AUS DER ABTEILUNG FÜR PLASTISCHE-,
HAND-, UND WIEDERHERSTELLUNGSCHIRURGIE
(PROF. DR. DR. MED. LUKAS PRANTL)
DER FAKTULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

Semi-automated extraction of stromal vascular fraction for autologous cell therapy

Inaugural - Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Alexander Hanke

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2. Summary (German)

Einleitung: Die stammzellreiche Stromal Vascular Fraction (SVF) kann aus Lipoaspirat oder Fettgewebe durch enzymatische Verdau und anschließender Zentrifugation gewonnen werden. Bisher hat sich jedoch weder ein einheitliches Extraktionsverfahren, noch eine gängige Methode zur Anwendung am Patienten durchgesetzt. Ein neues kommerziell erhältliches halbautomatisches System zur Herstellung von SVF verspricht Sterilität, konstante Ergebnisse und Anwendbarkeit im klinischen Alltag. Ziel dieser Arbeit war es die Menge und Qualität der SVF, welche mit diesem System gewonnen werden kann, mit einer etablierten manuellen Labormethode zu vergleichen.

Material und Methodik: Die SVF wurde aus Lipoaspirat sowohl mit einem Prototyp der halbautomatischen UNiStation (NeoGenesis, Seoul, Korea) als auch mittels einer etablierten manuellen Labormethode extrahiert. Nach Lyse der verbliebenen Erythrozyten in der SVF erfolgte eine Messung mittels multiparametrischer Durchflusszytometrie (FACSCanto-II, BD Biosciences). Von Interesse war vorrangig die (Quantität) Gesamtzellzahl des gewonnenen Materials. Zusätzlich wurde die Qualität der SVF anhand des Stammzellmarkers CD34, dem Leukozytenmarker CD45 und dem Marker CD271 für hochproliferative Stammzellen untersucht. Des Weiteren wurden die prozentuale Verteilung dieser Marker in der SVF, doppelt positive Zellen und der stain index ermittelt.

Ergebnisse: Aus Lipoaspirat von sechs Patienten wurde sowohl mit der Maschine (d für „device“) als auch der (manuellen) Labormethode (h für „hand preparation“) eine makroskopisch sichtbare SVF erzeugt. Mit der maschinellen Extraktion war die Zellausbeute pro ursprünglichem Gramm Lipoaspirat jedoch tendenziell geringer (d: $1.1*10^5 \pm 1.1*10^5$ vs. h: $2.0*10^5 \pm 1.7*10^5$; p=0.06). Bei der Zusammensetzung der SVF zeigte sich der Anteil an CD34+ Zellen nach maschineller Extraktion signifikant reduziert (d: 57.3±23.8% vs. h: 74.1±13.4%; p=0.02). Im Gegensatz dazu lag der Anteil an CD45+ Leukozyten tendenziell höher (d: 20.7±15.8% vs. h: 9.8±7.1%; p=0.07). Die Fraktion hochproliferativer CD271+ Zellen zeigte unabhängig von der Extraktionsmethode vergleichbarer Ergebnisse ohne signifikanten Unterschied (M: 13.4±11.6% vs. L: 12.9±9.6%; p=0.74). Es konnte kein Unterschied bzgl. des Anteils doppelt positiver Zellen für CD34+/CD45+ (d: 0.5±0.6% vs. h: 0.3±0.2%; p=0.21)

sowie CD34+/CD271+ (d: 1.9±2.3% vs. h: 2.4±2.0%; p=0.42) festgestellt werden. CD45+/CD271+ doppelt positive Zellen konnten in keiner Probe nachgewiesen werden. Außerdem gab es keinen signifikanten Unterschied bezüglich des Stain Index (p>0.12).

Diskussion und Schlussfolgerung: Das halbautomatisierte System ermöglicht auf kleinstem Raum nennenswerte Mengen an SVF steril zu gewinnen. Diese SVF unterscheidet sich nur geringfügig in der Zusammensetzung gegenüber der manuellen SVF Extraktion. Insgesamt decken sich die Ergebnisse beider Methoden sehr gut mit den aus der Literatur bekannten Werten. Das halbautomatisierte System bietet die Möglichkeit, Forschung und Anwendung der SVF einen Schritt näher in die Klinik zu bringen.

3. Summary

Introduction: The stem cell rich Stromal Vascular Fraction (SVF) can be obtained by enzymatic digestion with a collagenase followed by centrifugation from patients' lipoaspirate or fat tissue. To date neither a standardized extraction method nor a generally accepted application procedure exists for common use on patient. A novel commercially available semi-automated device for the extraction of SVF promises sterility, consistent results and usability in the clinical routine. The aim of this work was to investigate the quantity and quality of the SVF obtained by a semi-automated process in comparison to an established manual laboratory method.

Material and Methods: SVF was extracted from lipoaspirate by a prototype of the semi-automated UNiStation (NeoGenesis, Seoul, Korea) as well as by hand preparation with common laboratory equipment. The SVF was measured by multi-parametric flow-cytometry (FACSCanto-II, BD Biosciences) following the lysis of the remaining erythrocytes. The primary interest was the total cell number (quantity) of the extracted cells. In addition, the quality of the SVFs was investigated using the stem cell marker CD34, the leucocyte marker CD45 and the marker CD271 for highly proliferative stem cells. Furthermore, the distribution of these markers, double positive cells and the stain index were investigated.

Results: Lipoaspirate obtained from six patients was processed with both the novel device (d) as the hand preparation using laboratory equipment (h), always resulting in a macroscopically visible SVF. However, there was a tendency of a fewer cell yield per gram of used lipoaspirate with the device (d: $1.1*10^5 \pm 1.1*10^5$ vs. h: $2.0*10^5 \pm 1.7*10^5$; p=0.06). Regarding the composition of the SVF, the percentage of CD34+ cells was significantly reduced with the device (d: 57.3±23.8% vs. h: 74.1±13.4%; p=0.02). On the contrary there was a tendency to a higher percentage of CD45+ leukocytes (d: 20.7±15.8% vs. h: 9.8±7.1%; p=0.07). The percentage of highly proliferative CD271+ cells was comparable for both methods (d: 13.4±11.6% vs. h: 12.9±9.6%; p=0.74). No significant difference was identified regarding the double positive cell fraction for CD34+/CD45+ (d: 0.5±0.6% vs. h: 0.3±0.2%; p=0.21) and CD34+/CD271+ (d: 1.9±2.3% vs. h: 2.4±2.0%; p=0.42). Double positive cells for CD45+/CD271+ were not detected in any sample. The stain index did not show a significant difference between the two extraction methods (p>0.12).

Discussion: The semi-automated system was able to provide considerable amounts of sterile SVF without requiring much space. The SVF extracted by the semi-automated process showed only little difference in its composition compared with the SVF obtained by the hand preparation. Taken together both methods showed comparable extraction results which are in accordance with the data from literature. This semi-automated system offers an opportunity to take research and application of the SVF one step further to the clinic.

4. Abbreviations

AB	Antibody
APC	Allophycocyanin
ASCs	Human adipose tissue-derived stem cells
ATMP	Advanced therapy medicinal products
CV	Crystal violet
CAL	Cell assisted lipotransfer
DAPI	4′,6-Diamidin-2-phenylindol
FBS	Fetal bovine serum
FSC	Forwardscatter (Flow Cytometry)
HGF	Hepatocyte growth factor
IDO	indoleamine 2,3-dioxygenase
IGF1	Insulin-like growth factor 1
IL10	Interleukin 10
ISCT	International Society for Cellular Therapy
MEM	Minimum eagle medium
mg	Milligram
ml	Milliliter
µg	Microgram
µl	Microliter
P	Passage
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
rpm	Rounds per minute
SVF	Stromal vascular fraction
SSC	Sidewardscatter (Flow Cytometry)
TGFβ	Tissue growth factor β
U	Unit
V	Volume
VEGF	Vascular endothelial growth factor

5. Introduction

5.1. Stem Cells

A defining feature of stem cells is their self-renewal while maintaining the potential to differentiate into various cell types and lineages. However, the potential of stem cells depends on their origin and possible lineage specification during early stages of differentiation: Therefore, zygotes are referred as totipotent, capable of giving rise to any cell of an embryo including extra-embryonic tissue like the placenta. The potential of zygotes to differentiate during embryonic development becomes limited to one of the three germ layers (i.e. ectoderm, mesoderm, endoderm (1)) which is defined as pluri-potency. The multi-potent character of differentiation defines cells that are only able to differentiate into cell types of one germ layer. The following cells are only capable of creating a specific tissue which makes them progenitor cells. This leads to tri-, bi- and finally uni-potential cells which are the basis for mature tissue cells. By reaching the stage of a progenitor cell, they lose their telomerase activity and thus their potential for self-renewal. This restricts them to the Hayflick limit of 50-70 population doublings before cell senescence and programmed cell death (2). However, a limited number of cells keep their properties of self-renewal and differentiation. They are required for continual maintenance and repair of tissues and organs throughout the organism's lifespan and are specified as adult stem cells (3).

5.2. Mesenchymal Stem Cells

There is a hierarchy between adult stem cells, equal to the hierarchy during embryogenesis. Most of them are tissue specific precursor cells with low differentiation potential. They can differentiate into a few cell types only. Some other stem cells have a higher differentiation potential and can differentiate into a variety of cells types. Between the 1960s and 1970s one type of adult stem cells was identified as a subpopulation of bone marrow cells, however different from hematopoietic stem cells. They demonstrated a fibroblast like morphology, culture plastic adherence and osteogenic differentiation potential (4). In 1990, these cells were termed

"mesenchymal stem cells" (MSCs) for the first time by Caplan (5). In 2006, a concise definition was established by the International Society for Cellular Therapy (ISCT). Mesenchymal stem cells were defined as plastic adherent cells with a special phenotype (positive for $CD105^+$, $CD73^+$, $CD90^+$ and negative for $CD45^-$, $CD34^-$, $CD14^-$ or $CD11b^-$, $CD79a^-$ or $CD19^-$ and $HLA\text{-}DR^-$) and with the ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro (6). Nowadays, it is known that MSCs exist in bone marrow and many other tissues, e.g. in adipose tissue, skin, muscle, kidney, dental pulp and the heart (7). Moreover, recent in vitro experiments consistently provided evidence that MSCs can not only differentiate into mesenchymal cell lineages (as the previously named osteoblasts, adipocytes, and chondrocytes) but also into non-mesenchymal cell lineages as skeletal muscle cells, hepatocytes, endothelial or neuronal cells under appropriate culture conditions (8). Growing evidence suggests that MSCs might have a close relation to pericytes (9,10). Their exact origin still remains unclear. However, the interest in MSCs for novel cellular therapies grew over the last decade, accompanied by an increasing number of scientific publications.

5.3. Sources of Mesenchymal Stem Cells

MSCs can be found in various tissues (11) and have been investigated for their capacity of self-renewal and differentiation. For research purpose, and especially clinical application, a minimal invasive, easily accessible and abundant source of MSCs is required. Over the past decades MSCs were primarily isolated from bone marrow aspirates and are referred to as bone marrow mesenchymal stem cells (BMSCs). In 2002 Zuk et. al. (12) published the successful isolation of MSCs from adipose tissue for the first time, and provoked great interest in these cells and especially in their potential for clinical applications. The isolated heterogeneous cell mixture is commonly referred to as stromal vascular fraction (SVF) and the plastic adherent subpopulation as adipose tissue-derived stem cells (ASCs).

There exist various methods and variants for harvesting fat tissue. Direct fat resection from the abdomen is reported to have the highest cell yield compared to axilla and flank adipose tissue (13). However, liposuction appears to be the more suitable extraction technique given the fact that this technique is less invasive for the

patient, along with higher and maintained viability of cells (14). Thus, adipose tissue represents a valuable source for mesenchymal stem cells irrespective of the extraction method.

5.4. MSCs from Bone Marrow and Adipose Tissue

MSCs from bone marrow and adipose tissue share similar cell properties, but are not exactly identical. Therefore, direct comparison of published experimental outcomes is limited. On the one hand, BMSCs and ASCs demonstrate plastic adherence and spindle-like morphology (15), and are both able to differentiate into non-mesenchymal cell lineages as skeletal muscle cells, heart muscle cells and neuronal cells in vitro (16). On the other hand, they show a similar, however not identical expression of surface proteins. For example CD36 and CD49d is expressed on ASCs only, while CD49f, CD104 and CD106 are only present on BMSCs (17). Moreover, another difference is the expression of CD34. While cells of fresh isolated SVF are highly positive for CD34, the resulting ASCs stop expressing this marker and tend to be negative for this surface protein (18), as reported for BMSCs. A new and distinct definition of the SVF and ASCs was established by the International Society for Cellular Therapy in 2013 based on these new insights (19): General markers for stem cells were defined in the SVF (positive for $CD34^+$ and negative for $CD31^-$, $CD45^-$, $CD235a^-$) and ASCs (positive for $CD73^+$, $CD90^+$ and negative for $CD31^-$, $CD45^-$). However, similar beneficial potential have been reported for BMSCs and ASCs despite the differences in surface antigen expression. The beneficial effect of these cells has been explained not only by their differentiation potential but even more by the secretion of soluble factors. On the one hand, there are growth factors like insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF), which are produced by BMSCs as well as by ASCs (20). On the other hand, there are immune-modulative and immune-regulative factors like indoleamine 2,3-dioxygenase (IDO), tissue growth factor β (TGFβ), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF) and interleukin 10 (IL10). These factors are capable to modulate the adaptive immune and innate immune system in various ways (21). Thus, BMSCs and ASCs alter the micro-environment by secreting bioactive proteins

which induce an anti-inflammatory response, and is currently investigated for regenerative therapies (3,22).

A tremendous interest on a regenerative therapeutic strategy exists for example for ischemic diseases, like myocardial infarction. A myocardial infarction causes a cascade of inflammation, scarring and heart failure. MSCs have been reported to limit the extend of cardiac remodeling and improve the ejection fraction in vitro and in vivo, independent of MSCs origin (23). It has been shown, that only 1% of the injected cells are present at the injection site after 24h (24). Therefore, it is suggested that the positive effects are not mainly caused by differentiation of MSCs or contribution of contractile elements. The beneficial effect might be related to the MSCs' paracrine mechanisms that regulate the immune response and also to the secretion of IGF1 and VEGF preventing apoptosis of cardiomyocytes under hypoxia (25).

5.5. Advantages of Stem Cells from Adipose Tissue

Bone marrow is commonly obtained by punctuation of the iliac crest or vertebral bodies. Aspirations of high amounts up to more than one liter are possible (26). However, bone marrow is connected to the blood circulation and higher aspiration volumes cause dilution of the bone marrow with peripheral blood (27). This results in a lower concentration of nucleated cells while harvesting huge volumes of bone marrow. Therefore, it has been suggested, that bone marrow should be aspirated carefully in multiple small fractions of each 1ml to 4ml at every spot and level in the bone marrow (28). In addition, high volume aspirations require general anesthesia since the aspiration volume in local anesthesia is limited to about 40ml (16). Further aspiration is associated with high anxiety and pain of the patient (29). Bone marrow aspirates show a range of $1*10^7$ to $5*10^7$ nucleated cells/ml varying due to dilution (30,31).

The liposuction procedure can be performed to obtain tissue amounts starting from 50ml up to 3000ml whereas the amount of harvested tissue determines anesthesia (i.e. local or general anesthesia) (32). The lipoaspirate (or excised fat tissue) is digested with a collagenase, resulting in a heterogeneous cell mixture which is

referred as stromal vascular fraction (SVF). The concentration of nucleated cells per gram fat or milliliter lipoaspirate has been reported as $1*10^5$ to $5*10^5$ which is lower than reported yields in bone marrow (33–35). However, only 0,001%-0,002% of the nucleated cells in bone marrow represent MSCs, while the SVF contains 1-2% of MSCs (36). Thus, a total number of 100 to 1000 MSCs can be obtained from 1ml bone marrow aspirate but 1g fat tissue or 1ml lipoaspirate can yield 5000 MSCs (16). Taken together, the easy and safe access to fat tissue, the possibility of large-volume harvests and the higher concentration of MSCs underline the favorable usage of adipose tissue for regenerative therapies and tissue engineering.

5.6. SVF and ASCs for autologous therapy

Mesenchymal stem cells based therapies originated from adipose tissue are currently under investigation in pre-clinical and clinical trials. Direct application of heterogeneous SVF after extraction is currently investigated but also ASCs which are selected as a subpopulation from the SVF by cell culture. The application of a selected subpopulation requires a second therapeutically intervention but enables preservation for later application and also in vitro tissue engineering purposes. However, cell culturing bears the risk of contamination and induces significant changes in the expression of surface proteins (18) and the cell phenotype (37). Furthermore, these changes are depending on cell culture supplements or cell culture plate, leading to a high variability between different culture protocols (38). The use of the SVF has become an even more attractive alternative for autologous cell-based therapies since immediate application does not require cell culture procedures.

The application of freshly isolated SVF in a rat model of a chronic myocardial infarction provided evidence that the left ventricular ejection fraction significantly improved from 26.46% (95% confidence interval: 17.48% - 32.02%) to 38.25% (95% confidence interval: 28.77% - 47,73%) three months after treatment. No significant results were documented for the control group in a three month follow up (39).

An increased interest amongst orthopedic and trauma surgeons for ASCs was caused by the discovery of osteogenic differentiation potential of these cells.

Currently, bone autografts from the iliac crest are used for augmentation of damaged or lost bone. The iliac crest represents a limited source and therefore, alternative resources are under investigation. Different types of three dimensional ceramic and biodegradable scaffolds were combined with ASCs and tested in different animal models. An increased vascularization and a better distribution of applied cells within the scaffold has been reported. This effect was also apparent when the scaffolds were combined with freshly isolated SVF highlighting the advantages of SVF for future bone tissue engineering (40).

A positive effect on hypertrophic scars has been shown for both SVF and cultured ASCs in a humanized skin graft model of nude mice. A significant reduction of the scar thickness and the amount of collagen was evident two weeks after the application of either SVF or ASCs compared to a control group. However, the beneficial effect was higher for ASCs due to the fact that the heterogeneous SVF contains cells that have an impact on the scar remodeling capacity of stem cells itself (41).

In addition, freshly isolated SVF has been used for cell assisted lipotransfer (CAL) for soft tissue augmentation. The reason for this is mainly that solely re-injection of lipoaspirate is associated with an unpredictable loss of graft volume between 20% to 90% due to a lack of vascularization accompanied with central necrosis and fibrosis (42). The SVF supported lipo-autograft has been reported with better graft survival, enhanced vascularization and low complication rate (43). Therapeutic options are currently tested for breast reconstruction, breast augmentation, facial lipoarthrophy and other aesthetic or reconstructive indications. Recently, Yoshimura et al. reported promising results concerning engraftment for the treatment of breast implant complications (44). However, further research has to be performed in order to determine reasonable clinical application with standardized protocols and safety guidelines, especially regarding CAL in patients with a tumor history (45).

5.7. Rationale for the use of a Medical Device

SVF-based therapies for clinical use require standardized and predictable results in cell quality and quantity. In addition, extraction of the SVF has to be performed in an operating room with a sterile system preventing contamination (46).

The aim of a medical device for SVF extraction is to provide well defined and even automated extraction steps. This assures that all procedures will be conducted in accordance with the laws for autologous tissue transplantation since National regulations and a European regulation (1394/2007/EC) on advanced therapy medicinal products (ATMPs) exist (45,47). But in every case it is necessary that the application has to be performed in the shortest possible time to reduce the time of the lipo-aspirate and the SVF cells outside the body. Finally, there is a financial aspect regarding the costs of the device and also the consumables that are needed for every single treatment.

In summary, the following demands are required from a medical device:

- Standardized protocol
- Sterility and usage in the operating room
- Fast application regarding patient's safety and law regulations
- Reasonable price for the device and consumables

5.8. Medical devices

Various medical devices were launched during the past years to satisfy the requirements for a cell therapy, whereas every device is based on a different extraction strategy. Aronowitz et Ellenhorn (48) compared four commercially available systems for cell assisted lipotransfer (CAL): In particular, an open manual system including a biosafety hood (Multi Station, PNC), a closed semi-automated processing system (Cha-Station, CHA Biotech), a closed fully automated processing system (Celution, Cytori) and a closed manual processing system (Lipokit, Medi Khan) were compared with each other. While all systems were able to provide a SVF, the overall cell yield and the reproducibility of the results varied significantly between the systems. The best and most constant results ($2{,}41*10^5$ cells per gram

lipoaspirate) were achieved with the fully automated system. The variability of the extracted cell product can be minimized but requires the highest price for the device and the consumables.

