiation potential illustrated by strong collagen type II and alcian blue staining of micromass pellets (Figure 7A–D). Analysis of the diameter area of the three-dimensional micromass pellets after 7, 14, 21, 28 and 35 days did not show significant differences for healthy and lipedema patients (Figure 7E).

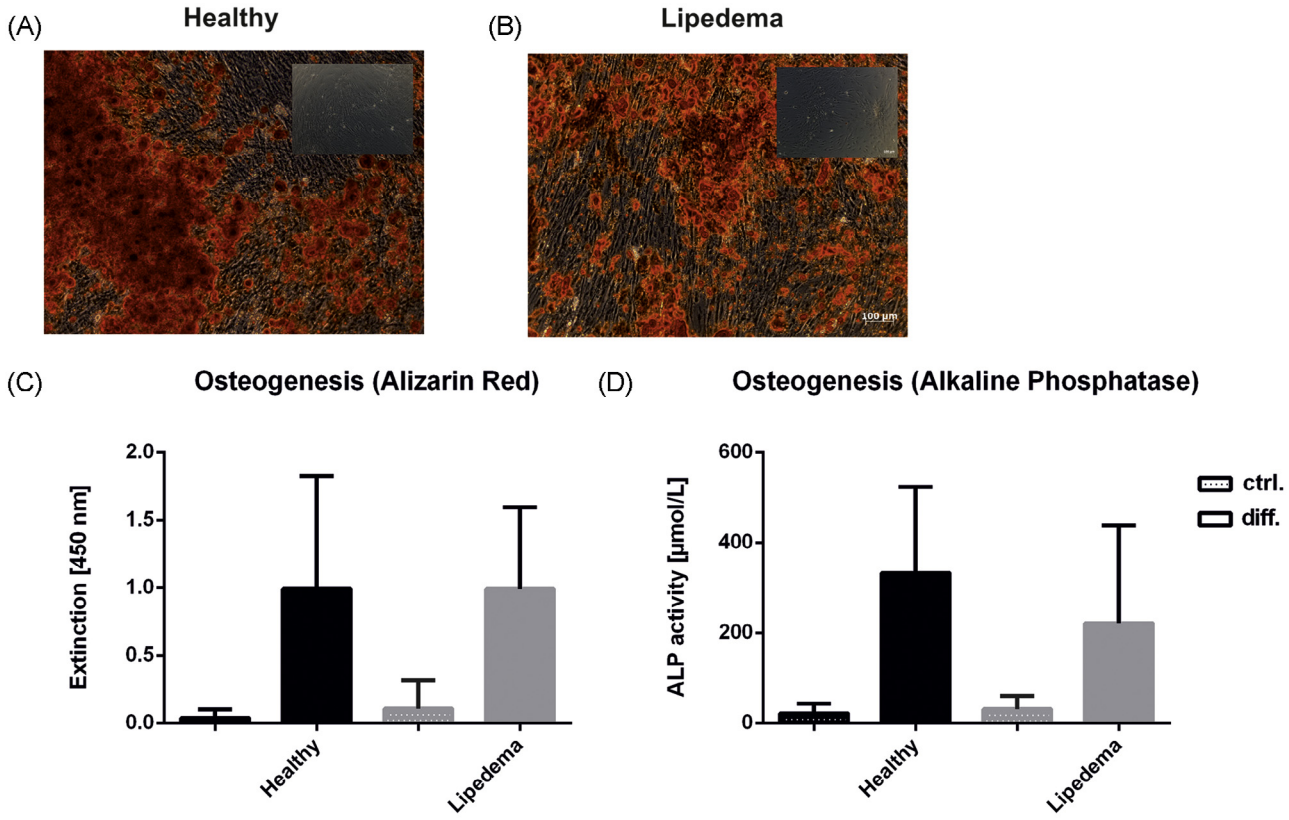

Figure 6. Osteogenic differentiation potential of isolated cells. *In vitro* osteogenic differentiation analyzed by alizarin red staining after 3 weeks of induction was similar for healthy and lipedema patients (A, B), which was confirmed through quantitative analysis of the staining (C) (n = 11 and 14) and alkaline phosphatase activity (D) (n = 11 and 13). Small inserts show cells in control media (A, B). Cells in differentiation conditions showed a significant enhancement compared to undifferentiated cells in control media (C, D). ctrl., cells in control media without growth factors and stimuli; diff., cells in differentiation media. Size bar = 100 µm.