The medical device UniStation (NeoGenesis Co. Ltd, Seoul, Korea) which is a semi-automated system for SVF extraction and CAL was characterized and evaluated in the present study. The Extraction results were compared to a common laboratory hand preparation method using identical samples of lipo-aspirate for direct reference purpose. Advantages of this new device are the compact design and the few and easy to handle consumables, which enable the application in every operating room or laboratory. Furthermore, a high amount of 800ml lipoaspirate can be processed at the same time which might be supportive for further research and a more standardized development of cell-based therapies.

5.9. Molecular markers

The main aim of this work was to characterize and compare the extracted SVF population between the novel medical device and a hand preparation method. The identification of stem cells was performed by multi-parametric flow cytometry using different fluorescent dye conjugated antibodies. As proposed by the ISCT (19), a combination of positive and negative molecular markers was chosen in order to obtain as much information as possible with a limited number of antibodies. Antibodies against CD34 and CD271 were chosen since these molecular markers are known to be expressed on mesenchymal stem cells in adipose tissue (49). Furthermore, CD45 was used as a negative control for mesenchymal stem cells since this leucocyte marker is not expressed on stem cells but on leucocytes (6). All applied molecular markers for characterization of extracted cells are described in detail in the following sections.

5.9.1. CD34

The surface protein CD34 is mainly expressed on hematopoietic stem cells and on several other cell lineages and precursor cells (50). 60-80% of the SVF cells are positive for CD34 and are reported to play an important role in vasculogenesis (51).

Interestingly, a CD34 down-regulated expression becomes apparent during in vitro cultivation of fresh isolated SVF (18).

5.9.2. CD45

The transmembrane phosphatase CD45 can be found on all mature blood cells, except erythrocytes and platelets (52) and therefore is often referred to as the leukocyte common antigen. This is why the CD45 antibody was used to identify leucocytes in the SVF but also to confirm no expression on CD34 positive MSCs as reported in various studies (6,53). Thus possible influences of the preparation method on the number of leukocytes could be investigated.

5.9.3. CD271

CD271, also known as LNGFR (low affinity nerve growth factor) or p75NTR (neutrophin receptor), is a surface protein expressed on MSCs with a high proliferative potential (54). This marker was proposed as suitable for the identification of MSCs originating from adipose tissue since $CD271^+$ positive subpopulations were identified in the SVF (55). However, the number of $CD271^+$ positive cells decreases in adipose tissue and consequently in the SVF with patients' age (56).

6. Aim of the study

The aim of the study was to investigate the influence of a new semi-automated process on the cell quality and quantity of stromal vascular fraction (SVF) and adipose tissue-derived stem cells (ASCs) from lipoaspirates. SVF was either isolated by a semi-automated process using a new medical device or by conventional hand preparation using laboratory equipment. The total number of cells was determined in the SVF extracted using both methods. In addition, cell populations of the extracted cells were investigated for three cell surface markers (i.e. CD34, CD45, CD271) by flow cytometry. Moreover, ASCs were cultured from both extraction techniques and characterized for their CFU capacity.

7. Material and methods

7.1. Materials

7.1.1. Cells and tissues

Lipoaspirate	Gained with informed consent from elective tumescent liposuction
Stromal vascular fraction (SVF)	Extracted from lipoaspirate (described in 7.2 Extraction of the stromal vascular fraction)
Human adipose-tissue derived stem cells (ASCs)	Defined as all cells of the SVF that adhere to cell culture plastic

7.1.2. Culture media and supplements

DMEM, Low Glucose, Pyruvate (Gibco®)	Life technologies, Darmstadt, Germany
Minimum Essential Medium Eagle Alpha Modification (α-MEM)	Sigma-Aldrich, Taufkirchen, Germany
Fetal bovine serum (FBS): South America Premium	PAN Biotech, Aidenbach, Germany
Penicillin (10000U/ml) Streptomycin (10mg/ml)	Sigma-Aldrich, Taufkirchen, Germany
GlutaMAX™ Supplement	Life technologies, Darmstadt, Germany

7.1.3. Fluids and other reagents

Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich, Taufkirchen, Germany
Paraformaldehyde (PFA) Powder	Sigma-Aldrich, Taufkirchen, Germany
Crystal Violet Powder	Carl Roth, Karlsruhe, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
Ammonium chloride (NH_2Cl)	Sigma-Aldrich, Taufkirchen, Germany
Potassium hydrogencarbonate ($KHCO_3$)	Sigma-Aldrich, Taufkirchen, Germany
Ethylenediaminetetraacetic (EDTA)	Sigma-Aldrich, Taufkirchen, Germany

7.1.4. Cell Culture equipment

Filtertop cell culture flask, 175cm², sterile	Greiner Bio One, Frickenhausen, Germany
Polystyrene Culture Dish 60X15mm	BD Falcon, Heidelberg, Germany

Polystyrene Culture Dish 100X20mm	BD Falcon, Heidelberg, Germany
Cryogenic Vial self standig 2ml	Simport Scientific, Beloeil, Canada
CoolCell® Alcohol-Free Cell Freezing Containers	PELOBiotech, Planegg, Germany
Tubes, 15 or 50ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio One, Frickenhausen, Germany
Serological pipette 5ml, 10ml or 20ml individually packed	Greiner Bio One, Frickenhausen, Germany
Eppendorf Easypet	Eppendorf, Wesseling-Berzdorf, Germany
Steriflip Filter Unit 50ml 100µm	MERCK Millipore, Schwalbach, Deutschland

7.1.5. Enzymes

Trypsin (EC 3.4.21.4)	PAN Biotech, Aidenbach, Germany
Liberase MNP-S (EC 3.4.24.-)	Roche, Mannheim, Germany

7.1.6. Antibodies and Kits

CD34 R-PE-conjugated mouse-anti-human monoclonal Antibody (clone 563)	BD Pharmingen, Heidelberg, Germany
PE Mouse IgG1, κ Isotype Control	BD Pharmingen, Heidelberg, Germany
PE-Cy™7conjugated Mouse Anti-Human CD45 (clone HI30)	BD Pharmingen, Heidelberg, Germany
PE-Cy™7 conjugated Mouse IgG1 κ Isotype Control	BD Pharmingen, Heidelberg, Germany
CD271 (LNGFR) APC antibodies Human (clone ME20.4-1.H4)	Miltenyi Biotec, Bergisch Gladbach, Germany
IgG1 APC isotype control antibodies	Miltenyi Biotec, Bergisch Gladbach, Germany
AccuCheck Counting Beads	Life technologies, Darmstadt, Germany
5ml Polystyrene Round-Bottom Tube with Cell-Strainer Cap	BD Bioscience, Heidelberg, Germany

7.1.7. Equipment for preparation with a medical device

UNiStation	Neogenesis, Seoul, Korea
UNiSyrige	Neogenesis, Seoul, Korea
Steel Cap for syringe	Neogenesis, Seoul, Korea
Steel transfer piece	Neogenesis, Seoul, Korea
Original-Perfusor Syringe 50ml (Luer Lock)	B Braun, Melsungen, Germany

7.1.8. Software

ScopePhoto (version: 3.0.12.792)	ScopeTek
BD FACSDiva (version: 7.0)	BD Biosciences, Heidelberg, Germany
Office 2007	Microsoft
SPSS Statistics (version 20)	IBM, Chicago, USA
Graph Pad Prism (version 5.01)	Graph Pad Software

7.1.9. General Equipment

Laminar Flow Fume Hood M18	Schulz, Sprockhövel, Germany
Heracell™ 240i CO_2 Incubator	Thermo Scientific, Dreieich, Germany
Inverse mikroscope “Wilovert S” with 8M Pixels camera	Hund, Wetzlar, Germany
BD FACSCanto II Flow Cytometer	BD Biosciences, Heidelberg, Germany
Water bath WB10	Memmert, Schwabach, Germany
Tubes, 50ml, PP, graduated, conical bottom, blue screw cap, sterile	Greiner Bio One, Frickenhausen, Germany
Serological pipette 5ml, 10ml or 20ml individually packed	Greiner Bio One, Frickenhausen, Germany
Eppendorf Easypet	Eppendorf, Wesseling-Berzdorf, Germany
Steriflip Filter Unit 50ml 100µm	MERCK Millipore, Schwalbach, Deutschland
Shaker ("Schüttler") SM30 with Incubator ("Inkubationshaube") TH 30	Edmund Bühler, Hechingen, Germany
Precicion scale EW 6200-2NM	Kern, Balingen, Germany
Multifuge 3s, table centrifuge	Thermo Scientific, Dreieich, Germany
Biofuge fresco, for 24x2ml	Thermo Scientific, Dreieich, Germany
Vortex Genie 2	Scientific Industries, Bohemia, New York, USA
Neubauer improved, depth 0,1mm	Marienfeld Superior, Lauda-Königshofen, Germany

7.2. Extraction of the Stromal Vascular Fraction

Patients undergoing elective liposuction gave written consent for harvesting subcutaneous fat tissue which was in accordance with the guidelines of the Declaration of Helsinki for biomedical research and approved by the institutional ethics committee of the University Medical Center of Regensburg (Nr. 08/117). Lipoaspirate was obtained by tumescent infiltration followed by liposuction (Figure 1).

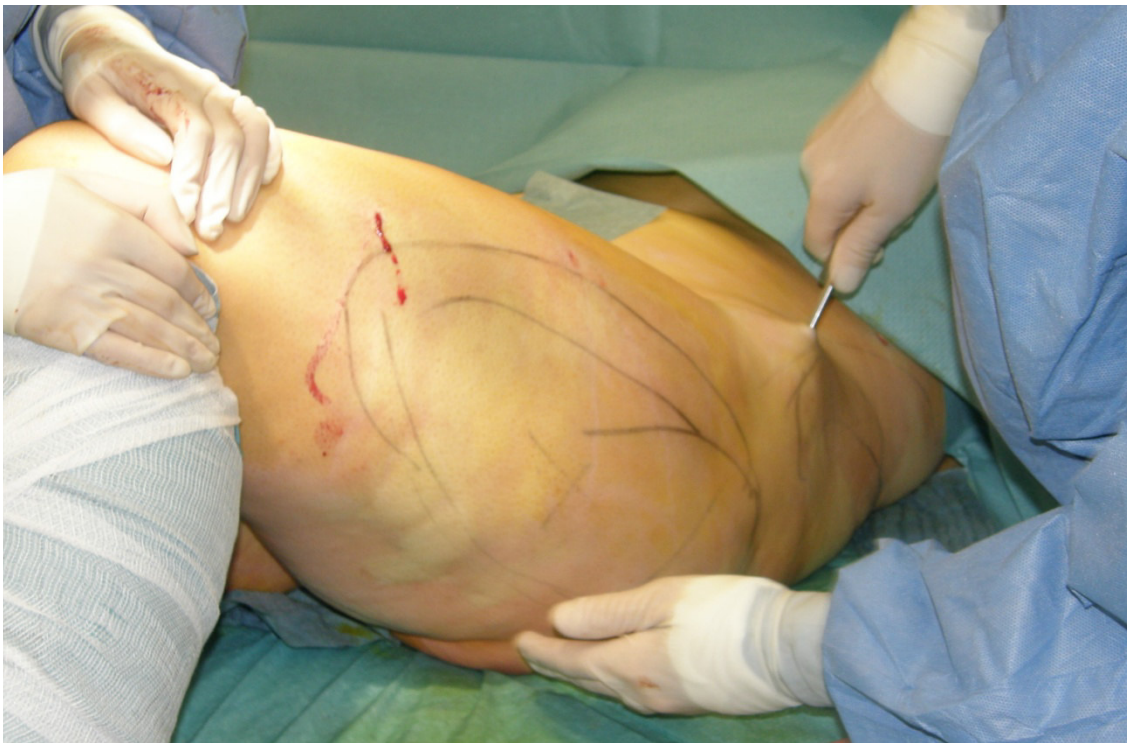

Figure 1 Liposuction procedure after tumescent infiltration at the left outer thigh. The area of liposuction is marked prior to the operation in accordance with patients request for aesthetic outcome. The liposuction cannula is maintained parallel to the surface during the suction procedure while the left hand is providing direction guidance.

The lipoaspirate contains rests of tumescent, debris, blood, oil and various cells integrated in fat tissue. These cells are released after tissue digestion with the MNP-S Liberase (Roche). Washing and centrifugation of digested tissue provide an inhomogeneous cell product consisting of lymphocytes, progenitor cells, mesenchymal stem cells and erythrocytes which is referred to as stromal vascular fraction (SVF).

The extraction was performed by "Hand preparation" with common laboratory equipment and by a "Preparation with a medical device" using the UniStation

(NeoGenesis Co. Ltd, Seoul, Korea), a prototype of a heatable centrifuge with a shake setting. Both will be described precisely in the following sections and are based on two crucial steps:

1. Disintegration of the cells by digestion with a collagenase (MNP-S Liberase, Roche)
2. Isolation of the cells by centrifugation

SVF sample pairs were obtained since 80 ml lipoaspirate from each patient was extracted by both methods using 40 ml by each method. Flow cytometry was applied for all samples and pairs. The extraction methods were compared regarding overall cell number, the percentage of single positive cells, double positive cells and the stain index of the investigated markers (i.e. CD34, CD45, CD271 as described previously in 5.9 Molecular markers used in this work). In addition, SVF samples from both extraction procedures were cultured in order to obtain ASCs which were characterized by their CFU Capacity.

The order of the methods and the outcome parameters used in this work are shown below in a flow diagram (Figure 2).

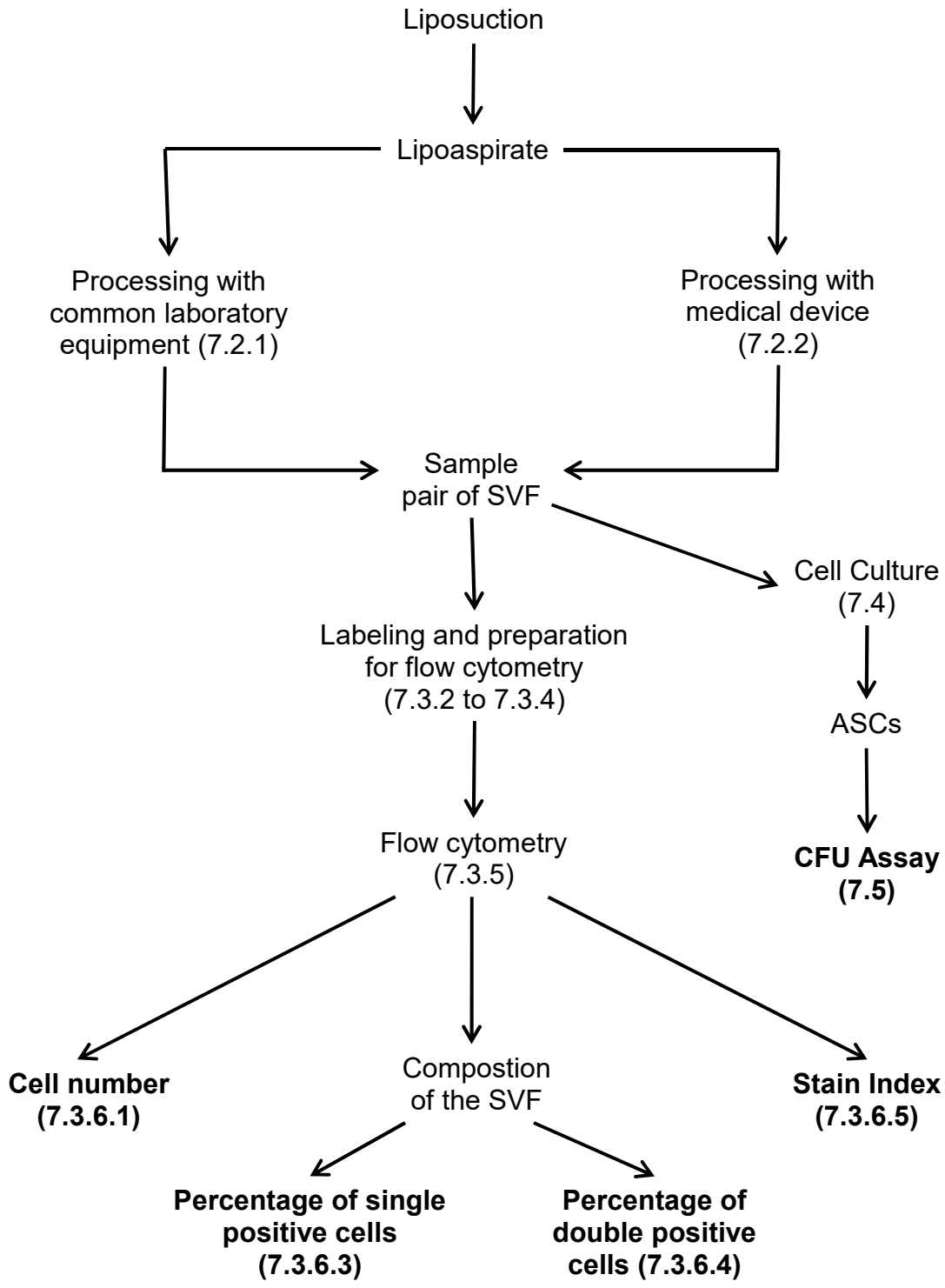

Figure 2 Flow diagram of the used methods and outcome parameters (marked bold).

7.2.1. Hand preparation

7.2.1.1. Description

Common laboratory equipment was used, in particular a sterile fume hood, a shaking incubator, a sterile filtering system, sterile serological pipettes and sterile centrifugation tubes.

7.2.1.2. Protocol

A total of 40ml lipoaspirate was processed and for better handling separated into 20ml portions. Each of the 20 ml portions was placed into sterile centrifugation tubes of 50ml volume and equal volume of DMEM mixed with 2.5 mg to 5 mg of the MNP-S Liberase were added (i.e. 0.125mg-0.25mg MNP-S Liberase per 1 ml of lipoaspirate). Immediately after incubation, the sample was incubated on a shaker with 100rpm at 37°C for 45min. The digested tissue was vigorously pipetted up and down ten times with a 25ml serological pipette to release remaining tissue bound cells. The suspension was filtered through a 100µm sterile filter system and centrifuged at 500g for 5min. The supernatant containing tumescent, debris and blood with an oil layer on top was carefully removed with a serological pipette without disturbing the cell pellet. The remaining pellet containing the SVF was re-suspended in 10ml of PBS. The steps of centrifugation, discarding of the supernatant and re-suspension in PBS were performed two more times until the SVF pellet was clean and neither oil nor tissue residue remained.

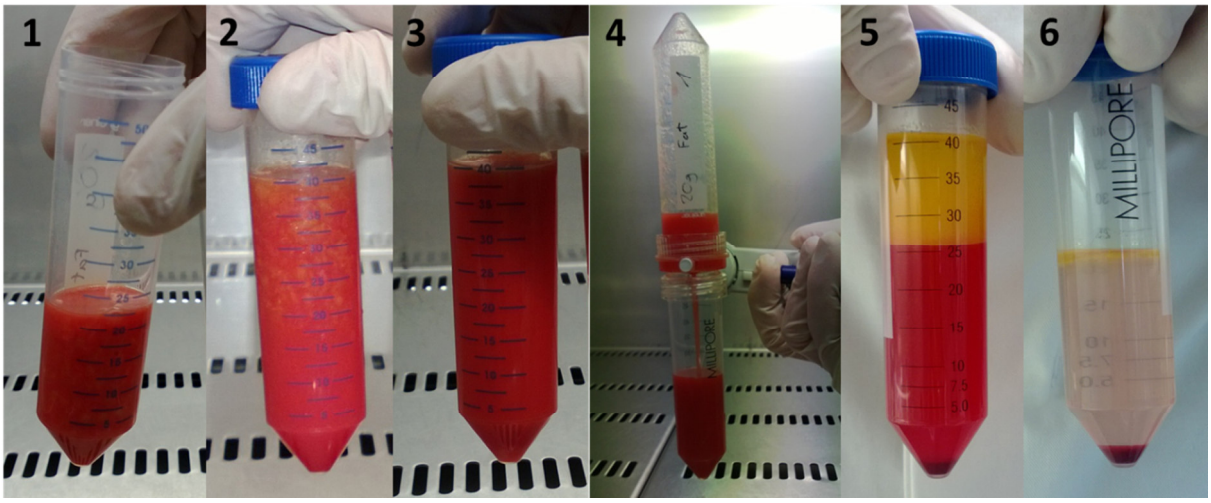

Figure 3 Hand preparation. 20ml of lipoaspirate (1) are mixed with equal volume of 20ml DMEM and 2.5-5mg of MNP-S Liberase (2). The tissue is digested after 45min of incubation at 37°C and shaking at 100rpm (3) and can be filtered through a sterile 100µm filter system (4). After the following centrifugation, the oily and fluid phase on the top can be clearly distinguished (5). After discarding of the supernatant and washing with PBS, the pellet representing the SVF is clearly visible on the bottom of the centrifuge tube (6).

Afterwards, 5ml of culture medium (αMEM containing 20% FBS) were added to inhibit the activity of the MNP-S Liberase. The tube was centrifuged at 300g for 5 minutes, and the supernatant was removed with a serological pipette. The resulting SVF pellet was immediately re-suspended in PBS for further experiments or in culture medium for in vitro experiments. After re-suspending in culture medium, the cell suspension was plated in a cell culture flask and handled as described in 7.4 Cell culture. Cells from the SVF which were able to adhere to cell culture plastic under cell culture conditions (7.4.1 Medium and culture conditions) were defined as ASCs.

7.2.2. Preparation with a medical device

The medical prototype device UniStation (NeoGenesis Co. Ltd, Seoul, Korea) for semi-automated extraction of the SVF was used in order to investigate consistent extraction results (i.e. cell count, cell composition, cell viability).

7.2.2.1. Description of the used tools

The medical device contains a heatable centrifuge (Figure 4) with a shaking plate that can be placed on top of the centrifuge (Figure 5). The sterile syringes used for processing provide a standard Luer Lock (ISO 594-1:1986) for connection with sterile

needles, sterile caps, and a sterile transfer piece to connect two syringes (Figure 6). Therefore, a closed sterile compartment can be established between the syringes and the transfer piece. A detachable handpiece can be connected to the plunge by a thread and is removed prior to centrifugation to fit into the centrifuge.

A four sequence program was used for the extraction of the SVF and is specifid in detail in Table 1. The medical device is preheated to 37°C prior to extraction process and temperature is maintained at this level for all steps.

Figure 4 The interior of the medical device shows the centrifuge with 16 slots for syringes. The heater is located behind the silver surroundings.

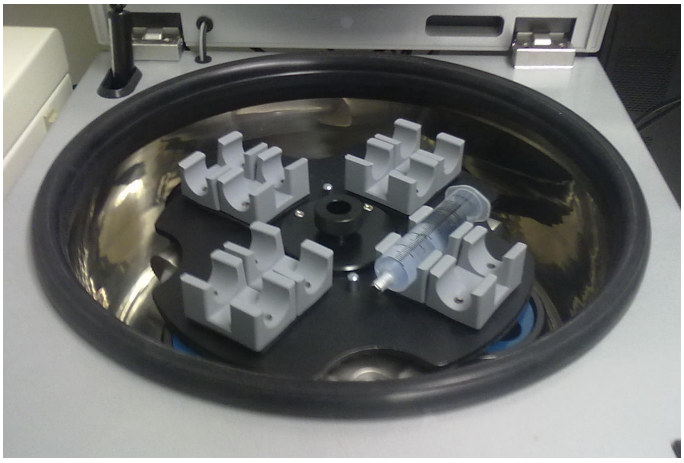

Figure 5 Shaking plate placed on top of the centrifuge for the incubation step (program A2) offering space for eight syringes. No centrifugation steps can be performed when the shaker is installed.

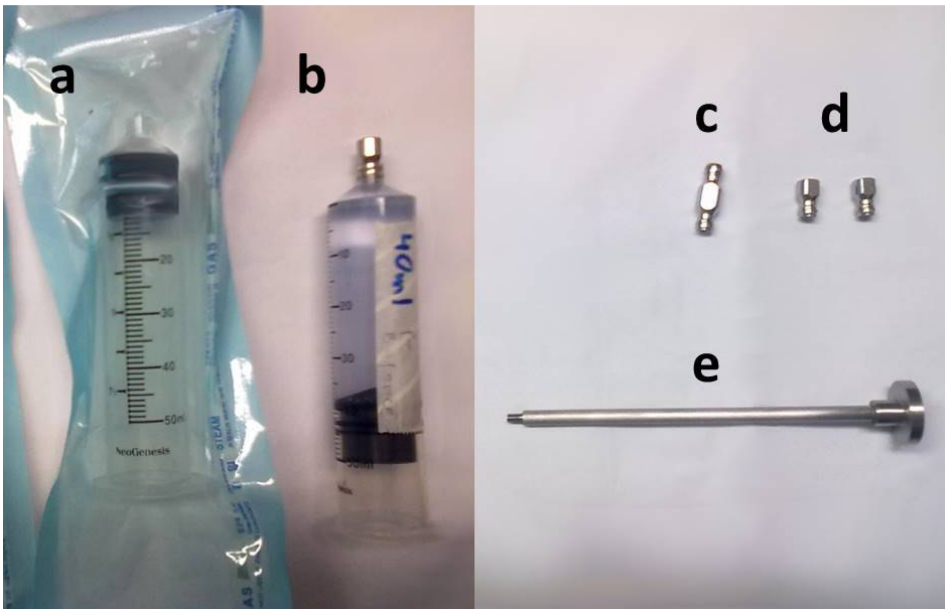

Figure 6 Syringe and transfer system to establish a sterile compartment for the extraction of the SVF. The syringes are separately packed (a) and can be filled with up to 50ml (b). The plunge (e) can be unscrewed to save space in the centrifuge. The Caps (d) and the transfer piece (c) all have the standardized Luer Lock system.

Table 1 Different programs / steps that are performed for the extraction of the SVF with short description of the action.

Program / Step	Action
A1 - Fat washing	Centrifugation at 700g for 5min
A2 - Shaking incubation	Shaking incubation: The shaking plate is added to the top of the centrifuge and is alternately turning at 2g for 30min
A3 - Separation of the SVF	Centrifugation at 800g for 5min
A4 - Washing the SVF	Centrifugation at 800g for 3min

7.2.2.2. Protocol

40ml of lipoaspirate were transferred into a sterile syringe and closed with a cap. After the centrifugation (Program A1) the lower part containing blood and tumescent was discarded (Figure 7) whereas solid tissue and oil remained in the syringe. 5-10mg of the MNP-S Liberase was dissolved in an extra syringe with as much DMEM was required to reach the syringe volume of 40ml after discarding the lower part. Thereafter, both syringes were connected with a transfer piece and the solution containing the MNP-S Liberase was added to the remaining tissue (Figure 8).

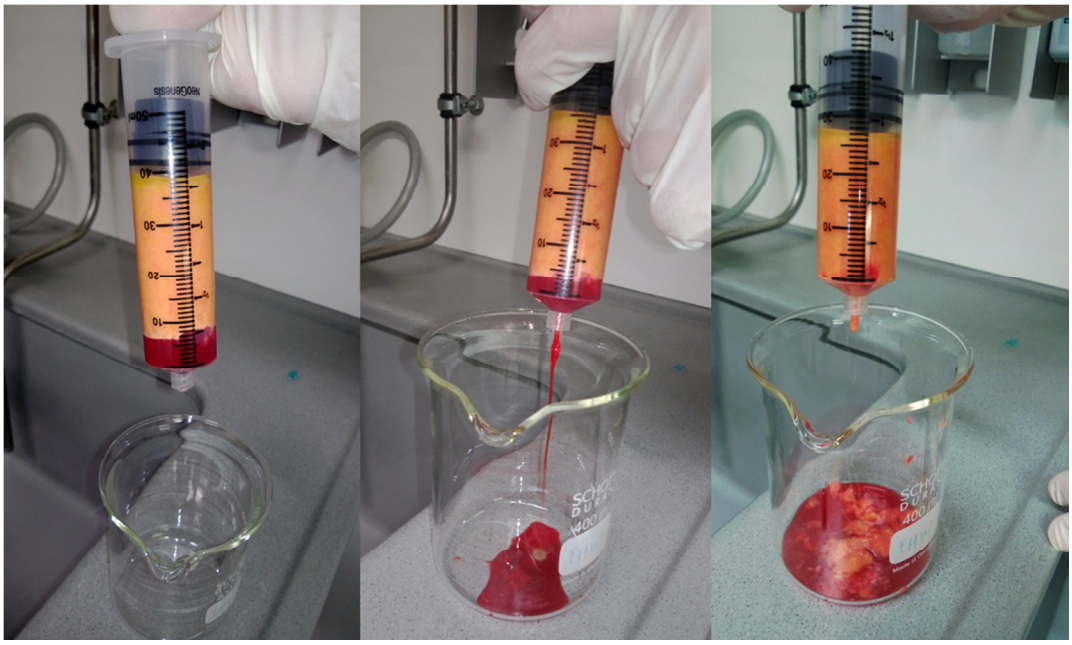

Figure 7 Discarding of tumescent solution and blood accumulated in the lower part of the syringe after the first centrifugation step of the lipoaspirate. Solid tissue and oil remained in the syringe for further processing.

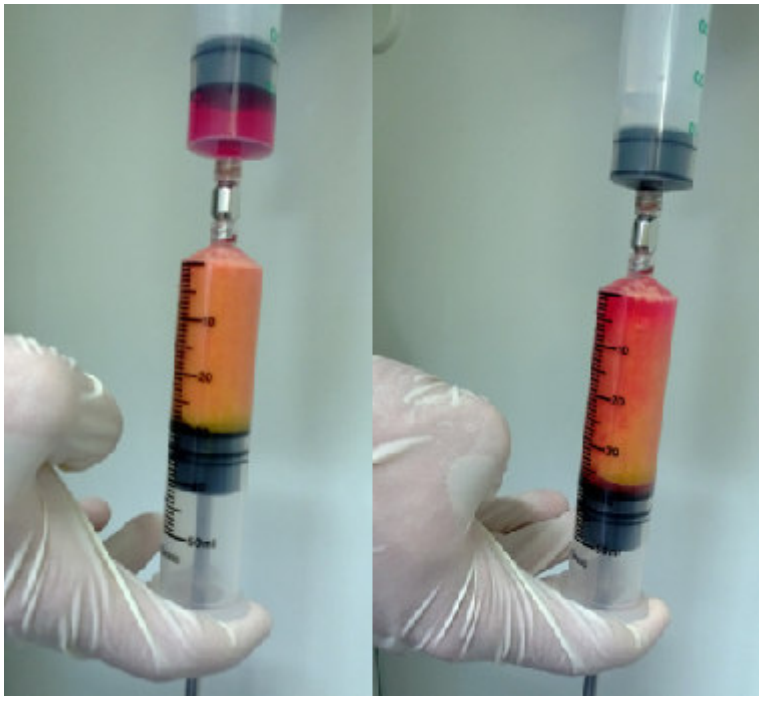

Figure 8 The collagenase was added to the centrifuged lipoaspirate: The upper syringe contains DMEM with 10mg of MNP-S Liberase with a volume that is required to obtain 40ml in the lower syinge containing the lipoaspirate after the first centrifugation step and removal of the lower part. Both are connected via the silver transfer piece and the MNP-S Liberase solution is added by applying slight pressure on the plunge of the upper syringe.

The shaking plate was installed to the centrifuge in order to start the digestion of the tissue with Program A2. Afterwards, the shaking plate was removed and the syringe was inserted into the centrifuge again (Program A3). After this centrifugation, the oil was separated from the layer of tumescent, blood and the SVF at the bottom (Figure 9).

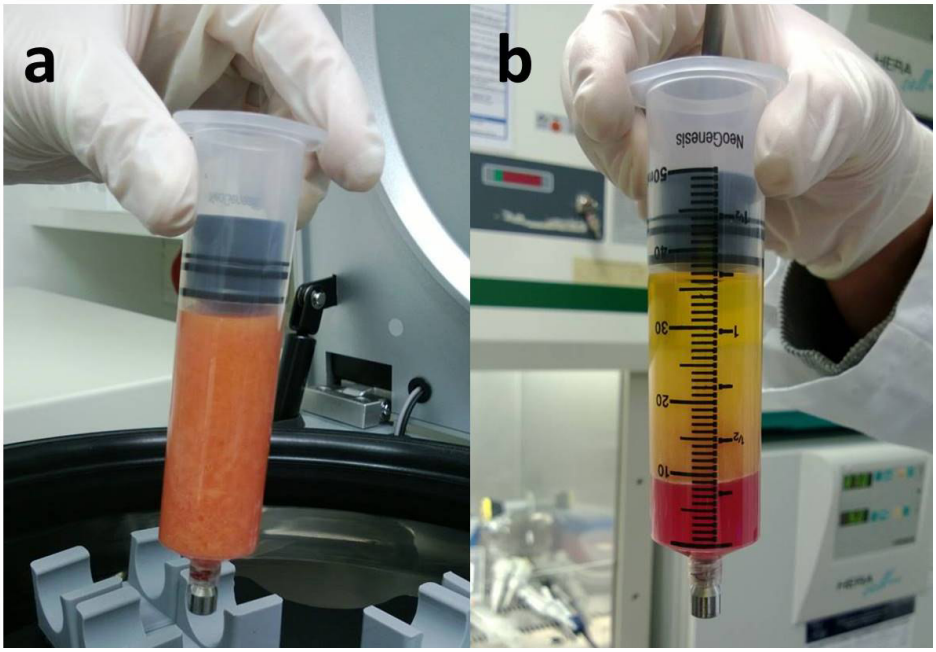

Figure 9 Digested tissue before (a) and after centrifugation (b). The content is separated into an upper oil layer, a layer of tumescent, blood and a small SVF pellet at the bottom.

Thereafter, the syringe was uncapped without disturbing the separated layers. The plunge was pulled up 1mm to prevent cell loss in the syringe cap while uncapping as described in the manual and demonstrated in an instructional video by the manufacturer (57). The syringe was carefully connected to the transfer piece with a second empty syringe. The lower 5ml containing the SVF were transferred to the new syringe and the primary syringe was removed with the transfer piece from the secondary syringe. The remaining tissue in the primary syringe was discarded. 35ml of PBS were added to the secondary syringe, containing the SVF. The final volume reached 40ml and the syringe was centrifuged for the last time (Program A4).

After centrifugation, the SVF was apparent in the lower 5ml of the syringe and was transferred to a sterile 50ml centrifugation tube (Figure 10). The MNP-S Liberase activity was inhibited by adding 5ml of culture medium (αMEM containing 20% FBS). The tube was centrifuged at 300g for 5minutes, and the supernatant was removed

with a serological pipette. The resulting SVF pellet was immediately re-suspended in PBS for further experiments or in culture medium for cultivation purpose as described in 7.4 Cell culture. Cells from the SVF that were able to adhere to cell culture plastic under cell culture conditions (7.4.1 Medium and culture conditions) were defined as ASCs.

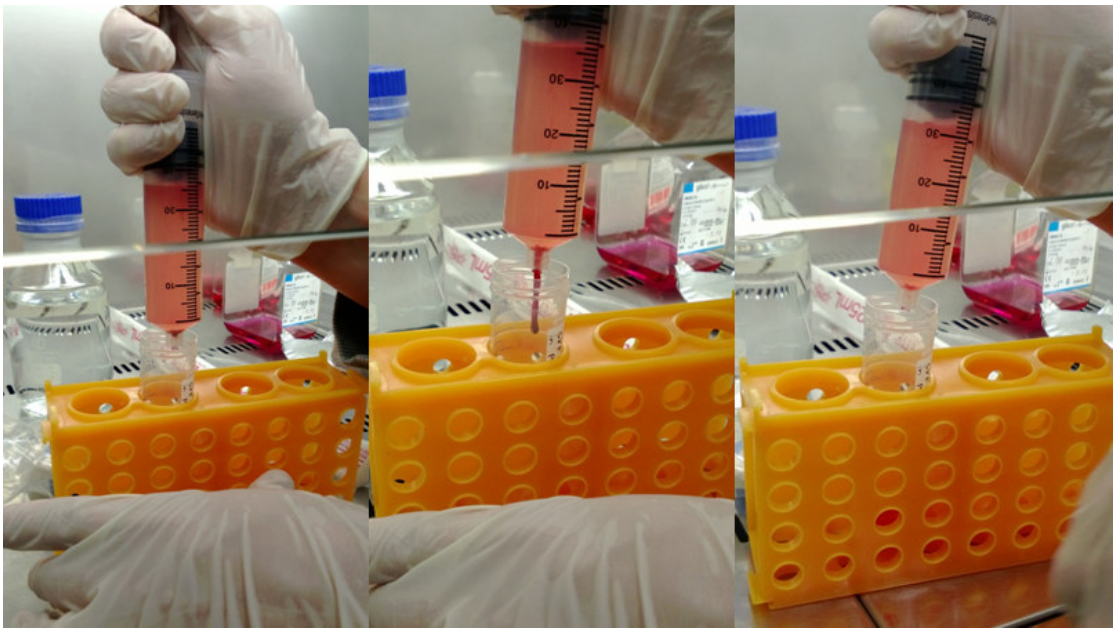

Figure 10 Transferring the SVF into a 50ml centrifugation tube. The SVF is evident as a small red pellet at the bottom of the syringe and is released with the lower 5ml of PBS.

7.3. Flow Cytometry of the SVF

7.3.1. Technique of Flow Cytometry

The cell pellet is resuspended and the cell suspension is pumped through a steadily reducing cannula to a diameter where all cells are tightly connected. A laser beam of defined wavelength is adjusted to the cannula and deflected by passing cells. Different detectors are able to measure the resulting light or fluorescence. One detector, directed along with the laser beam, called forward scatter (FSC), and another detector, directed perpendicular to the laser beam, called sideward scatter (SSC). Moreover, fluorescence detectors are included into the system to detect different wavelengths (multi-parameter flow cytometry). While the FSC signal correlates mainly with the volume, the SSC signal correlates with the granularity of the passing cells. The fluorescence detectors can provide further information about the cell, when fluorescent substances or antibody markers are used. All detector information, gained from a single passing cell, is combined and described as an event. The result is an accumulation of events corresponding to the cells in the suspension.

7.3.2. Experimental setting

Three antibody markers were used to determine the different surface proteins expressed by cells of the SVF. In particular, CD34, CD45 and CD271 were examined and are summarized in Table 2. Each marker was conjugated to a different fluorescent dye. R-Phycoerythrin (PE) Phycoerythrin-Cy7 (PE-Cy7) and Allophycocyanin (APC) were used because of their different emission spectrum. The combinations of antibodies with the corresponding fluorescence and the excitation and emission wavelengths are listed in Table 2.

Table 2 Short description of the examined surface proteins and specifications of the fluorescents conjugated to the used antibodies.

Surface protein	Short description of the surface protein	Fluorescent conjugated to the surface protein antibody	Excitation wavelength (nm)	Emission wavelength (nm)
CD 34	Expressed mainly by hematopoietic progenitor cells but also different kinds of mesenchymal stem cells.	R-Phycoerythrin (PE)	480, 565, 743	767
CD 45	Called the "leucocyte common antigen" (LCA) and is expressed an all human leukocytes.	Phycoerythrin-Cy7 (PE-Cy7)	480, 565	578
CD 271	Also known as LNGFR (low-affinity nerve growth factor receptor) it can be found on mesenchymal stem cells with high proliferative potential	Allophycocyanin (APC)	650	660

Counting beads were added since the concentration of cells in the SVF was of interest and the flow cytometer only counts events. Counting beads have a special FSC and SSC signal that made bead-events clearly distinguishable from cell-events. Two different types of beads with different FSC and SSC signals were used in a 1:1 ratio. To calculate the cell concentration (C_{cells}), the number of cell-events (N_{cells}), the number of bead-events (N_{Beads}) and the known concentration of beads in the suspension (C_{Beads}) are required according to the following formula:

$$\mathbf{(1)} \quad C_{cells} = N_{cells} * C_{Beads} / N_{Bead}$$

Cell concentration per gram original lipoaspirate ($C_{cells/gram\ lipo}$) was of interest for better comparability given the fact that the cell concentration varies by the processed amount of lipoaspirate. Thus, the volume of PBS in which the SVF was re-suspended (V_{PBS}) and the initial mass of the lipoaspirate (m_{lipo}) was recorded besides the previously identified cell concentration (C_{cells}). In case only a part of the SVF was used, the partition of the SVF used for the experiment (p) was also taken into

account. In case the whole SVF was used the variable p equals 1. The cells per gram lipoaspirate were calculated using the following equation:

$$(2) \quad C_{cells/gram\ lipo} = C_{cells} * V_{PBS} / p * m_{lipo}$$

By applying formula (1) into formula (2) the final equation is obtained and further used in 7.3.6.1 Cell concentration of single positive cells:

$$C_{cells/gram\ lipo} = N_{cells} * C_{Beads} * V_{PBS} / N_{Bead} * p * m_{lipo}$$

7.3.3. Preparation of the samples

The SVF was extracted for each sample of lipoaspirate with two methods using a hand preparation or medical device. In this work, six pairs of corresponding SVFs were obtained and treated equally as described in the following:

The freshly isolated SVF was re-suspended in PBS. The cell suspension was centrifuged for 5min at 500g and the supernatant was discarded to remove residual red blood cells. The remaining cell pellet was re-suspended in 15ml of erythrocyte lysis buffer, containing 168 mM Ammoniumchloride (NH_2Cl), 10 mM Potassiumhydrogencarbonate ($KHCO_3$), 1 mM Ethylenediaminetetraacetic acid (EDTA lysed at pH 8.0) and 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI). The suspension was kept at room temperature for 15min and afterwards centrifuged for 10min at 300g. The color of the SVF pellet changed from red to white when erythrocytes were appropriate lysed (Figure 11). The supernatant containing the lysed erythrocytes was discarded.