Adipose tissue biopsies from healthy and lipedema patients

Adipose tissue biopsies were excised from healthy and lipedema patients and analyzed for their structure. H/E staining of adipose tissue biopsies from lipedema patients shows typical crown-like structures (Figure 8B) compared with biopsies from healthy patients (Figure 8A).

Discussion

Investigating the cell composition and property of lipedema compared with healthy adipose tissue might provide further understanding of this disease and insights into the possible etiology. In this comparison study, liposuction material from 52 women was enzymatically digested and the SVF isolated. Comparison of the SVF cell yield revealed a significantly increased cell number when isolated from lipedema patients, whereas viability and ATP concentration showed no difference compared with healthy patients.

The potential of ASC to differentiate into the adipogenic, osteogenic and chondrogenic lineage (as defined by IFATS and ISCT) [28] could be demonstrated with cells from lipedema as well as healthy patients. However, regarding the adipogenic differentiation potential the degree of lipid vesicle accumulation upon adipogenic induction was significantly reduced in lipedema patients compared with healthy patients. Within the yielded SVF of lipedema patients, significantly higher cell numbers expressing the mesenchymal stromal cell marker CD90 and the endothelial/pericytic marker CD146 were found. In particular, approximately half of these cells are double positive expressing $CD90^{+}CD146^{+}$ (data not shown) indicating the presence of a subpopulation of adipose stem cells originating from perivascular cells [30] and approximately 20% of these cells are $CD146^{+}CD31^{-}CD45^{-}$ (data not shown), a set of markers representing pericyte-like cells [31]. The enhanced number of $CD90^{+}$ and $CD146^{+}$ cells might explain the increased cell yield because the other tested surface marker were not reduced in lipedema patients.

Li *et al.* previously demonstrated that $CD146^{+}$ cells (pericyte subpopulation) exhibit a very low adipogenic differentiation potential compared with $CD31^{-}/CD34^{+}$