The erythrocyte free pellet was re-suspended in PBS and divided to the required sample size in 2ml Eppendorf cups. The cells were stored in PBS at 4°C up to a maximum of 24h when not immediately processed for flow cytometry.

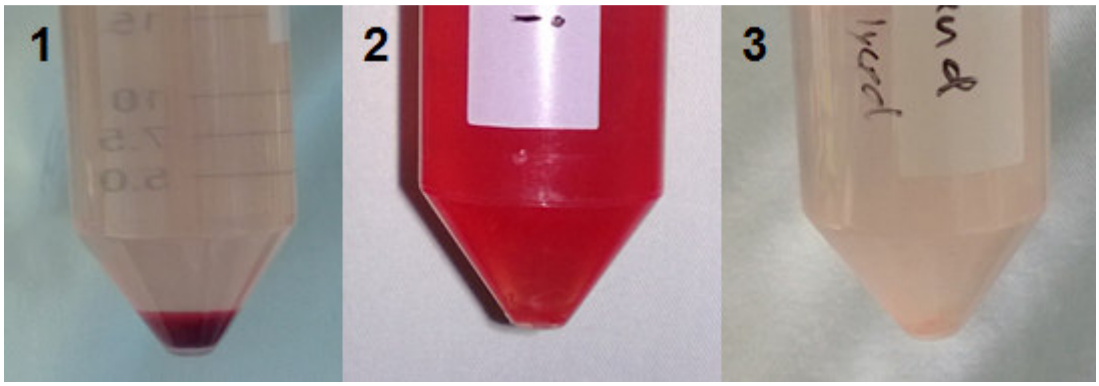

Figure 11 Deep red color of the SVF pellet in PBS after isolation (1). Re-suspended pellet in erythrocyte lysis buffer (2). The white SVF pellet is barely visible after erythrocyte lysis in PBS (3).

7.3.4. Labeling

For every flow cytometric analysis, different samples from one SVF were required. Hence the SVF was split into four 2ml Eppendorf cups. The following procedure was applied for both SVF preparation methods.

Three isotype control samples with antibodies of no specific binding were prepared to detect background-events. In addition, one test sample containing all three antibodies and counting beads was used to gather the desired information about the SVF. Samples with single antibody controls and unstained controls were measured first to calibrate and adjust gate settings of the flow cytometer.

Every 2ml Eppendorf cup contained a defined part of the SVF re-suspended in PBS after erythrocyte lysis. All cups were centrifuged at 300g for 5 minutes, the supernatant was discarded and the pellets were re-suspended in 100µl PBS. The antibodies were added in the dark as shown in Table 3 and incubated for 30min at room temperature.

Table 3 Description of the samples and volume of antibody solution added.

Name of the sample (pellet re-suspended in 100µl PBS)	Antibodies added	Volume
Control Isotype PE	PE isotype control (BD Pharmingen)	5µl
Control Isotype PE-Cy7	PE-Cy7 isotype control (BD Pharmingen)	5µl
Control Isotype APC	APC isotype control (Miltenyi Biotec)	10µl
Sample with all Antibodies	Anti-human CD34 R-PE conjugated (BD Pharmingen)	20µl
	Anti-human CD45 PE-Cy7 conjugated (BD Pharmingen)	5µl
	Anti-human CD 271 APC conjugated (Miltenyi Biotec)	10µl

All steps after the incubation were performed in dark environment. 1.5ml PBS were added to each cup, and mixed gently by pipetting up and down. Thereafter, each cup was centrifuged for 5min at 300g. The supernatant with non-bound antibodies was discarded and the cell pellets of the isotype control samples were re-suspended in 800µl PBS. The test sample with the three antibodies was re-suspended in 700µl of PBS and 100µl of the counting bead solution was added. Each sample was loaded with a total volume of 800µl.

All samples were transferred into polystyrene round-bottom tubes through a cell-strainer cap retaining all particles which were too large to pass through the flow cytometer. All samples were stored on ice in the dark until the measurement with the flow cytometer.

7.3.5. Measurement

Each sample was vortexed for 10 seconds prior to flow cytometry, to ensure the homogeneity of its suspension. The measurement was finished when at least 30.000 events of the three isotype control samples and at least 250.000 events of the test

sample were recorded. The pressure was adjusted, so that the flow rate never exceeded 3000 events per second to ensure precise measurement.

7.3.6. Gating strategy

All recorded events were depicted in a dot plot diagram whereas the forwardscatter (FSC) signal represents the X-axis and the sidewardscatter (SSC) signal displays the Y-axis. The counting beads for determination of the cell concentration can easily be distinguished on the plot as they have a defined FSC-SSC pattern. This pattern can be clearly separated from the remaining events. These remaining events showed an inhomogeneous distribution and the amount of populations varied between the samples. While starting the evaluation by investigating the cell concentration for single positive cells (7.3.6.1 Cell concentration of single positive cells) the distribution of the events became more apparent. A main population was detected with positive signals for both stem cell markers (i.e. CD34 and CD271) (7.3.6.2 Definition of the main population) which was used for the investigation of single and double positive cells.

7.3.6.1. Cell concentration of single positive cells

The gating started with analyzing the number of cells per gram lipoaspirate which were positive for the investigated markers. First the counting beads were gated (Figure 12, number 1) resulting in the number of beads (N_{Bead}). Secondly the remaining population was gated and thereafter shown in a histogram for the three markers. Positive events were gated due to the equal isotype controls of every one of the three markers (Figure 12, number 2a, 3a and 4a). This procedure ensured that only events with sufficient fluorescence intensity were taken into consideration and counted as positive. The positive events for $CD34^+$, $CD45^+$ and $CD271^+$ were plotted in three further FSC-SSC dot plots. Thereby the subpopulations were identified and finally gated (Figure 12, number 2b, 3b and 4b) resulting in the number of cells for each marker (N_{cells}). Finally, the concentration of cells per gram lipoaspirate was calculated by the formula previously explained in 7.3.2 Experimental setting using obtained results of N_{beads} and N_{cells}.

$$\mathbf{C_{cells/gram\ lipo} = N_{cells} * C_{Beads} * V_{PBS} / N_{Bead} * p * m_{lipo}}$$

The sum of all cells positive for the three markers was calculated to estimate the overall cell number as well the overall cell concentration.

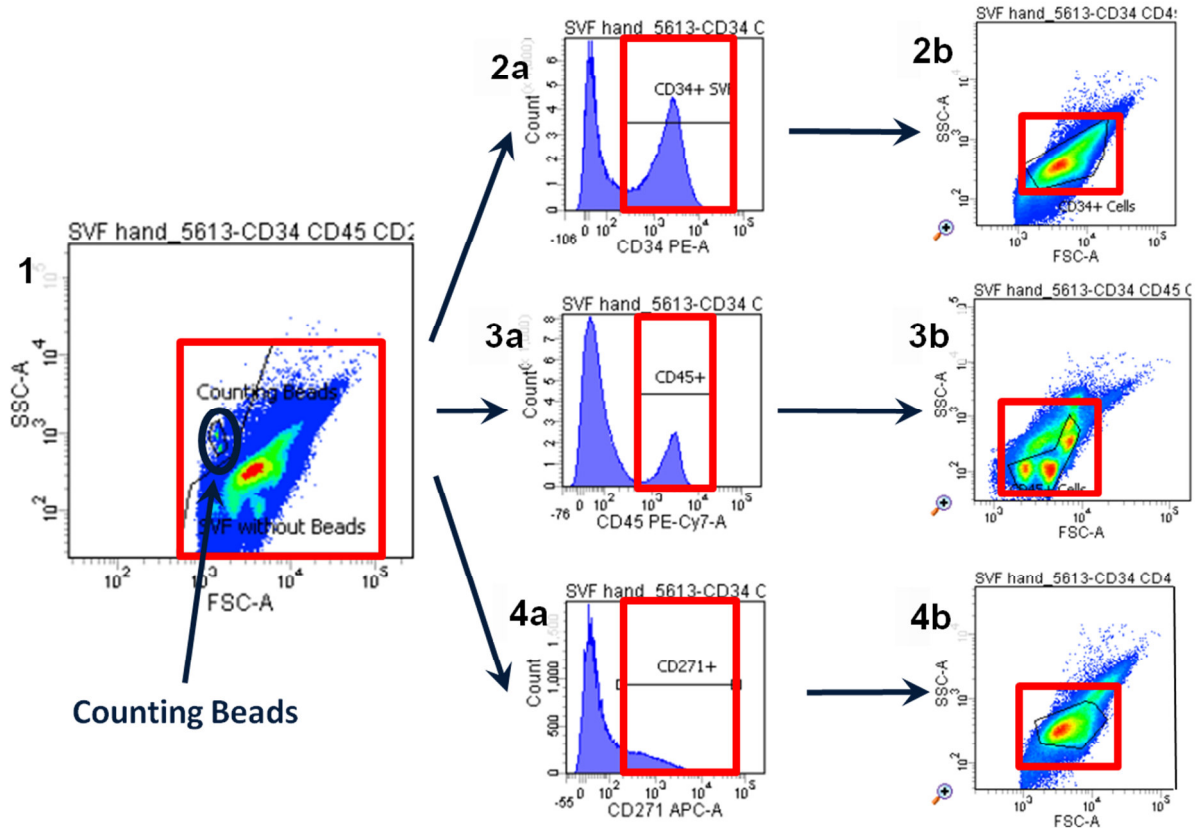

Figure 12 Gating strategy to obtain the cell concentration of the cells positive for one marker in the lipoaspirate. The complete SVF is shown in a FSC-SSC dot plot (1). All events are gated and shown in histograms for CD34 (2a), CD45 (3a) and CD271 (4a). All positive cells are drawn back to a FSC-SSC dot plot (2b, 3b, 4b) and gated for the cell population. The concentration could be calculated with the help of counting beads (marked blue in 1).

7.3.6.2. Definition of the main population

The main population was gated based on two principles: Firstly, cells' physical appearance in the FSC-SSC dot plots indicating viability. Secondly, the population was defined when the criteria for stem cells were fulfilled (19). Thus, the population of interest included cells which were positive for $CD34^+$ and negative for $CD45^-$. Furthermore high proliferative cells positive for $CD271^+$ (54) were included. The different markers were gated separately and shown in a FSC-SSC dot plot in the process of finding the cell concentration. Thereby the $CD34^+$ and $CD271^+$ positive events representing the stem cells arranged in one population in the same area of

the dot plots. On the other hand the $CD45^+$ positive events representing the lymphocytes were located in different subpopulations. Based on these finding, a new gate was created in the initial dot plot containing viable mesenchymal stem cells and were termed main population and used for the analyses described in the following.

7.3.6.3. Percentage of single positive cells

The composition of the stem cells in the SVF was determined by investigating the percentage of single positive cells for the markers within the previously defined main population. Histograms for each marker were created for this specific cell population. A gate was set in every histogram which symbolize positive cells for each marker (Figure 13) and were adjusted in reference to the isotype control samples. Finally, the percentage of events shown as positive in the matching isotype control was subtracted from the percentage of positive cells in the sample in order to reduce background noise. In case of a negative difference, no positive cells were assumed and the percentage was set to zero.

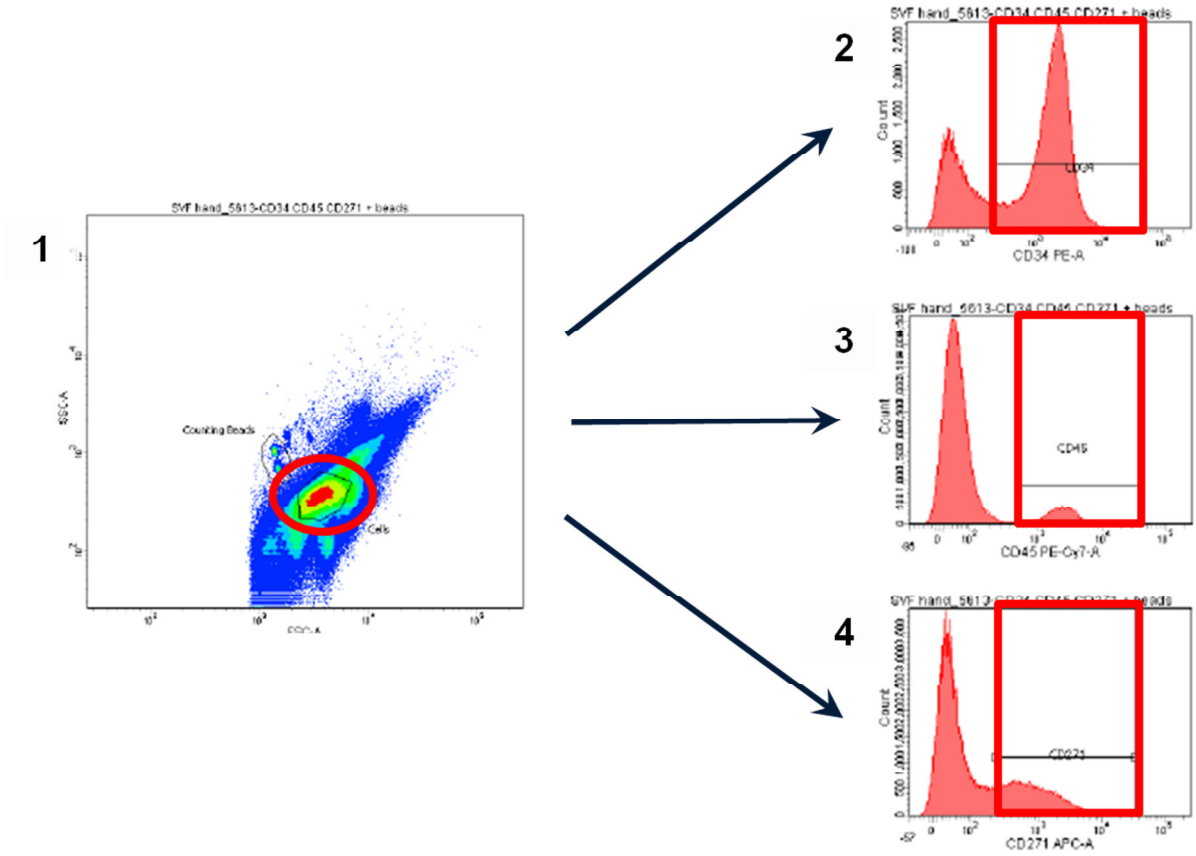

Figure 13 Gating strategy for the percentage of single positive cells within the defined main cell population. The FSC-SSC dot plot shows a main cell population circled red and two smaller populations on the left upper side that are the two different counting beads (1). The histograms on the right show the fluorescence of PE associated with CD34 (2), PE-Cy7 associated with CD45 (3) and APC associated with CD271 (4). The gates for positive cells were set in reference to the isotype controls, so that almost no events of the isotype control could be found in this gate.

7.3.6.4. Percentage of double positive cells

Following the gating of the percentage of single positive cells in the defined main population cells were investigated for the remaining other two markers. Thus all cells gated for a particular marker were depicted in a dot plot whereas each axis was labelled with the two other markers (e.g. all $CD34^+$ cells were shown in a CD45 and CD271 dot plot). The plot was split up into four different gates: One gate representing only single positive (Figure 14, 3c), two containing double positive (Figure 14, 3a and 3d), and the last triple positive cells (Figure 14, 3b) for the investigated markers. The gates were set according to the isotype controls. The percentage of double positive cells in the single positive cell population was analyzed.

While the percentage of double positive cells in the main population was investigated two values resulted for every marker combination, sample and preparation method due to the gating procedure. For example, one marker was gated and further investigated for the other two markers. The same procedure was performed two times starting with a different marker (e.g. the percentage of $CD34^+/CD271^+$ double positive cell can be obtained by first gating for CD34 and then CD271 or the other way around).

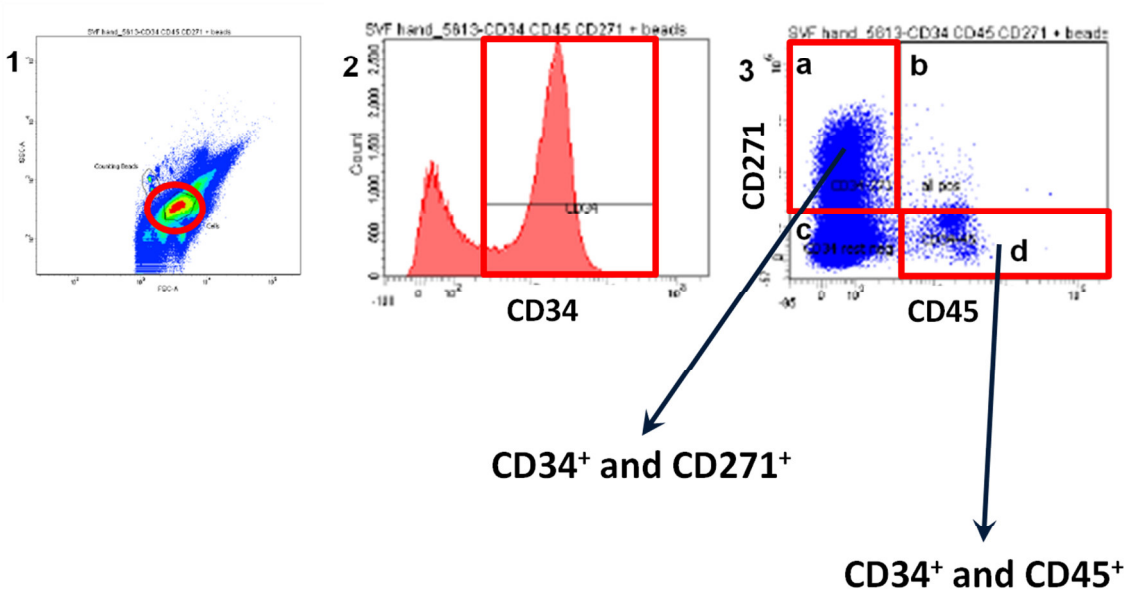

Figure 14 Example for gating double positive cells. The events gated as cells in the FSC-SSC dot plot (1) are further gated in a histogram to obtain the CD34 positive cells (2). Those are shown in a dot plot with the two axes defined by the two other markers: CD45 and CD271 (3). The cross divides the plot defining four new gates. The cells of interest lay in gate 3a and 3d. They show $CD34^+$ and $CD271^+$ cells (3a) and $CD34^+$ and $CD271^+$ cells (3d). Just CD34 single positive cells can be found in gate 3c, while cells positive for all three markers lay in gate 3b.

7.3.6.5. Stain index

Autofluorescence, background noise and the dyes used for the multiparameter flow cytometry can influence the results. A different approach to the comparison of the main population of the SVF samples was the stain index (58). The stain index is a normalization of the fluorescence signals and gives information about the fluorescence intensity.

The stain index uses three values per sample (Figure 15) all resulting from the gate set around the samples main population: The mean fluorescence intensity of the sample (MFI_{sample}). And two values origin from the matching isotype control: The mean fluorescence intensity of the isotype control ($MFI_{isotype}$) and its width represented by its standard deviation ($SD_{isotype}$). With these three values the stain index is calculated for every single marker as shown below:

Stain Index = (MFI_{sample} - $MFI_{isotype}$) / 2* $SD_{isotype}$

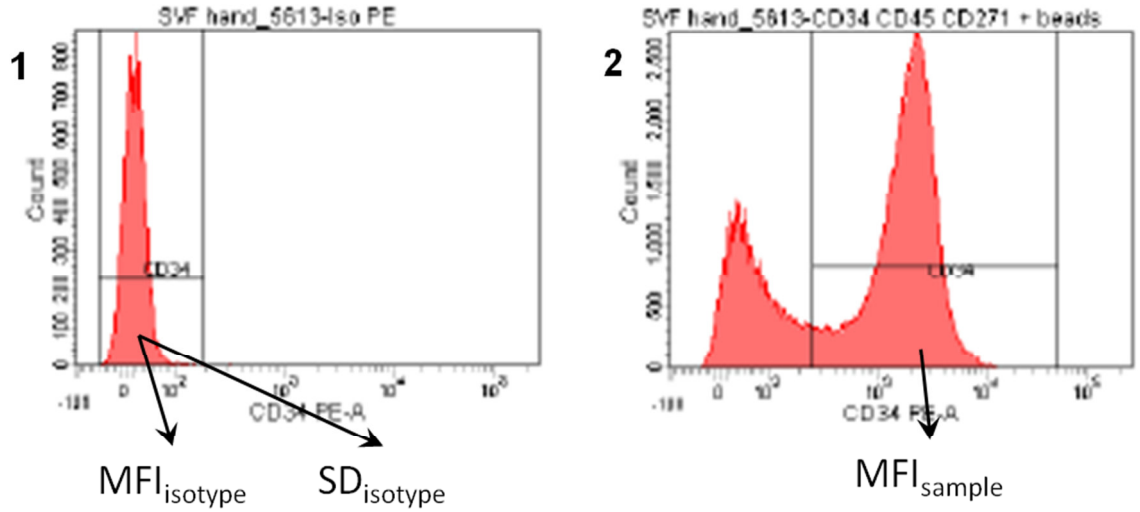

Figure 15 Obtaining the stain index: The mean fluorescence intensity ($MFI_{Isotype}$) and its standard deviation ($SD_{isotype}$) are taken from the istoype control of one marker (1). From the same marker the mean fluorescence intensity of the sample (MFI_{Sample}) is obtained (2). The stain index is calculated: *Stain Index = (MFI_{sample} - $MFI_{isotype}$) / 2* $SD_{isotype}$* (58).

7.4. Cell culture

7.4.1. Medium and Culture Conditions

Plastic adherent cells from the SVF, termed ASCs, were expanded in filter top cell culture flasks at 37°C containing 5% carbon dioxide (CO_2). Cell culture medium α-MEM was supplemented with 20% Fetal Bovine Serum (FBS), 1% GlutaMAX and 100U/ml of Penicillin and 0.1mg/ml Streptomycin and changed every 3-4 days.

7.4.2. Trypsination and Splitting

The cells were split before reaching confluence. The cells were washed twice with PBS to remove all residuals of FBS and incubated with a trypsine solution for 5 minutes. Afterwards, the cells were released from the culture plastic by gently tapping the culture flask. Their detachment was verified under the microscope. When adherent cells were remaining, the incubation time was prolonged for some minutes until all cells were finally detached. The cells were re-suspended in fresh culture medium containing FBS which stopped the trypsination process. The detached cells were counted, and used for further experiments or split into new culture flasks at a ratio of 1:3. With every trypsination and splitting, the passage number of the cells increased by one digit.

7.4.3. Counting

A small volume of the suspension of non-adherent cells (e.g. after trypsination) was counted in a Neubauer chamber. It offers a defined depth of 0.1mm between two parallel glass plates and a counting grid with a big square in each corner covering a space of 1mm². All visible cells found in the four big edge squares were counted.

The volume over a square results from its measurements and represents 0.1mm³ = 0.1µl = 10^{-4}ml. Since four squares were counted, the cell concentration was obtained as following:

Cell concentration = Number of counted cells / $4*10^{-4}$ ml

7.4.4. Freezing

Cells were preserved in a freezing medium, slowly and continually frozen and finally stored in a liquid nitrogen tank for further experiments at about -160°C.

The adherent cells were detached from the cell culture flasks by trypsination (7.4.2 Trypsination and Splitting). The overall number of cells was determined with a Neubauer Chamber (7.4.3 Counting). The cell suspension was transferred into a centrifugation tube and centrifuged for 5min at 500g. The supernatant was removed, while the cell pellet was re-suspended in freezing media containing 90% FBS and 10% DMSO. The volume of the freezing media was adapted to the cell number in order to create a concentration of $2.5*10^6$ cells/ml. 200µl of this suspension (equals $5*10^5$ cells) were used per cryogenic vial to enable fast freezing and thawing. In between of 5 minutes the cryogenic vials were inserted into a cell freezing container with a controlled freezing rate of -1°C/min and stored at -80°C. This procedure prevents cell damage by the freezing process or freezing media. After 24 hours, the frozen vials were transferred to the liquid nitrogen tank.

7.4.5. Thawing

At the beginning, a new culture flask was filled with ASCs culture medium and pre-warmed for 15min in the incubator at 37°C for equilibration. Thereafter, the selected cryogenic vial was taken from the nitrogen tank and transferred into a water bath with a controlled temperature of 37°C. After thawing, the cell suspension in the cryogenic vial was re-suspended with 1ml of the pre-warmed media and transferred to the remaining pre-warmed media in the culture flask. This process did not last longer than 5 minutes to prevent cell damage by the freezing media. After 24 hours the medium was changed, to remove residuals of the DMSO and non-vital cells.

7.5. Colony Forming Unit (CFU) Assay

The CFU assay determines the cell number required to generate a cell colony with more than 50 cells and is calculated as a ratio between counted colonies and seeded cells. Cells were seeded at a low density on culture plastic tissue in order to prevent initial contact between cells. After a cultivation period of 14 days, cells were stained to expose colonies (Figure 16). Thereafter, the colonies were counted. The CFU capacities of ASCs were compared between the hand preparation and the medical device procedure.

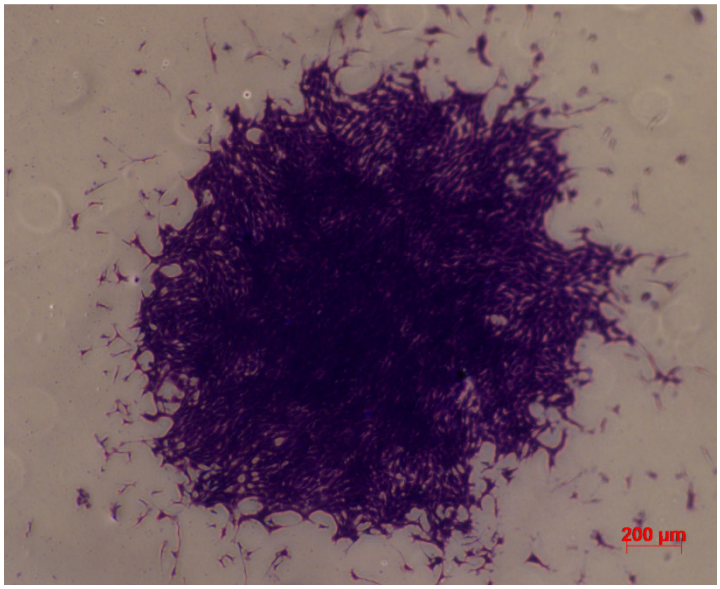

Figure 16 A model colony of ASCs stained with 0.05% crystal violet. Further expansion of the cells can be seen at the border of the colony.

The SVF was extracted out of lipoaspirate with the use of the novel medical device and by hand preparation. Thereafter, the SVF was cultured in cell culture medium (described in 7.4.1 Medium and culture conditions). The resulting ASCs were all cultured to passage P2 and frozen in 90% FBS and 10% DMSO (described in 7.4.4 Freezing and 7.4.5 Thawing).

After thawing, the cells were incubated for two days without further manipulation. To detach the cells, they were trypsinated and the reaction inhibited by re-suspending the cell suspension in α-MEM culture medium. Cells were counted in a Neubauer chamber and seeded with defined densities of 5, 10, 15, 20, 25 and 30 cells/cm² onto polystyrene culture dishes with 100mm diameter. All experiments were repeated

three times for both preparation techniques. The dishes were incubated in culture medium at 37°C with an atmosphere containing 5% CO_2 for 14 days. The medium was changed every third day. On day 15, the cells were fixed for 5 min with a 4% Paraformaldehyde (PFA) solution and stained for 30 min with a 0.05% Crystal Violet solution (CV). Excess stain solution was removed by washing the culture dishes twice with distilled water. Finally, the dishes were air-dried in inverted position at room temperature under non-sterile conditions.

All visible colonies were counted (Figure 17) and marked with a pen to prevent miscounts of colonies. Consequently, the CFU capacities were calculated.

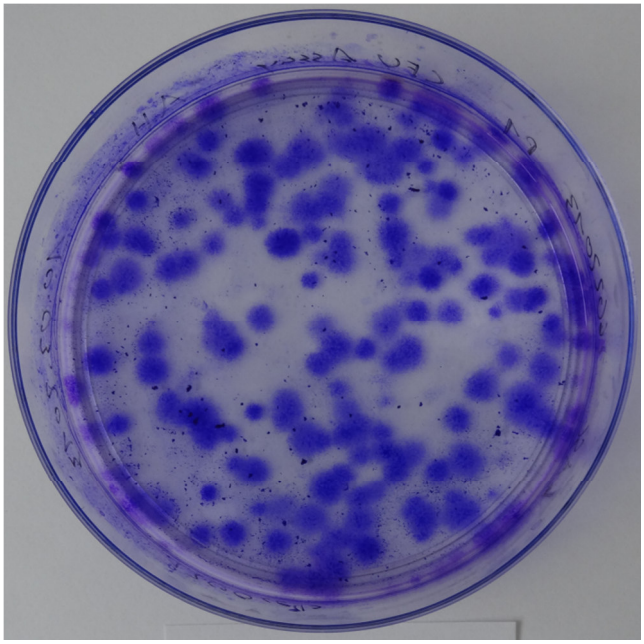

Figure 17 Example CFU Assay ready for counting, after two weeks of incubation, fixation with 4% PFA and staining with 0.05% crystal violet.

7.6. Statistical analysis

All tests were carried out using IBM SPSS Statistics 20 (IBM, Chicago, USA). Normal distribution was tested using the Kolmogorov-Smirnov Test.

The results are shown as means with standard deviation (SD). All samples of lipoaspirate were processed by the hand preparation (h) or using the medical device (d). Therefore, paired samples were created and tested using the paired t-test. Unpaired data was compared with unpaired t-test. Differences between all samples processed by the hand preparation (h) or using the medical device (d) were tested by the one-way analysis of variance (one way ANOVA). P-Values <0.05 were considered statistically significant, p-Values $0.05<p<0.08$ were considered as tendency. Linear correlations are described with the Pearson's r.

8. Results

8.1. Flow Cytometry of the SVF

8.1.1. SVF

The SVF was extracted from lipoaspirate of six patients. Each sample was prepared by hand preparation (h) and with the use of the medical device (d). Finally, six sample pairs were obtained and analyzed by muliparameter flow cytometry using fluorescent antibodies against the cell surface markers CD34, CD45 and CD271 and counting beads.

8.1.2. Cell concentration

The counting beads were properly distinguishable from the rest of the SVF events in the FSC-SSC plot. With their established concentration, the cell number was calculated as described in the methods part (7.3.2 Experiment setting and 7.3.6.1 Cell concentration of single positive cells). The overall cell number, originating from a gram of lipoaspirate did not show a significant difference between hand preparation ($2.0\pm1.7*10^5$) and the medical device procedure ($1.1\pm1.1*10^5$) ($p=0.0623$, data shown in Table 4 and Figure 18). Furthermore, the concentration of cells positive for the different markers was determined (Table 4 and

Figure 19) and demonstrated no significant difference between both preparation methods but a slight tendency towards less $CD34^+$ positive cells extracted with the medical device ($p>0.0596$).

Table 4 Cell concentration of the SVF shown for the hand preparation and the preparation with a medical device. The overall value is shown and also the cell concentration of the different markers. The results of both methods were compared with a paired two tailed T-Test.

Cell concentration in cells/gram lipoaspirate			
Marker	Hand preparation	Medical device	paired two tailed T-Test (p value)
Overall / Sum	**$2.0*10^5$ ($\pm1.7*10^5$)**	**$1.1*10^5$ ($\pm1.1*10^5$)**	**0.0623**
$CD34^+$	$1.3*10^5$ ($\pm1,1*10^5$)	$5.7*10^4$ ($\pm6.0*10^4$)	*0.0596*
$CD45^+$	$3.3*10^4$ ($\pm2.5*10^4$)	$3.1*10^4$ ($\pm3.6*10^4$)	0.8244
$CD271^+$	$3.4*10^4$ ($\pm4.0*10^4$)	$1.7*10^4$ ($\pm2.2*10^4$)	0.1143

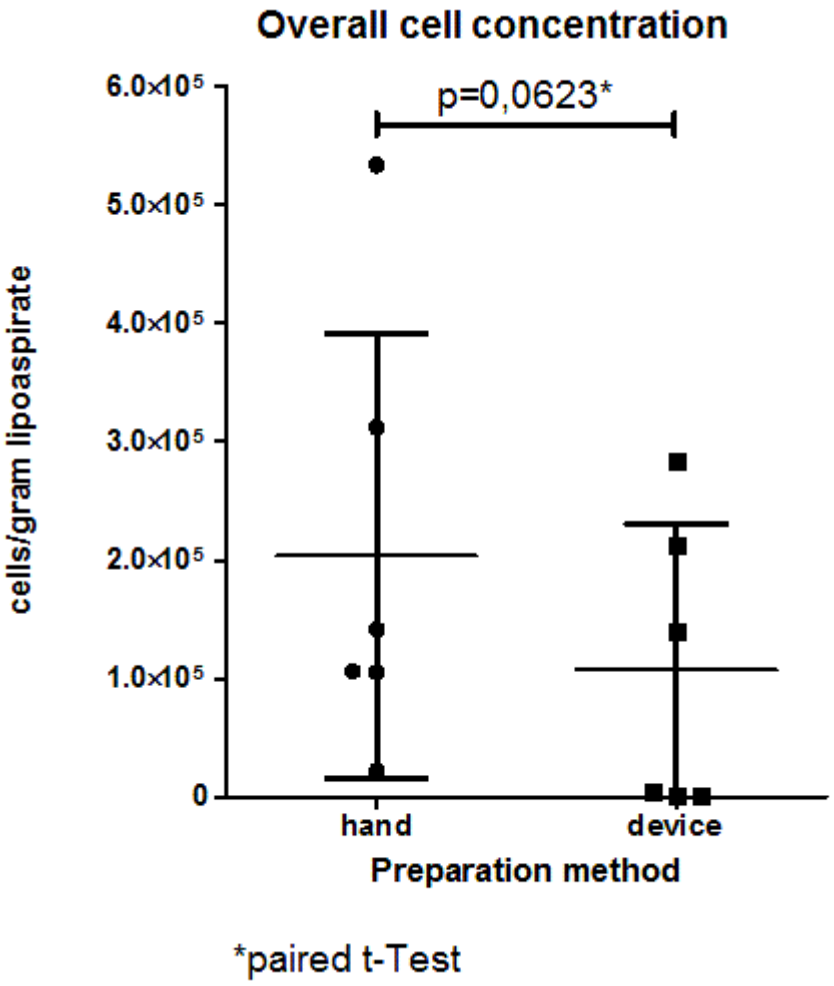

Figure 18 Overall cell number of the SVF per gram of lipoaspirate for both preparation methods. The hand preparation resulted in $2.04\pm1.88 *10^5$ and the preparation with the use of the medical device in $1.07\pm1.0 *10^5$ cells/gram lipoaspirate. No significant difference was found (p=0.0623).

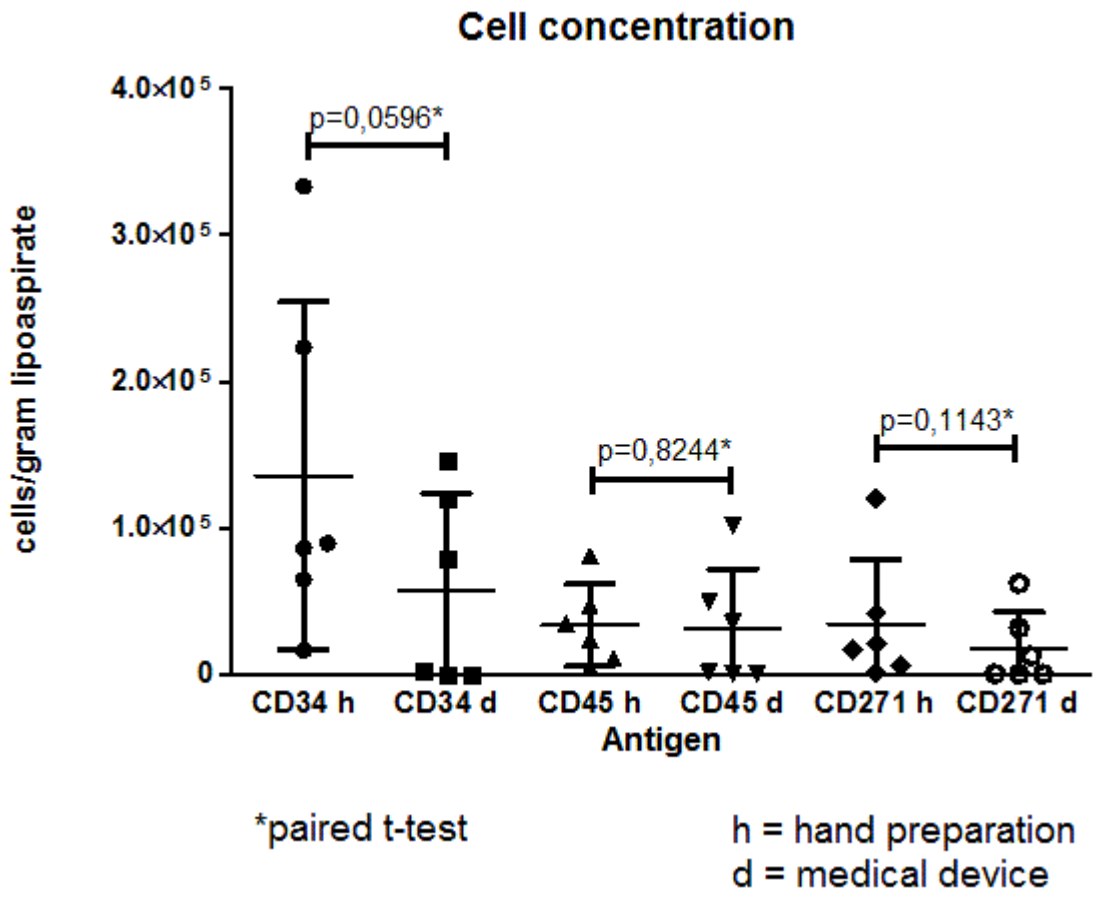

Figure 19 Cell concentration per gram lipoaspirate of the investigated markers CD 34, CD 45 and CD 271 in the SVF for hand preparation (h) and preparation with the use of the medical device (d). Both preparation methods demonstrated no significant difference in the expression of the investigated markers, but a slight difference with the marker CD34 (p=0.0596).

8.1.3. Single positive Cells

The main cell population was identified in the FSC-SSC plot based on the distribution and the pattern of the events (7.3.6.2 Definition of the main population). The percentage of single positive cells for each of the markers was investigated. The novel medical device demonstrated a significantly lower percentage of $CD34^+$ positive cells (57.3±23.8%) compared to the hand preparation (74.1±13.4%) (p=0.0185). The percentage of $CD45^+$ positive cells was similar in both preparation methods with a tendency towards a higher percentage in the samples created with the medical device (h: 9.8±7.1% vs. d: 20.7±15.8%, p=0.0742). Moreover, the percentage of $CD271^+$ cells demonstrated no significant difference device preparation (12.9±9.6%) and hand preparation (13.4±11.6%) (p=0.7408). The results are depicted in Table 5 and Figure 20.

Table 5 Percentage of positive cells in the previously defined main cell population for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test.