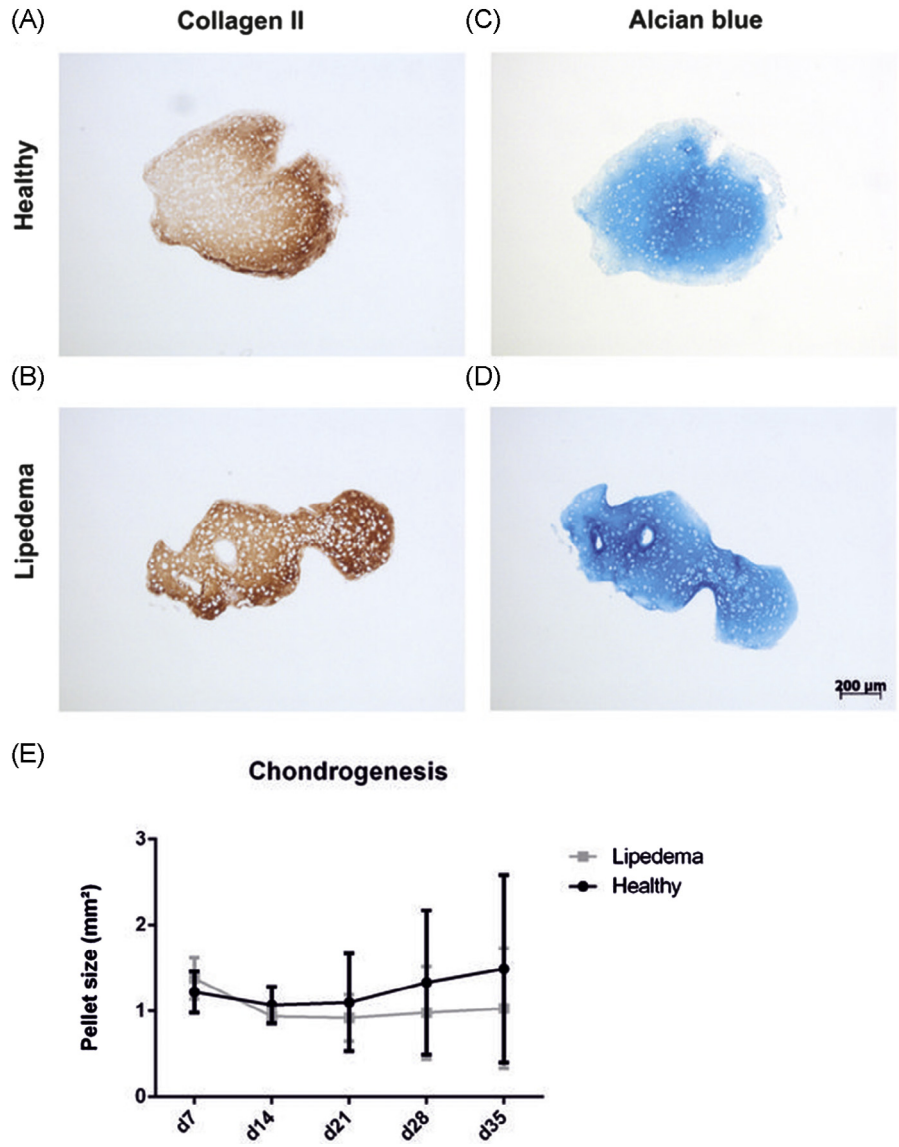

Figure 7. Chondrogenic differentiation potential of isolated cells. Cells derived from both healthy and lipedema patients showed a high chondrogenic differentiation potential when cultured in micromass pellets under inductive conditions for 5 weeks, as illustrated by strong collagen type II (A, B) and Alcian blue (C, D) staining. Analysis of the diameter area of the 3D micromass pellets after 7, 14, 21, 28 and 35 days did not show significant differences for healthy and lipedema patients (E). Size bar = 100 µm; n = 8.

cells (preadipocyte subpopulation) from adipose tissue [32]. Similarly, Hu *et al.* observed that the $CD146^+$ subpopulation of $CD31^-/CD34^+$ cells ($CD31^-/CD34^+/CD146^+$) showed a lower adipogenic potential compared with the $CD146^-$ subpopulation ($CD31^-/CD34^+/CD146^-$) [33]. In contrast, James *et al.* found a higher osteogenic differentiation potential when sorting the SVF for $CD146^+$ cells from adipose tissue of healthy patients [34].

Adipocyte death and subsequent macrophage infiltration in lipedema could be explained by hypertrophy-induced hypoxia [25]. Bilkovski *et al.* also showed in human adipose tissue biopsies that adipocytes are surrounded by macrophages in crown-like structures. These observations were also found in our analyzed lipedema tissue biopsies showing typical crown-like structures. Moreover, macrophages were also found responsible for inhibiting adipogenesis of a mesenchymal precursor cell line via Wnt signaling molecules [35]. CD146 is up-regulated by enhanced tumor necrosis factor (TNF)-alpha level [36], which is secreted by macrophages and adipocytes shown in obese patients [37]. This is consistent with our findings about the increased number of $CD146^+$ cells and enhancement of crown-like structures in adipose tissue biopsies of lipedema patients.

These observations might be due to the fact that edematous tissue contains leaky lymphatic vessels. This

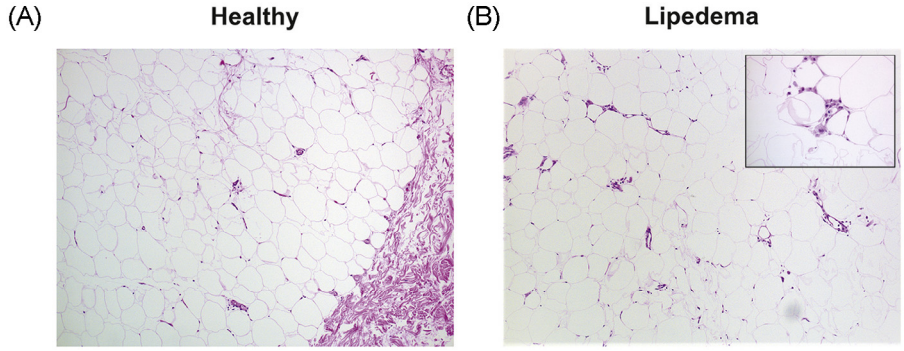

Figure 8. Adipose tissue from healthy and lipedema patients. H/E staining of adipose tissue biopsies from lipedema patients shows typical crown-like structures (B) compared with biopsies from healthy patients (A). Main images are 40× magnification; the small insert is 100× magnification.

leads to enhanced interstitial lymphatic fluid concomitant adipocyte growth, resulting in hypoxia and then microangiopathy, microaneurysm and lymphedema [38–41]. It has long been known that TNF-alpha and interleukin-6 are associated with obesity and type 2 diabetes by suppressing the insulin pathway [42]. Lipedema is a chronic injury of the capillaries that could be demonstrated by enhanced pericyte-like markers, which are responsible for repair of vessel damage after injuries [43].

In conclusion, we demonstrate that SVF cells from lipedema patients meet the characterization criteria for adipose tissue–derived cells postulated by ISCT and IFATS [28]. These cells may hence be a suitable option for autologous tissue regeneration strategies in lipedema patients.

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