Percentage of positive cells			
Marker	Hand preparation	Medical device	paired two tailed T-Test (p value)
$CD34^+$	74.1 (±13.4)	57.3 (±23.8)	**0.0185**
$CD45^+$	9.8 (±7.1)	20.7 (±15.8)	*0.0742*
$CD271^+$	13.4 (±11.6)	12.9 (±9.6)	0.7408

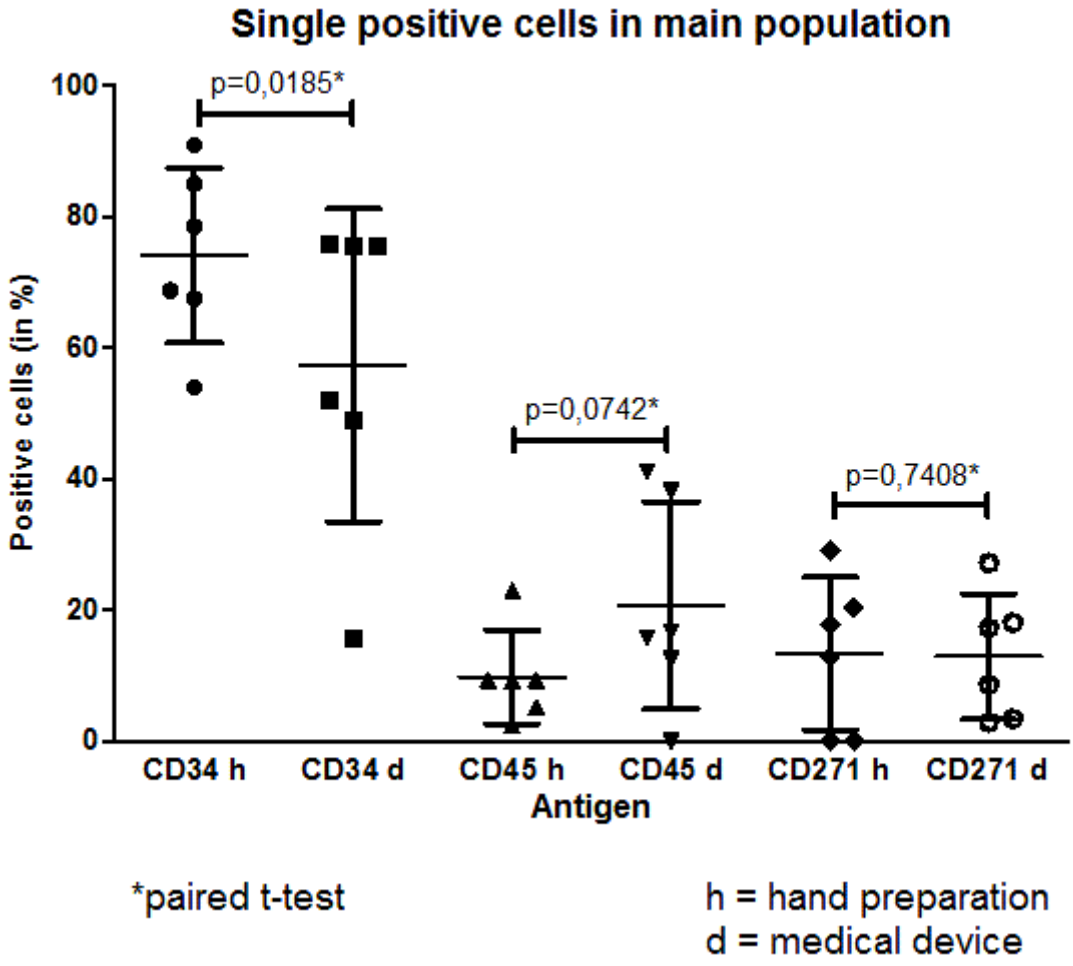

Figure 20 Percentage of single positive cells for the investigated markers in the previously defined main cell population in the FSC-SCC plot of the SVF. A significant difference between the hand preparation (h) and the preparation with the medical device (d) was found only for the marker CD34 (p=0.0185) and a tendency for the marker CD45 (p=0.0742).

8.1.4. Double positive Cells

Single positive cells were further investigated with flow cytometry to identify double positive cells. The percentages of single positive cells further positive for another marker are shown in Table 6. No significant differences were found. $CD45^+$ positive cells were not positive for $CD271^+$ or the other way round.

No significant difference between both methods was for $CD34^+/CD45^+$ double positive cells (h: 0.3±0.2% vs. d: 0.5±0.6%; p=0.2701) and $CD34^+/CD271^+$ (h: 2.4±2.0% vs. d: 1.9±2.3%; p=0.4150). The results for the main population are shown in Table 7 and Figure 21.

Table 6 The percentage of cells being positive for two markers in the population of single positive cells, for hand preparation and preparation with the medical device. The percentages of single positive cells are not shown here and marked with a star (*) and can be seen in 8.1.3 Single positive Cells. Cells double positive for $CD45^+$ and $CD271^+$ did not exist and are not shown in this table.

Percentage of single positive cells being positive for a second marker			
Marker	Hand preparation	Medical device	paired two tailed T-Test (p value)
$CD34^+$	*	*	*
$CD45^+$	1.67 (±1.11)	5.9 (±5.9)	0.130
$CD271^+$	14.2 (±8.46)	24.1 (±12.0)	*0.070*
$CD\ 45^+$	*	*	*
$CD34^+$	15.0 (±11.7)	15.9 (±7.5)	0.889
$CD271^+$	*	*	*
$CD34^+$	76.4 (±20.2)	83.8 (±11.4)	0.131

Table 7 Percentage of double positive cells in the previously defined main cell population for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test. Because no $CD45^+$ / $CD271^+$ double positive cells existed in both preparation methods no T-Test could be performed (#). They show the same result.

Percentage of double positive cells in the main population			
Marker	Hand preparation	Medical device	paired two tailed T-Test (p value)
$CD34^+/CD45^+$	0.3 (±0.2)	0.5 (±0.6)	0.2071
$CD34^+/CD271^+$	2.4 (±2.0)	1.9 (±2.3)	0.4150
$CD45^+/CD271^+$	0	0	#

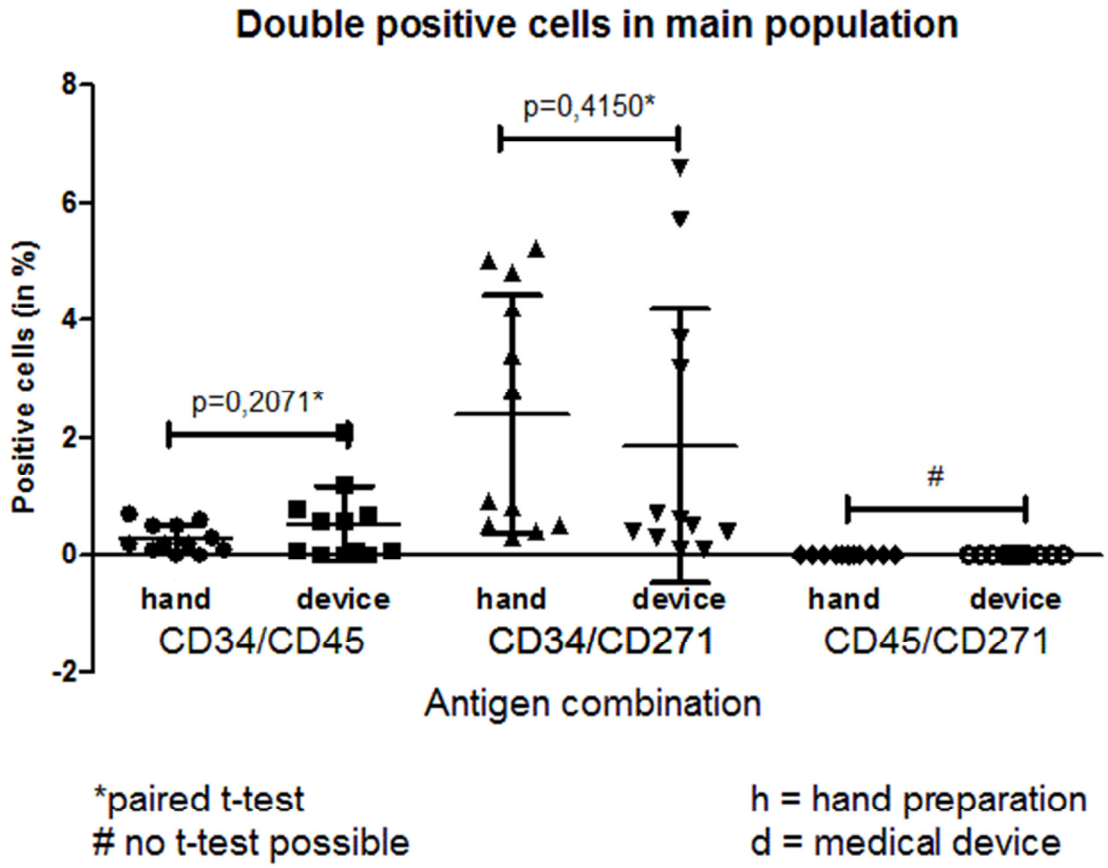

Figure 21 Percentage of double positive cells for the investigated markers in the previously defined main cell population in the FSC-SCC plot of the SVF. Based on the gating method, the number of values for double positive cells (n=12) is double of the number of samples (n=6). No significant difference between the hand preparation (h) and the preparation with the use of the medical device (d) was observed. In particular, for the marker combinations $CD34^+/CD45^+$ (p=0.2071) and $CD34^+/CD271^+$ (p=0.4150). No $CD45^+/CD271^+$ double positive cells were detected and therefore, no statistical analysis could be performed (#).

8.1.5. Stain index

The stain index was determined of the main population in the SVF in order to reduce the background noise and to investigate the fluorescence intensity of the events. No significant difference was apparent when comparing both methods (p>0.1210). The marker CD34 had the highest stain indices (h: 63.3±38.3 vs. d: 111.0±72.5; p=0.1210), while CD271 had the lowest (h: 16.5±10.5 vs. d: 15.5±7.1; p=0.6207). The stain index for CD45 was 22.2±9.2 for the hand preparation method and 28.5±19.3 when extracted with the device (p=0.3336). The results are also depicted in Table 8 and Figure 22.

Table 8 Stain Index in the main population of the SVF for the three markers for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test.

Stain Index			
Marker	Hand preparation	Medical device	paired two tailed T-Test (p value)
$CD34^+$	63.3 (±38.3)	111.0 (±72.5)	0.1210
$CD45^+$	22.2 (±9.2)	28.5 (±19.3)	0.3336
$CD271^+$	16.5 (±10.5)	15.5 (±7.1)	0.6207

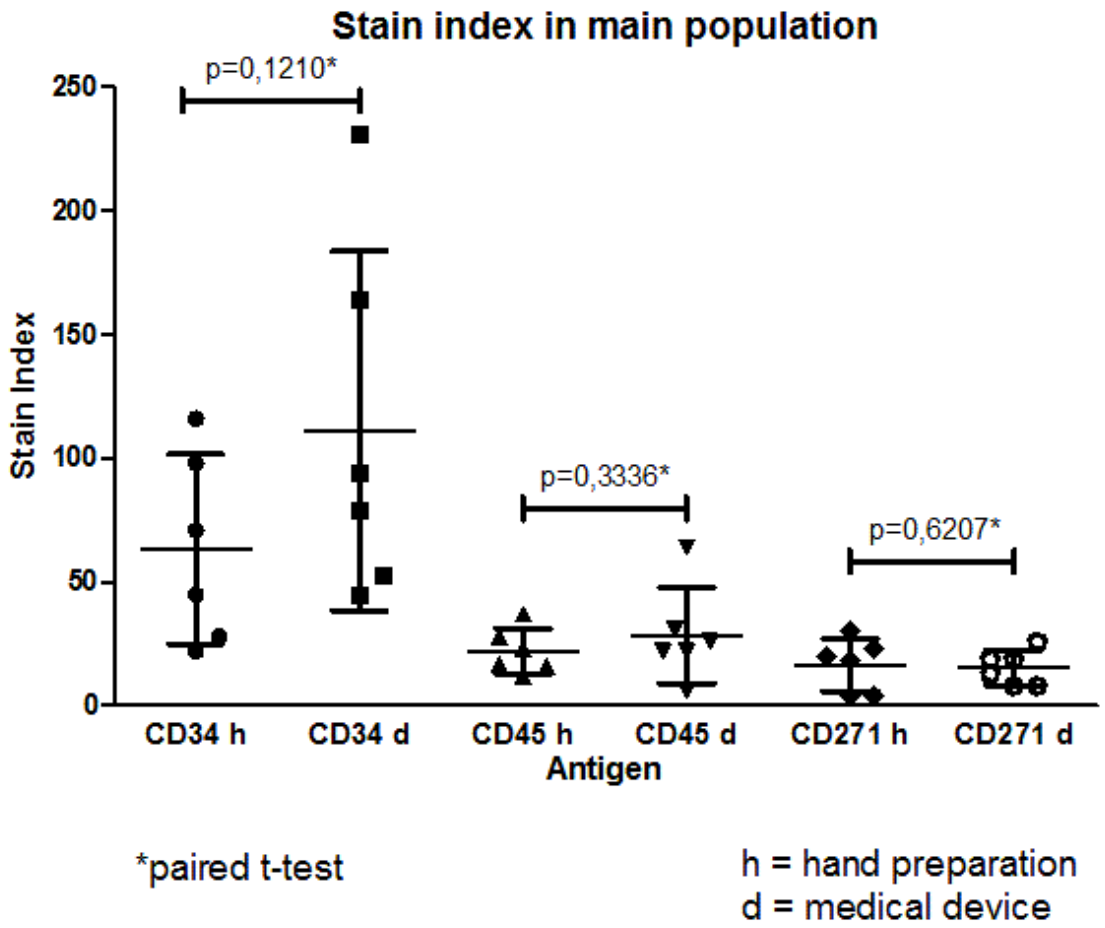

Figure 22 The stain index of the complete SVF regarding the investigated surface markers for the hand preparation (h) and the preparation with the use of the medical device (d). Both methods demonstrated no significant difference in their stain index for CD34 (p=0.1210), CD45 (p=0.3336) and CD271 (p=0.6207).

8.2. Colony Forming Unit (CFU) Assay

CDU assays were performed for ASCs derived from the lipoaspirate of three different donors extracted with both preparation methods. They were stained and counted after two weeks as described in the method section (7.5 CFU Assay). The number of visible colonies increased with the cell density of the initially seeded concentration (Figure 23). Overlapping colonies limited the accuracy of discrimination of different colonies.

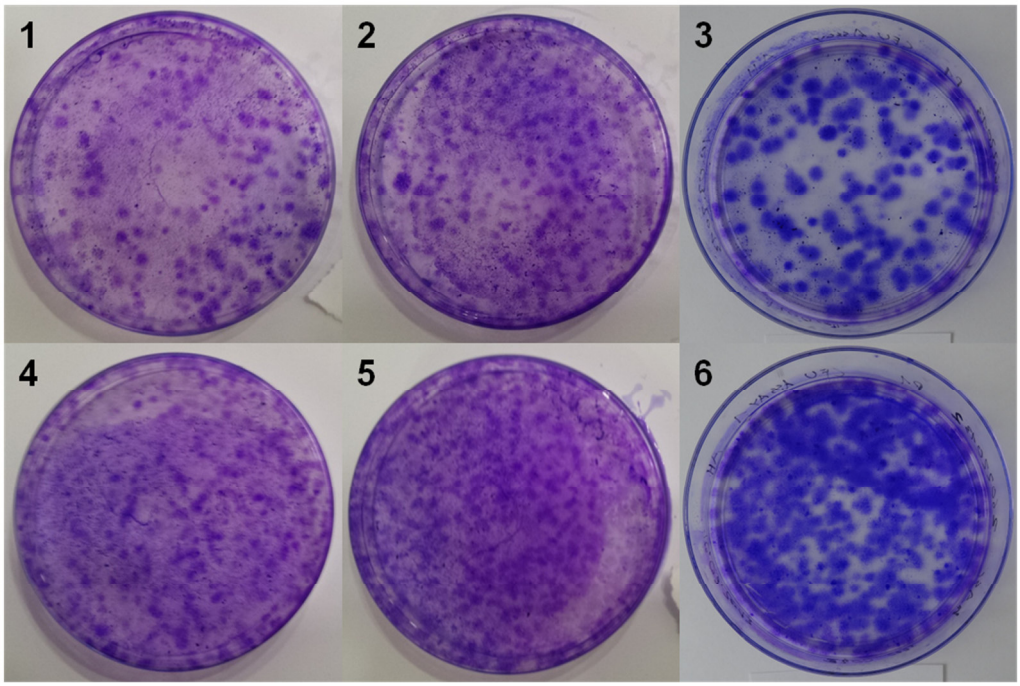

Figure 23 CFU Assays with ASCs of different concentration after two weeks of incubation, fixation and staining with crystal violet. The seeding concentration was 5 cells/cm² (1), 10 cells/cm² (2), 15 cells/cm² (3), 20 cells/cm² (4), 25cells/cm² and 30 cells/cm² (6). Different colonies could be separated best when initially seeded at lower concentrations (1-3), while when seeded at higher concentrations the colonies were overlapping a lot (5 and 6).

The lowest seeding concentration of 5 cells/cm² yielded the least amount of colonies, in particular 0.63±0.45 colonies/cm² for hand preparation and 0.60±0.35 colonies/cm² for preparation with a medical device. The resulting colony densities differ according to the initial seeding concentrations for both methods ($p<0.003$) and correlated with a rising tendency with a Spearman's correlation of $r=0.855$ for the hand preparation and $r=0.939$ for the preparation with a medical device.

The CFU capacity (colonies per initial seeded cells) was independent of the seeding concentration (h: $p=0.931$ and d: $p=0.503$), but was associated with a slight linear Spearman's correlation of $r=-0.287$ for the hand preparation and $r=-0.341$ for preparation with a medical device. The overall CFU capacity was 10.4±4.71 % for hand prepared cells and 12.45±3.70 % for the cells extracted with the use of the medical device. The overall CFU capacity showed no significant difference between both preparation methods ($p=0.147$). All results are shown in Table 8, Figure 24 and Figure 25.

Table 9 Colony density after two weeks and the resulting CFU capacity for hand preparation and the preparation with the use of the medical device resulting from the initial seeding concentration. Because colony density and CFU capacity can be transformed into each other the p values are the same and are shown in the right column. No significant difference could be detected.

Results of the CFU Assays					
Seeding concentration (cells/cm²)	Colony density after two weeks (colonies/cm²)		CFU capacity (in %)		T-Test (p value)
	hand preparation	medical device	hand preparation	medical device	
5	0.63 ± 0.45	0.60 ± 0.35	12.6 ± 8.92	11.9 ± 6.94	0.667
10	1.19 ± 0.43	1.58 ± 0.26	11.9 ± 4.32	15.8 ± 2.58	0.312
15	1.47 ± 0.37	2.10 ± 0.09	9.8 ± 2.51	14.0 ± 0.57	0.095
20	1.96 ± 0.50	2.35 ± 0.10	9.8 ± 2.51	11.7 ± 0.50	0.313
25	2.24 ± 0.22	2.77 ± 0.20)	9.0 ± 0.86	11.1 ± 0.79	0.137
30	2.70 ± 0.42	3.05 ± 0.09	9.0 ± 1.41	10.2 ± 0.29	0.300
One way ANOVA (p value)	**0.003**	**<0.001**	0.931	0.503	
Spearman's correlation	**0.855**	**0.939**	**-0.287**	**-0.341**	
Middle			10.4 ± 4.71	12.45 ± 3.70	0.147

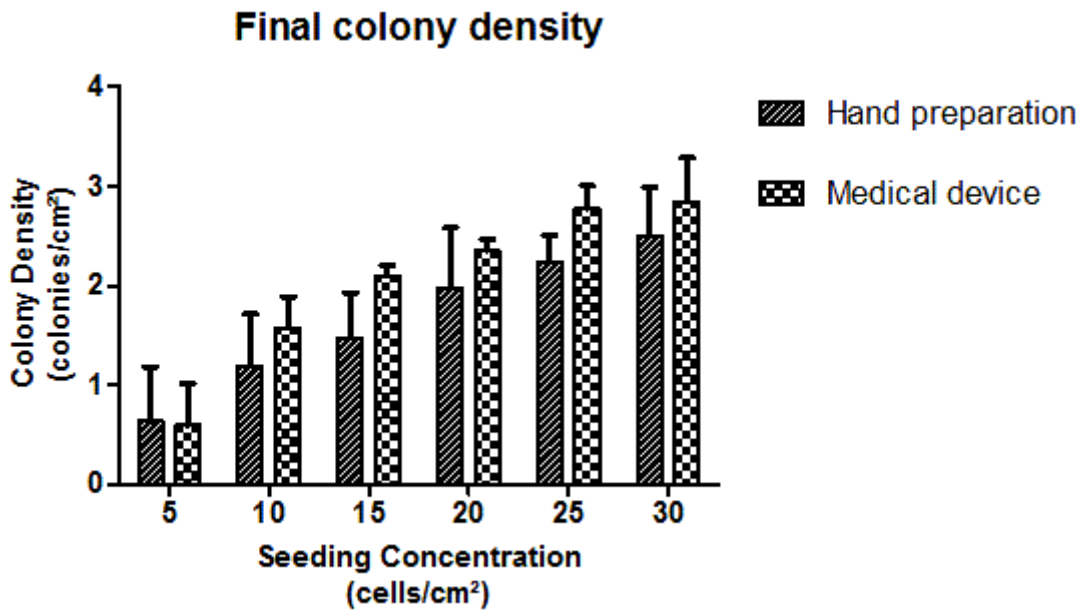

Figure 24 Seeding concentration and correlating colony density of both hand preparation and the medical device. A rising tendency can be seen with a Spearman's correlation of r=0.855 for hand preparation and r=0.939 for preparation with the medical device.

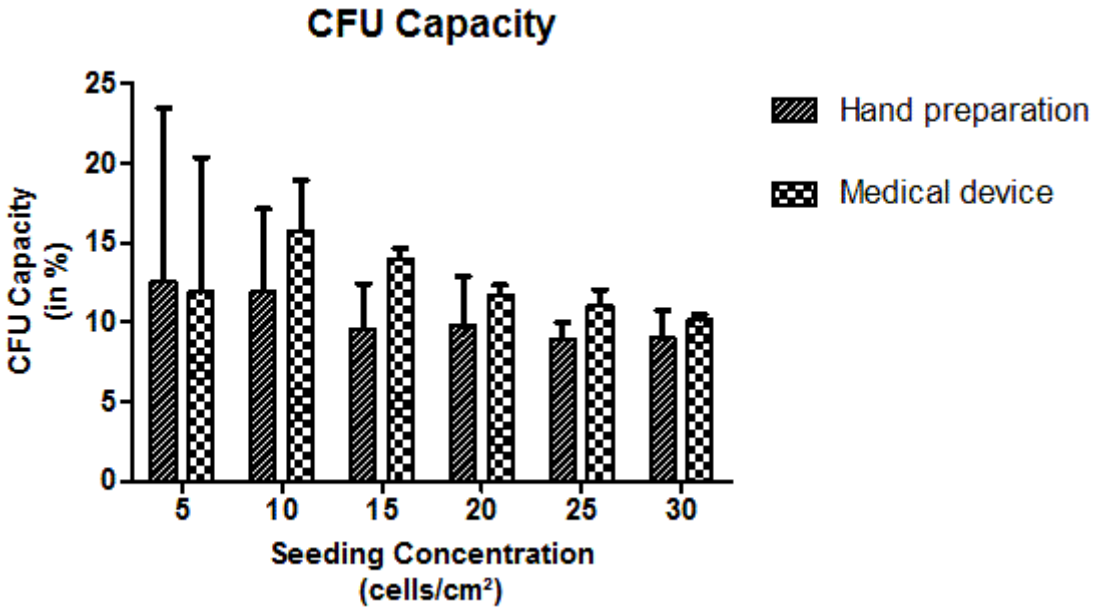

Figure 25 Seeding concentration and the CFU capacity of both hand preparation and preparation with a medical device. No significant difference between the CFU capacities of each method could be found (p>0.503).

9. Discussion

In the present study, the stromal vascular fraction (SVF) extracted from lipoaspirate by two different extraction methods was investigated regarding cell quantity and cell quality. The first method was performed with common laboratory equipment and is referred to as hand preparation in this work. The second method used a novel medical device consisting of a heatable centrifuge with a shaking option and a sterile system created with syringes and a transfer piece. Both preparation methods were able to create a visible amount of SVF and showed no significant difference in the cell number extracted (p=0.0623), although there is an indication for preparation failures caused by a technical issue with the syringe cap of the novel medical device, which are discussed in the paragraph 9.1. Cell concentration varies between both methods.

The composition of the cells in the SVF was investigated in the main population for the markers CD34, CD45 and CD271. There was a significant difference between both methods regarding the percentage $CD34^+$ single positive cells, while there was none for the other single and double positive cells as well for the stain index.

Finally the ASCs resulting from the SVF extracted by both methods were compared by CFU-Assays revealing a similar CFU capacity (p=0.147).

9.1. Cell concentration varies between both methods

The cell numbers per gram of lipoaspirate for the SVF reported in the literature varies due to different extraction methods and the biological variability of lipoaspirate. The typical range is from $1*10^5$ to $5*10^5$ cells per gram lipoaspirate, as shown in detail in Table 10. In the present study, a number of 2,03±1,88 $*10^5$ cells per gram lipoaspirate was extracted by hand preparation and 1,07±1,23 $*10^5$ per gram lipoaspirate using the novel medical device. Both values are in the range with those reported in the literature (Table 10 and Figure 26). No significant difference, but a tendency, for the overall cell number could be found comparing both methods in the present study (p=0.0623). Moreover, no significant difference was apparent regarding

the number of single positive cells for the different markers, but a slight tendency for the marker CD34 (p=0.0596) as shown in Figure 27 on the upper graph (a).

Taking a closer look at the cell number, it can be observed that three of the six preparations with the use of the medical device have a very low cell yield as marked red in Figure 26 (a) for the overall cell number and Figure 27 (a) divided for the three different markers. Because of this very low SVF cell yield, these three preparations with the use of the medical device can be considered preparation failures. Comparing these three preparation failures with the three successful preparations all performed by the use of the medical device, a significant difference in the cell concentration of the SVF could be observed with $1.7 \pm 1.9 * 10^{3}$ vs. $2.12 \pm 0.71 * 10^{5}$ cells per gram lipoaspirate (p=0.007).

After removing the preparation failures from the statistical analysis, the overall cell number of the successful three samples increased to $2.12 \pm 0.71 * 10^{5}$ cells per gram lipoaspirate. Consequently, the mean values observed from both preparation methods were comparable, without a significant difference (p=0.940), as shown in Figure 26 (b). In addition, this finding could be confirmed for the single positive cells: For CD34 (p=0.78), CD271 (p=0.97) and CD45 (p=0.22) no significant difference between both preparation methods could be found, as shown in Figure 27 (b).

Table 10 SVF cell numbers reported in the literature compared to the results in this study. Preparation methods and repeats are shown. # = manual preparations with laboratory equipment use different protocols, § = only valid preparations taken into consideration

Cell number (cells per gram lipoaspirate)	Repeats (n)	Preparation method	Source
$6.24*10^5$	5	Lipokit (Medi-Khan)	Aronowitz et al., 2016 (46)
$5.35*10^5$	5	Multi Station (PNC)	Aronowitz et al., 2016 (46)
$4.04\pm2.06*10^5$	18	Laboratory Equipment#	Aust et al., 2004 (59)
$3.6\pm1.8*10^5$	31	Celution System (Cytori)	Fraser et. al., 2014 (60)
$3.08\pm1.40*10^5$	44	Laboratory Equipment#	Mitchell et al., 2006 (61)
$2.84*10^5$	5	GID SVF-2 (The GID Group)	Aronowitz et al., 2016 (46)
$2.6\pm1.2*10^5$	11	Sepax device (Biosafe SA)	Güven et. al., 2012 (62)
$2.41*10^5$	5	Celution System (Cytori)	Aronowitz & Ellenhorn, 2013 (63)
$1.6\pm0.9*10^5$	11	Laboratory Equipment#	Güven et. al., 2012 (62)
$1.17\pm0.5*10^5$	11	Automated System (patent pending)	SundarRaj et al., 2015 (64)
$1.15\pm0.3*10^5$	11	Laboratory Equipment#	SundarRaj et al., 2015 (64)
$1.07*10^5$	5	Multi Station (PNC)	Aronowitz & Ellenhorn, 2013 (63)
$1.01*10^5$	5	Cytori Stem Source 900/MB (Cytori)	Aronowitz et al., 2016 (46)
$0.35*10^5$	5	Lipokit (Medi-Khan)	Aronowitz & Ellenhorn, 2013 (63)
$0.05*10^5$	5	Cha-Station (CHA Biotech)	Aronowitz & Ellenhorn, 2013 (63)
$2.03\pm1.88*10^5$	6	"hand preparation" (equals laboratory equipment#)	/
$1.07\pm1.23*10^5$	6	UNiStation (Neogenesis)	/
$2.12\pm0.71*10^{5\S}$	3§	UNiStation§ (Neogenesis)	/

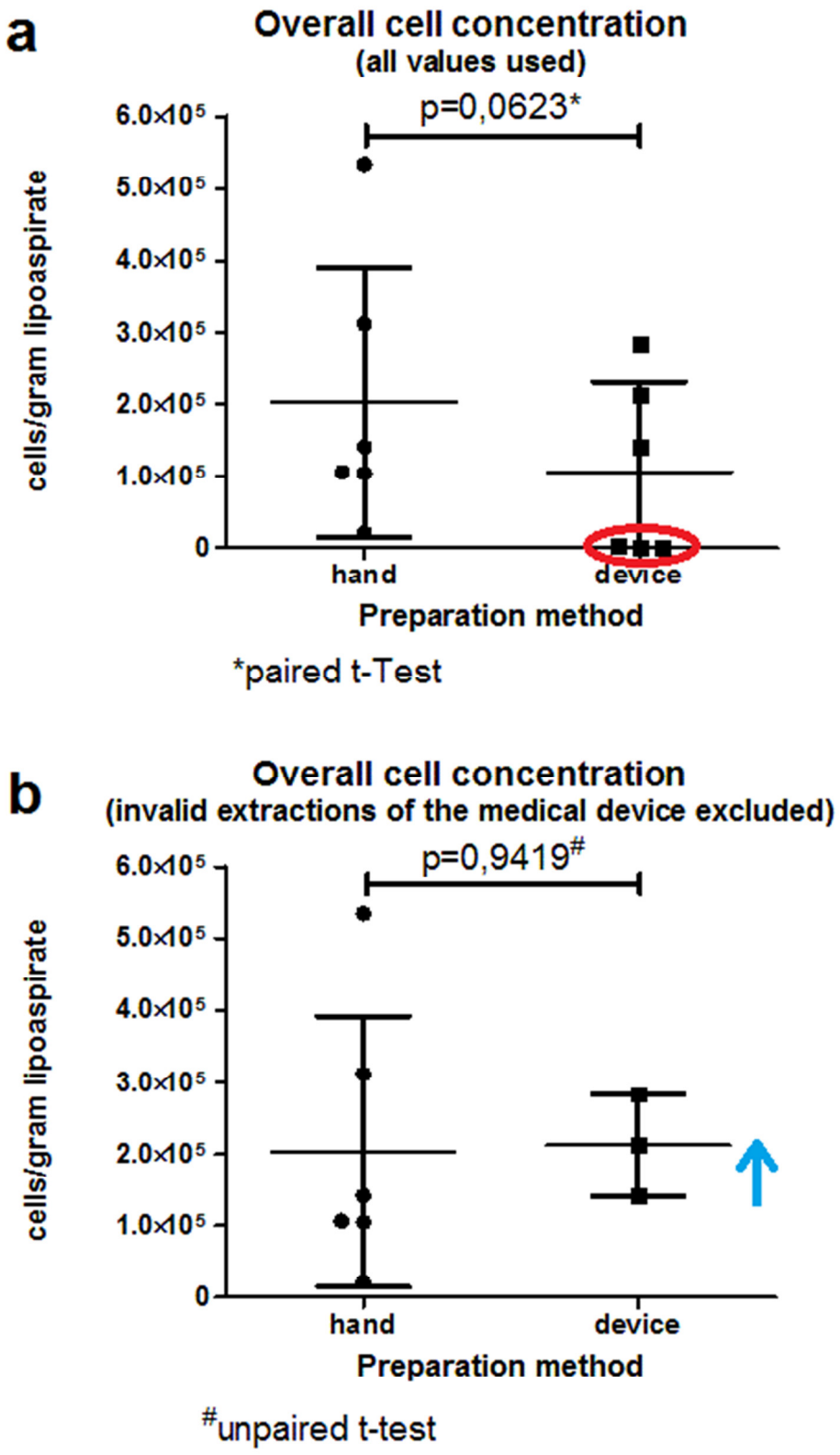

Figure 26 Cell concentration of the SVF obtained by the hand preparation and with the use of the medical device in cells per gram lipoaspirate. On the upper (a) all values are shown. The preparations that produced almost no cells are circled red and marked. On the lower (b) all invalid preparation failures were removed.

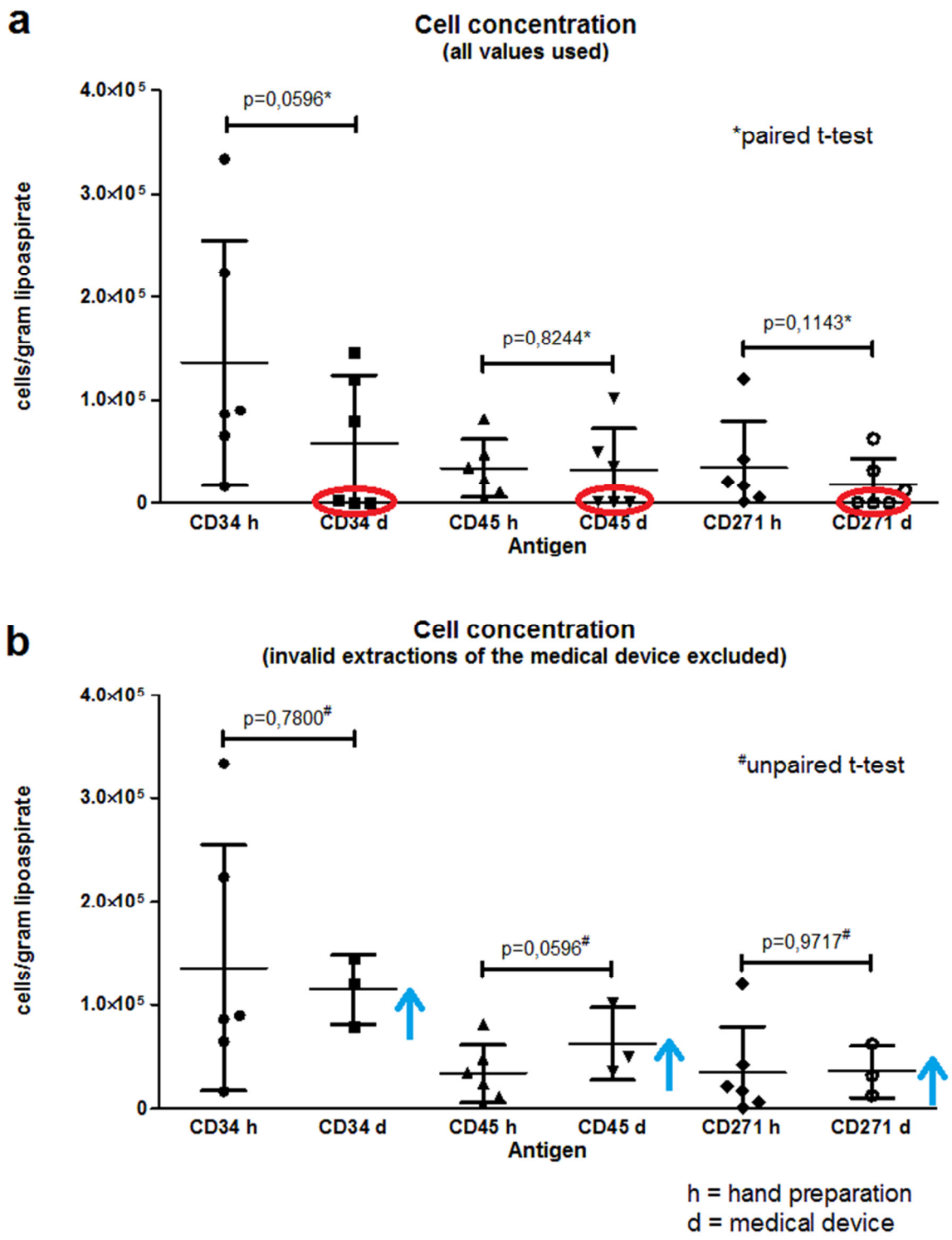

Figure 27 Cell concentration of the SVF for the markers CD34, CD45 and CD271 obtained by hand preparation (h) and preparation with the use of the medical device (d). On the upper (a) all values are shown. The preparations that produced almost no cells are circled red and marked. On the lower (b) all invalid preparation failures were removed. This results in an increase of all middles and an approximation of the results of both preparation methods.

9.1.1. Explanation of the Preparation Failures

We performed a detailed inspection of all consumables needed for the novel medical device. Thereafter, we traced the preparation failures back to the cap of the syringes used in the extraction with the medical device. During the preparation process, the SVF is centrifuged to the bottom of the syringes and hereby into the syringe cap, which is attached to the bottom of the syringes. The cap has to be removed for the final isolation of the SVF. During this process, a slightly negative pressure is created and the small SVF can be vacuumed into the syringe cap as shown in Figure 28 (b). This problem is known to the manufacturer as it is described in the an instructional video by the manufacturer (57). Their proposed solution is to pull up the plunge of the syringe about 1mm while removing the syringe cap, which was also tried during the extractions in this work (described in 7.2.2.2 Protocol). During the experiments for this work it was difficult to apply the right amount of negative pressure to the plunge or moving it exactly 1mm. In case of too much negative pressure air was sucked into the syringe while removing the cap. This caused an air bubble wandering through the syringe, taking the SVF up and away from its wanted low position and mixing the layers. If this happened the centrifugation had to be repeated again. In the other case of too low or no negative pressure the SVF cannot be removed properly and a large amount of cells is lost as shown in Figure 28 (c). This would offer an explanation for the lower cell numbers that were found in three of the SVF isolations with the use of the medical device. Thus, this sensitive step during the extraction of the SVF is not applicable for the daily routine in the operating room. Therefore, a more technical solution for high and constant cell yields is suggested in the following section.

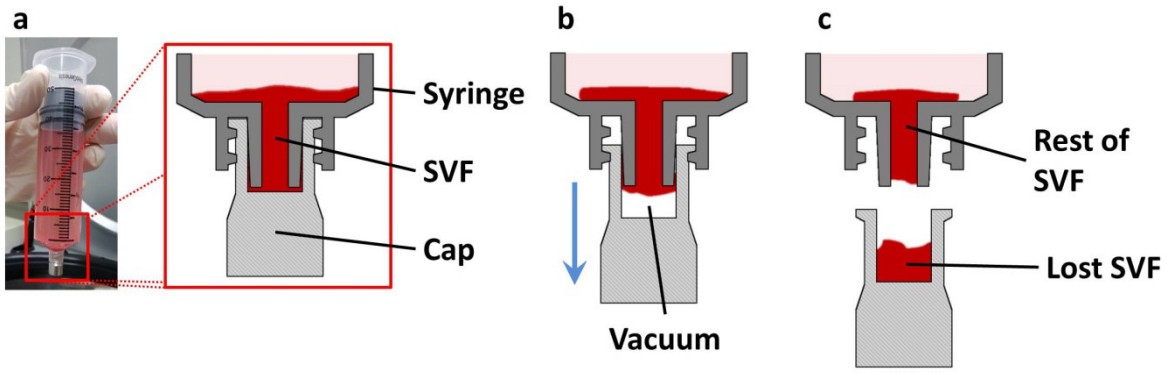

Figure 28 Explanation of the preparation failures with the medical device. The bottom of the syringe with the SVF is illustrated (a). When the cap is removed a slightly negative pressure is created (b) and this can be enough to vacuum off a great part of the SVF into the cap (c). This part of the SVF is lost and cannot be used further.

9.1.2. Suggestion for solution

The novel medical device could be improved by using a different syringe cap. A counterpart for the lumen of the syringe should be added as it is shown in Figure 29. Thereby the SVF could not be centrifuged into the cap. During the removal of the improved cap, a vacuum would be still created. However, because of the counterpart there would be no space for the SVF to get vacuumed into the cap. Thus the whole SVF should stay in the syringe even applying any negative pressure to the plunge making the handling much easier. This might be a solution to increase the cell yield and create a consistent extraction results with the medical device.

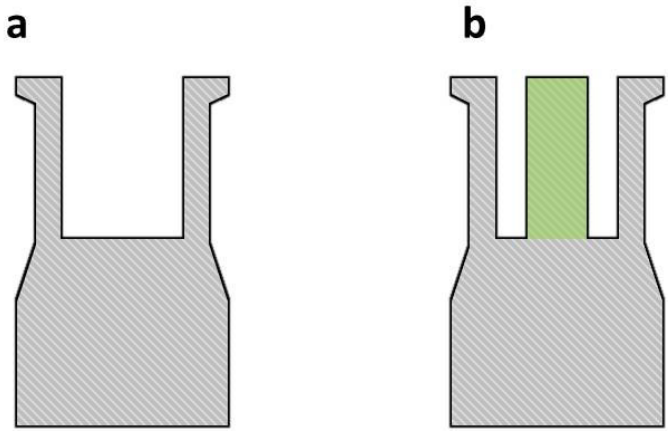

Figure 29 The currently used syringe cap (a) and a suggestion for an improved syringe cap (b) with a counterpart for the syringe shown in green.

9.2. Percentage of single and double positive cells

While the number of the cells within the SVF varies between samples and extraction methods, the composition of the SVF should remain similar, independent of the amount of SVF that was prepared. Therefore, the smaller amounts of SVF generated during the preparation failures of the novel medical device can also be taken into consideration for the surface marker characterization. Consequently, the defined main SVF cell population was investigated for the percentage of cells positive and double positive for the three different markers (i.e. CD34, CD45 and CD271).

The percentage of $CD34^+$ positive cells showed a significant difference between both extraction methods ($p=0.0185$) and for the percentage of $CD45^+$ positive cells a tendency was apparent ($p= p=0.0742$). For both the percentage of $CD271^+$ and the double positive cells, which were overall low and not exceeding 5%. No significant differences were detected ($p>0.2071$). All of that is discussed in the following paragraphs.

9.2.1. CD34

Despite the fact, that CD34 was not defined as a marker for MSCs (65), it can be commonly found on freshly isolated SVF cells (19). $CD34^+$ cells were reported to have haematopoetic (50) and vasculogenetic potential (66) which might offer new thearapies for example for vascular diseases (67). 30 to 80% of freshly isolated SVF cells are referred to as $CD34^+$ positive (66,68). This is in line with the findings of this study, both for the SVF preparation by hand (74.1±13.4%), and with the use of the novel medical device (57.3±23.8%). However, using the medical device resulted in a significantly lower percentage of $CD34^+$ cells ($p=0.0185$). This indicates a lower yield of endothelial progenitor cells in the SVF created by the medical device, but is still sufficient for further research and potential therapies.

9.2.2. CD45

In contrast to the lower percentage of $CD34^+$ positive cells extracted with the medical device, the number of extracted $CD45^+$ positive cells is increased (20.7±15.8%) compared with the preparation by hand (9.8±7.1%). This tendency could be observed without reaching statistical significance ($p=0.0742$). However, the results for both

preparation methods are comparable to the values known from literature (68). And although, the leukocyte common antigen (CD45) was used to identify leukocytes on the one hand and on the other hand as a negative control for adipose tissue-derived stem cells (19), a pro angiogenetic effect and a higher formation of neo-vessels was found in SVF containing $CD45^+$ cells compared to CD45 deprived SVF (68).

9.2.3. CD271

The marker CD271 was described as suitable for identification of a highly proliferative subpopulation in MSCs (55) with high differentiation potential (69) and immunomodulative abilities (70). Bone marrow MSCs selected as $CD271^+$ positive were reported to have a higher CFU capacity and a higher in vitro wound healing capacity than cells selected by plastic adherence (71). Ishimura et al. reported that 23.6±6.8% of the SVFs cells are positive for $CD271^+$ (72). This is in accordance with the results of the hand preparation and the medical device, as both had the same partition of $CD271^+$ positive cells in the main population, respectively 13.4±11.6% and 12.9±9.6% (p=0.7408). Therefore, the medical device is also able to create a reasonable amount of those cells which are currently in the focus of regenerative medicine.

9.2.4. CD34/CD45 double positive cells

In this work the marker CD34 was used as positive control and the marker CD45 as negative control for MSC, as proposed by the ISCT (19). But a small amount of cells being double positive for those both markers were though found in the SVF's main population. In particular, 2.4%±2.0% for hand preparation and 1.9%±2.3% for preparation with the medical device were $CD34^+/CD45^+$ double positive cells. The preparation method had no influence on those results (p=0.2071). Such cells are known to be found in peripheral blood (73), being hematopoietic stem cells with the ability to create endothelial structures such as blood vessels (74). Navarro et al. (68) reported that such cells can be found in the SVF and that 29%±13% of $CD45^+$ positive cells were further positive for $CD34^+$. This shows a similarity with our findings, which were lower but still in the same range: In particular, 15.0%±11.7% for hand preparation and 15.9±7.5% for preparation with the medical device, showing no difference between both methods (p=0.889). In summary, this shows that this subpopulation of $CD34^+/CD45^+$ cells is extracted in the same amount by the novel

medical device as hand preparation. Those cells might contribute to the SVFs beneficial effects.

9.2.5. CD34/CD271 double positive cells

There were no significant differences regarding both preparation methods for the $CD34^+/CD271^+$ double positive cells in the main population (p=0.4150). The effect of those double positive cells is not researched yet. However, it is described that the plastic adherent hASCs lose the marker CD34 in cell culture while CD271 is preserved, leaving only 3.33±1.94% $C271^+$ positive cells coexpressing CD34 hASCs after three to four days of in vitro cultivation (69). Thus CD271 might play greater role in the beneficial potential of cultured hASCs than CD34.

Quirici et al. (49) used a different approach by separating the cells expressing CD34 and CD271 prior to flow cytometry, showing that 78.0%±10.6% of the $CD271^+$ positive cells were positive for $CD34^+$. In this work the direct flow cytometric approach showed similar results for SVFs prepared by hand preparation (76.4±20.2%) and preparation with the medical device (83.8±11.4%), with no significant difference between the methods (p=0.131). However, there was a tendency (p=0.070) towards more $CD34^+$ cells being further positive for $CD271^+$ for the preparation with the medical device (24.1±12.0%) compared to the hand preparation (14.2±8.46%). Apart from this tendency, both values are in concordance with the value of 28.0±37.4% given also by Quirici et al. (49).

9.2.6. CD45/CD271 double positive cells

No $CD45^+/CD271^+$ double positive cells were detected in the samples of both preparation methods. As $CD271^+$ positive cells were reported to be negative for $CD45^-$ in 99.6% (56), this leaves almost no room for cells being double positive for the markers concerned. This is in accordance with the initial idea that CD271 is a positive marker for stem cells, while CD45 is a negative marker, as previously described in 5.9 Molecular markers.

9.3. Stain index

The stain index is used to quantitate the brightness of an fluorescent dye over the background (58). In this work it was used as a different approach to compare the samples created by hand preparation and preparation with a medical device. The stain index was investigated for the main population of the SVF and determined for all three markers used. No significant differences between both preparation methods could be found for all three markers (CD34: p=0.19; CD45: p=0.48; CD271: p=0.84). This leads to the conclusion that the brightness of the cells over the background in the flow cytometer is similar due to the amount of bonded antibodies binding to them. This is another aspect where both methods are creating SVF of similar quality.

9.4. Colony Forming Unit (CFU) Assay

CFU assays were performed with ASCs derived from three samples of lipoaspirate using both extraction methods. The preparation method had no influence on the CFU capacity of the cultured SVF cells, resulting in 10.40±4.71% for hand prepared ASCs and 12.45±3.70% for ASCs extracted with the use of the medical device (p=0.147). This goes along with the CFU capacity of >5% for ASCs (19) reported in literature and indicates that neither of the two tested preparation methods has an influence on the proliferation of the resulting ASCs.

9.5. Application of the novel device for cell assisted lipotransfer (CAL)

For CAL application an ideal dose of SVF cells per reinjected milliliter of lipoaspirate has not been established yet, but one- to four-fold better engraftments were found with concentrations differing from 10^5 to 10^7 cells (75). Yoshimura et al. (44) proposed a one to one ratio of lipoaspirate for reinjection and lipoaspirate for SVF extraction. This is also implemented in the novel medical device where a maximum of 800ml lipoaspirate can be processed at the same time: While one half of this centrifuged lipoaspirate is saved for reinjection the other half (i.e. up to 400ml) can be used to create SVF. Adding the SVF to the saved lipoaspirate results in a maximum

of roughly 400ml SVF augmented lipoaspirate for reinjection. Consequently, if just the SVF is needed for treatments it can be extracted out of a maximum of 400ml lipoaspirate at the same time.

If the beneficial effects of the SVF of better graft retention and less scarring in CAL treatment (75) can be attributed to solely to their differentiation potential is currently under discussion. Because recently their paracrine secretion which inhibits inflammatory response at first and promotes tissue repair afterwards is coming more into the focus of research (76,77). Furthermore, there is growing evidence that the SVF is superior to selected subpopulations like ASCs, as higher paracrine secretion was found in the whole SVF rather than in selected subpopulations of the SVF (78). This leads to the assumption that the absolute number of SVF cells is of lesser importance than the concentration of their produced cytokines.

9.6. Limitations of this work and outlook

Six tissue samples were obtained from different patients in order to have sufficient number of replications for statistical calculation. Three preparation failures with the medical device led to lesser repetitions regarding the devices cells number. More repetitions would be beneficial; especially ones using the proposed syringe cap (9.1.2 Suggestion for solution). Thereby could be tested, if its usage leads to better results. However, such a modified syringe cap was not provided during the formation of this work.

Releasing the cells from the adipose tissue was acquired by the use of the MNP-S Liberase, which is for research purpose only and not suitable for a clinical setting. A GMP approved collagenase is needed for clinical application. Furthermore the collagenases activity was inhibited with FBS. For clinical application FBS has to be replaced by patients' blood serum, as described in the manufacturers instructional video (57). Furthermore, the rest activity of the remaining collagenase has to be explored for patients' safety as also the sterility of the final product (46).

Each sample of lipoaspirate was prepared at the same time with both preparation methods. A measurement of preparation time was not possible since extraction steps overlapped. In addition, only small amounts of lipoaspirate (40-80ml) were processed

that suited into one or two syringes. The novel medical device offers the opportunity of processing 800ml of lipoaspirate in one session, needing a handling of 16 syringes, which result in a longer processing time than in this work.

During the flow cytometric analysis the physical appearance in the FSC-SSC dot plot was used to determine vital cells. A more precise method would have been the a viability staining using 4′,6-Diamidin-2-phenylindol (DAPI) or another appropriate kit.

9.7. Conclusion

The present study characterized the SVF and ASCs obtained with the use of a novel medical device (UniStaion, Neogenesis), and compared it to the common laboratory SVF preparation by hand. The obtained results reveal that the novel medical device offers a considerable amount of SVF, comparable to the common laboratory preparation, when handled correctly. A suggestion for a new syringe cap, offering better handling, was made to avoid preparation failures using the novel medical device.

The composition of the SVF extracted by the novel device slightly has a smaller number in $CD34^+$ positive cells, however tends to a larger number in $CD45^+$ positive leukocytes than the hand preparation. However, the number of $CD34^+$ positive progenitor cells is still very high. There is no indication for any differences comparing the percentage of double positive cells and the fluorescence intensity measured with the stain index. Moreover, the common laboratory preparation shows no difference with regard to the CFU capacity of the cultured ASCs, thereby indicating a comparable proliferation potential.

To conclude, the novel medical device (UNiStaion, Neogenesis) can be a useful tool in the extraction of the SVF and ASCs, both in a research, and a clinical setting. It offers a SVF comparable in quantity and quality to the results by preparation with common laboratory equipment and values known from literature. Besides, the semi-automated process needs little space and is applicable to an operating theater. Most important, due to a closed syringe and transfer system, the novel medical device contributes to ensuring sterility in a clinical setting for future cell therapies in patient care.

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11. Declaration (German)

ERKLÄRUNG ZUM PROMOTIONSVERFAHREN

nach § 3 Abs. 3 und 4 der Promotionsordnung
der Fakultät für Medizin der Universität Regensburg

Name: Hanke

Vorname: Alexander

geb. am: 18. Juli 1991

in: Regensburg

Ich erkläre,

- dass ich den **Doktorgrad der Medizin** nicht schon an einer Hochschule der Bundesrepublik Deutschland erworben habe.
- das ich nicht an anderer Stelle zu einem Promotionsverfahren zum Erwerb des **medizinischen Doktorgrades** zugelassen bin.
- dass ich die **medizinische Doktorprüfung** nicht schon an einer Hochschule der Bundesrepublik Deutschland endgültig nicht bestanden habe.

Außerdem erkläre ich,

- dass mir keine Tatsachen bekannt sind, die mich zur Führung eines akademischen Grades im Sinne des Gesetzes über die Führung akademischer Grade unwürdig erscheinen lassen.
- dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeit erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

_____ _____

(Ort, Datum) (Unterschrift)

12. Acknowledgements (German)

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13. Addendum

13.1. Figures

Figure 1 Liposuction procedure after tumescent infiltration at the left outer thigh. The area of liposuction is marked prior to the operation in accordance with patients request for aesthetic outcome. The liposuction cannula is maintained parallel to the surface during the suction procedure while the left hand is providing direction guidance. **24**

Figure 2 Flow diagram of the used methods and outcome parameters (marked bold). **26**

Figure 3 Hand preparation. 20ml of lipoaspirate (1) are mixed with equal volume of 20ml DMEM and 2.5-5mg of MNP-S Liberase (2). The tissue is digested after 45min of incubation at 37°C and shaking at 100rpm (3) and can be filtered through a sterile 100µm filter system (4). After the following centrifugation, the oily and fluid phase on the top can be clearly distinguished (5). After discarding of the supernatant and washing with PBS, the pellet representing the SVF is clearly visible on the bottom of the centrifuge tube (6). **28**

Figure 4 The interior of the medical device shows the centrifuge with 16 slots for syringes. The heater is located behind the silver surroundings. **29**

Figure 5 Shaking plate placed on top of the centrifuge for the incubation step (program A2) offering space for eight syringes. No centrifugation steps can be performed when the shaker is installed. **29**

Figure 6 Syringe and transfer system to establish a sterile compartment for the extraction of the SVF. The syringes are separately packed (a) and can be filled with up to 50ml (b). The plunge (e) can be unscrewed to save space in the centrifuge. The Caps (d) and the transfer piece (c) all have the standardized Luer Lock system. **30**

Figure 7 Discarding of tumescent solution and blood accumulated in the lower part of the syringe after the first centrifugation step of the lipoaspirate. Solid tissue and oil remained in the syringe for further processing. **31**

Figure 8 The collagenase was added to the centrifuged lipoaspirate: The upper syringe contains DMEM with 10mg of MNP-S Liberase with a volume that is required to obtain 40ml in the lower syinge containing the lipoaspirate after the first centrifugation step and removal of the lower part. Both are connected via the silver transfer piece and the MNP-S Liberase solution is added by applying slight pressure on the plunge of the upper syringe. **31**

Figure 9 Digested tissue before (a) and after centrifugation (b). The content is separated into an upper oil layer, a layer of tumescent, blood and a small SVF pellet at the bottom. **32**

Figure 10 Transferring the SVF into a 50ml centrifugation tube. The SVF is evident as a small red pellet at the bottom of the syringe and is released with the lower 5ml of PBS. **33**

Figure 11 Deep red color of the SVF pellet in PBS after isolation (1). Re-suspended pellet in erythrocyte lysis buffer (2). The white SVF pellet is barely visible after erythrocyte lysis in PBS (3). **37**

Figure 12 Gating strategy to obtain the cell concentration of the cells positive for one marker in the lipoaspirate. The complete SVF is shown in a FSC-SSC dot plot (1). All events are gated and shown in histograms for CD34 (2a), CD45 (3a) and CD271 (4a). All positive cells are drawn back to a FSC-SSC dot plot (2b, 3b, 4b) and gated for the cell population. The concentration could be calculated with the help of counting beads (marked blue in 1). **40**

Figure 13 Gating strategy for the percentage of single positive cells within the defined main cell population. The FSC-SSC dot plot shows a main cell population circled red and two smaller populations on the left upper side that are the two different counting beads (1). The histograms on the right show the fluorescence of PE associated with CD34 (2), PE-Cy7 associated with CD45 (3) and APC associated with CD271 (4). The gates for positive cells were set in reference to the isotype controls, so that almost no events of the isotype control could be found in this gate. **42**

Figure 14 Example for gating double positive cells. The events gated as cells in the FSC-SSC dot plot (1) are further gated in a histogram to obtain the CD34 positive cells (2). Those are shown in a dot plot with the two axes defined by the two other markers: CD45 and CD271 (3). The cross divides the plot defining four new gates. The cells of interest lay in gate 3a and 3d. They show $CD34^+$ and $CD271^+$ cells (3a) and $CD34^+$ and $CD271^+$ cells (3d). Just CD34 single positive cells can be found in gate 3c, while cells positive for all three markers lay in gate 3b. **43**

Figure 15 Obtaining the stain index: The mean fluorescence intensity ($MFI_{Isotype}$) and its standard deviation ($SD_{isotype}$) are taken from the istoype control of one marker (1). From the same marker the mean fluorescence intensity of the sample (MFI_{Sample}) is obtained (2). The stain index is calculated: $Stain\ Index = (MFI_{sample} - MFI_{isotype}) / 2 * SD_{isotype}$ (58). **44**

Figure 16 A model colony of ASCs stained with 0.05% crystal violet. Further expansion of the cells can be seen at the border of the colony. **47**

Figure 17 Example CFU Assay ready for counting, after two weeks of incubation, fixation with 4% PFA and staining with 0.05% crystal violet. **48**

Figure 18 Overall cell number of the SVF per gram of lipoaspirate for both preparation methods. The hand preparation resulted in $2.04 \pm 1.88 * 10^5$ and the preparation with the use of the medical device in $1.07 \pm 1.0 * 10^5$ cells/gram lipoaspirate. No significant difference was found (p=0.0623). **51**

Figure 19 Cell concentration per gram lipoaspirate of the investigated markers CD 34, CD 45 and CD 271 in the SVF for hand preparation (h) and preparation with the use of the medical device (d). Both preparation methods demonstrated no significant difference in the expression of the investigated markers, but a slight difference with the marker CD34 (p=0.0596). **52**

Figure 20 Percentage of single positive cells for the investigated markers in the previously defined main cell population in the FSC-SCC plot of the SVF. A significant difference between the hand preparation (h) and the preparation with the medical device (d) was found only for the marker CD34 (p=0.0185) and a tendency for the marker CD45 (p=0.0742). **53**

Figure 21 Percentage of double positive cells for the investigated markers in the previously defined main cell population in the FSC-SCC plot of the SVF. Based on the gating method, the number of values for double positive cells (n=12) is double of the number of samples (n=6). No significant difference between the hand preparation (h) and the preparation with the use of the medical device (d) was observed. In particular, for the marker combinations $CD34^+/CD45^+$ (p=0.2071) and $CD34^+/CD271^+$ (p=0.4150). No $CD45^+/CD271^+$ double positive cells were detected and therefore, no statistical analysis could be performed (#). **55**

Figure 22 The stain index of the complete SVF regarding the investigated surface markers for the hand preparation (h) and the preparation with the use of the medical device (d). Both methods demonstrated no significant difference in their stain index for CD34 (p=0.1210), CD45 (p=0.3336) and CD271 (p=0.6207). **57**

Figure 23 CFU Assays with ASCs of different concentration after two weeks of incubation, fixation and staining with crystal violet. The seeding concentration was 5 cells/cm² (1), 10 cells/cm² (2), 15 cells/cm² (3), 20 cells/cm² (4), 25cells/cm² and 30 cells/cm² (6). Different colonies could be separated best when initially seeded at lower concentrations (1-3), while when seeded at higher concentrations the colonies were overlapping a lot (5 and 6). **58**

Figure 24 Seeding concentration and correlating colony density of both hand preparation and the medical device. A rising tendency can be seen with a Spearman's correlation of r=0.855 for hand preparation and r=0.939 for preparation with the medical device. **60**

Figure 25 Seeding concentration and the CFU capacity of both hand preparation and preparation with a medical device. No significant difference between the CFU capacities of each method could be found (p>0.503). **60**

Figure 26 Cell concentration of the SVF obtained by the hand preparation and with the use of the medical device in cells per gram lipoaspirate. On the upper (a) all values are shown. The preparations that produced almost no cells are circled red and marked. On the lower (b) all invalid preparation failures were removed. **64**

Figure 27 Cell concentration of the SVF for the markers CD34, CD45 and CD271 obtained by hand preparation (h) and preparation with the use of the medical device (d). On the upper (a) all values are shown. The preparations that produced almost no cells are circled red and marked. On the lower (b) all invalid preparation failures were removed. This results in an increase of all middles and an approximation of the results of both preparation methods. **65**

Figure 28 Explanation of the preparation failures with the medical device. The bottom of the syringe with the SVF is illustrated (a). When the cap is removed a slightly negative pressure is created (b) and this can be enough to vacuum off a great part of the SVF into the cap (c). This part of the SVF is lost and cannot be used further. **67**

Figure 29 The currently used syringe cap (a) and a suggestion for an improved syringe cap (b) with a counterpart for the syringe shown in green. **67**

13.2. Tables

Table 1 Different programs / steps that are performed for the extraction of the SVF with short description of the action. **30**

Table 2 Short description of the examined surface proteins and specifications of the fluorescents conjugated to the used antibodies. **35**

Table 3 Description of the samples and volume of antibody solution added. **38**

Table 4 Cell concentration of the SVF shown for the hand preparation and the preparation with a medical device. The overall value is shown and also the cell concentration of the different markers. The results of both methods were compared with a paired two tailed T-Test. **51**

Table 5 Percentage of positive cells in the previously defined main cell population for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test. **53**

Table 6 The percentage of cells being positive for two markers in the population of single positive cells, for hand preparation and preparation with the medical device. The percentages of single positive cells are not shown here and marked with a star (*) and can be seen in 8.1.3 Single positive Cells. Cells double positive for $CD45^+$ and $CD271^+$ did not exist and are not shown in this table. **54**

Table 7 Percentage of double positive cells in the previously defined main cell population for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test. Because no $CD45^+$ / $CD271^+$ double positive cells existed in both preparation methods no T-Test could be performed (#). They show the same result. **55**

Table 8 Stain Index in the main population of the SVF for the three markers for hand preparation and preparation with the use of the medical device. The results of both methods were compared with a paired two tailed T-Test. **56**

Table 9 Colony density after two weeks and the resulting CFU capacity for hand preparation and the preparation with the use of the medical device resulting from the initial seeding concentration. Because colony density and CFU capacity can be transformed into each other the p values are the same and are shown in the right column. No significant difference could be detected. **59**

Table 10 SVF cell numbers reported in the literature compared to the results in this study. Preparation methods and repeats are shown. $^{\#}$ = manual preparations with laboratory equipment use different protocols, § = only valid preparations taken into consideration **63**

13.3. Curriculum Vitae (German)

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